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To cite this article: Joana Costa, Isabel Mafra, Isabel Carrapatoso & Maria Beatriz P. P. Oliveira (2016) Hazelnut Allergens: Molecular Characterization, Detection, and Clinical Relevance, Critical Reviews in Food Science and Nutrition, 56:15, 2579-2605, DOI: [10.1080/10408398.2013.826173](https://doi.org/10.1080/10408398.2013.826173)

To link to this article: <https://doi.org/10.1080/10408398.2013.826173>



Accepted author version posted online: 09 Mar 2015.  
Published online: 09 Mar 2015.



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# Hazelnut Allergens: Molecular Characterization, Detection, and Clinical Relevance

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*In last few years, special attention has been given to food-induced allergies, in which hazelnut allergy is highlighted. Hazelnut is one of the most commonly consumed tree nuts, being largely used by the food industry in a variety of processed foods. It has been regarded as a food with potential health benefits, but also as a source of allergens capable of inducing mild to severe allergic reactions in sensitized individuals. Considering the great number of reports addressing hazelnut allergens, with an estimated increasing trend, this review intends to assemble all the relevant information available so far on the following main issues: prevalence of tree nut allergy, clinical threshold levels, molecular characterization of hazelnut allergens (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 10, Cor a 11, Cor a 12, Cor a 14, and Cor a TLP) and their clinical relevance, and methodologies for detection of hazelnut allergens in foods. A comprehensive overview of the current data about the molecular characterization of hazelnut allergens is presented, relating to biochemical classification and biological function with clinical importance. Recent advances in hazelnut allergen detection methodologies are summarized and compared, including all the novel protein-based and DNA-based approaches.*

**Keywords** Food allergens, hazelnut allergy, *Corylus avellana* L., prevalence, threshold levels, detection

## INTRODUCTION

Hazelnut is classified as a tree nut that belongs to the botanical family of Betulaceae and genus *Corylus*. According to the United States Department of Agriculture – Germplasm Resources Information Network (USDA-GRIN), the genus *Corylus* encompasses more than 14 different species of hazels distributed all over the world (United States Department of Agriculture, 2013). Nuts from all hazel trees are considered edible; however, the most cultivated and consumed correspond to the seeds of the hazel species *Corylus avellana* L., usually known as “common hazelnut.” This hazel species is native of Europe and Western Asia (Caucasus region) but is also cultivated in North America. In general, alternative names, such as cobnut or filbert, are frequently used to designate common hazelnuts, although this classification is mostly attributed to the nuts of species *Corylus avellana* or *Corylus maxima*,

respectively. Apart from these, the nuts from other hazel species can also be consumed, although they do not represent any relevant interest in terms of trade. There are a large number of hazel (*Corylus avellana* L.) cultivars and selections (United States Department of Agriculture, 2013); Morell, Negret, Grossal, Buttler, Ennis, Pauetet, Fertile de Coutard, Segorbe, Sta María del Gésu, Tonda di Giffoni, Culplà, Camponica, Cosford, Gunslebert, Lansing, Palaz, Sivri, Tombul, and Tonda Romana being some examples of the varieties originated and/or cultivated in the south of Europe and the Caucasus region.

The edible part of the hazelnut is the kernel, which is consumed either raw or roasted (snacks), or included as an ingredient in a wide range of processed foods such as cakes, creams, chocolates, and confectionary products (Alasalvar and Shahidi, 2008). Subsequently, hazelnuts along with other tree nuts play an important role in economy since these are an integral part of human food supply (Costa et al., 2012a). Among the worldwide production of tree nuts in 2011, hazelnut represented the seventh most relevant culture, and in terms of global trade, these seeds occupied the fourth position just behind

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pistachio, almond, and cashew nut. In 2011, Western Asia and Europe retained almost 89% of the world's total production of hazelnut. The three major producers of this nut are Turkey, Italy, and Azerbaijan, ranking as the first, second, and third, respectively, since these countries alone are accountable for approximately 80% of hazelnut production each year (FAO-STAT, 2013).

For some years, hazelnuts and other tree nuts have been regarded as foods with potential health benefits, namely as "heart-protective," which have led to an increase in their consumption, especially in the developed countries (Food and Drug Administration (FDA), 2003). Apart from this recognition, tree nuts have also been pointed as likely to induce hypersensitivity in sensitized/allergic individuals. Therefore in 1985, the Codex Alimentarius Commission recommended the obligation to label foods susceptible of containing potentially allergenic ingredients and since 1993, tree nuts are defined as one of the eight groups responsible for almost 90% of human food allergies (CODEX STAN 1). Accordingly, the European Union (EU) has established some directives determining the clear obligation of food producers to declare all the ingredients present in pre-packaged foods commercialized inside the EU (Directive 2000/13/EC). Presently, tree nuts are included in a list of fourteen groups of certain substances or products causing allergies or intolerances that are required to be emphasized from the rest of the list of ingredients of processed foods, regardless of their quantity (Directive 2007/68/EC; European Union 2011).

Of all the tree nuts, hazelnut is probably the most well-studied nut regarding the impact of its presence in the life of sensitized individuals. The purpose of this review is to provide an actual and critical overview on the prevalence of hazelnut allergy, the molecular characterization of its allergens, and the available methodologies for its detection. Herein, important subjects, such as the clinical relevance of hazelnut allergy and the definition of threshold levels, will also be addressed.

### ***PREVALENCE OF TREE NUT ALLERGY: THE CASE OF HAZELNUT***

Food-induced allergy represents an emerging problem of public health affecting adults and children, and whose prevalence is estimated to be rising. By definition, food allergy is a reproducibly adverse health effect arising from a specific immunological response that occurs in sensitized individuals upon exposure to a given food (Boyce et al., 2010). General data seem to indicate that the number of sensitized/allergic patients affected by food allergy is more than 1–2% but less than 10% of the world's population (Chafen et al., 2010). Although the available data are considered rather imprecise, some studies suggest that as many as 3–4% of adult population and 5–6% of young children/adolescents can suffer from some type of allergy related to food (Sicherer and Sampson, 2009, 2010). In Europe alone, the number of patients allergic to

food is estimated to reach 17 millions, which represents approximately 2.3% of the European population. From these, 3.5 million of the European allergic patients are younger than 25 years, with the sharpest rise in food allergies being among children and young people (EAACI, 2013).

The true prevalence of food allergies has been very difficult to determine due to several inconsistencies regarding key issues such as those related to study design. The majority of the information about the prevalence of food allergies is based on self-reported reactions to foods (questionnaires/surveys) rather than using objective assessments as open and double-blind food challenge tests, or determined sensitization to foods by serum immunoglobulin E (IgE) and skin prick tests (SPT) (Zuidmeer et al., 2008). Consequently, prevalence data must be interpreted with caution and considered as mere indicators of the true incidence of food allergy.

In Europe, tree nuts are regarded as a common cause of food allergy (Ortolani et al., 2000), with hazelnut representing a significant part of those induced allergies, while in the United States, allergies to peanut and/or tree nuts, such as almond, walnut, or cashew, appear to be more relevant (Sicherer et al., 2003). In an 11-year follow-up self-reported study, conducted between 1997 and 2008 in the United States and encompassing three different surveys during this period (1997, 2002, and 2008), the prevalence of peanut and/or tree nut allergy was higher than 1.1%, which corresponded to more than three million individuals of the general US population. The same study also enabled to estimate that the increasing number of allergic patients to peanut and/or tree nut was more significant in individuals aged under 18 years than with walnut, cashew, pecan nut, and almond, presenting the highest allergic incidence in the US population (Sicherer et al., 2010). Regarding the same age-target population, in 2009, a randomized cross-sectional survey was administrated electronically to a representative sample of US households, which enabled in estimating an overall incidence of 8% of food allergic children (<18 years old). According to this study, the prevalence of nut allergy was approximately 1%, with more than 52% of the allergic children suffering from severe immunological reactions (Gupta et al., 2011). Another study performed in Canada in 2011, using a nationwide telephone survey of randomly selected households, reported an overall incidence of approximately 6.7% of individuals with allergy to at least one food. The prevalence data for this region was higher for children (7.1%) than for adult population (6.6%). The estimated incidence of tree nut allergy was approximately 1.22% for the entire population and 1.74% for children, confirming the prediction of overall indicators (Soller et al., 2012).

Usually, the prevalence studies involve only data from one country or region, thus in order to provide a broader overview about this topic, the European Commission funded in 2005 a large Europe-wide research project (EuroPrevall) specifically designated to evaluate the prevalence, the basis, and the cost of food allergies. This project included the participation of 56 partners from 21 countries, including 19 European countries,

Ghana, and India, as well as additional co-partners from the United States, Australia, and the New Zealand (Mills et al., 2007a). Study designs involved birth cohorts, community surveys, and outpatient clinical studies, with complementary information provided by SPT and double-blind placebo-controlled food challenges (DBPCFC). Part of the project tasks also covered the evaluation of the prevalence data available in the literature concerning food-induced allergies. In this context and on the basis of the comparison of studies published during the past 15 years, Zuidmeer et al. (2008) reported a prevalence of 0.03–0.2% of nut allergies in children aged up to 6 years, 0.2–2.3% in children/adolescents aged between 6 and 18 years, and 0.4–1.4% in adults. In accordance with challenge tests and sensitization assessed by SPT, the highest prevalence of nut allergies was estimated for hazelnut (4%). Adolescents and adults seem to be more affected by nut allergies, probably due to their late introduction into the diet. As nuts are often eaten separately, they are more easily identified as the possible cause of observable symptoms, rather than other fruits or vegetables that are usually consumed in mixed dishes, making it difficult to distinguish the allergenic ingredient (Zuidmeer et al., 2008). Included in the EuroPrevall project and involving several centers from 13 countries (the United States, Australia, and 11 countries from Europe), sera from test subjects was scanned using five allergen mixes from a total of 24 foods defined previously as priorities. Allergy induced by hazelnut presented the highest overall incidence, accounting for approximately 7.2% of the test population (Burney et al., 2010). Hazelnut allergy is often related to birch pollinosis, therefore some patients are commonly allergic to the nut itself, others are allergic to the pollen of hazel trees, but frequently patients are allergic to both (Roux et al., 2003). These facts seem to be well stated in the study reported by Burney et al. (2010), as after excluding the birch positive patients, the percentage of positive reactants to hazelnut kernel was reduced to approximately 3.1%. The United States presented the highest incidence (14.9%) of allergic patients to hazelnut, being closely followed by Germany (14.7%), Norway (12.8%), Switzerland (12.6%), and Sweden (11.8%). From a panel of 24 foods, hazelnut was also considered as the food with the highest prevalence of allergy, at least in 46% of the participating countries (Burney et al., 2010).

From all the information displayed, it is clear that more studies are needed aiming at establishing the prevalence of tree nut allergies, specially focusing on each of the most significant nuts.

#### **CLINICAL THRESHOLD LEVELS FOR HAZELNUT**

Presently, the only effective means of preventing any adverse immunological reactions in the allergic patients is based on the total avoidance of offending foods. Because of this protective measure, allergic individuals face several restrictions when carefully choosing processed foods that are

commercially available. In order to avoid probable legal actions against food-processing companies, products' labels are often excessively precautionary due to the potential risk of cross-contaminations in the production line or during storage. Thereby, the definition of clinical threshold values for allergenic foods would be of utmost interest since it could contribute to better risk management by regulatory authorities, providing more adequate guidelines to food industry. Not less relevant, information about personal thresholds would enable patients, caretakers, and physicians to establish adequate individual strategies aiming at preventing potential adverse immunological responses (Eller et al., 2012). The term "threshold" is attributed to the dose of allergenic food that lies between the highest amount of the offending food not eliciting any allergic response and the lowest observed adverse effect level (LOAEL). In general, the threshold dose is functionally defined as LOAEL or the no observed adverse effect level (NOAEL), being determined either on individual- or population basis (Taylor et al., 2009). The assessment of individual NOAEL and LOAEL parameters can be performed by clinical challenge trials such as open food challenges (OFC) and DBPCFC. In practice, these challenges cannot be carried out in all patients allergic to food. However, statistical models based on individual LOAEL have been conducted for the elaboration of a dose-response curve for a given allergen (Crevel et al., 2007).

Using clinical challenge tests (OFC and DBPCFC) on a large test population (487 food allergic patients), Eller et al. (2012) were able to establish threshold values for hazelnut, egg, peanut, and milk. In the case of hazelnut, the frequency of first-dose responders with objective symptoms corresponded to 8% of the test group when an initial dose of 1 mg was administered to those patients. Therefore, this dose (1 mg) was considered as the lowest amount of allergenic food inducing observed adverse immunological responses, and consequently defined as the threshold value for hazelnut. In the same study, Eller et al. (2012) were able to predict that 8.7 mg and 15.9 mg of hazelnut protein were sufficient to induce allergic reactions in 5% and 10% of the hazelnut allergic population, respectively. Another study estimating the threshold distribution of seven allergenic foods (egg, milk, peanut, hazelnut, walnut, cashew nut, and soy) was conducted using DBPCFC in children and adolescents (0–18 years old) as the test population (Blom et al., 2013). In this study, patients with hazelnut allergy were the most sensitized group since 5% and 10% of the tested population is likely to respond with objective symptoms to 0.3 mg (ED<sub>05</sub>) and 1.4 mg (ED<sub>10</sub>) of hazelnut protein, respectively. Moreover, the same target population evidenced any type of symptoms for an estimated threshold level of 0.05 mg of hazelnut protein (Blom et al., 2013).

From the evaluation of these two studies, the influence of different parameters, such as the size, age, and sex of the target allergic population as well as the type of food matrices and the type of food challenges used (OFC versus DBPCFC), is very clear. The distribution of the threshold level for hazelnut

reported by those studies is rather different, which suggests that further investigation regarding this topic is needed. However, these data represent a step forward in the elaboration of precautionary labeling action levels and for their incorporation in risk assessment for adverse immunological reactions in the allergic population upon eventual consumption of foods contaminated with allergens (Blom et al., 2013).

### ***Molecular Characterization of Hazelnut Allergens***

The majority of molecules defined as food allergens are biochemically classified as proteins or glycoproteins that are naturally present in foods (Boyce et al., 2010). In the specific case of hazelnut, several proteins have been recognized as allergens. Until now, 10 groups of allergenic proteins (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 10, Cor a 11, Cor a 12, Cor a 13, Cor a 14, and Cor a thaumatin-like protein (TLP)) have been identified and characterized in hazelnut. Of these, Cor a TLP has not been included in the World Health Organization and International Union of Immunological Societies (WHO-IUIS) list of allergens (ALLERGEN, 2013) but has been integrated in the Allergome database (ALLERGOME, 2013). All the other hazelnut allergens are included in the referred list with the classification of food allergens, with an exception of Cor a 10, since it is only present in the pollen of hazel trees (ALLERGEN, 2013).

### ***Cor a 1 (PR-proteins)***

The pathogenesis-related proteins comprise a collection of several unrelated families that are expressed in response to external factors such as environmental stress, pathogen infection, or antibiotic stimuli. These proteins are characterized by their small size, stability at low pH, and resistance to proteolysis, making them good candidates for inducing adverse immunological responses in sensitized individuals (Hauser et al., 2008). The PR-10 proteins are included in the PR protein superfamily, being commonly known as the Bet v 1-related proteins or Fagales group I because these are very common among the trees from the order Fagales. Cor a 1 allergens are classified as PR-10 proteins, which are abundant in reproductive tissues such as pollen, fruits, and seeds. As a consequence, Cor a 1 proteins are classified as both food allergens and aeroallergens.

Cor a 1 appears to comprise a complex set of proteins encoded by different nucleotide sequences ranging from 486 to 860 base pairs (bp) (NCBI, 2013), presenting distinct allergenicity among proteins (Lüttkopf et al., 2001). Cor a 1 exhibits four isoallergens designated by Cor a 1.01, Cor a 1.02, Cor a 1.03 in hazel pollen, and Cor a 1.04 in hazelnut seed, with molecular weight of approximately 17 kDa (Table 1) (Roux et al., 2003). These four sequences are

denominated isoallergens due to their similar molecular size, identical biological function, and 67% or more amino acid identity (Chapman et al., 2007). Isoallergen Cor a 1.04 comprise four variants or isoforms (Cor a 1.0401–Cor a 1.0404) with 161 amino acids (aa), a calculated molecular mass of 17.4 kDa, and an isoelectric point (pI) of 6.1 (Lüttkopf et al., 2001). These are polymorphic variants of the same allergen (Chapman et al., 2007) and among them exhibit 97–99% of amino acid identity with a maximum of six substitutions in five of the highly conserved regions. However, they only share 63% or more sequence identity with the other six hazel pollen isoforms (Table 1) (Lüttkopf et al., 2001). The amino acid sequence identity is higher between Cor a 1.04 and Bet v 1 (85%) than with the other pollen isoallergens (Cor a 1.01, Cor a 1.02, and Cor a 1.03) from the same tree. This fact seems to suggest that in some populations, the majority of the patients can be primarily sensitized to birch pollen (Bet v 1) (Roux et al., 2003). Cor a 1.01, which is classified as a hazel pollen isoallergen, also comprises four isoforms (Cor a 1.0101 to Cor a 1.0104) with sequence identity higher than 95% among variants.

In general, these allergens are considered to be heat-labile proteins, suggesting that they suffer unfolding when submitted to heat treatments such as food processing (Müller et al., 2000; Schocker et al., 2000; Pastorello et al., 2002; Hansen et al., 2003). The loss of the protein tri-dimensional structure enables the destruction of conformational epitopes (IgE-reactivity) and consequently the ability to trigger adverse reactions in sensitized individuals (Mills et al., 2007b). Dry heat processing, such as roasting, enables reducing significantly the allergenicity of hazelnuts in patients with positive diagnosis for birch-pollen allergy related to hazelnuts, although some sensitized individuals can still experience positive reactivity toward roasted hazelnuts (Hansen et al., 2003). Structure models for Cor a 1 proteins revealed that the most part of the amino acid sequence is well organized, with loops located almost exclusively at the apical structures of beta-turns and thus being very influenced by the beta arrangement itself. López et al. (2012) reported that the application of autoclave processing (121°C or 138°C, 15 or 30 min) to hazelnut samples allowed decreasing the allergenicity of Cor a 1, since the location of epitopes is mainly dependent on the conformational structure of the protein, which is affected by heat treatment. This finding is in good agreement with the data reported by Hansen et al. (2003), stating that Cor a 1 allergenicity is highly influenced by the tri-dimensional conformation of proteins. As an alternative to thermal processing, new technologies, such as high-pressure processing, have been exploited to evaluate their effect on the structural conformation of allergens and hence to decrease their allergenic activity (Mills et al., 2003). The application of high-pressure processing with increasing pressures, ranging from 300 to 600 Mpa, was performed aiming at testing its effect on the allergenicity of Cor a 1 proteins,

**Table 1** Identification of hazelnut allergens according to their biological function, clinical relevance, and respective accession numbers in the NCBI and UniProt databases

| Allergen  | Molecular weight (kDa)  | Isoallergens                                         | Isoforms or variants                                                                                                                                         | Nucleotide (NCBI)                                                                                                            | Protein (NCBI)                                                                                                                           | Protein (UniProt)                                                                                | Protein Families              | Biochemical classification               | Biological function                                                                                                                                     | Clinical relevance                                                                                              |
|-----------|-------------------------|------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|-------------------------------|------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|
| Cor a 1   | 17 (160 aa)<br>(161 aa) | Cor a 1.01<br>Cor a 1.02<br>Cor a 1.03<br>Cor a 1.04 | Cor a 1.0101<br>Cor a 1.0102<br>Cor a 1.0103<br>Cor a 1.0104<br>Cor a 1.0201<br>Cor a 1.0301<br>Cor a 1.0401<br>Cor a 1.0402<br>Cor a 1.0403<br>Cor a 1.0404 | X70999.1<br>X71000.1<br>X70997.1<br>X70998.1<br>Z72439.1<br>Z72440.1<br>AF136945.1<br>AF323973.1<br>AF323974.1<br>AF323975.1 | CAA50327.1<br>CAA50328.1<br>CAA50325.1<br>CAA50326.1<br>CAA96548.1<br>CAA96549.1<br>AAD48405.1<br>AAG40329.1<br>AAG40330.1<br>AAG40331.1 | Q08407<br>Q08407<br>Q08407<br>Q08407<br>Q39453<br>Q39454<br>Q9SWR4<br>Q9FPK4<br>Q9FPK3<br>Q9FPK2 | Pathogenesis-related proteins | PR-10                                    | Involved in mechanisms of defense and biotic stimulus responses                                                                                         | Mild symptoms, mostly related to OAS. (Major allergen)                                                          |
| Cor a 2   | 14 (131 aa)             | Cor a 2.01                                           | Cor a 2.0101<br>Cor a 2.0102                                                                                                                                 | AF327622.1<br>AF327623.1                                                                                                     | AAK01235.1<br>AAK01236.1                                                                                                                 | Q9AXH5<br>Q9AXH4                                                                                 | Profilin                      | Profilin                                 | Binds to actin and affects the structure of cytoskeleton<br>Transference of phospholipids and galactolipids across membranes.<br>Antimicrobial activity | Mild symptoms mostly related to OAS.<br>(Minor allergen)<br>Severe and systemic reactions.<br>(Major allergens) |
| Cor a 8   | 9 (115 aa)              | Cor a 8.01                                           | Cor a 8.0101                                                                                                                                                 | AF329829.1                                                                                                                   | AAK28533.1                                                                                                                               | Q9ATH2                                                                                           | Prolamin                      | PR-14                                    | Storage of nutrients for plant growth                                                                                                                   | Severe and systemic reactions.<br>(Major allergens)<br>No data available                                        |
| Cor a 9   | 40 (515 aa)             | Cor a 9.01                                           | Cor a 9.0101                                                                                                                                                 | AF449424.1                                                                                                                   | AAL73404.1                                                                                                                               | Q8W1C2                                                                                           | Cupin                         | 11S seed storage globulin (legumin-like) | Storage of nutrients for plant growth                                                                                                                   | Severe and systemic reactions.                                                                                  |
| Cor a 10  | 70 (668 aa)             | Cor a 10.01                                          | Cor a 10.0101                                                                                                                                                | AJ295617.1                                                                                                                   | CAC14168.1                                                                                                                               | Q9FSY7                                                                                           | Heat shock protein 70         | Luminal binding protein                  | Interacting selectively and non-covalently with ATP (binds ATP and nucleotides)                                                                         | No data available                                                                                               |
| Cor a 11  | 48 (448 aa)             | Cor a 11.01                                          | Cor a 11.0101                                                                                                                                                | AF441864.1                                                                                                                   | AAL86739.1                                                                                                                               | Q8S4P9                                                                                           | Cupin                         | 7S seed storage globulin (vicilin-like)  | Storage of nutrients for plant growth                                                                                                                   | Severe and systemic reactions.(Major allergens but classification should be revised)                            |
| Cor a 12  | 17 (159 aa)             | Cor a 12.01                                          | Cor a 12.0101                                                                                                                                                | AY224679.2                                                                                                                   | AAO67349.2                                                                                                                               | Q84T21                                                                                           | Oleosin                       | 17 kDa oleosin                           | Cellular component. Intervene in lipid metabolism and storage, regulation of intracellular trafficking and signal transduction                          | No data available.<br>(Suggested major allergen classification)                                                 |
| Cor a 13  | 14-16 (140 aa)          | Cor a 13.01                                          | Cor a 13.0101                                                                                                                                                | AY224599.1                                                                                                                   | AAO65960.1                                                                                                                               | Q84T91                                                                                           | Oleosin                       | 14-16 kDa oleosin                        | The same as Cor a 12                                                                                                                                    | No data available.<br>(Suggested major allergens classification)                                                |
| Cor a 14  | 15-16 (147 aa)          | Cor a 14.01                                          | Cor a 14.0101                                                                                                                                                | FI358504.1                                                                                                                   | ACO56333.1                                                                                                                               | D0PWG2                                                                                           | Prolamin                      | 2S albumin                               | Storage of nutrients for plant growth                                                                                                                   | Moderated to severe symptoms. (Minor allergens)                                                                 |
| Cor a TLP | Unknown                 | Unknown                                              | Unknown                                                                                                                                                      | Unknown                                                                                                                      | Unknown                                                                                                                                  | Unknown                                                                                          | Pathogenesis-related proteins | TLP (PR-5)                               | Anti-fungal activity                                                                                                                                    | No data available<br>(Suggested minor allergens classification)                                                 |

although resultant allergenic profile remained the same as that of raw hazelnuts (López et al., 2012).

### ***Cor a 2 (Profilins)***

Profilins are a family of cytosolic actin binding proteins with small molecular size (1215 kDa) and constituted by polypeptides ranging from 124 to 153 aa (Vieths et al., 2002). They are highly conserved molecules, sharing more than 75% of amino acid sequence identity with profilins from members of distantly related organisms (Hauser et al., 2010). The sequence conservation reflected among all eukaryotic cells is evidenced by similar tertiary structures and the identical biological functions of profilins (Hauser et al., 2008). These proteins are important mediators of membrane–cytoskeleton communication, being able to specifically bind to ligands such as actin, phosphatidylinositol-4,5-bisphosphate (PIP2), and poly-L-proline (Vieths et al., 2002). Such characteristics enable profilins to actively intervene in processes associated with cell motility (regulation of the actin microfilament polymerization) and interact with the PIP2 pathway of signal transduction (Valenta et al., 1992; Vieths et al., 2002). As components of many essential cellular processes, profilins are ubiquitously spread through nature. Therefore, these can be considered as pan-allergens that are responsible for several of the observed cases of cross-reactivity between inhalant and food allergens (Valenta et al., 1992).

One group of allergens identified in hazelnut comprises a family of profilins named *Cor a 2*, which were characterized as a relevant IgE-binding protein for a minority of pollen–nut allergic individuals (Hirschwehr et al., 1992). *Cor a 2* proteins are classified as pollen and food allergens since they both are present in the pollen of hazel trees and in their respective seeds/nuts (hazelnuts) (Hauser et al., 2010). Two variants of *Cor a 2* allergens, encoded by nucleotide sequences of 396 bp, have been described (NCBI, 2013), namely *Cor a 2.0101* and *Cor a 2.0102* (Table 1). Allergen isoforms present sequences with 131 aa, similar molecular size (14.0 kDa and 14.1 kDa), and acidic proprieties (pI of 4.9 and 4.7) for *Cor a 2.0101* and *Cor a 2.0102*, respectively (Vieths et al., 2002). Sequence identity between the two variants (*Cor a 2.0101* and *Cor a 2.0102*) is higher than 98%, and approximately 77% toward *Bet v 2*, which states that *Cor a 2* is one of the *Bet v 2*-related allergenic food profilins. Similar to isoallergen *Cor a 1.04*, the allergenicity of *Cor a 2* seems to decrease with food processing (roasting) in patients sensitized to birch-pollen hazelnut allergens, suggesting that profilins *Cor a 2* are also heat-labile proteins (Hansen et al., 2003).

### ***Cor a 8 (nsLTP)***

*Cor a 8* is another group of allergenic proteins present in hazelnut, and belongs to the family of non-specific lipid

transfer proteins (nsLTP) that are included in the prolamin superfamily. Along with the family of nsLTP, this superfamily comprises several groups of proteins that contain many plant food allergens such as 2S albumins and inhibitors of alpha-amylase and trypsin from cereals (Breiteneder, 2006). The family of nsLTP is characterized by monomeric proteins of low molecular size, revealing primary sequences with a high content of cysteine residues, thus contributing to secondary structures comprising alpha-helices that involve a lipid binding cavity in the core. Members from this family share common structural features that include eight cysteine residues bonded in four disulphide bridges, basic isoelectric points, and high similarity in amino acid sequences (Kader, 1996). One of the biological functions of nsLTP is related to their ability to transport different types of lipids (fatty acids, phospholipids, glycolipids, and sterols) through membranes comprising two subfamilies of 9 kDa proteins (nsLTP 1) or 7 kDa proteins (nsLTP 2) (Hauser et al, 2010; UniProt, 2013). The nsLTP are also interveners in other functions such as those associated with plant defense (antifungal and antibacterial activities) (Ebner et al., 2001) or potential involvement in plant growth and development (embryogenesis and germination) (Kader, 1996; Salcedo et al., 2007), being widely distributed throughout the plant kingdom (Hauser et al, 2010). According to these facts, the nsLTP were included in the class of pathogenesis-related proteins, representing the PR-14 family.

In hazelnut, *Cor a 8* is classified as a food allergen due to its exclusive presence in nutritive tissues (seed). With a polypeptide chain of 115 aa and a molecular weight of 9 kDa, *Cor a 8* is encoded by a nucleotide sequence of 348 bp (NCBI, 2013). Considering that the immature protein contains a signal peptide of 23 aa, the mature *Cor a 8* allergen presents a total of 92 aa (Schocker et al., 2004) with alpha-helical structure (Rigby et al., 2008). According to the NCBI database, upon protein alignment of relevant allergenic nsLTP from different species, *Cor a 8* exhibited 60% of sequence identity with *Pru du 3* (almond, ACN11576), 59% with *Mal d 3* (apple, AAR22488), and *Pru ar 3* (apricot, ADR66948), 58% with *Pru av 3* (cherry, AAF26449), 56% with *Pru p 3* (peach, ACE80969), and 54% with *Pyr c 3* (pear, AAF26451) (NCBI, 2013), which indicates their structural relationship (Salcedo et al., 2007). Therefore, IgE cross-reactivity is expected to occur between *Cor a 8* and the allergens from fruits of the Rosaceae family (Schocker et al., 2004). Several factors are known to affect the allergenic potential of nsLTP, namely the location of proteins and the stability of these to thermal or proteolytic processing. In fruits from the Rosaceae family, the nsLTP are predominantly accumulated in the outer epidermal layers, being responsible for the stronger allergenicity of peels in comparison with inner pulps (Fernández-Rivas and Cuevas, 1999). Studies evidenced that some LTP-sensitized individuals can tolerate fruits (apple, peach) after peeling (Fernández-Rivas and Cuevas, 1999); however, they are still at a risk of developing adverse reactions upon the ingestion of nuts. The nsLTP are regarded as true food allergens considering that these molecules are capable of

eliciting severe allergic responses after resisting to food processing (thermal treatments and abrupt pH changes) and to the inhospitable environment of the gastrointestinal tract (proteolysis) (Zuidmeer and van Ree, 2007). Similar to other nsLTP, Cor a 8 was also found to be resistant to the activity of gastric and intestinal enzymes, which justify its capacity to induce severe allergic reactions in some sensitized individuals (Schulten et al., 2011a). In contrast with the 2S albumin family, the nsLTP are slightly less stable when submitted to the temperatures of over 90°C, probably due to the existence of the lipid-binding tunnel (Sancho et al., 2005; Mills et al., 2007b). As for Cor a 1, the allergenicity of Cor a 8 was significantly affected when submitting hazelnuts to high temperatures and wet processing, such as autoclave (121°C and 138°C, for 15 and 30 min), since autoclaving induces the disorganization of almost all possible epitopes in this protein. The application of high-pressure processing (300 to 600 Mba) to hazelnut samples did not affect the IgE-binding capacity of Cor a 8 in the test population (López et al., 2012). Similar to profilins, the nsLTP are also considered pan-allergens, suggesting that these proteins are well spread throughout the nature. The highly conserved regions and tri-dimensional structures seem to ensure the functionality of nsLTP, even those originating from unrelated sources, enabling to satisfy the requisites of IgE recognition (Hauser et al., 2010).

### ***Cor a 9 (11S Globulin – legumin)***

Cor a 9 proteins constitute another group of hazelnut allergens that belong to the cupin superfamily. It encompasses a large and multifunctional variety of proteins sharing a common origin, as their evolution can be traced from bacteria to eukaryotes, including animals and superior plants (Dunwell et al., 2004; Hauser et al., 2008). The cupin superfamily comprises two functional classes of proteins, namely the monocupins and the dicupins, containing one or two conserved cupin domains respectively. The dicupin class includes the 7S and 11S globular seed storage proteins, which represent major components of human diet. In tree nuts as well as in several legumes, the seed storage globulins represent almost 50% of the total seed proteins that contain the resources needed for plant germination. Globulins are further divided in two groups according to their sedimentation coefficient: the 7S vicilin-type globulins and the 11S legumin-type globulins (Breiteneder, 2006).

Cor a 9, also known as corylin, is classified as an 11S legumin-type globulin (Beyer et al., 2002; Guo et al., 2009). It is expressed by a gene with 1767 bp encoding a protein of 515 aa with a theoretical molecular mass of 59 kDa. Each isoform of 11S globulin is apparently coded by a single gene producing a precursor that is post-transnationally split in the asparaginyl endopeptidase site. The functional 11S legumins are non-glycosylated proteins, forming hexameric structures comprising six subunits interacting non-covalently and arranged in an open ring conformation with 360 kDa (Breiteneder, 2006).

Each subunit is constituted by an acidic polypeptide (30–40 kDa) linked to a basic polypeptide (~20 kDa) by a disulphide bond. 11S globulins from several tree nuts display 8 to 15 linear epitope-bearing peptide regions that are scattered along the length of acidic and basic subunits (Robotham et al., 2009). Functional Cor a 9 also comprises acidic and basic polypeptide chains linked by a disulphide bond (Beyer et al., 2002). The cleavage site in the peptide bond seems to be well conserved among a wide variety of plant species. Cor a 9 and several other legumins possess the NGXEET motif: NGFEET in Cor a 9 from hazelnut, NGLEET in Pru du 6 from almond and Jug r 4 from walnut, and NGIEET in Ana o 2 from cashew and Ara h 3 from peanut (Albillos et al., 2008). The identified allergen with a molecular weight of 40 kDa is an acidic subunit of Cor a 9 after cleavage and reduction of protein (Table 1) (Beyer et al., 2002). A BLAST search evidenced sequence identities ranging from 46 to 70% between Cor a 9 and other plant allergens such as Gly m 6 from soybean, Ara h 3 from peanut, Ana o 2 from cashew, Ber e 2 from Brazil nut, Pru du 6 from almond, and Pis v 2 from pistachio. Belonging to legumins, corylin (Cor a 9) also presents a hexameric form organized in a quaternary structure, which is in good agreement with the functional structure attributed to other 11S globulins, namely Pru du 6 from almond (Albillos et al., 2008). The IgE binding epitope(s) have not yet been identified; however, Cor a 9 presents 67% of sequence identity with the corresponding region of the linear IgE binding epitope of peanut allergen Ara h 3 (Beyer et al., 2002).

Legumins are thermostable proteins, only suffering partial unfolding of their conformational structures at temperatures of above 94°C (Mills et al., 2007b). However, even after being submitted to high temperatures, the secondary structures of these proteins remain unchanged or have minor modifications, suggesting that the characteristic beta-barrel motif is highly stable. Preliminary studies of raw hazelnuts submitted to dry heat treatment at 170°C indicated that the protein profile and the amount of Cor a 9 were not affected until after 20 min of roasting (Dooper et al., 2008). More recently, López et al (2012) verified that Cor a 9 is a highly well-structured protein, enriched by a beta-sheet core and with long unstructured loops. These loop regions are estimated not to be modeled due to their lack of stable structure, but are predicted to exhibit linear epitopes located at the external faces of protein, and thereby being exposed to solvent. However, after submitting hazelnut samples to autoclaving (121°C or 138°C, 15 or 30 min), the allergenicity of Cor a 9 seemed to be affected by this procedure since no comparable size band corresponding to this protein was visible by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. This finding suggests that the allergenicity of Cor a 9 is predominantly related to structural conformation and not to linear epitopes (López et al., 2012). In the same study, other food processing procedures were tested, namely the application of high-pressure processing (300 to 600 Mba) to hazelnut samples, which did not induce any effects on the IgE binding capacity of Cor a

9 in the test population. By means of SDS-PAGE analysis, the protein pattern of Cor a 9 subjected to high-pressure processing remained similar to the profile presented by the allergen of raw hazelnuts (López et al., 2012).

### ***Cor a 10 (Luminal Binding Protein)***

Cor a 10 is an airborne allergen present in hazel trees. The nucleotide sequence contains 2007 bp of open reading frame encoding a protein of 668 aa, with a molecular mass of 73.5 kDa and acidic properties (pI 4.8) (Table 1). The deduced primary sequence exhibits 14 potential phosphorylation sites, namely one for tyrosine kinase, five for protein kinase C, and eight for casein kinase II. A BLAST search evidences that Cor a 10 has high sequence identity with other luminal binding proteins. As an example, protein Cor a 10 showed 90% of sequence identity with BLP-4 and BLP-5 from *Nicotiana tabacum* (CAA42659.1, CAA42660.1), with a 70 kDa heat shock protein 12 from *Arabidopsis thaliana* (AAB86942.1) and with the endoplasmic reticulum HSC70-cognate binding protein from *Glycine max* (BAA12348.1) (Gruehen et al., 2003; NCBI, 2013). Cor a 10 belongs to the 70 kDa heat shock proteins (Hsp70) that comprise a family of molecular chaperones ubiquitously expressed in nature. The Hsp70 proteins can be found in nearly all living organisms, presenting similar structures and functions, namely participating in protein biogenesis, transport, and degradation mechanisms (Morano, 2007). As member of the Hsp70 family, Cor a 10 is described as stress-related protein with the capacity to bind other peptides, and thus assisting the conformational protein-folding events. Cor a 10 interaction is adenosine 5'-triphosphate (ATP) dependent and the functional protein structure is contained within the N-terminal ATPase region of 45 kDa (1–435), being followed by a 141 aa binding domain (436–577) and a C-terminal regulatory site of 75 aa (578–654) (Gruehen et al., 2003). Physicochemical properties of Cor a 10 allergen are according to those attributed to other known allergens, namely polymorphism, acidic isoelectric point, IgE-binding capacity, and cross-reactivity (Stanley and Bannon, 1999; Gruehen et al., 2003). The high sequence identity of Cor a 10 with other Hsp70 chaperones seems to help explaining the ubiquitous expression of chaperone homologues and their relation to protein synthesis rates in different tissues. Pollen, fruits, and nuts are naturally resistant to adverse environmental conditions, thus presenting high amounts of Hsp70 stress proteins. This evidence suggests that pollen-sensitized patients with specific IgE against Cor a 10 could develop allergy toward the consumption of plant foods. Molecular chaperons such as Cor a 10 have a key role in the expression and structural integrity of several proteins, which might contribute to maintain the molecular integrity of allergens in different plant tissues. In addition, Cor a 10 possesses IgE-binding activity, which transform this protein in a potential pan-allergen (Gruehen et al., 2003).

### ***Cor a 11 (7S Globulin – vicilin)***

The vicilin-like proteins belong to the cupin superfamily. Like the legumins, the vicilins also have two conserved domains that classify them as bicupins. Mature 7S globulins are trimeric proteins ranging from 150 to 190 kDa, with subunits exhibiting a molecular weight of 40–80 kDa each (Breiteneder and Radauer, 2004). Both 11S and 7S globulins show similar conformational structures, although the primary sequence of vicilins does not contain residues of cysteine (Shewry et al., 1995). The structural integrity of vicilins is guaranteed by non-covalent hydrophobic interactions, hydrogen bonds, and van der Waals interactions, while in legumins, the tri-dimensional structure is ensured by disulphide bonds. With two structurally equivalent N- and C-terminal domains, legumins and vicilins comprise the cupin beta-barrel conformation, although the vicilins are usually glycosylated proteins with one or two N-linked glycosylation sites in the C-terminal domain (Mills et al., 2002). Classified as vicilins, Cor a 11 has been identified as one of the allergenic groups of proteins in hazelnut. This allergen is encoded by the *Corylus avellana* 48 kDa glycoprotein precursor with 1347 bp and evidences a primary sequence containing 448 aa (Table 1). The mature Cor a 11 has 401 aa with two potential glycosylation sites (Asn<sup>38</sup> and Asn<sup>254</sup>) and a signal (leader) peptide of 47 aa (Lauer et al., 2004). This protein reveals high sequence identity with several other plant allergens, namely 67% with Ses i 3 (sesame, AAK15089.1), 64% with Ara h 1 (peanut, AAB00861.1), 52% with Ana o 1 (cashew nut, AAM73730.2), 47% with Jug r 2 (walnut, AAF18269.1), and 46% with 7S vicilin (pecan nut, ABV49593.1) (NCBI, 2013). In addition, two IgE-binding regions of allergens Ara h 1 and Ana o 1 were compared with the corresponding sequences of other 7S globulins. From this assessment, one IgE-binding epitope of Ara h 1 evidenced approximately 67% of similarity with Cor a 11, and the other IgE-binding epitope evidenced 45% identity with this vicilin (four out of five critical amino acids from IgE of Ara h 1 were identical in Cor a 11). The high degree of similarity between Cor a 11 and Ara h 1 partial sequences seems to suggest that they are potential epitopes of Cor a 11 allergen (Lauer et al., 2004).

In general, almost all globulin storage proteins share high predisposition to form large thermally induced aggregates, with propensity to form stable gels and act as emulsifiers, thus their widespread application in food industry (Mills et al., 2007b). The vicilins are considered thermostable proteins with major thermal transition of around 70–75°C, whereas the legumins unfold at higher temperatures (>94°C) as determined by differential scanning calorimetry, the precise values ranging between plant species, protein concentration, and ionic force (Mills et al., 2002, 2007b). When submitted to high temperatures, proteins can suffer conformational disruptions and covalent modifications with special emphasis on those involved in glycation processes or Maillard rearrangements (Mills et al., 2002). Recently, Iwan et al. (2011) evaluated the

immunoreactivity and degranulation capacity of Cor a 11 using different temperatures for glycation (37°C, 60°C, and 145°C), which resulted in three types of Maillard reaction products. Glycation at 37°C during seven days did not allow the formation of colored products, suggesting that the susceptibility of Cor a 11 for pepsin hydrolysis or its capacity to bind human IgE was not affected. Maillard products from glycation at 60°C for three days and at 145°C for 20 min resulted in decreased hydrolysis by pepsin, indicating that the protein suffers tri-dimensional alterations in the presence of glucose. The physicochemical properties of Cor a 11 were affected after heat treatment at 60°C and at 145°C in the presence of glucose. Under these conditions, reduced or even no reactivity could be observed between Cor a 11 and IgG/IgE (Iwan et al., 2011). Hazelnut processing using wet heat treatment (121°C or 138°C, for 15 or 30 min) also enables to confirm the existence of structure arrangement for the NAG group attached to Asn<sup>301</sup> residue as the initial site for the glycosylation of protein in presence of sugar (López et al., 2012). Glycation of Cor a 11 suggested a decrease of allergenicity (López et al., 2012), which is in good agreement with the previous study (Iwan et al., 2011). As for other allergens (Cor a 1, Cor a 8, and Cor a 9), high-pressure processing (300–600 Mba) of hazelnuts did not affect the IgE-binding capacity of Cor a 11 allergens. After high-pressure processing, the Cor a 11 profile was identical to the protein extracted from raw hazelnuts (López et al., 2012).

### ***Cor a 12 and Cor a 13 (Oleosins)***

Oleosins are an intriguing new set of proteins that have been classified as novel allergens in peanut and sesame seeds (Pons et al., 2002; Leduc et al., 2006). The biological functions of these proteins are mainly centered in stabilizing lipid bodies (oil bodies) by means of preventing their coalescence during the desiccation of seeds. They possibly interact with the lipidic fraction (lipids and phospholipids) of oil bodies. Oleosins comprises three distinct domains: a highly conserved hydrophobic central domain of approximately 70 aa (predominantly rich in aliphatic amino acids) flanked by N- and C-terminal domains presenting more hydrophilic affinity and less conserved sequences (Hauser et al., 2008). The C-terminal flanking sequence has an amphipathic alpha helix that is conserved in several oleosins (Tzen et al., 1992). The central core of these proteins is constituted by one of the longest hydrophobic domains with natural occurrence (Napier et al., 1996). Oil bodies contain triacylglycerols that represent the source of energy for seed germination and growth, thus large amounts of oleosins are required (Akkerdaas et al., 2006).

The allergenic properties of oleosins are not well defined; however, their privileged location in oil bodies prevents their detection and identification in nuts and seeds, since mostly diagnostic tools use defatted material. In hazelnut, two

representative allergens, Cor a 12 and Cor a 13, have been classified as oleosins. These proteins comprising primary structures of 159 aa and 140 aa, and molecular sizes of 16.7 kDa and 14.7 kDa, and presenting basic properties (pI of 10.5 and 10.0) result from the expression of two nucleotide sequences containing 480 bp and 423 bp, respectively, for Cor a 12 and Cor a 13 (Table 1) (Akkerdaas et al., 2006; NCBI, 2013). The sequence identity between those oleosins is only 36%; however, both present high homology with other oleosins from different plant sources (Akkerdaas et al., 2006). Cor a 12 presented the highest degree of similarity with oleosins classified as allergens, such is the case of sesame (50% identity) and peanut (48% identity). Cor a 13 also evidenced a high degree of homology with oleosins from almond (73% identity) and maize (55% identity); however until now, none of these has been related to food allergy. The assembly of oleosins with lipid fraction may be determined to prevent the protection of this allergen toward rapid proteolysis in the gastrointestinal tract (Akkerdaas et al., 2006). To verify these data, further studies about this topic should be in pursuit for better evaluation of the effect of food processing, namely thermal treatment, on the allergenicity of these oleosins.

### ***Cor a 14 (2S Albumins)***

Belonging to the prolamin superfamily, hazelnut allergen Cor a 14 is included in 2S albumins, which along with the globulins comprise the major group of seed storage proteins of dicotyledonous plants (Shewry et al., 1995). The 2S albumins are water-soluble proteins at low salt concentrations, presenting a high content of arginine, glutamine, asparagines, and cysteine residues. They are small globular proteins that are subjected to sequence modifications after their synthesis. Most of these proteins are cleaved into a large and small subunits (heterodimers) held together by conserved inter-chain disulphide bonds. Similar to LTP, the 2S albumins also contain eight cysteine residues that ensure four disulphide bonds. The amino acid composition of 2S albumins, their high abundance in seed cells, and their mobilization during germination suggest that these proteins act as important nitrogen and sulfur donors (Breteiner and Ebner, 2000; Hauser et al., 2008). Additional functions, such as antifungal properties, have also been attributed to some 2S albumins.

The nucleotide sequence containing 633 bp expresses a protein defined as Cor a 14 with a primary structure of 147 aa and a molecular size of 17.1 kDa (ALLERGEN, 2013; NCBI, 2013). Recombinant 2S albumin from hazelnut has already been cloned from a nucleotide sequence of 441 bp, which allowed encoding a similar 147 aa polypeptide (Garino et al., 2010). This primary sequence comprises a signal peptide of 22 aa, a linker peptide of 20 aa, and a mature protein comprising 105 aa. The native protein is predicted to have a molecular size of 12.6 kDa after post-translational clipping of a

N-terminal and internal peptide as described for other 2S albumins. Recombinant and native 2S albumins exhibited similar IgE activity, suggesting that the availability of these recombinant proteins might help in establishing the importance of Cor a 14 regarding hazelnut allergy (Garino et al., 2010). A Blast search evidenced the high sequence identity of Cor a 14 with other allergenic 2S albumins from different tree nuts, namely 63% of similarity with Jug r 1 from walnut (AAB41308.1) and 62% with Car i 1 from pecan nut (AAO32314.1) (NCBI, 2013). These evidences implicate possible cross-reactivity between Cor a 14 and other allergenic 2S albumins from different plant species. Regarding the allergenicity of 2S albumins, not only conformational epitopes but also shared linear epitopes are apparently related to cross-reactivity phenomena among these proteins (Moreno and Clemente, 2008).

The secondary organization of 2S albumins, their compact and rigid structure dominated by a well-conserved skeleton of cysteine residues are probably responsible for their high stability to the harsh conditions of the gastrointestinal tract (Moreno and Clemente, 2008), thus preserving their allergenic activity. When compared with the nsLTP, the 2S albumins present higher resistance to thermal processing, maintaining their original folding at temperatures of up to 90°C (Mills et al., 2007b).

#### *Cor a TLP (Thaumatococcus-like Protein)*

The thaumatins are included in the PR-5 group of pathogenesis-related proteins of the defense system, mainly involved in antifungal activity (Ebner et al., 2001). These proteins evidence a structure containing 16 cysteine residues linked to form eight disulphide bridges, probably contributing to their high resistance to proteases and pH- or heat-induced denaturation (Breiteneder, 2004). Normally, these proteins are divided in three groups according to their biological role that could include responses to pathogen infection, fungal infection, or osmotic stress. Cor a TLP was very recently identified as an allergen present in hazelnut (Palacín et al., 2012), although it has not yet been included in the WHO-IUIS list of allergens (ALLERGEN, 2013). Palacín et al. (2012) purified 16 different TLP, in which is comprised the hazelnut TLP with the NCBI accession number P83336. However, this accession number identifies a protein with 212 aa as TLP 1b expressed from a nucleotide sequence with 696 bp of *Malus domestica* organism. With the available data from this study, it is not possible to state other relevant information about the biochemical properties of hazelnut TLP (Palacín et al., 2012), rather than the recognition of these proteins by less than 10% of the tested population that included several patients from seven regions of Spain. Lack of more reliable information suggests that further research work is still needed to correctly identify these proteins. The designation of Cor a TLP can be found in Allergome database, which also categorizes their presence in seed tissues, being by ingestion the natural route of exposure to this food allergen.

#### **CLINICAL RELEVANCE OF HAZELNUT ALLERGY**

Food allergens are defined as natural food components (proteins/glycoproteins) that are recognized by the immune system and can elicit immunologic reactions in sensitized individuals, resulting in characteristic symptoms (Boyce et al., 2010). A number of specific clinical syndromes may occur as a result of food allergy, which are classified on the basis of inter-related immunologic causes and the organ(s) or system(s) affected (Sicherer and Sampson, 2006; Boyce et al., 2010). These disorders can vary in intensity, targeting one or more organs/systems simultaneously. Clinical manifestations of food allergy can include cutaneous reactions (dermatitis, urticarial, angioedema), gastrointestinal disorders (oral allergy syndrome (OAS), eosinophilic gastroenteritis and esophagitis, immediate gastrointestinal hypersensitivity), respiratory syndromes, and anaphylaxis (Boyce et al., 2010).

Common clinical symptoms related to hazelnut allergy are often described as mild to potentially life-threatening (anaphylaxis), according to the severity of the elicited response. Allergy to hazelnut is especially frequent in individuals presenting respiratory disorders associated with allergy to pollens from birch, hazel, or alder (Ortolani et al., 2000). This fact is linked to high homology among the allergenic PR-10 proteins, which are known to be responsible for a wide frequency of cross-sensitization to multiple PR-10 proteins from different fruits, seeds, pollens, and nuts. In northern Europe, most cases of fruit or nut allergy seem to be connected with birch pollinosis, while in southern Europe, non-pollen-related allergens play an important role in hazelnut allergy, suggesting the existence of different patterns of sensitization (Hirschwehr et al., 1992; Akkerdaas et al., 2000; Schocker et al., 2000). Belonging to the PR-10 proteins, Cor a 1 are classified as both inhalant and food allergens, being also regarded as major allergens, since more than 50% of the allergic patients are skin test reactant to this allergen (Chapman, 2008). In majority of the cases, clinical manifestations associated with this class of proteins are typically mild and frequently related to OAS exclusively. These facts were demonstrated in a recent study conducted in a birch-endemic region, where 97% of the tested population with OAS was sensitized to Cor a 1.04 and Cor a 1.0101, probably as a result of cross-reactivity with Bet v 1. However, the same study also reported that approximately 24% of preschool children and 50% of school-age children and adults with severe systemic reactions were sensitized to Cor a 1.04 or Cor a 1.0101, thus evidencing the importance of this group of allergens (De Knop et al., 2011).

Classified as pan-allergens due to their widespread distribution throughout nature, Cor a 2 (profilin) and Cor a 8 (nsLTP) are regarded as important allergens in hazelnut, although with very different clinical profiles. Profilins are generally considered as minor (less than 20% of positive skin test responses) (Chapman, 2008) but rather highly relevant allergens such as the case of Cor a 2. The most common route of sensitization to this allergen is by inhalation and not

ingestion since these proteins are greatly affected by heat processing and gastric digestion (García and Lizaro, 2011). The clinical symptoms related to profilins are considered mild and mainly restricted to the oral cavity (OAS) because of the ingestion of raw foods. Since profilins can be virtually found in almost all tissues, namely pollen and nuts/seeds, the risk of multiple sensitization to different pollens and fruits is probabilistically elevated (Hauser et al., 2010). The clinical relevance of profilin sensitization is still a matter of discussion, as clinical studies seem to suggest that patients displaying profilin-specific IgE antibodies are often asymptomatic or at risk of evolving multiple pollen-related food allergies (Costa et al., 2012a). Moreover, other studies advocate that profilins can be considered as food allergens with clinical relevance in specific food allergic patients (Asero et al., 2003, 2008), evidencing that further research is obviously needed. While profilins are considered as minor allergens, the classification of major allergens can be attributed to some nsLTP. In general, the nsLTP are major cross-reactive allergens present in majority of plant-derived foods as well as in pollens of diverse plants; nevertheless, the route of sensitization to these proteins is presumably dependent on geographical differences. The clinical symptoms associated with nsLTP are normally classified as severe immunological responses (Hansen et al., 2009; Hauser et al., 2010).

As other allergenic nsLTP, Cor a 8 have also been reported to induce severe anaphylactic reactions in seven out of 65 patients with allergy to hazelnut (Pastorello et al., 2002). In another study performed in a birch-endemic area, regarding hazelnut allergic patients with systemic reactions, sensitization to Cor a 8 allergens was observed in 12% of preschool children, 17% of school-age children, and 6.7% of individuals over 18 years old. In patients with mild responses (OAS) or in birch pollen allergic individuals without hazelnut allergy, no sensitization to this allergen could be perceived (De Knop et al., 2011). Recently, it was evidenced that Pru p 3 allergens from peach, which share 59% of sequence identity with Cor a 8, are estimated to function as a primary sensitizer to nsLTP from a large amplitude of unrelated plant-derived foods, including Cor a 8 (Hartz et al., 2010; Asero, 2011; Schulten et al., 2011b). In addition, the level of IgE to peach LTP is regarded as a key issue associated with cross-reactivity and subsequent clinical allergy to foods from different botanical origins (Asero, 2011). Although the classification of major allergens has not yet been attributed to Cor a 8 as for other allergenic nsLTP, the severity of the reactions triggered by these type of proteins cannot be underestimated. The pattern of IgE sensitization to hazelnut is probably affected by geographical differences, once in the northern Europe the rate of sensitization to hazelnut is more frequently attributed to Cor a 1 allergens (specifically Cor a 1.04), whereas in the Mediterranean, the IgE toward Cor a 8 prevailed (Hansen et al., 2009).

Similar to nsLTP and as an important member of prolamin superfamily, the 2S albumin Cor a 14 was suggested to be connected with moderate to severe hazelnut allergy (Pastorello

et al., 2002; Garino et al., 2010). Although the WHO-IUIS identifies Cor a 14 with an IgE-binding prevalence of about 33% (ALLERGEN, 2013), suggesting its classification as a minor allergen, relevant information about clinical manifestations and pattern of sensitization to this allergen remain scarce (Ebo et al., 2012).

Proteins from the cupin superfamily, with biological functions mainly related to nutrient storage, are classified as major components in nuts/seeds. Because of their abundance in these tissues, the proteins with allergenic properties are frequently considered as major allergens in foods. Cor a 9 and Cor a 11, i.e. 11S legumin- and 7S vicilin-like proteins respectively, are associated with severe hazelnut allergy. The exact route of sensitization to Cor a 9 is still not very well defined but the clinical symptoms regarding this allergen are rather important, since systemic and potentially life-threatening allergic reactions are normally attributed to it (Beyer et al., 2002; Ebo et al., 2012). This fact was demonstrated by Beyer et al. (2002), who reported systemic reactions in 86% of the test population (predominantly children), and by Hansen et al. (2009), who found sensitization to Cor a 9 in four of the seven patients with clear severe allergy to hazelnut. De Knop et al. (2011) also reported that in a birch-endemic region, the majority of hazelnut allergic children (65% of preschool children and 50% of school-age children) revealed systemic reactions upon the consumption of processed hazelnut, mostly being sensitized by Cor a 9 with no relation to birch pollen allergy. Sensitization to Cor a 9 seems to demonstrate a distinct clinical pattern and age-related distribution (Ebo et al., 2012), evidencing that it can occur in very young children prior to pollen sensitization or allergy, independent of cross-reactivity with other homologues in legumes (soy and peanut) (Verweij et al., 2011). In the same context, a recent study performed among hazelnut allergic Dutch adults and children with objective symptoms evidenced highly specific sensitization to Cor a 9 and Cor a 14, whereas sensitization to Cor a 8 was rare. Although still needing more supporting evidences, this study suggests Cor a 9 and Cor a 14 as possible markers for the evaluation of a more severe hazelnut allergic phenotype (Masthoff et al., 2013).

Regarding Cor a 11 protein, the sensitization to this allergen has been reported in hazelnut allergic patients presenting mild immunological responses, mainly related to OAS (Lauer et al., 2004) or those experiencing severe systemic reactions (Ebo et al., 2012). Similar to Cor a 9, sensitization to Cor a 11 was more prominent in children with systemic responses than in adults with the same clinical symptoms (Ebo et al., 2012). The route of sensitization to this allergen, as for the Cor a 9, is also not defined, although it seems to follow the same pattern as that of the latter. The classification of major allergens has also been advanced for Cor a 11 (Rigby et al., 2008; Cucu et al., 2012a), nonetheless this designation seems to be overestimated since in most of the reports the percentage of positive reactions to Cor a 11 is lower than 50% (Lauer et al.,

2004; Ebo et al., 2012; Verweij et al., 2011). Hence, the designation of minor allergen should be more adequate to classify Cor a 11.

The clinical relevance and the pattern of sensitization of oleosins Cor a 12 and Cor a 13 are not yet defined. As described for other oleosins, namely from peanut (Ara h 10 and Ara h 11) and sesame (Ses i 4 and Ses i 5) (Pons et al., 2002; Leduc et al., 2006), the oleosins from hazelnut also reveal IgE-binding activity (Akkerdaas et al., 2006). Recent data about the allergenicity of Cor a 12 and Cor a 13 estimated an IgE prevalence of 63% corresponding to about 118 individuals with positive immunological responses in a total of 185 patients with hazelnut and/or peanut allergies (ALLERGEN, 2013). Although the IgE-binding capacity seems to be rather elevated, the purified fraction of oleosins from hazelnut also contained an unidentified 27 kDa protein and 11S globulin (ALLERGEN, 2013), which might affect the estimate of IgE activity of these oleosins. Taking into consideration the high percentage of positive reactants to Cor a 12 and Cor a 13, it is possible to suggest that these proteins could be classified as major allergens.

Presently, relevant information correlating different groups of allergenic proteins in hazelnut and their respective symptoms/clinical relevance, as well as sensitization patterns, is slowly becoming available. Still, further research based on multidisciplinary teams (clinicians, immunologists, and researchers) must be in pursuit to enable better tools for the management of food allergies, namely hazelnut allergy.

### **STRATEGIES FOR DETECTING HAZELNUT ALLERGENS IN FOODS**

The need for adequate methodology to evaluate the presence of allergenic ingredients and hidden allergens in foods has been, for a long time, a source of extensive discussion and contradictory opinions among researchers. Lack of harmonization regarding the most suitable methodology to verify labeling compliance and the absence of available testing/reference materials contribute to the generalized controversy among researchers and represent key issues in the management of food allergens. While in the opinion of some, the direct monitoring of offending proteins should always be addressed, others defend that alternative methodologies via indirect assessment of allergenic foods (DNA) can also be considered as valuable tools. Presently, there are a great number of methods, either based on proteins or DNA, available for the detection of hazelnut as an ingredient or a potential hidden component in foods.

#### ***Protein-based Methods***

The most representative and widely used techniques for allergen monitoring in foods are the immunochemical assays

such as enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD), dipstick tests, immunoblotting, and biosensors. All these assays are based on the same principle, which consists of the interaction between an antibody (Ig) and an antigen (protein). Thus, protein-based techniques allow detecting the offending food directly, either targeting the allergenic protein itself or other marker(s) protein.

#### ***ELISA Systems***

ELISA is considered as the most largely applied type of immunoassay for the detection of allergenic ingredients/non-ingredients in foods with the advantage of providing quantitative information. In general, the most common format of ELISA is the sandwich type, but these immunoassays can also present other forms that are included in two groups: competitive assays and non-competitive assays. For the detection and quantification of hazelnut in foods, several ELISA kits are commercially available, and listed in Table 2. The application of ELISA kits to food analysis presents the advantages of rapid performance and versatility, high reproducibility, and reliable detection of trace amounts of hazelnut proteins in foods down to 0.3 mg/kg (Table 2). These limits of detection (LOD) are considered low and very adequate to trace hazelnut in foods, once it is known that minute amounts of this nut could induce allergic reactions in sensitized individuals.

Although with very well-established advantages, it is important to mention that the performance of ELISA kits can be affected by the composition of foods (e.g. matrix effects) and by the effect of food processing (e.g. heat treatments, formation of Maillard products, fermentation, and partial hydrolysis) (Pele et al., 2007; Roder et al., 2009; Garber and Perry, 2010; Cucu et al., 2011; Platteau et al., 2011a, 2012; Cucu et al., 2013). To address the referred issues, some studies have been conducted aiming at evaluating the performance of commercial kits from different brands to detect hazelnut traces in processed foods (Garber and Perry, 2010; Cucu et al., 2011). For the same set of samples spiked with hazelnut (chocolate, baked muffins, and cooked oatmeal), three different brands of commercial ELISA kits were tested by Garber and Perry (2010), reporting that each kit performed very distinctly. When applied to processed foods, such as baked muffins, the three kits revealed poor recoveries and dynamic ranges, which seem to indicate that heat treatment led to possible alterations on the structural conformation of target proteins. The modification of the natural folding of native protein structures is quite frequent with processing at elevated temperatures. The same results regarding the negative effect of heat treatment toward the detection of hazelnut proteins by different commercial ELISA kits were also reported by Cucu et al. (2011).

Proteins are also susceptible of suffering severe chemical modifications as a consequence of Maillard reactions, thus resulting in a distinct tri-dimensional conformation of native protein. Most of the available ELISA kits are probably

**Table 2** Commercial ELISA, LFD, and real-time PCR kits for the detection and quantification of hazelnut allergens

| Commercial kits/brand                                                | Assay type                                                                                         | Cross-reactivity                                                                    | LOD                       | LOQ       | Estimated time to perform assay                   |
|----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|---------------------------|-----------|---------------------------------------------------|
| Lateral flow hazelnut (R-Biopharm AG Darmstadt, Germany)             | LFD                                                                                                | Walnut: 0.1%, pumpkin seed: 0.01%                                                   | 1.5 mg/kg                 | 2.5 mg/kg | ~10 min (sample preparation)                      |
| Reveal 3-D for hazelnut (NEOGEN, MI, USA)                            | LFD                                                                                                | No available information about specificity                                          | 5–10 mg/kg                | —         | ~5 min                                            |
| AgraStrip Hazelnut (Romer Labs Division Holding GmbH, Austria)       | LFD                                                                                                | No available information about specificity                                          | —                         | —         | —                                                 |
| Ridascreen Fast Hazelnut (R-Biopharm AG Darmstadt, Germany)          | Specific antibody against hazelnut proteins, sandwich ELISA                                        | No apparent cross-reactivity with 31 plant-derived foods and 2 animal-derived foods | 1.5 mg/kg                 | 2.5 mg/kg | ~40 min (10-min sample preparation, 30-min ELISA) |
| AgraQuant Hazelnut Assay (Romer Labs Division Holding GmbH, Austria) | Quantitative–sandwich ELISA                                                                        | No cross-reactivity with 30 plant-derived foods                                     | 0.3 mg/kg                 | 1 mg/kg   | ~60 min                                           |
| DIA hazelnut (Diagnostic Automation Inc., CA, USA)                   | Quantitative–sandwich ELISA                                                                        | No cross-reactivity with 31 plant-derived foods                                     | 0.33 mg/kg                | 1 mg/kg   | ~50 min (applied to extracted sample)             |
| ELISA Systems Hazelnut (Queensland, Australia)                       | ELISA                                                                                              | ELISA Systems                                                                       | —                         | —         | ~35 min (applied to extracted sample)             |
| Veratox for hazelnut allergen (NEOGEN, MI, USA)                      | Quantitative–Sandwich ELISA                                                                        | No available information about specificity                                          | 2.5 mg/kg                 | 2.5 mg/kg | ~30 min                                           |
| SureFood Allergen Hazelnut (R-Biopharm AG Darmstadt, Germany)        | Real-time PCR (qualitative)                                                                        | No available information about specificity                                          | ≤5 DNA copies, ≤0.4 mg/kg | —         | ~30 min (applied to extracted sample)             |
| SureFood Allergen Quant Hazelnut (R-Biopharm AG Darmstadt, Germany)  | Real-time PCR using the laboratory reference material Surefood Quantard Allergen 40 (quantitative) | No available information about specificity                                          | 0.4 mg/kg                 | 0.4 mg/kg | ~40 min (applied to extracted sample)             |

developed upon antibodies that were raised against raw hazelnuts. This fact can conduct to a reduced capacity of antibodies to recognize proteins modified by Maillard reactions and, consequently, provide erratic results when analyzing processed foods (Cucu et al., 2011). While the native structure of proteins is affected by heat treatment that destroys conformational epitopes, linear epitopes are mainly altered by the interactions between lipids or carbohydrates and proteins through partial hydrolysis (Cucu et al., 2013). This procedure can be used in food processing, contributing to a general decrease in allergenicity. Different ELISA kits were tested with extracts of hazelnut proteins submitted to partial hydrolysis. In general, the results evidenced a reduction in the recognition of hydrolyzed proteins by antibodies, although one of the tested kits exhibited good performance toward the target proteins (Cucu et al., 2013). All these facts seem to emphasize that the choice of an ELISA kit is highly dependent on the purpose of analysis, therefore reliant on the type of food matrices and on the kind of processing the samples were submitted to (Cucu et al., 2013).

In addition to commercial kits, in the last decade, other ELISA tests have been proposed regarding the detection and quantification of hazelnut in foods (Table 3). Most of the developed ELISA employed polyclonal antibodies (IgG from rabbit and sheep or IgY from hen's eggs) that were generally raised against raw and/or roasted hazelnut protein extracts or purified allergen fractions (e.g. corylin). Table 3 assembles all the proposed ELISA systems reported in the literature. Those assays presented elevated performance with LOD ranging from 0.1–3 mg/kg of hazelnut protein in foods, being applied to a wide variety of food samples, namely cookies, biscuits, chocolates, breakfast cereals, ice cream, cereal bars, and olive oils. The specificity of ELISA using polyclonal antibodies is usually good, but in some cases some cross-reactivity can be observed with other nuts or legumes. The ELISA protocols employing anti-hazelnut monoclonal antibodies are commonly less prone to cross-reaction with other species. However, since monoclonal antibodies have poorer affinities than the corresponding polyclonal antibodies, the assays using monoclonal antibodies are frequently less sensitive (Table 3). In sandwich ELISA format, mono- and polyclonal antibodies could be combined, aiming at developing a more sensitive and specific assay.

### ***Lateral Flow Devices and Immunoblotting***

Lateral flow devices or dipstick assays are another type of immunochemical tests applied to the detection of hazelnut in processed products. In food industry, these assays are commonly used for rapid screening of possible cross-contaminations in production lines. This type of test provides qualitative or semi-quantitative information that can be easily interpreted visually. In addition, LFD present the advantage of being rapid and simple to perform, without the need of using specialized

equipment or personnel (Schubert-Ullrich et al., 2009). Similar to ELISA, there are two types of LFD: the sandwich format and the competitive format. The application of commercial LFD enables detecting down to 1.5 mg/kg of hazelnut proteins in foods in approximately 5–10 min (Table 2), which represents a major benefit for food industry. Using LFD from three distinct brands, Roder et al. (2009) reported the detection of hazelnut with two of the tested LFD in spiked chocolate and cookie dough down to 3.5 mg/kg and 2.6 mg/kg, respectively. As for ELISA kits, the choice of adequate LFD should be performed carefully, taking in consideration the finality of the test. Concerning all the advantages, LFD also pose some drawbacks, such as the lack of quantitative information, and the susceptibility of providing false-negative results (Diaz-Amigo, 2010).

Immunoblotting assays are also used to detect allergens in foods, although these are not considered suitable methods for routine analysis. The application of this technique enables the evaluation of antibody specificity and the occurrence of cross-reactivity between non-target proteins and the applied antibody. Scheibe et al. (2001) have reported a sensitive protocol to trace hazelnut in chocolate using SDS immunoblot with chemiluminescence detection method with a LOD of 5 mg/kg of hazelnut protein in chocolates. Due to the lack of reliable quantification data, immunoblotting is rather used as a confirmatory analytical tool during the development of other immunochemical assays.

### ***Biosensors***

The application of biosensors as alternative platforms for the detection of allergens in foods has become one of the most emerging and attractive fields, whose advances and future trends have been recently highlighted by Pilolli et al. (2013). When compared with other protein-based methods, such as ELISA, biosensors are regarded as one of the most promising ways to solve some issues concerning simple, fast, reproducible, and low-cost multitarget detection. In addition to these advantages, biosensors are featured to be of high speed in execution, ease to use, and feasible for automation. Considering all the potentials attributed to these analytical devices, it is expected that biosensors could be applied at industrial scale for direct and real-time monitoring of allergens along a production line (Pilolli et al., 2013). Biosensors base their principle on the direct recognition of a biological interaction between an antibody and a target protein by a transducer that produces a measurable signal. This interaction can be monitored by different types of transducers (optical, acoustical, amperometrical, or potentiometrical), generating a signal that is further processed to give a proportional output to the concentration of specific target. According to the type of transducer, biosensors can be classified as optical, piezoelectric, or electrochemical. The detection of allergenic material in foods (e.g. hazelnut) using biosensor technology has been restricted

**Table 3** Summary of the reported protein-based methods for the detection and quantification of hazelnut allergens in foods

| Protein-based methods                   | Antibody/target protein (immunization)                                                                                                                     | Cross-reactivity                                                                                                                                                                                                      | Sensitivity levels                                                             | Application to food matrices                                                    | References                   |
|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|---------------------------------------------------------------------------------|------------------------------|
| Sandwich ELISA (Quantitative)           | Polyclonal rabbit and sheep antibodies (raised against raw and toasted hazelnut, mainly corylin fraction)                                                  | 3/39 animal- and plant-derived foods. Cross-reactivity with cashew (2.1 mg/kg), pumpkin seeds (6.4 mg/kg), and walnut (4.6 mg/kg)                                                                                     | 10 mg/kg of hazelnut (1 mg/kg of hazelnut protein)                             | Chocolates, breakfast cereals, bar cereals, and cookies (spiked and commercial) | Holzhauser and Vieths (1999) |
| Sandwich ELISA                          | Rabbit anti-hazelnut IgG (raised against crude hazelnut protein)                                                                                           | 6/27 plant-derived foods. Cross-reactivity for cashew (34 mg/kg), walnut (787 mg/kg), Brazil nut and almond (28 mg/kg), pine (10 mg/kg), and peanut (11 mg/kg)                                                        | 10 mg/kg of hazelnut (1 mg/kg of hazelnut protein)                             | Cakes, chocolates, cookies (commercial)                                         | Koppelman et al. (1999)      |
| Sandwich enzyme immunoassay (EIA)       | Polyclonal hen's egg yolk IgY (raised against hazelnut globulin fraction)                                                                                  | 0/4 almond, pecan, walnut, and sesame. No cross-reactivity observed                                                                                                                                                   | <1 mg/kg of hazelnut protein                                                   | Cakes, ice cream, chocolate, fruit bars (spiked and commercial)                 | Blais and Phillippe (2001)   |
| Sandwich ELISA and dipstick immunoassay | Polyclonal rabbit antibody (raised against raw hazelnut – corylin fraction), polyclonal sheep antibody (raised against native and heated hazelnut corylin) | 5/23 plant-derived foods. Weak cross-reactivity with kidney beans, pine, coconut and mix cereals (wheat, rye, maize, rice, oat, and barley). Strong cross-reactivity with walnut                                      | 10 mg/kg of hazelnut (1 mg/kg of hazelnut protein) for both ELISA and dipstick | Chocolates creams, chocolate bars, cereal bars, cookies (commercial)            | Stephan et al. (2002)        |
| Competitive ELISA                       | Polyclonal rabbit IgG (raised against defatted roasted hazelnut protein extracts)                                                                          | 0/39 plant- and animal-derived foods. No cross-reactivity observed at 1 mg/mL protein                                                                                                                                 | 0.25 mg/kg of hazelnut protein                                                 | Chocolates (spiked and commercial)                                              | Rejeb et al. (2003)          |
| Multiplex EIA system                    | Polyclonal hen's egg IgY (raised against defatted raw hazelnut – mainly globulin fraction)                                                                 | 0/2 peanut and Brazil nut. No cross-reactivity with peanut or Brazil nut at 1 mg/kg of protein                                                                                                                        | 0.1 to 1 mg/kg of hazelnut protein                                             | Chocolate, ice cream, and cookies (spiked)                                      | Blais et al., (2003)         |
| Indirect competitive ELISA              | Polyclonal hen's egg IgY (raised against defatted raw hazelnut)                                                                                            | 13/29 plant- and animal-derived foods. Cross-reactivity with chestnut, Brazil nut, chickpea, green pea, bean, sunflower, sesame, poppy seed, wheat, corn, oats, barley, yeast ranging from 0.30 to 8.09%              | 10 µg/L of hazelnut protein (estimated as the baseline of the method)          | Cookies (spiked)                                                                | Drs et al. (2004)            |
| Sandwich ELISA                          | Polyclonal rabbit IgG (raised against hazelnut pepsin digest and hazelnut not digested extracts)                                                           | 9/10 plant-derived foods. Cross reactivity with peanut (340 mg/kg), walnut (170 mg/kg), almond (80 mg/kg), and sesame (20 mg/kg). Cross-reactivity with wheat, Brazil nut, pine, barley, and birch pollen (<10 mg/kg) | 1 mg/kg of hazelnut                                                            | Chocolate (spiked) chocolates and cookies (commercial)                          | Akkerdaas et al. (2004)      |

(Continued on next page)

**Table 3** Summary of the reported protein-based methods for the detection and quantification of hazelnut allergens in foods (*Continued*)

| Protein-based methods                               | Antibody/target protein (immunization)                                                                                                                                | Cross-reactivity                                                                                                                  | Sensitivity levels                                                                        | Application to food matrices                                                       | References             |
|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|------------------------|
| Competitive ELISA (multitarget)                     | Polyclonal rabbit IgG (raised against defatted roasted hazelnut protein extracts)                                                                                     | 5/24 plant- and animal-derived foods. Cross-reactivity with almond, cashew, egg, and lobster. Weak cross-reactivity for chocolate | 1 mg/kg of hazelnut protein                                                               | Milk and dark chocolates (spiked)                                                  | Rejeb et al. (2005)    |
| Sandwich ELISA                                      | Monoclonal mouse IgG, polyclonal rabbit IgG (raised against roasted hazelnut extracts)                                                                                | 4/21 plant-derived foods. Cross-reactivity with macadamia (1.1 mg/kg), almond and cashew (1.2 mg/kg), and walnut (12 mg/kg)       | 0.2–1.2 mg/kg                                                                             | Dark and milk chocolates, cereals, cookies, and ice cream (spiked and commercial). | Kiening et al. (2005)  |
| Sandwich ELISA                                      | Polyclonal rabbit IgG (raised against native hazelnut – corylin fraction)                                                                                             | 1/26 plant- and animal-derived foods. Cross-reactivity with walnut (1.1 mg/kg)                                                    | 0.1 mg/kg hazelnut protein                                                                | Cereals, cookies, dark and milk chocolates (spiked and commercial)                 | Faeste et al. (2006)   |
| Sandwich ELISA                                      | Polyclonal hen's egg yolk IgY (raised against Cor a 9 allergen)                                                                                                       | 4/22 plant-derived foods. Cross-reactivity with pecan (80 mg/kg), coconut and beans (20 mg/kg), and macadamia (17 mg/kg)          | 0.1 mg/kg of Cor a 9 in food                                                              | Cookies (spiked)                                                                   | Trashin et al. (2011)  |
| Indirect competitive ELISA                          | Polyclonal hen's egg yolk IgY (raised against modified hazelnut proteins – raw and roasted hazelnut protein extract + glucose + sunflower oil)                        | 2/22 plant- and animal-derived foods. Cross-reactivity with walnut (8.2%) and pecan (5.9%)                                        | 3 mg/kg of hazelnut protein in blank cookies                                              | Baked cookies (spiked)                                                             | Cucu et al. (2012b)    |
| SPR biosensor (optical transducer)                  | Polyclonal rabbit IgG and hen's egg yolk IgY (raised against hazelnut proteins with molecular weight ranging from 15–30 kDa and predominant band at 18 kDa – corylin) | Not verified                                                                                                                      | 5 mg/kg of hazelnut                                                                       | Dark and milk chocolates, ice creams, bread, biscuits, pasta (commercial)          | Yman et al. (2006)     |
| Direct biosensor (optical transducer)               | Monoclonal mouse IgG (raised against raw and roasted hazelnut extracts)                                                                                               | 0/40 plant- and animal-derived foods. No cross-reactivity observed                                                                | 0.08 mg/kg of hazelnut proteins                                                           | Olive oils (spiked)                                                                | Bremer et al. (2009)   |
| SPR biosensor (optical transducer) (multitarget)    | Monoclonal mouse IgG (raised against raw and roasted hazelnut extracts)                                                                                               | 0/40 plant- and animal-derived foods. No cross-reactivity observed                                                                | 1.5 mg/kg and 4.6 mg/kg of hazelnut proteins in cookies and dark chocolates, respectively | Dark chocolates and cookies (commercial)                                           | Rebe Raz et al. (2010) |
| Electrochemical biosensor (amperometric transducer) | Polyclonal hen's egg yolk IgY (raised against Cor a 9 allergen)                                                                                                       | 4/22 plant-derived foods. Cross-reactivity with pecan (80 mg/kg), coconut and beans (20 mg/kg), and macadamia (17 mg/kg)          | 1 mg/kg of Cor a 9 in food                                                                | Cookies (spiked)                                                                   | Trashin et al. (2011)  |
| Immunoblotting                                      | Polyclonal rabbit antibody (raised against hazelnut and almond proteins)                                                                                              | 0/3 peanut, cocoa, and milk. No cross-reactivity observed                                                                         | 5 mg/kg of hazelnut proteins                                                              | Chocolates (spiked)                                                                | Scheibe et al. (2001)  |

|                                           |                                                                                               |                                                                                                                                                          |                                                                                                              |                                                        |                        |
|-------------------------------------------|-----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|--------------------------------------------------------|------------------------|
| MALDI-TOF/TOF-MS                          | Not applicable/peptides from hazelnut (oleosins and Cor a 9)                                  | Not verified                                                                                                                                             | 1% of hazelnut oil (peptides from oleosins and Cor a 9)                                                      | Extra virgin olive oil                                 | Arlorio et al. (2010)  |
| LC-ESI-LIT-MS/MS (multiallergen approach) | Not applicable/peptides from hazelnut (1 Cor a 9 peptide)                                     | Not verified                                                                                                                                             | 30 or 35 mg/kg of Cor a 9 according to the MS <sup>2</sup> or MS <sup>3</sup> acquisition mode, respectively | Biscuits (spiked), cereal mixes, biscuits (commercial) | Bignardi et al. (2010) |
| LC-MS/MS (multiallergen approach)         | Not applicable/peptides from hazelnut (4 Cor a 9 peptides)                                    | Not verified                                                                                                                                             | 5 mg/kg of hazelnut                                                                                          | Bread and flour (spiked)                               | Heick et al. (2011a)   |
| LC-MS/MS (multiallergen approach)         | Not applicable/peptides from hazelnut (4 Cor a 9 peptides)                                    | Not verified                                                                                                                                             | 5 mg/kg of hazelnut                                                                                          | Bread material (spiked)                                | Heick et al. (2011b)   |
| LC-MS/MS                                  | Not applicable/8 peptides from hazelnut (1 from Cor a 8, 4 from Cor a 9, and 3 from Cor a 11) | One out of four peptides from Cor a 9 occurs in walnut, pecan, pistachio, and cashew. One out of three peptides from Cor a 11 occurs in walnut and pecan | Not defined                                                                                                  | Not tested in food samples                             | Ansari et al. (2012)   |

to a small number of antibody-based applications (Table 3). Of these, the optical biosensors are the most commonly used, relying their function on the phenomenon of surface plasmon resonance (SPR) that measures changes in refractive index when the antibody binds the target protein (Diaz-Amigo, 2010; Pilolli et al., 2013). Employment of an optical biosensor was successfully achieved by Yman et al. (2006) for the detection of hazelnut in diverse food matrices, namely chocolates, ice-creams, bread, pasta, and biscuits. For the elaboration of this biosensor, Yman et al. (2006) used polyclonal rabbit IgG and hen's egg IgY raised against corylin fraction, allowing to trace minute amounts of hazelnut until the spiked level of 5 mg/kg. The application of optical biosensors based on the same principle was also successfully reported by Bremer et al. (2009) and Rebe Raz et al. (2010). Both optical biosensors evidenced high specificity for hazelnut detection since the monoclonal IgG from mouse used in those systems did not cross react with any of the 40 tested foods. The biosensor proposed by Bremer et al. (2009) presented an LOD of 0.08 mg/kg of hazelnut proteins in spiked olive oil, with recoveries ranging from 93 to 109% and an assay time of about 4.5 min. Using an optical biosensor based on SPR, but with multitarget approach (microarray), Rebe Raz et al. (2010) showed the applicability of this system for the simultaneous detection of several allergenic foods. In a specific case of hazelnut, LOD of 1.5 mg/kg and 4.6 mg/kg of hazelnut proteins in cookies and in dark chocolates, respectively, were attained by Rebe Raz et al. (2010), which were also in good agreement with the sensitivities reported for ELISA or LFD systems (Tables 2 and 3).

Trashin et al. (2011) described the development of an electrochemical biosensor as an alternative approach to ELISA, using in both methods the same type of polyclonal hen's egg yolk IgY raised against Cor a 9 fraction. The system allowed detecting down to 1 mg/kg of Cor a 9 in spiked cookies, although the antibodies evidenced some cross-reactivity in four (pecan nut, coconut, beans, and macadamia) of the 22 plant-derived foods tested. When compared with the ELISA system, the LOD attained with electrochemical biosensor was approximately 10-fold higher, however, with the strong advantage of reducing the analysis time from 4 to 1 h. Therefore, the developed biosensor proved to be adequate for the detection of minute amounts of hazelnut in food samples with possible application in food industry (Trashin et al., 2011). These findings seem to support the emergent potential of biosensors for the detection of allergenic ingredients in processed foods, especially if displayed in platforms to simultaneously, target multiple allergens.

### **Mass Spectrometry (MS)-based Methods**

The development of novel strategies for allergen detection, quantification, and characterization is in constant demand. Recently, the application of proteomic methodologies (allergenomics) for the analysis of food allergens has been addressed,

especially centered on core technologies such as MS-based platforms. This technology evidences several advantages because it allows proteins to be analyzed quickly with high sensitivity, accuracy, specificity, and reproducibility (Picariello et al., 2011). In addition, MS methods can overcome the problems of cross-reactivity phenomena, often linked to immunoassays, allowing unequivocal confirmation of the identity of tested proteins/peptides (Monaci and Visconti, 2009). Several MS-based methods can be used for relative and absolute quantification of proteins (e.g. allergens), but all these methods rely on one of the two approaches. In the first one, the analysis is performed using intact proteins (analyte and reference standards), while in the second approach, the target analytes are peptides obtained from protein digestion by proteolytic enzymes (Picariello et al., 2011). The identification of allergens or protein markers by MS technology is commonly performed in "bottom-up" mode that is conducted on the basis of the digestion of proteins by a protease, typically trypsin (Monaci and Visconti, 2009). Prior to mass analysis and data recording, proteolytic fragments are separated by reverse-phase liquid chromatography (LC) (Harrer et al., 2010). Due to the complexity of proteins, the purification process has to be specifically developed to ensure reliable recognition of molecule via the generation of a peptide mass fingerprinting. With respect to the detection of proteins/allergens in processed foods, MS methods are the effective means of providing reliable insights about any protein/peptide modification or changes in their conformation resulting from food processing (Monaci and Visconti, 2009).

Some applications using MS techniques have been reported for the detection and quantification of hazelnut in foods, being listed in Table 3. Using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOP MS), Arlorio et al. (2010) described the identification of two oleosin isoforms and Cor a 9. With this method, the authors were able to detect the addition of 1% of hazelnut oil to extra-virgin olive oils, evidencing that this adulteration might also represent a potential risk for allergic patients (Arlorio et al., 2010). Application of a system based on liquid chromatography–electrospray–linear ion trap–tandem mass spectrometry (LC-ESI-LIT-MS/MS) was successfully achieved by Bignardi et al. (2010) for the simultaneous detection of five nut allergens: Ana o 2 (cashew), Ara h 3/4 (peanut), Pru 1 (almond), Jug r 4 (walnut), and Cor a 9 (hazelnut). In the specific case of hazelnut, Bignardi et al. (2010) reported the sensitivities of 30 mg/kg or 35 mg/kg of Cor a 9 in spiked biscuits and limits of quantification (LOQ) of 90 mg/kg or 110 mg/kg of Cor a 9 in the same matrix, respectively, using MS<sup>2</sup> or MS<sup>3</sup> acquisition modes. No cross-reactivity assessments with peptide from different plant- or animal-derived foods were described (Bignardi et al., 2010). Development of a multiallergen approach for the simultaneous detection of soy, milk, egg, peanut, walnut, almond, and hazelnut using LC-MS/MS was also successfully achieved by Heick et al. (2011a, 2011b). For the identification of hazelnut, four Cor a 9 peptides were targeted in spiked

matrices of bread and flour. The method allowed tracing 5 mg/kg of Cor a 9 in both incurred bread and flour, demonstrating elevated sensitivity of the proposed method for raw and processed food samples. A similar LC-MS/MS system was also reported by Ansari et al. (2012) using eight different peptides from Cor a 8, Cor a 9, and Cor a 11 allergens for the unequivocal identification of hazelnut. Although without reporting the level of sensitivity, Ansari et al. (2012) evaluated the potential application of selected hazelnut peptides for the identification of this nut in processed foods. One of the four Cor a 9 peptides also occurs in pecan, walnut, pistachio, and cashew, and one of the three Cor a 11 peptides is present in walnut and pecan, which evidenced that similar peptides can be found in different foods. Therefore, the use of single peptides should be avoided for the unambiguous identification of hazelnut from other nuts or foods as demonstrated by Ansari et al. (2012). Mass spectrometry methodologies have been applied very recently but with high potentiality in the field of food allergen assessment. Further research using MS platforms is expected shortly.

### ***DNA-based Methods***

The detection of food allergens by DNA-based methods has been extensively applied in the last decade in spite of some criticisms. In opposition to the immunochemical assays that exhibit some relevant disadvantages, the only drawback pointed out to the use of molecular approaches for allergen detection is their indirect assessment in food matrices. In fact, if any protein can act as a marker for species' identification, then DNA encoding an allergenic or a marker protein can also play the same role. The elevated stability of DNA upon thermal treatment, pH alterations, or partial hydrolysis, which are processes frequently used in food industry, elect this molecule as a favored target for allergen evaluation. In addition, the molecular methods can be established in routine analysis and function as confirmatory tools for the identification of allergenic foods.

### ***Polymerase Chain Reaction (PCR) Systems***

Polymerase chain reaction-based techniques are among the most widely exploited approaches for the detection and quantification of hazelnut in foods (Table 4) in addition to the fact of being the DNA approaches for which few commercial kits are available (Table 2). High specificity is one main advantage attributed to these techniques. This characteristic is well evidenced in Table 4, since in the majority of the developed methods, no cross-reactivity with other plant or animal species could be observed. In most of the cases, the selected nucleotide sequences encode allergens (e.g. Cor a 1) rather than other proteins as potential targets for hazelnut identification. In addition, since no official reference or testing materials are

available for hazelnut evaluation, almost all authors choose to elaborate their own set of reference mixtures for the development of molecular approaches as stated in Table 4. Thus, depending on the aim of the research work, model mixtures for PCR-based systems have been prepared using raw, defatted, toasted, roasted, or autoclaved hazelnut in food matrices that are also as diversified as wheat material, cookies, chocolate, pasta, walnut, or peanut flour (Table 4). The relative limits of detection and quantification of hazelnut in different food matrices range from 100 mg/kg down to 1 mg/kg (Table 4), which are slightly higher than the LOD reported for protein-based methods (Table 3). In spite of this fact, the LOD are in good agreement with the intervals considered ideal for allergen evaluation (Poms et al., 2004). In terms of absolute LOD, hazelnut can be targeted in processed foods down to one DNA copy (Costa et al., 2012b), which is theoretically enough to identify the presence of this ingredient. Unlike proteins that can be affected by several variations at the expression level, the DNA complement in a tissue is usually very stable. Thus, it is possible to establish a consistent correlation between the amount of DNA detected and the amount of allergen-containing tissue (Johnson et al., 2011).

The first studies regarding the detection of hazelnut reported the development of methods based on the application of qualitative PCR systems, being the amplified PCR products evaluated on the basis of their differential migration through agarose gel electrophoresis (Holtzhauser et al., 2000; Herman et al., 2003). Other qualitative PCR systems coupled with peptide nucleic acid (PNA) HPLC (Germini et al., 2005) or with PNA-array (Rossi et al., 2006) have also been employed. Lately, due to recent advances in high-resolution instrumentation and with the arising of more specialized fluorescent DNA-binding dyes, the development of real-time PCR systems aiming at quantifying hazelnut allergens in foods has been preferably applied. Using the classical SYBR Green I or the enhancement of SYBR GreenER, several real-time PCR systems have been described with high specificity and sensitivity to trace hazelnut allergens in foods (D'Andrea et al., 2009; Pafundo et al., 2010; D'Andrea et al., 2011; Iniesto et al., 2013;). As an alternative and for the unequivocal identification of hazelnut in complex food matrices, other studies have demonstrated the use of specific hydrolysis probes to enhance the specificity of reaction (Arlorio et al., 2007; Píknová et al., 2008; Köppel et al., 2010; Platteau et al., 2011a, 2011b; Costa et al., 2012a, 2012b; Köppel et al., 2012) based on the complementarity of the third hybridization oligonucleotide during amplification.

In the past few years, special emphasis has been devoted to the development of multitarget approaches, namely duplex, tetraplex, and hexaplex real-time PCR systems for simultaneous detection and quantification of several allergenic foods, including hazelnut (Schöringhumer et al., 2009; Köppel et al., 2010; Pafundo et al., 2010; Köppel et al., 2012). In the same sense, other developed multitarget systems were proposed based on ligation-dependent probe amplification (LPA). Ehlert et al. (2009) developed

**Table 4** Summary of the reported molecular-based methods for the detection and quantification of hazelnut in foods

| Molecular method                                     | Target gene (NCBI accession number) | Fragment size (bp) | Cross-reactivity                                                                      | Reference standards or model mixtures (range)                                                                                                          | Sensitivity levels                           | Application to commercial foods                             | References                  |
|------------------------------------------------------|-------------------------------------|--------------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|-------------------------------------------------------------|-----------------------------|
| Qualitative PCR, gel electrophoresis                 | Cor a 1.04 (AF136945)               | 182                | 0/32 plant- and animal-derived foods                                                  | Food matrix spiked with hazelnut (100,000–10 mg/kg)                                                                                                    | 10 mg/kg                                     | Chocolate bars, cereal bars, cookies                        | Holzhauser et al. (2000)    |
| PCR-ELISA using specific hybridization probe         | Cor a 1.04 (AF136945)               | 152                | 0/35 plant- and animal-derived foods                                                  | Hazelnut genomic DNA serially diluted (100–1 pg)<br>Food matrix spiked with hazelnut (100,000–10 mg/kg)                                                | <10 mg/kg, 2pg                               | Chocolates, cream desserts, cereals, cookies, biscuits      | Holzhauser et al. (2002)    |
| Qualitative PCR, gel electrophoresis                 | NadI, mitochondrial (AJ428871)      | 294                | 4/33 plant- and animal-derived foods. Cross-reactivity with 4 different hazel species | Hazelnut genomic DNA serially diluted (15,000–30 pg)<br>Chocolates spiked with hazelnut paste (50,000–5 mg/kg)                                         | 10 mg/kg, 300 pg                             | No data                                                     | Herman et al. (2003)        |
| PCR coupled with HPLC using specific PNA probe       | Cor a 1.03 (Z72440)                 | 156                | 0/14 plant-derived foods                                                              | Hazelnut genomic DNA serially diluted (150,000–1 pg)                                                                                                   | 5 pg                                         | Chocolates, cereals, snacks                                 | Germini et al. (2005)       |
| PCR-array using specific PNA probe                   | Cor a 1.03 (Z72440)                 | 156                | 0/14 plant-derived foods (reported by Germini et al., 2005)                           | Hazelnut genomic DNA serially diluted (150,000–1 pg)                                                                                                   | 50 pg                                        | Chocolates, cereals, snacks                                 | Rossi et al. (2006)         |
| Real-time PCR using specific hydrolysis probe        | Cor a 1 (Z72440)                    | 82                 | 0/14 plant-derived foods                                                              | Hazelnut genomic DNA serially diluted (98,000–49 pg)                                                                                                   | 100 pg                                       | Creams, chocolates, biscuits, corn flakes                   | Arlorio et al. (2007)       |
| Real-time PCR with hydrolysis probe                  | Hsp1 (AF021807)                     | 100                | 0/19 plant- and animal-derived foods                                                  | Walnut kernel spiked with hazelnut (100,000–100 mg/kg)<br>Hazelnut genomic DNA serially diluted                                                        | 100 mg/kg, 13 pg                             | Wafers, chocolates, biscuits, corn flakes                   | Piknová et al. (2008)       |
| Real-time PCR with SYBR-Green I, melt curve          | Cor a 8 (AF329829)                  | 78                 | 0/18 plant-derived foods                                                              | Wheat flour spiked with defatted hazelnut (100,000–10 mg/kg)<br>Hazelnut genomic DNA serially diluted (30,000–9.6 pg)                                  | 10 mg/kg, 9.6 pg                             | Creams, wafers, dark chocolates, biscuits                   | D'Andrea et al. (2009)      |
| Duplex real-time PCR with hydrolysis probe           | Cor a 1 (Z72440)                    | 109                | 0/25 plant-derived foods                                                              | Cookies spiked with sesame/hazelnut (10,000–10 mg/kg)<br>sesame/hazelnut DNA serially diluted (100,000–10 pg/ $\mu$ L)                                 | 50 mg/kg, 50 pg                              | Cereals, cookies<br>chocolates, snacks, muesli bars, creams | Schöringhumer et al. (2009) |
| Ligation-dependent probe amplification (multitarget) | Cor a 1.04 (AF136945)               | 104                | 0/48 plant- and animal-derived foods                                                  | Chocolate spiked with hazelnut (20–5 mg/kg)<br>Walnut cookies spiked with 1% of 5 nut mix (pecan, hazelnut, cashew, macadamia, walnut (10,000–1 mg/kg) | 5 mg/kg in chocolate<br>100 mg/kg in cookies | Chocolates, yogurts, cookies, spreads                       | Ehlert et al. (2009)        |

|                                                                          |                                                                                                                   |                          |                                                                         |                                                                                                                    |                                                                 |                                                |                         |
|--------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|--------------------------|-------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|------------------------------------------------|-------------------------|
| Tetraplex real-time PCR with hydrolysis probe                            | Cor a 1 (Z72440)                                                                                                  | 85                       | 1/44 plant- and animal-derived foods. Cross-reactivity with peach (10%) | Rice cookies spiked with hazelnut (50 and 10 mg/kg)                                                                | 50 mg/kg                                                        | Cookies, cakes, cereal bars, biscuits, snacks  | Köppel et al. (2010)    |
| Multiplex real-time PCR with SYBR-GreenER, melt curve                    | No data                                                                                                           | 54                       | No data                                                                 | Mix containing six species with 1%. Mix serially diluted (50-0.5 pg of each species)                               | 5 pg                                                            | Biscuits, crackers, chocolates                 | Pafundo et al. (2010)   |
| Real-time PCR with hydrolysis probe                                      | Cor a 8 (AF329829)                                                                                                | 218                      | No data                                                                 | Defatted hazelnut in flour/cookies dough/baked cookies (10,000–1 mg/kg)                                            | 100 mg/kg (flour and dough), 1000 mg/kg (baked cookies), 3.2 pg | No data                                        | Platteau et al. (2011a) |
| Real-time PCR with hydrolysis probe                                      | Cor a 1 (Z72440)<br>Cor a 8 (AF329829)                                                                            | 101<br>218               | 0/29 plant- and animal-derived foods                                    | Hazelnut genomic DNA serially diluted (50,000-0.256 pg)                                                            | No data                                                         | No data                                        | Platteau et al. (2011b) |
| Real-time PCR with SYBR Green I, melt curve                              | Cor a 1 (AF136945)<br>Cor a 8 (AF329829)<br>Cor a 14 (FJ358504)                                                   | 105<br>78<br>116         | 0/18 plant-derived foods                                                | Wheat flour spiked with defatted hazelnut (100,000–1 mg/kg) Hazelnut genomic DNA serially diluted (30,000-1.92 pg) | 10 mg/kg, 9.6 pg                                                | Creams, dark chocolates, wafers, biscuits      | D'Andrea et al. (2011)  |
| Multiplex ligation-dependent probe amplification (multitarget)           | Cor a 1 (AF136945) and (Z72440)                                                                                   | 117                      | 0/27 plant foods                                                        | No data                                                                                                            | 48 pg (105 DNA copies)                                          | No data                                        | Mustorp et al. (2011)   |
| Hexaplex real-time PCR with hydrolysis probe                             | Cor a 1 (Z72440)                                                                                                  | 85                       | 1/44 plant- and animal-derived foods. Cross-reactivity with peach (10%) | Sausages spiked with hazelnut (3160–32 mg/kg)                                                                      | 32 mg/kg                                                        | Sandwiches, lasagne, spices, chocolates, pasta | Köppel et al. (2012)    |
| Single-tube nested real-time PCR (real-time PCR coupled with nested PCR) | Hsp1 (AF021807)                                                                                                   | 126<br>97                | 0/25 plant-derived foods                                                | Wheat material spiked with hazelnut (100,000–10 mg/kg) Hazelnut genomic DNA serially diluted (50,000-0.5 pg)       | 50 mg/kg, 0.5 pg                                                | Chocolates, breakfast cereal                   | Costa et al. (2012b)    |
| Real-time PCR with SYBR Green I, melt curve                              | Cor a 9, (JN674437-JN674440)<br>Cor a 11, (JN674445-JN674448)<br>Cor a 13, (JN674441-JN674444)<br>Hsp1 (AF021807) | 101<br>101<br>101<br>100 | 0/7 plant-derived foods                                                 | Peanut flour spiked with defatted hazelnut (500,000–1 mg/kg) Hazelnut genomic DNA serially diluted                 | 1 mg/kg, 2.16 pg                                                | Snacks, biscuits, chocolates                   | Iniesto et al. (2013)   |
| Real-time PCR with hydrolysis probe                                      | Hsp1 (AF021807)                                                                                                   | 100                      | 0/19 plant- and animal-derived foods (reported by Piknová et al., 2008) | Model chocolates spiked with hazelnut (100,000–1 mg/kg)                                                            | 50 mg/kg                                                        | No data                                        | Costa et al. (2015)     |

(Continued on next page)

**Table 4** Summary of the reported molecular-based methods for the detection and quantification of hazelnut in foods (*Continued*)

| Molecular method                          | Target gene (NCBI accession number)          | Fragment size (bp) | Cross-reactivity                     | Reference standards or model mixtures (range) | Sensitivity levels | Application to commercial foods                  | References                     |
|-------------------------------------------|----------------------------------------------|--------------------|--------------------------------------|-----------------------------------------------|--------------------|--------------------------------------------------|--------------------------------|
| Electrochemical DNA-array (genosensor)    | Cor a 1.03 (Z72440)<br>Cor a 1.04 (AF136945) | 156<br>233         | No data                              | No data                                       | No data            | Chocolates, snacks, creams, cereals, biscuits    | Bettazzi et al. (2008)         |
| Optical thin-films biochips (multitarget) | Oleoin (AY224599)                            | 67                 | No data                              | No data                                       | No data            | Bread sticks, wafers, biscuits, cookies, noddles | Wang et al. (2011)             |
| Optical DNA-array (multitarget)           | Cor a 1 (Z72440)                             | 109                | 0/25 plant- and animal-derived foods | No data                                       | 1 mg/kg            | Cereal bars, cookies chocolates, pasta           | Tortajada-Genaro et al. (2012) |

an LPA technique for simultaneous identification of 10 allergenic foods that allowed detecting down to 5 mg/kg of hazelnut in chocolates and 100 mg/kg of hazelnut in walnut cookies, thus evidencing its adequacy for the analysis of processed foods. Still based on a similar approach, Mustorp et al. (2011) demonstrated that only ligated probes are amplified by PCR, which ensures high specificity and efficiency of method. Using the proposed system, Mustorp et al. (2011) were able to detect down to 48 pg of hazelnut DNA (105 DNA copies).

### **Genosensors and Microarrays**

Multiplex methods offer the opportunity of detecting several allergens in a single run with the additional benefits of saving time, reducing reagent costs, and decreasing the occurrence of possible cross-contaminations. In the case of food safety agencies and food-processing industries that are subjected to detailed examination of their control programmes, these features are of extreme importance for the rapid assessment of allergenic ingredients in processed foods (Tortajada-Genaro et al., 2012). Considering all the benefits of multiplex analysis, some studies have been conducted aiming at developing microarrays and DNA chips for simultaneous detection of several allergens in a single assay. Bettazzi et al. (2008) described the application of an electrochemical genosensor platform for the detection of PCR fragments obtained from the cDNA of Cor a 1.04 and Cor a 1.03 isoallergens. Development of a silicon-based optical thin-film biochip was proposed by Wang et al. (2011), which enabled to simultaneously detect eight food allergens, including hazelnut, based on two tetraplex PCR systems. Using a different analytical platform, the digital versatile disk technology (drivers and disks), Tortajada-Genaro et al. (2012) reported the successful application of an optical DNA microarray for the detection of PCR fragments from hazelnut, peanut, and soybean in foods. With the proposed method, Tortajada-Genaro et al. (2012) obtained sensitivities of 1 mg/kg of each allergenic target. These findings highlight the elevated potential of this technology for the assessment of multiple allergenic foods with virtual application in food industry. However, much effort is still required for its full development and to comply with this main goal.

### **FINAL REMARKS**

From the available reports, it is clear that tree nuts are regarded as a common cause of food allergy in Europe, from which hazelnut is responsible for a significant part. Common clinical symptoms related to hazelnut allergy are often described as mild to potentially fatal, being frequently associated with allergy to birch pollen. Presently, 10 groups of hazelnut allergens have been identified and characterized, from which relevant information regarding their biological function

and clinical significance as well as sensitization patterns has been advanced. From the identified allergens, the nsLTP Cor a 8, and the seed storage proteins Cor a 9, Cor a 11, and Cor a 14 have been associated with severe allergic reactions.

So far, the only actual means of preventing allergic reactions in sensitized individuals consists mainly of the total avoidance of offending food. Thus, adequate food labeling plays a crucial role in the safeguard of hazelnut allergic patients' health. In this sense, a need for proficient tools to verify labeling compliance has prompted the development of several protein- and DNA-based methods. In spite of great advances, no official method is yet available for the detection and quantification of hazelnut in foods. This means that efforts are still required to accomplish harmonization regarding the most suitable methodology to detect hazelnut and other food allergens.

Considered as an important allergenic food, hazelnut is one of the most well-studied nuts. The number of publications addressing issues related to hazelnut allergy is high, and it is estimated to increase due to the importance of this topic. Until now, no effective treatments concerning hazelnut allergy are available. More recently, clinical trials using oral immunotherapy have been performed, aiming at inducing desensitization or even tolerance to certain allergenic foods. Although such interventions are still at an early stage and limited to foods such as milk, egg, or peanut, their success could represent a clear improvement in the quality of life of allergic patients. Similar treatments are expected for hazelnut allergy in the near future.

### **FUNDING**

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. UID/QUI/50006/2013. Joana Costa is grateful to FCT PhD grant (SFRH/BPD/102404/2014) financed by POPH-QREN (subsidized by FSE and MCTES).

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