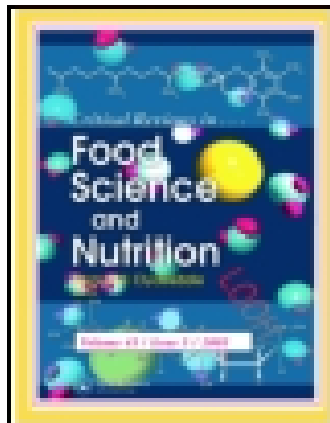


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### Hazelnut Allergens: Molecular Characterisation, Detection and Clinical Relevance

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## **Hazelnut Allergens: Molecular Characterisation, Detection and Clinical Relevance**

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## ***ABSTRACT***

In the last years, special attention has been devoted to food-induced allergies, from which hazelnut allergy is highlighted. Hazelnut is one of the most commonly consumed tree nuts, being largely used by the food industry in a wide 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 sensitised 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 main issues: prevalence of tree nut allergy, clinical threshold levels, molecular characterisation 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 hazelnut allergen detection in foods. A comprehensive overview on the current data about the molecular characterisation of hazelnut allergens is presented, relating biochemical classification and biological function with clinical importance. Recent advances on hazelnut allergen detection methodologies are summarised and compared, including all the novel protein- 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 to the 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 (USDA, 2013). The nuts from all the 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 from 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 those, the nuts from other hazel species can also be consumed, though they do not represent any relevant interest in terms of trade. There is a large number of hazel (*Corylus avellana* L.) cultivars and selections (USDA, 2013), being Morell, Negret, Grossal, Buttler, Ennis, Pautet, Fertile de Coutard, Segorbe, Sta María del Gésu, Tonda di Giffoni, Culplà, Camponica, Cosford, Gunslebert, Lansing, Palaz, Sivri, Tombul and Tonda Romana, some examples of the varieties originated and/or cultivated in the south of Europe and Caucasus region.

The edible part of the hazelnut is the kernel, which can be 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 they 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 pistachio, almond and cashew nut, respectively. 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 the first, second and third places, respectively, since these countries alone are accountable for approximately 80% of hazelnut production in each year (FAOSTAT, 2013).

From some years, hazelnuts as well as 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 (FDA, 2003). Apart from this recognition, tree nuts have also been pointed as likely to induce hypersensitivity in sensitised/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 commercialised 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 emphasised from the rest of the list of ingredients of processed foods, regardless of their quantity (Directive 2007/68/EC, Regulation (EU) 1169/2011).

From all tree nuts, hazelnut is probably the most well studied nut, regarding the impact of its presence in the life of sensitised individuals. The purpose of this review is to provide an actual and critical overview on the prevalence of hazelnut allergy, the molecular characterisation 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 sensitised individuals upon exposure to a given food (Boyce et al., 2010). General data seem to indicate that the number of sensitised/allergic patients affected by food allergy is higher than 1-2%, but less than 10% of the world's population (Chafen et al., 2010). Although the available data is 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; Sicherer and Sampson, 2010). In Europe alone, the number of food allergic patients is estimated to reach the 17 millions of individuals, which represents approximately 2.3% of the European population. From those, 3.5 million of the European allergic patients are younger than 25 years old, with the sharpest rise in food allergies being among children and young people (EAACI, 2012).

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 sensitisation 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 USA, allergies to peanut and/or tree nuts such as almond, walnut or cashew, appear to be more relevant (Sicherer et al., 2003). In an eleven-year follow up self-reported study, conducted between 1997 and 2008 in the USA 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 millions of 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 under 18 years-old and with walnut, cashew, pecan nut and almond, presenting the highest allergy incidence among the US population (Sicherer et al., 2010). Regarding the same age-target population, a randomised cross-sectional survey was administrated electronically to a representative sample of US households in 2009, which enable estimating an overall incidence of 8% of food allergic children (<18 years-old). According to this study the prevalence of nut allergy was approximately 1.0% with more than 52% of the allergic children suffering from severe immunological reactions (Gupta et al., 2011). Another study performed in Canada, using a nationwide telephone survey of randomly selected households in 2011, 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 among 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 the 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, basis and cost of food allergies. This project included the participation of 56 partners from 21 countries, being from 19 European countries, Ghana and India, as well as additional co-partners from USA, Australia and 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 range of prevalence regarding nut allergies of 0.03-0.2% in children up to 6 years-old, 0.2-2.3% in children/adolescents between 6-18 years and 0.4-1.4% in adults. In accordance with challenge tests and sensitisation 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 for the 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 centres from a total of 13 countries (USA, Australia and eleven countries from Europe),



sera from test subjects were scanned using 5 allergen mixes from a total of 24 foods previously defined as priorities. Allergy induced by hazelnut presented the highest overall incidence, accounting with 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%. USA 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 the panel of 24 foods, hazelnut was also considered the food with the highest prevalence of allergy at least in 46% of the participating countries (Burney et al., 2010).

From all the exposed information, it is clear that more studies aiming at establishing the prevalence of tree nut allergies are still needed, 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 the offending foods. As a consequence of this protective measure, those 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 a better risk management by the 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 the 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 the LOAEL or the no observed adverse effect level (NOAEL), being determined either on an individual or on a 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 food-allergic patients. 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) was 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 administrated to those patients. Therefore, this dose (1 mg) was considered the lowest amount of allergenic food inducing observed adverse immunological responses and consequently defined as the threshold value for hazelnut. In the same study, those authors 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 (Eller et al., 2012). 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 test-population (Blom et al., 2013). In this study, patients with hazelnut allergy were the most sensitised 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 the estimated threshold level of 0.05 mg of hazelnut protein (Blom et al., 2013).

From the evaluation of these two studies, it is very clear 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). The distribution of the threshold level for hazelnut reported by those studies is rather different, which suggests that further investigation regarding this topic is still needed. However, these data represent a step forward in the elaboration of precautionary labelling action levels and for the incorporation in the risk assessment for adverse immunological reactions in the allergic population, upon eventual consumption of foods contaminated with allergens (Blom et al., 2013).

### ***MOLECULAR CHARACTERISATION OF HAZELNUT ALLERGENS***

The majority of the 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 the hazelnut, several proteins have been recognised as allergens. Until now, ten 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 TLP) have been identified and characterised in hazelnut. From those, Cor a TLP has not yet been comprised in the WHO-IUIS list of allergens (ALLERGEN, 2013), but has already 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 for 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 characterised by their small size, stability at low pH and resistance to proteolysis, making them good candidates for inducing adverse immunological responses in sensitised 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 they are very common among the trees from the order Fagales. Cor a 1 allergens are classified as PR-10 proteins, which are greatly abundant in the reproductive tissues such as pollen, fruits and seeds. As consequence, Cor a 1 proteins are classified both as food allergens and as aeroallergens.

Cor a 1 appear 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 exhibit 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 weights 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). They 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 sensitised to birch pollen (Bet v 1) (Roux et al., 2003). Cor a 1.01, which is classified as 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 (Hansen et al., 2003; Müller et al., 2000; Pastorello et al., 2002; Schocker et al., 2000). The loss of the protein tri-dimensional structure enables the destruction of the conformational epitopes (IgE-reactivity) and, consequently, the ability to trigger adverse reactions in sensitised 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 few sensitised individuals can still experience positive reactivity towards roasted

hazelnuts (Hansen et al., 2003). Structure models for Cor a 1 proteins revealed that most part of the amino acid sequence is well organised, 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 the epitopes is mainly dependent on the conformational structure of the protein, which is affected by heat treatment. This finding is in good agreement with data reported by Hansen et al. (2003), stating that Cor a 1 allergenicity is highly influenced by the tri-dimensional conformation of the 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 Mba, was performed aiming at testing its effect on the allergenicity of Cor a 1 proteins, though resultant allergenic profile remained the same as from raw hazelnuts (López et al., 2012).

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

Profilins are a family of cytosolic actin binding proteins with small molecular size (12-15 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 (PIP<sub>2</sub>) 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 polymerisation) and interact with the PIP<sub>2</sub> 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 they 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 characterised 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 are both 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 properties (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% towards Bet v 2, which states that Cor a 2 is one of the Bet v 2-related allergenic food profilins. Similarly to isoallergen Cor a 1.04, the allergenicity of Cor a 2 seems to decrease with food processing (roasting), in patients sensitised 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, which belongs to the family of the non-specific lipid transfer proteins (nsLTP) that is included in the prolamin superfamily. Along with the family of the nsLTP, this superfamily comprises several groups of proteins that contain many plant food allergens such as the 2S albumins and inhibitors of alpha-amylase and trypsin from cereals (Breiteneder, 2006). The family of nsLTP is characterised by monomeric proteins of low molecular size, revealing primary sequences with a high content of cysteine residues, thus, contributing to secondary structures composed by 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 the 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), respectively (Hauser et al, 2010; UniProt, 2013). The nsLTP are also interveners in other functions such as those associated with plant defence (antifungal and antibacterial activities) (Ebner et al., 2001) or potential involvement in plant growth and development (embryogenesis, germination) (Kader, 1996; Salcedo et al., 2007), being widely distributed throughout the kingdom of plants (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 the 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 contain 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, it is expected to occur IgE cross-reactivity 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 the nsLTP, namely the location of the proteins and the stability of those 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 the peels in comparison with the inner pulps (Fernández-Rivas and Cuevas, 1999). Studies evidenced that some LTP-sensitised individuals can tolerate fruits (apple, peach) after peeling (Fernández-Rivas and Cuevas, 1999), however they are still at risk of developing adverse reaction 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). Like for 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 sensitised individuals (Schulten et al., 2011a). In contrast with the 2S albumin family, the nsLTP are slightly less stable when submitted to temperatures over 90°C, probably due to the existence of the lipid-binding tunnel (Mills et al., 2007b; Sancho et al., 2005). 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 disorganisation 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). Like the 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 for 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). 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 the 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, the 7S vicilin-type globulins and the 11S legumin-type globulins, according to their sedimentation coefficient (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-translationally split in the asparaginyl endopeptidase site. The functional 11S legumins are non-glycosylated proteins, forming hexameric structures composed by 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 from 8 to 15 linear epitope-bearing peptide regions that are scattered along the length of the acidic and the basic subunits (Robotham et al., 2009). Functional Cor a 9 is also composed of 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 molecular weight of 40 kDa is the acidic subunit of Cor a 9 after cleavage and reduction of the 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 the legumins, Corylin (Cor a 9) also presents a hexameric form organised 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 above 94°C (Mills et al., 2007b). However, even after being submitted to high temperatures, the secondary structures of these proteins remain unchanged or with minor modifications, suggesting that the characteristic beta-barrel motif is highly stable. Preliminary studies on raw hazelnuts submitted to dry heat treatment at 170°C, indicated that the protein profile and the amount of Cor a 9 was 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. Those loop regions are estimated not to be modelled due to their lack of stable structure, but they are predicted to exhibit linear epitopes located at the external faces of the 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 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 2,007 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 example, protein Cor a 10 showed 90% of sequence identity with BLP-4 and BLP-5 from *Nicotiana tabacum* (CAA42659.1, CAA42660.1), with the heat shock 70 kDa 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 (Gruehen et al., 2003; Stanley and Bannon, 1999). 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-sensitised patients with specific IgE against Cor a 10 could developed allergy towards 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, though 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). Additionally, 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 (4 out of 5 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 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 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; Mills et al., 2007b). When submitted to high temperatures, proteins can suffer conformational disruptions and covalent modifications with special emphasis for 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 7 days did not allow the formation of coloured products, suggesting that the susceptibility of Cor a 11 for pepsin hydrolysis or its capacity to bind human IgE were not affected. Maillard products from glycation at 60°C for 3 days and 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. In those 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 glycosylation of the protein by the presence of a 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). Like 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 (Leduc et al., 2006; Pons et al., 2002). The biological functions of these proteins are mainly centred in stabilising 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 are composed by 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 prevent 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 composed by primary structures of 159 aa and 140 aa, 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 those have been related to food allergy. The assembly of oleosins with lipid fraction may be determined to prevent the protection of this allergen towards the rapid proteolysis in the gastrointestinal tract (Akkerdaas et al., 2006). To verify these data, further studies about this topic should be 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 the 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, asparagine 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 subunit (heterodimers) held together by conserved inter-chain disulphide bonds. Like the LTP, the 2S albumins also contain eight cysteine residues that ensure four disulphide bonds. The amino acid composition of the 2S

albumins, their high abundance in seed cells and their mobilisation during germination suggest that these proteins act as important nitrogen and sulphur donors (Breiteiner 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 composed of 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 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 organisation 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 to the nsLTP, the 2S albumins present higher resistance to thermal processing, maintaining their original folding at temperatures up to 90°C (Mills et al., 2007b).

### ***Cor a TLP (Thaumatococcus like protein)***

The thaumatococins are included in the PR-5 group of the pathogenesis-related proteins of the defence 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 sixteen different TLP in which comprised the hazelnut TLP with the NCBI accession number P83336. However, this accession number identifies a protein with 212 aa as the thaumatococin-like protein 1b expressed from a nucleotide sequence with 696 bp of the *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. The 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 categorises 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 recognised by the immune system and can elicit immunologic reactions in sensitised 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 or system(s) affected (Boyce et al., 2010; Sicherer and Sampson, 2006). Those 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 elicit 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 the high homology among the allergenic PR-10 proteins, which are known to be responsible for the wide frequency of cross-sensitisation to multiple PR-10 proteins from different fruits, seeds, pollens and nuts. In the 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 sensitisation (Akkerdaas et al., 2000; Hirschwehr et al., 1992; Schocker et al., 2000). Belonging to the PR-10 proteins, Cor a 1 are classified both as 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 the majority of the cases, clinical manifestations associated with this class of proteins are typically mild and frequently exclusively related to OAS. These facts were demonstrated in a recent study conducted in a birch-endemic region, where 97% of the tested population with OAS were sensitised 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 sensitised 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 sensitisation 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), as the result 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 sensitisation to different pollens and fruits is probabilistically elevated (Hauser et al., 2010). The clinical relevance of profilin sensitisation 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; Asero et al., 2008), evidencing that further research is obviously still 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 the majority of the plant-derived foods as well as in pollen of diverse plants, nevertheless, the route of sensitisation 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, sensitisation 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 sensitisation 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 to Cor a 8 (Asero, 2011; Hartz et al., 2010; Schulten et al., 2011b). Additionally, the level of IgE to peach LTP is regarded as the key issue associated with cross-reactivity and subsequent clinical allergy to foods from different botanical origin (Asero, 2011). Although the

classification of major allergen 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 sensitisation to hazelnut is probably affected by geographical differences, once in the northern Europe the rate of sensitisation to hazelnut is more frequently attributed to Cor a 1 allergens (specifically Cor a 1.04), whereas in the Mediterranean prevailed the IgE towards Cor a 8 (Hansen et al., 2009).

Like the nsLTP and as an important member of the prolamin superfamily, the 2S albumin Cor a 14 was suggested to be connected with moderate to severe hazelnut allergy (Garino et al., 2010; Pastorello et al., 2002). Although the WHO/IUIS identifies Cor a 14 with an IgE-binding prevalence of about 33% (ALLERGEN, 2013), suggesting its classification as minor allergen, relevant information about clinical manifestations and pattern of sensitisation 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. Due to their high abundance in those tissues, the proteins with allergenic properties are frequently considered major allergens in foods. Cor a 9 and Cor a 11, that are 11S legumin- and 7S vicilin-like proteins, respectively, are associated with severe hazelnut allergy. The exact route of sensitisation 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) that reported systemic reactions in 86% of the test population (predominantly children) and by Hansen et al. (2009) that found sensitisation to Cor a 9 in four out 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 the hazelnut allergic children (65% of preschool children and 50% of school-age children) revealed systemic reactions upon the consumption of processed hazelnut, mostly being sensitised by Cor a 9 with no relation to birch pollen allergy. The sensitisation 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 sensitisation or allergy, independently from 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 sensitisation to Cor a 9 and Cor a 14, whereas sensitisation 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 sensitisation to this allergen has been reported in both hazelnut allergic patients presenting mild immunological responses, mainly related to OAS (Lauer et al., 2004) or experiencing severe systemic reactions (Ebo et al., 2012). Like for Cor a 9, the sensitisation 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 sensitisation to this allergen, as for the Cor a 9, is also not defined though it seems to follow the same pattern of the latter. The classification of major allergen has also been advanced for Cor a 11 (Cucu et al., 2012a; Rigby et al., 2008), 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% (Ebo et al., 2012; Lauer et al.,

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

The clinical relevance and the pattern of sensitisation of the 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) (Leduc et al., 2006; Pons et al., 2002), 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 the 11S globulin (ALLERGEN, 2013), which might affect the estimative of the 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 the different groups of allergenic proteins in hazelnut and their respective symptoms/clinical relevance as well as sensitisation patterns, is slowly becoming available. Still further research studies based on multidisciplinary teams (clinicians, immunologists, researchers) must be pursued in order 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 the researchers. The lack of harmonisation regarding the most suitable methodology to verify labelling compliance and the absence of available testing/reference materials contribute to the generalised controversy among researchers and represent key issues in the management of food allergens. While in the opinion of some, the direct monitoring of the offending proteins should always be addressed, others defend that alternative methodologies via the indirect assessment of allergenic foods (DNA) can also be considered 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 on 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 protein marker(s).

*ELISA systems*

ELISA is considered 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: the competitive and the 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 sensitised individuals.

Although with very well established advantages, is important to mention that the performance of the 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, partial hydrolysis) (Cucu et al., 2011; Cucu et al., 2013; Garber and Perry, 2010; Pele et al., 2007; Platteau et al., 2011a; Platteau et al., 2012; Roder et al., 2009). 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 (Cucu et al., 2011; Garber and Perry, 2010). 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 the heat treatment led to possible alterations on the structural conformation of the 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 towards 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 the native protein. Most of the available ELISA kits are probably developed upon antibodies that were raised against raw hazelnuts. This fact can conduct to a reduced capacity of the antibodies to recognise proteins modified by Maillard reactions and, consequently, provide erratic results when analysing 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 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 of the 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 the hydrolysed proteins by the antibodies, though one of the tested kits exhibited good performance towards the target proteins (Cucu et al., 2013). All these facts seem to emphasise that the choice of an ELISA kit is highly dependent on the purpose of the 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 the 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-react 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 the production lines. This type of tests 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 specialised equipment or personnel (Schubert-Ullrich et al., 2009). Like the ELISA, there are two types of LFD: the sandwich and the competitive formats. 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. (2011) 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 the 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 it is not considered a suitable method for routine analysis. The application of this technique enables the evaluation of the 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 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 to 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 of execution, ease to use and feasible for automation. Considering all the potential attributed to these analytical devices, it is expected that biosensors could be applied at industrial scale for the direct and in 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 a 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 to a small number of antibody-based applications (Table 3). From those, the optical biosensors are the most commonly used, relying their function on the phenomenon of surface plasmon resonance (SPR) that measures the changes in the refractive index when the antibody bonds to the target protein (Diaz-Amigo, 2010; Pilolli et al., 2013). The 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, those authors used polyclonal rabbit IgG and hen's egg IgY raised against the 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 forty tested foods. The biosensor proposed by Bremer et al. (2009) presented a 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 the 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 those authors, which were also in good agreement with the sensitivities reported for ELISA or LFD systems (Table 2 and Table 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 with four (pecan nut, coconut, beans and macadamia) out of the 22 plant-derived food tested. When compared with the ELISA system, the LOD attained with the electrochemical biosensor was approximately ten-fold higher, however with the strong advantage of reducing the analysis time from four to one hour. Therefore, the developed biosensor proved to be adequate for the detection of minute amounts of hazelnut in food samples with possible application for the 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, targeting multiple allergens.

### *Mass spectrometry (MS)-based methods*

The development of novel strategies for allergen detection, quantification and characterisation is a constant demand. Recently, the application of proteomic methodologies (allergenomics) for the analysis of food allergens has been addressed, especially centred on core technologies such as the MS-based platforms. This technology evidences several advantages because it allows proteins to be quickly analysed 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 the unequivocal confirmation of the identity of the tested proteins/peptides (Monaci and Visconti, 2009). Several MS-based methods can be used for the relative and absolute quantification of proteins (e.g. allergens), but all of those rely on one of 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 the proteins, the purification process has to be specifically developed to ensure reliable recognition of the molecule via the generation of a peptide mass fingerprinting. With

respect to the detection of proteins/allergens in processed foods, MS methods are 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, those 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). The 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, those authors reported sensitivities of 30 mg/kg or 35 mg/kg of Cor a 9 in spiked biscuits and 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). The 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) and Heick et al. (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, both in 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 sensitivity, those authors evaluated the potential application of the selected hazelnut peptides for the identification of this nut in processed foods. One out of four Cor a 9 peptides also occurs in pecan, walnut, pistachio and cashew, and one out 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). MS methodologies have been applied only 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, despite 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 regards their indirect assessment in food matrices. In fact, if any protein can act as a marker for species identification, the 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 favoured 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.

### *PCR systems*

Polymerase chain reaction (PCR) 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). The 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. Additionally, 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 so 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). Despite 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 (Herman et al., 2003; Holtzhauser et al., 2000). 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 the recent advances in high resolution instrumentation and with the arising of more specialised 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 (DøAndrea et al., 2009; DøAndrea et al., 2011; Iniesto et al., 2013; Pafundo et al., 2010). As 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 the reaction (Arlorio et al., 2007; Costa et al., 2012b; Costa et al., submitted; Köppel et al., 2010; Köppel et al., 2012; Píknová et al., 2008; Platteau et al., 2011a; Platteau et al., 2011b), based on the complementarity of a third hybridisation oligonucleotide during the 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 the simultaneous

detection and quantification of several allergenic foods including hazelnut (Köppel et al., 2010; Köppel et al., 2012; Pafundo et al., 2010; Schöringhumer et al., 2009). In the same sense, other developed multitarget systems were proposed based on ligation-dependent probe amplification (LPA). Ehlert et al. (2009) developed a LPA technique for the 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 the ligated probes are amplified by PCR, which ensures the high specificity and efficiency of the method. Using the proposed system, those authors 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 detail 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 the multiplex analysis, some studies have been conducted aiming at developing microarrays and DNA chips for the 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. The 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, on the basis of 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 those authors 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 the 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 from 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, ten groups of hazelnut allergens have been identified and characterised, from which relevant information regarding their biological function and clinical significance as well as sensitisation patterns have 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 to severe allergic reactions.

So far, the only actual means of preventing allergic reactions in sensitised individuals consists mainly on the total avoidance of the offending food. Thus, adequate food labelling plays a crucial role in the safeguard of hazelnut allergic patients' health. In this sense, the need for



proficient tools to verify labelling compliance has prompted the development of several protein- and DNA-based methods. In spite of the great advances, no official method is yet available for the detection/quantification of hazelnut in foods. This means that many efforts are still required to accomplish harmonisation regarding the most suitable methodology to detect hazelnut and other food allergens.

Considered 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 desensitisation 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 the allergic patients. Similar treatments are expected for hazelnut allergy in the near future.

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**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, Michigan, USA)	LFD	No available information about the specificity	5-10 mg/kg	-	~5 min
AgraStrip Hazelnut (Romer Labs Division Holding GmbH, Austria)	LFD	No available information about the 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., California, 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, Michigan, USA)	Quantitative - Sandwich ELISA	No available information about the 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 the specificity	Ö5 DNA copies, Ö0.4 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 the specificity	0.4 mg/kg	0.4 mg/kg	~40 min (applied to extracted sample)

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

Protein-based methods	Antibody/target protein (immunisation)	Cross-reactivity	Sensitivity levels	Application to food matrices	Reference
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 $\phi$ 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 $\phi$ 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 $\phi$ 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 $\mu$ g/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)
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 $\phi$ 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 henø 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 henø 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 henø 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)	1 out of 4 peptides from Cor a 9 occurs in walnut, pecan, pistachio and cashew. 1 out of 3 peptides from Cor a 11 occurs in walnut and pecan.	Not defined	Not tested in food samples	Ansari et al. (2012)

**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	Reference
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 hybridisation probe	Cor a 1.04 (AF136945)	152	0/35 plant- and animal-derived foods.	Hazelnut genomic DNA serially diluted (100-1 pg) 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	Nad1, 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) 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) 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) 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) sesame/hazelnut DNA serially diluted (100,000-10 pg/ $\mu$ L)	50 mg/kg 50 pg	Cereals, cookies 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) Walnut cookies spiked with 1% of 5 nut mix (pecan, hazelnut, cashew, macadamia, walnut (10,000-1 mg/kg)	5 mg/kg in chocolate 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 6 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)	No data	Platteau et al. (2011a)
Real-time PCR with hydrolysis probe	Cor a 1 (Z72440) Cor a 8 (AF329829)	101 218	0/29 plant- and animal-derived foods	Hazelnut genomic DNA serially diluted (50,000-0.256 pg)	3.2 pg	No data	Platteau et al. (2011b)
Real-time PCR with SYBR Green I, melt curve	Cor a 1 (AF136945) Cor a 8 (AF329829) Cor a 14 (FJ358504)	105 78 116	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 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) Cor a 11, (JN674445-JN674448) Cor a 13, (JN674441-JN674444)	101 101 101	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 Píknová et al., 2008)	Model chocolates spiked with hazelnut (100,000-1 mg/kg)	50 mg/kg	No data	Costa et al. (submitted)
Electrochemical DNA-array (genosensor)	Cor a 1.03 (Z72440) Cor a 1.04 (AF136945)	156 233	No data	No data	No data	Chocolates, snacks, creams, cereals, biscuits	Bettazzi et al. (2008)
Optical thin-films biochips (multitarget)	Oleosin (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)