

REVIEW

Walnut allergens: molecular characterization, detection and clinical relevance

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Summary

Food-induced allergies have been regarded as an emergent problem of public health. Classified as important allergenic ingredients, the presence of walnut and other nuts as hidden allergens in processed foods constitutes a risk for sensitized individuals, being a real problem of allergen management. Attending to the increasing importance dedicated to walnut allergy, this review intends to provide the relevant and up-to-date information on main issues such as the prevalence of walnut allergy, the clinical threshold levels, the molecular characterization of walnut allergens and their clinical relevance, as well as the methodologies for walnut allergen detection in foods. As the walnut used in human diet comes from *Juglans regia* and *Juglans nigra*, the molecular characterization of the allergens from both species included in the prolamins (Jug r 1, Jug n 1 and Jug r 3), cupins (Jug r 2, Jug n 2 and Jug r 4) and profilins (Jug r 5), together with respective clinical relevance, were compiled in this review. The most recent progresses on walnut allergen detection techniques (protein- and DNA-based) are described and critically compared, including the emergent multitarget approaches.

Introduction

Walnut belongs to the botanical family of Juglandaceae, and it is the seed of the trees of the genus *Juglans*, which encompass 24 different species. The nuts from all the species included in this genus are edible, but only two have economical interest, namely the *Juglans regia* (also designated as common, Persian, English, California, or Carpathian walnut) and the *Juglans nigra* (Eastern black walnut) [1]. Both species are well spread around the globe, being predominantly cultivated in temperate climates. The most well-known species of walnut is the common walnut (*Juglans regia*) that is native from the Balkans region in southeast Europe, southwest and central Asia to the Himalaya and southwest China. The black walnut (*Juglans nigra*) is native from the eastern North America, but also widely cultivated in other regions [1]. There is a large number of walnut cultivars, being Chandler, Hartley, Tulare, Howard, Ashley, Payne, Lara, Franquette, Marbot, Parisienne, Serr, Vina and Valcor, some examples of the varieties originated and/or cultivated in Europe and USA [1, 2].

Like other tree nuts, walnuts have also attained a special interest among world's population as their consumption is frequently associated with healthy habits and equilibrated food diets. The regular consumption of walnut seems to improve body's lipid profile, contributing to the decrease of cholesterol levels and thus reducing the risk of coronary heart diseases. Additionally, other positive beneficial health effects can be attributed to the intake of walnut and other nuts, such as their active intervention on inflammatory processes, oxidative stress, vascular reactivity and glycemic control (see review [3]). The edible part of walnuts is the kernels, though the hulls also present elevated commercial interest, especially those from *Juglans nigra* due to its high content in pigments (juglone and plumbagin) and tannins. As consequence, walnut hulls have multiple applications, not only as dyes and inks, but also as potent antibacterial, antimicrobial and antifungal agents [4].

Taking into consideration all the human health benefits and the numerous industrial uses attributed to walnut, the demand for this nut has been increasing, which is supported by the growth of its global production

(approximately 270%) during the past 20 years [5]. In 2011, the world's total production of walnut corresponded to 3 418 502 tonnes, ranking this nut in the second place in terms of total tree nut production quantity, just behind cashew nut. In the same year, the major walnut producers were China and Iran, accounting for almost 63% of the world's production [5].

The recognized health effects of walnut, combined with its pleasant taste, have led to its inclusion (as seed or oil) in several cookery dishes and pastry products (e.g. cakes, biscuits, ice-creams) [6]. The walnut consumption is frequent and mostly appreciated by the majority of the individuals. However, for a part of the general population, the ingestion of walnut can represent a health risk due to the possibility of inducing hypersensitivity in sensitized/allergic individuals. As preventive measure since 1985, the Codex Alimentarius Commission suggested the mandatory labelling of foods susceptible of containing potentially allergenic ingredients. From 1993, walnut and other tree nuts were defined as one of the eight groups accountable for almost 90% of human food allergies [7]. Since then, legislation has been issued and further updated to protect sensitized/allergic individuals. Inside the European Union (EU), food producers are obligated to declare all the ingredients present in prepackaged foods [8]. Currently, EU legislation establishes a list of fourteen groups of certain substances or products causing allergies or intolerances that are required to be emphasized from the rest of the ingredients enumerated in processed foods, regardless of their quantity [9, 10]. In the referred list, walnut together with other tree nuts, represent one of the fourteen groups of potentially allergenic foods, which also includes soybean, cereals containing gluten, sesame, mustard, celery, peanuts, milk, eggs, fish, molluscs, crustaceans, lupine and sulphites.

Over the past years, tree nuts have been the focus of several studies about food allergies. With this review, it is intended to provide a broad and updated overview about some of the most relevant issues regarding walnut as an allergenic food. Topics such as the prevalence of walnut allergy, the molecular characterization of identified allergens (from both *Juglans regia* and *Juglans nigra*) and the available analytical methods for its detection will be focused in this review. In addition, other pertinent subjects concerning the clinical relevance of walnut allergy, the definition of threshold levels and an insight into forthcoming potential immunotherapies for food allergies will also be addressed.

General considerations about food allergy

Food-induced allergies can be classified as immunoglobulin-E (IgE) mediated, non-IgE mediated, or a combination of both [11]. Most of IgE-mediated reactions are

featured by a series of symptoms (involving skin, gastrointestinal, cardiovascular and/or respiratory tracts) that commonly appear during the first 2 h upon ingestion of the offending food. After the allergic sensitization, food-specific IgE antibodies are produced and bounded on the surface of the mast cells and blood basophils. Upon re-exposure to the given food, the allergenic proteins are recognized by the specific IgE antibodies, which are responsible for activating the release of mediators such as leukotrienes, prostaglandins and histamine [11, 12]. Without observable clinical symptomatology, the sensitization alone is not sufficient to classify as food allergy [13].

The cell-mediated immunological reactions (non-IgE mediated) have different clinical presentations often associated with proctocolitis, enterocolitis and enteropathy syndromes that affect primarily infants and young children. In those cases, patients frequently exhibit abdominal cramps, diarrhoea and/or vomiting. Clinical syndromes related to eosinophilic esophagitis and atopic dermatitis are examples of food allergy caused by mixed IgE- and cell-mediated disorders [12, 13]. Abnormal immunological responses can be catalogued in different types, namely immediate and delayed hypersensitivity reactions. The immediate hypersensitivity reactions are mediated by IgE and clinical symptoms ensue, generally, within 2 h after ingestion or exposure to the offending food [11]. Despite being very rare, the appearance of observable symptoms after 24 h or more, upon the ingestion of the allergenic food are classified as delayed hypersensitivity reactions. In these conditions, the adverse responses are cell-mediated and normally activate the T cells [14]. Although the cell-mediated responses, such as allergic contact dermatitis, are catalogued as immune-mediated reactions, they are the most uncommon form of food allergy [11]. For this reason, they are classified of small clinical relevance when compared to other immune-mediated adverse food reactions.

The severity of the allergic reactions varies from mild to potentially life-threatening, and it is dependent on a variety of internal and external factors [15]. Several features have been investigated and are pointed out as possible risks and causes for the development of food allergies. Accordingly, genetic aspects (familial associations and specific genes), association with atopic diseases (e.g. atopic dermatitis) and pre-existing conditions (e.g. asthma), timing and route of exposure to allergen (e.g. topical/respiratory exposure may be sensitizing), components of diet (reduced consumption of ω -3 polyunsaturated fatty acids, vitamin D), diet during pregnancy and medication (e.g. antibiotics, antacids) have been highlighted as potentially increasing risk factors for developing food allergy [16]. The severity of the allergic reactions is highly dependent on the amount of food ingested, on the type of food processing used

(cooked, raw, or processed) and on the possible interactions with other foods [13]. In addition, factors such as patient's age, speed of food absorption and ingestion of food close to a time of exercise can also contribute to the severity of the allergic reaction. Although all those conditions are known to enhance the severity of an allergic response, its degree of intensity is very difficult to predict [11].

In the past few years, the knowledge about different aspects of food allergies have been actively used to protect and enhance the quality of life of the sensitized/allergic individuals, though much work is needed in this field.

Prevalence of walnut allergy

Food-induced allergy is regarded as an emerging problem of public health affecting adults and children, whose prevalence seems to be rising, not only in the industrialized countries, but also in developing and emerging economies [17]. The true prevalence of food allergies has been very difficult to estimate. Until now, data suggest that 3–4% of adults and 5–6% of young children/adolescents can suffer from some type of allergy related to food [18, 19]. Although the available data is considered rather inaccurate, general numbers estimate that 1–2% up to 10% of the world's population can be affected by food allergy [20].

Most of the studies designed to evaluate the prevalence of food allergies are frequently based on self-reported reactions to foods such as questionnaires and surveys, rather than objective assessments as open food challenges (OFC), double-blind placebo-controlled food challenge tests (DBPCFC), or determined sensitization to foods by serum IgE and skin prick tests (SPT) [21]. Although questionnaires and surveys could represent helpful tools, respective data must to be carefully interpreted as individuals often tend to overestimate virtual symptoms. With respect to some of these facts, the prevalence data are still far from being accurate and should always be regarded as mere indicators of the true incidence of food allergies [22].

Although some studies have been advanced estimating the prevalence of tree nut allergies [23–27], the information concerning the specific incidence of walnut allergy is still very scarce. Walnut allergies seem to be more prevalent in the USA along with allergies towards almond and cashew, in opposition to Europe, where hazelnut allergy is more frequently reported [25, 28].

On the basis of a large Europe-wide research project (EuroPrevall) funded by the European Commission that was specifically elected to evaluate the prevalence, basis and cost of food allergies [29], data about the prevalence of walnut allergy were recently provided. In this project, involving numerous centres from a total of 13

countries (USA, Australia and eleven countries from Europe), 5 allergen mixes from a total of 24 foods previously defined as priorities (including walnut) were tested against the sera of sensitized/allergic subjects. Walnut allergy presented an overall incidence of 2.2% and a prevalence of 1.8% after excluding the birch positive individuals [30]. France exhibited the highest incidence of walnut-allergic patients (3.7%), closely followed by Germany (3.3%), Italy (3.1%) and Spain (3.1%). From the total of 24 foods surveyed in the referred study, only two nuts (hazelnut and walnut) were evaluated. Accordingly, walnut allergy ranked fifteenth, being the first position occupied by hazelnut [30]. This ranking evidenced the relevance of hazelnut allergy in Europe, considering that most of the countries participating in this wide project were European.

With respect to the presented information, it becomes clear that more extensive studies are needed to support the estimated data for the prevalence of walnut allergy. In addition, it would also be very important to include objective information regarding walnut and other nut allergies involving more countries from different regions (e.g. Africa and Asia).

Clinical threshold levels for walnut

In spite of the severity, the prevalence and the impact of food allergies on daily life of the sensitized/allergic individuals, there is still no cure for them. Only preventive and therapeutic measures are available to help these patients managing their food allergies. Therefore, for the sensitized/allergic individuals, the only effective means of preventing an adverse reaction is the total avoidance of the offending food or a therapeutic treatment (antihistaminic, corticosteroids, bronchodilators and/or epinephrine) in the case of an accidental exposure to the allergenic food [15]. As food-allergic consumers represent a small, but rather significant growing portion of the general population [31], protective measures such as precautionary labelling have been widely adopted by food-processing industry to prevent possible legal actions. The use of precautionary labelling safeguards the allergic population, but also restricts their choices when acquiring processed foods. In this sense, the knowledge of the minimum dose that can elicit a reaction is of great interest to all food allergy stakeholders (caretakers, health professionals, food industry) [32, 33]. The definition of clinical thresholds for allergenic foods is fundamental to evaluate their risk at both individual and population levels. While the information about individual thresholds permits allergic patients and health professionals to better manage food allergy, the insight about population thresholds should help food industry and regulatory authorities assessing appropriate guidelines for risk management of aller-

genic foods [32]. The clinical threshold level for an allergenic food lies between the highest dose observed, not inducing any adverse effect (no observed adverse effect level – NOAEL), and the lowest dose of food triggering an observed adverse effect (lowest observed adverse effect level – LOAEL) [34]. Considering that this limit is difficult to delineate, threshold is often defined as the LOAEL or the NOAEL on both population and individual basis [34].

When in contact with the offending food, allergic individuals can respond differently to a very large range of doses. As a result, the assessment of individual threshold levels can be determined by clinical challenge trials such as OFC and DBPCFC. Results from those challenges can be extrapolated to provide a dose–response curve to a given allergen by means of statistical models based on individual LOAEL parameters [35]. Nevertheless, these tests must involve the cooperation of the allergic patients during small or long periods, which is not always easily carried out.

The determination of clinical thresholds for allergenic foods is still at a very preliminary stage. In the particular case of the group of tree nuts, only primary data have been advanced for hazelnut, cashew nut and walnut [36, 37]. Regarding walnut, a single study based on DBPCFC was reported by Blom *et al.* [36], in which the retrieved information was analysed to determine individual LOAEL and NOAEL for both subjective and objective symptoms. The DBPCFC was applied to a total of 363 allergic children/adolescents with ages ranging from 2 to 16 years-old to trace a profile of threshold levels for 7 different allergenic foods, namely peanut, egg, milk, cashew nut, hazelnut, soybean and walnut. Statistical models were applied to define population thresholds using LOAEL and NOAEL values expressed as discrete doses in milligram of total protein of the allergenic food [36]. Threshold levels were established for 5 major allergenic foods (cashew nut, hazelnut, egg, milk and peanut). However, the number of individuals with positive objective symptoms for walnut was too small to fit into the distribution models and only individual LOAEL and NOAEL were defined for this allergenic ingredient. The positive LOAEL with objective symptoms exhibited a wide scope of doses, ranging from 0.9 to 350 mg of walnut total protein in baked cookies [36].

From the exposed facts, it is well reinforced that much research is still needed to establish clear clinical threshold levels for walnut and other allergenic foods such as different nuts.

Allergen characterization and clinical relevance

In the last years, significant research has been conducted to characterize the allergens involved in tree nut allergy. The categorization of the specific allergens,

both from a biochemical and from an immunological point of view, allowed them to be classified into a very restrict number of families and superfamilies [38, 39]. In addition to this classification, food allergens can be further catalogued as class I or class II, according to their subjacent pattern of sensitization. While class I food allergens evidence similar characteristics such as high stability to food processing and gastric digestion, enabling them to induce allergic sensitization via the gastrointestinal tract, class II food allergens are considered to be more sensitive to heat and digestive enzymes. As consequence, class II food allergens do not cause sensitization via the oral route. Instead, they are more likely to provoke allergic reactions in patients with previous sensitization to inhalant allergens, with pollen acting as primary sensitizer, potentially leading to pollinosis-associated food allergies [40–42]. Thus, parameters, such as stability to acidic environment, thermal denaturation and gastrointestinal proteases, are highly relevant for the classification of class I or class II food allergens [40].

Currently, a clear and distinct classification of class I or II food allergens is not always possible, although evidences suggest that class I allergens can only be found in foods (not in pollen), in contrast to class II allergens that are commonly present in both [42]. In terms of clinical relevance, class I food allergens are often considered of major importance because they are correlated with severe clinical presentations. In the case of class II food allergens, allergic reactions are generally classified as mild, although severe adverse immunological responses can also be attributed to them. Class I and II food allergens can be found in the tree nut group, being both of critical importance in nut allergy. Therefore, 2S albumins, legumins and vicilins are classified as important tree nut seed storage proteins, representing relevant class I food allergens, while class II allergens from pathogenesis-related (PR) proteins (Bet v 1-homologous family) and structural profilins are minor components in nuts.

A number of specific clinical syndromes can occur as a result of food allergies, which are rated on the basis of inter-related immunologic causes and the organ or system(s) affected. As disorders can vary in intensity targeting one or more organs/systems, clinical manifestations of food allergy are known to affect the skin, as well as the gastrointestinal, cardiovascular and/or respiratory tracts [13]. The most common symptoms related to IgE-mediated food allergies are urticaria, angioedema, pruritus, nausea and vomiting, abdominal pain or cramping, and diarrhoea. Respiratory symptoms such as sneezing, rhinorrhoea, nasal congestion, coughing, stridor, wheezing and ocular injection, often occur associated with cutaneous and gastrointestinal complaints [11]. In adults, the most usual clinical manifestation

concerns the oral allergy syndrome (OAS) also referred as pollen-associated food allergy syndrome, which ordinarily affects patients with pollen allergies [13].

Among the observable IgE-mediated responses to food, the most severe is anaphylaxis that is a systemic and potentially fatal allergic reaction. Anaphylactic shocks can ensue suddenly upon the ingestion of the offending food [43] and are frequently reported to occur in children and adolescents. IgE-mediated reactions to peanut and tree nuts are usually related to this severe clinical manifestation, often resulting in fatalities [11, 44]. In the tree nut group, walnuts, hazelnuts and almonds are among the most usually cited as being the cause of systemic allergic reactions [45]. Walnut allergy is typically classified as severe and potentially life-threatening, occurring with complex clinical presentations [39].

The recent advances in recombinant DNA technology for allergen research, the application of recombinant allergens and synthetic sequential epitopes have been contributing for the detection of sensitization profiles, with IgE specific to several allergens/substructures, now being pointed out as possible markers of severity and persistence. Thereby, the use of novel technologies (e.g. microarrays) for the simultaneous measurement of multiple IgE reactivity concerning specificity, abundance, reactivity, or interaction has been regarded as excellent tools to improve allergy diagnosis procedures. In spite of this, clinical studies on cross-reactivity between tree nuts are still scarce.

In the specific case of walnut, different allergenic proteins have been identified and characterized, concerning the two species *Juglans regia* and *Juglans nigra* owing to their involvement in walnut allergy. Common and black walnut allergens were included in two superfamilies of proteins, namely the prolamin (Jug r 1, Jug n 1 and Jug r 3) and the cupin (Jug r 2, Jug n 2 and Jug r 4). More recently, another allergen (Jug r 5) was identified in walnut as belonging to a different family of proteins, the profilins.

At the time, proteins Jug r 1, Jug r 2, Jug r 3 and Jug r 4 from common walnut and Jug n 1 and Jug n 2 from black walnut were already included and classified as food allergens in the WHO-IUIS list of allergens [46]. The profilin Jug r 5 was not yet encompassed in the referred list, but it was already comprised in the Allergome database [47]. In this context, the allergens identified so far, as well as their clinical relevance concerning walnut allergy, will be extensively discussed in this review.

Prolamin superfamily

This superfamily of proteins was initially characterized by its solubility in alcohol/water mixtures, either in its

native state or after the reduction of interchain disulphide bonds and by its high content in amino acids such as proline and glutamine, thus justifying its designation (prolamin) [48, 49]. It encompasses three major groups of food allergens: the 2S albumins, the nonspecific lipid transfer proteins (nsLTP) and the cereal alpha-amylase/trypsin inhibitors. The members of the prolamin superfamily present a highly conserved pattern of cysteine residues located within a sequence of about 100 amino acids (aa). They exhibit six or eight cysteine residues, forming three or four intrachain disulphide bonds, respectively [49]. In general, these proteins have low molecular weight, high cysteine content, similar three-dimensional structures rich in alpha-helices and high stability to thermal processing and proteolysis [50]. Due to these characteristics, many of the allergens belonging to this superfamily are considered important class I food allergens responsible for inducing severe allergic reactions (e.g. anaphylaxis) in sensitized individuals [51].

2S Albumin family

Included in the prolamin superfamily of proteins, Jug r 1 and Jug n 1 were identified as allergens from common and black walnut, respectively. Sharing the same general characteristics of the prolamin superfamily members, the 2S albumins are considered water-soluble proteins at low salt concentrations, with a primary structure presenting high content in arginine, glutamine, asparagine and cysteine residues. After their synthesis, these small globular proteins (12–15 kDa) are subjected to sequence modifications that include the cleavage of 2S albumins into a large and small subunits (heterodimers) linked by conserved interchain disulphide bonds [52]. Owing to the presence of eight cysteine residues that are distributed in a conserved pattern, the conformational structure of 2S albumins is ensured by four disulphide bonds. During germination, the 2S albumins are thought to act as important nitrogen and sulphur donors, as a result of the amino acid composition of these proteins, their high abundance and their mobilization in the seed cells [38, 42]. In addition to their active role in the germination of seeds, other functions have also been attributed to some 2S albumins, namely antifungal properties.

Jug r 1

Jug r 1 protein presents a primary sequence of 139 aa and an estimated molecular weight of 16.4 kDa, being encoded by the nucleotide sequence *Juglans regia* 2S albumin seed storage protein precursor mRNA with 649 bp (Table 1) [46, 53]. The native walnut Jug r 1 is composed of two polypeptide chains of 3.5 kDa (light

Table 1. Identification of common and black walnut allergens according to their biological function, clinical relevance and respective accession numbers [53, 62]

Allergen	MW (kDa)	Isoallergens	Isoforms or variants	Biochemical classification	Biological Function	Clinical relevance	Nucleotide (NCBI) [53]	Protein (NCBI) [53]	Protein (UniProt) [62]
Jug r 1	16.4 (139 aa)	Jug r 1.01	Jug r 1.0101	2S albumin (prolamin superfamily)	Storage of nutrients for plant growth	Major allergen. Severe and systemic allergic reactions	U66866.1	AAB41308.1	P93198
Jug r 2	44 (593 aa)	Jug r 2.01	Jug r 2.0101	Vicilin (cupin superfamily)	Storage of nutrients for plant growth	Major allergen. Severe and systemic allergic reactions	AF066055.1	AAF18269.1	Q9SEW4
Jug r 3	11.8 (119 aa)	Jug r 3.01	Jug r 3.0101	Non-specific lipid transfer protein 1 (prolamin superfamily)	Transference of lipids across membranes. Antimicrobial activity	Major allergen. Severe and systemic allergic reactions	EU780670.1	ACI47547.1	C5H617
Jug r 4	58 (507 aa)	Jug r 4.01	Jug r 4.0101	Legumin (cupin superfamily)	Storage of nutrients for plant growth	Major allergen. Severe and systemic allergic reactions	AY692446.1	AAW29810.1	Q2TPW5
Jug r 5	No data	No data	No data	Profilin	Binds to actin and affects the structure of the cytoskeleton	No data	No data	No data	No data
Jug n 1	18.9 (161 aa)	Jug n 1.01	Jug n 1.0101	2S albumin (prolamin superfamily)	Storage of nutrients for plant growth	Major allergen Severe and systemic allergic reactions	AY102930.1	AAM54365.1	Q7Y1C2
Jug n 2	55.7 (481 aa)	Jug n 2.01	Jug n 2.0101	Vicilin (cupin superfamily)	Storage of nutrients for plant growth	Major allergen Severe and systemic allergic reactions	AY102931.1	AAM54366.1	Q7Y1C1

chain) and 8 kDa (heavy chain), respectively, coupled to two interchain disulphide bridges and noncovalent bonds, resulting from the processing of a single-chain precursor of 14 kDa [54]. Besides the two interchain disulphide bridges, the protein has two additional intra-chain disulphide bonds in the heavy chain, contributing to its globular conformation. Similarly to other 2S albumins, the tri-dimensional structure attributed to the native Jug r 1 consists of four alpha-helices connected by loops to spiral regions. Overlapping peptide scans prepared by Spot synthesis have been used to map interaction sites in Jug r 1. Accordingly, four epitopic regions have been identified and characterized in native Jug r 1, corresponding to stretches ⁶IDNPRR¹¹ (epitope #1, with 6 aa), ⁴²YDEDNQRQH⁵⁰ and ⁷²QVVRQQQ⁸⁰ (epitopes #2 and #3, respectively, with 9 aa each), and ¹⁰²CGISSQRCEIRR¹¹³ (epitope #4, with 12 aa) [54]. These epitopic regions have charged residues that coincide with the exposed electropositively (epitopes #1, #3 and #4) and electronegatively (epitope #2) charged areas on the surface of the allergenic protein. Other small IgE-binding epitopic stretches were identified during the epitope mapping of Jug r 1, namely ¹⁸EQI²⁰, ²⁴QNL²⁶, ³⁰QYY³² and ⁵⁴CCQ⁵⁶, although residues ²⁴QNL²⁶ and ³⁰QYY³² have little exposure on the surface of the protein, thus being considered of poor contribution to the allergenicity of this protein. The other two regions (¹⁸EQI²⁰ and ⁵⁴CCQ⁵⁶) are located near the epitopes #1 and #2, respectively, suggesting a more close participation of these residues in the IgE binding of walnut-allergic patients [54].

Teuber et al. [55] were the first to report the successful cloning of the recombinant Jug r 1 from common walnut into the frame-shifted plasmid expression vector using the Jug r 1 cDNA, with a purified fusion protein detected as a band of approximately 42 kDa. The recombinant Jug r 1 presented a deduced molecular weight of about 15–16 kDa, considering that the synthesis of foreign proteins expressed by the plasmid expression vector as a fusion product with a 26 kDa glutathione-S-transferase. The 2S albumin seed storage protein precursor (recombinant Jug r 1) with 139 aa exhibited a significant homology with the allergenic methionine-rich 2S albumin seed storage precursor from Brazil nuts, revealing 46% of identity with Ber e 1 [55]. Epitope mapping of the recombinant Jug r 1 evidenced that a linear stretch of 12 aa (³³QGLRGEEMV⁴⁴) of the large subunit was responsible for the strong IgE reactivity observed with the sera of walnut-allergic patients. Moreover, the presence of the core amino acids ³⁶RGE³⁹ and an additional residue of glutamic acid at position 42 are determinant for IgE binding to occur [56].

More recently, Sordet et al. [54] described the production of a correctly folded and fully functional recombinant Jug r 1. The recombinant allergen

consisted of an uncleaved single-chain precursor, thus differing from the native protein that is composed of two covalently linked light and heavy chains, resulting from the *in planta* processing of the single chain precursor. Although not submitted to processing, the recombinant Jug r 1 precursor evidenced an overall fold and a disulphide bonding profile that is virtually identical to the native protein. In accordance with these structural similarities, both native and recombinant proteins exhibited a very close IgE-binding reactivity suggesting that the recombinant Jug r 1 could be a useful tool for replacing native Jug r 1 in the component-resolved diagnosis of walnut allergy. Jug r 1 presented high sequence identity with other 2S albumins, namely with Ana o 3 from cashew nut (identity of 55% and similarity of 91%) [57] and with Car i 1 from pecan nut (identity of 88% and similarity of 92%) [58]. The high degree of sequence identity among 2S albumins from different plant species contributes to the immunological cross-reactivity between Jug r 1 and other allergenic 2S albumins. In this case, it is possible that both linear and conformational epitopes can play a role in the cross-reactivity phenomena observed among these proteins [59]. 2S Albumins present high pH and thermal stability (original folding at temperatures >90°C), mostly attributed to their compact and globular conformational structure, which is probably responsible for the resilient allergenic activity related to them [59, 60]. Supporting these evidences, the native Jug r 1 proteins reveal high resistance to enzymatic activity (trypsin/chymotrypsin) at basic pH (8.0), although they progressively lose allergenicity at acidic conditions (pH 1.3) in the presence of pepsin. In addition, native Jug r 1 molecules preserve their alpha-helical fold after heating at 90°C, being subsequently cooled at 20°C, which indicates a good resistance to heat denaturation [54]. Attending to these facts, the poor proteolysis of Jug r 1 at basic pH could contribute to the allergenic activity attributed to this allergen as well as to other 2S albumins.

Jug r 1 allergen is considered of great clinical importance because it has been suggested as being associated with severe allergic symptoms in walnut-allergic patients. To support this evidence, in a study reported by Teuber et al. [55], the sera of 12 of 16 walnut-allergic patients with severe clinical presentations demonstrated IgE binding to the 2S albumin seed storage precursor fusion protein (Jug r 1). Considering that more than 50% of sera from walnut-allergic patients were reactive to Jug r 1, this protein was defined as a major allergen in walnut [55, 61]. The high stability of their intrinsic protein structure enables the 2S albumins to cause sensitization directly *via* the gastrointestinal tract, which classify them as potent class I food allergens. Allergic reactions to 2S albumins from different plant sources, such as Brazil nut, mustard, sesame and

almond, have been described with increasing frequency [59]. Although mild symptoms have also been associated with this class of proteins, it is important to emphasize the relatively high frequency of the occurrence of severe reactions, including laryngeal angioedema and anaphylaxis.

Jug n 1

Jug n 1 is an allergen identified in black walnut, whose primary sequence is constituted by 161 aa (Table 1). Classified as a 2S albumin, this protein is encoded by a nucleotide sequence of 574 bp with an estimated molecular weight of 18.9 kDa [46, 62]. A BLAST search indicates that this allergen presents high sequence identity with other 2S albumins from nuts, namely 96% with Jug r 1 (*Juglans regia*), 87% with Car i 1 (*Carya illinoensis*) and 57% with Cor a 14 (*Corylus avellana*) [46, 53]. The shared homology of those allergens could partly explain the cross-reactivity observed among these different nuts. Although the information about this allergen is very scarce, Ling et al. [63] described the cloning of a protein with 234 aa (Jug n 1) from a cDNA clone of black walnut library with 749 bp, presenting IgE-reactivity with sera from walnut-allergic patients. In addition, this cloned protein also evidenced 83%, 82% and 81% of sequence homology with rubber tree manganese superoxide dismutase (MnSOD), rice MnSOD and tobacco MnSOD, respectively. Still, more detailed information is needed for a more precise biochemical characterization of Jug n 1 allergen from black walnut.

From a clinical point of view, Jug n 1 should be considered as important as Jug r 1 as both belong to the family of 2S albumin and share high degree of homology (>97%). Although black walnut is clearly less consumed, most of walnut-allergic patients could experience adverse immunological responses to this species as the result of prior sensitization to common walnut [39]. Preliminary reports seem to suggest a classification of major allergen in case of black walnut as the sera of 8 of 12 walnut-allergic patient tested positively for Jug n 1 [39]. In an interesting study performed by Comstock et al. [64], extensive *in vitro* cross-reactivity to seed storage proteins, among different walnut cultivars and species, was demonstrated. Consequently, patients with severe allergy to common walnut are expected to be clinically allergic to all commercial walnut cultivars and to other closely related species from *Juglans* genus.

nsLTP family

As part of the prolamin superfamily, the nsLTP are classified as important plant seed allergens [51, 65]. They

are biochemically defined as monomeric proteins of low molecular size, with primary sequences rich in cysteine residues that contribute to secondary structures composed of alpha-helices, involving a lipid binding cavity in the core. The members of this family share common structural features encompassing a characteristic eight cysteine motif (four disulphide bonds) and basic isoelectric points (pI~9) [66, 67]. The nsLTP are divided into two subfamilies of 9 kDa (nsLTP 1) and 7 kDa proteins (nsLTP 2), both with biological functions related to the transport of different classes of lipids (fatty acids, phospholipids, glycolipids and sterols) through membranes [65, 67]. Although performing identical tasks, proteins from the two subfamilies present a low overall similarity regarding the amino acid sequence, sharing only approximately 30% of identity [67]. To the nsLTP, other biological functions can also be attributed, namely active roles in plant protection (antifungal and antibacterial properties) [68] or potential involvement in plant growth and development (embryogenesis, germination) [66, 69]. With a wide distribution throughout nature [65], the nsLTP are commonly stimulated by biotic and abiotic plant stress factors, which is a typical trait of the superfamily of PR proteins. Therefore, the nsLTP are also known as the PR-14 protein family [51].

The nsLTP are extremely resistant to heat treatment and proteolytic digestion, which enables them to remain intact during food processing and to survive to the harsh environment of the gastrointestinal tract [70, 71]. As a result, LTP are classified as class I food allergens, indicating a possible direct pattern of sensitization via oral ingestion. Still, it is also important to refer that some pollen LTP have been reported to behave as primary sensitizing agents in patients presenting IgE reactivity to both mugwort and peach LTP [72], thus suggesting a possible involvement of these proteins in class II food allergies [40].

The sensitization to nsLTP appears to be affected by geographical differences and dietary habits [73], being frequently associated with severe symptoms of food allergy. In this regard, LTP from Rosaceae fruits have been considered as major allergens in the Mediterranean area, whereas in Central and Northern Europe, allergy to fruits from this family seems to be more closely related to birch pollinosis (sensitization to Bet v 1-homologues proteins) rather than to LTP. Therefore, LTP are thought to induce food allergies through different sensitization pathways, namely primary sensitization to a food LTP without pollen allergy; primary sensitization to a food allergen with pre-existing pollen allergy or primary sensitization to a pollen allergen. Regardless the sensitization pathway, individuals may suffer from food allergy, though with distinct clinical symptoms [74].

Taking into consideration these characteristics, together with their great dissemination among organisms, nsLTP have been regarded as pan-allergens, being even proposed as model allergens for true food allergy [70, 74, 75].

Jug r 3

Included in the nsLTP, the Jug r 3 protein has been classified as a food allergen in walnut seeds [46, 47]. It presents a primary structure of 119 aa (Table 1) encoded by the nucleotide sequence *Juglans regia* non-specific lipid transfer protein mRNA with 360 bp [53]. With an isoelectric point of 9.45 and a molecular size of 9.2 kDa, the purified LTP seems to exclude any glycosylation. The N-terminal sequence of the first 30 amino acids exhibited a high degree of homology (80%) with other fruits, namely peach and apricot [76]. According to the NCBI database [53], upon protein alignment of relevant allergenic nsLTP from different species, Jug r 3 exhibited a sequence identity of 60% with Cor a 8 from hazelnut (accession no. AAK28533.1), 59% with Pru av 3 from cherry (accession no. AAF26449.1), 57% with Ara h 9 from peanut (accession no. ABX56711.1) and 53% with Pru du 3 from almond (accession no. ACN11576.1). Owing to this apparent relationship between Jug r 3 and other allergens from fruits/seeds of the Rosaceae family (almond, cherry, peach), cross-reactivity among them is potentially high [77]. The close sequence homology of LTP allergens from Jug r 3 and peach was evidenced by the IgE cross-reactivity between these proteins, suggesting the IgE-binding epitopes of walnut LTP are also present in peach LTP [76]. The nsLTP are mainly located in the outer epidermal layers of fruits such as peach, which in some cases allow allergic patients to tolerate peel-off fruits. However, in the case of nuts that are commonly eaten without removing the outer layer (peel), the allergic patients are still at risk of developing adverse immunologic reactions upon their consumption [78]. The effect of food processing on Jug r 3 allergen is not yet clear, although heat treatments above 90°C during long periods appear to reduce the allergenicity of some nsLTP such as Mal d 3 from apple [79] and Cor a 8 from hazelnut [80].

In terms of clinical relevance, the nsLTP are normally associated with severe and systemic allergic reactions that can be potentially life-threatening [65, 81]. In a study performed by Pastorello *et al.* [76], which was specifically designed to identify the allergens involved in walnut allergy of Italian patients, confirmed that this nut is able to provoke very severe allergic reactions. Besides the fact of many of the selected patients had a convincing history of life-threatening reactions to walnut, some of the individuals with history of oral itching

(oral allergy syndrome – OAS grade I) evidenced aggravated symptoms at the oral provocation test (OAS grade II). From a total of 46 Italian patients with mild (OAS) or severe clinical symptoms, 36 individuals evidenced IgE reactivity to LTP (Jug r 3). This protein exhibited IgE cross-reactivity with peach major allergen LTP, suggesting that all of the IgE-binding epitopes of walnut LTP are also present in peach LTP. This fact indicates that many of the patients allergic to walnut were primarily sensitized to peach and only secondarily to walnut. According to these evidences, Jug r 3 has been proposed as a major allergen [76]. Additionally, the walnut-allergic individuals reacting to LTP often exhibited severe clinical presentations, thus confirming the findings of previous reports [82, 83]. Regarding those patients, they are most likely to be also allergic to other fruits containing LTP, such as apricot, cherry, plum, apple, grape, maize and hazelnut. As a consequence, allergy to walnut can be considered a clinical manifestation of the LTP syndrome [76].

In the Mediterranean area, IgE reactivity to walnut is strongly associated with sensitization to LTP [73]. More recently, in a study described by Asero et al. [84] for the evaluation of genuine allergy to walnut and hazelnut, skin prick tests were performed and sera was collected from Italian allergic patients, excluding those sensitized to cross-reacting plant-food allergens (PR-10, profilins and LTP). In the specific case of walnut, IgE reactivity can be related to other allergens rather than those already identified so far [84]. In the same report, the authors also verified that walnut allergy was mainly observed among young patients, indicating that in most of the cases sensitization to genuine nut allergy occurs at a rather young age.

Cupin superfamily

This superfamily encompasses a large and multifunctional variety of proteins that are estimated to be originated by divergent evolution from a common ancestor, whose presence is transversal to different kingdoms (Bacteria, Plants and Animals) [42, 85]. These proteins were collectively designated as cupins due to the existence of a beta-barrel motif in their tri-dimensional structure [86]. Based on the presence of one or two cupin domain(s), the members of this superfamily are classified as mono- or dicupins, respectively. The functional class of dicupins includes the 7S and 11S globular seed storage proteins, which represent major protein components of several plant foods, namely tree nuts. Owing to their different sedimentation coefficients, globulins are divided in two groups: the vicilins (trimeric 7S globulins) and the legumins (hexameric 11S globulins) [48, 87]. Among tree nuts, as the edible part lies on the kernel, seed storage proteins are major

components of the nutrient reservoir. As consequence, both legumins and vicilins are considered important allergens in this group of seeds.

Vicilin family (7S globulins)

The vicilin-like proteins belong to the cupin superfamily, presenting a structure of two conserved beta-barrel motifs classifying them as bicupins. Included in the vicilin family of proteins, the Jug r 2 and Jug n 2 were identified as allergens of *Juglans regia* and *J. nigra*, respectively. The 7S globulins are proteins with a total molecular weight of 150–190 kDa and a trimeric structure, being composed of three subunits with molecular weights ranging from 40 to 80 kDa each [88]. Vicilins lack residues of cysteine in the primary sequence [89], and thus, their structural stability is ensured by non-covalent hydrophobic interactions, hydrogen bonds and van der Waals interactions. The 7S globulins are structurally composed of N-terminal and C-terminal domains, containing a beta-barrel motif. Unlike the 11S globulins, the vicilins are commonly glycosylated, with one or two N-linked glycosylation sites located in the C-terminal domain [90]. In legumes, vicilins are considered important seed storage proteins, playing a role as nitrogen donor during seed germinating and acting as plant protection proteins (namely antifungal activity) [91].

Jug r 2

The Jug r 2 protein has been identified as an important allergen in common walnut [46]. The primary structure of this protein presents 593 aa (Table 1) encoded by the nucleotide sequence *Juglans regia* vicilin-like protein precursor, mRNA with 2057 bp [53]. The recombinant protein Jug r 2 was first reported by Teuber et al. [91], who subcloned the Jug r 2 cDNA into the frame-shifted plasmid vector pGEX-4T-3. Considering that the recombinant Jug r 2 is expressed in a fusion protein (92 kDa) containing a glutathione-S-transferase of 26 kDa, the estimated molecular weight for the resulting Jug r 2 was 66 kDa. As Jug r 2 cDNA encodes a proprotein, the molecular weight of the mature vicilin is about 47 kDa (44 kDa in 12% acrylamide) after the cleavage of the precursor at amino acid position 173 [91]. Jug r 2 is considered a complex trimeric protein like several other plant vicilins. Similarly to the 7S globulins of soybean (Gly m 5) and peanut (Ara h 1), Jug r 2 from walnut was also classified as an allergen. A BLAST search evidences a high sequence similarity of this protein, not only with the allergic vicilin from black walnut (Jug n 2), but also with vicilins from different plant sources, namely other tree nuts. Accordingly, Jug r 2 exhibits a sequence identity of 97% with Jug n 2 (black walnut,

accession no. AAM54366.1), 47% with Cor a 11 (hazelnut, accession no. AAL86739.1), 43% with Ses i 3 (sesame, accession no. AAK15089.1) and 38% with Pis v 3 (pistachio, accession no. ABO36677.1) [53]. Jug r 2 also presents 92% of sequence similarity with Car i 2 allergen (accession no. ABV49590.1), though this vicilin from pecan nut was not yet included in the WHO-IUIS list of allergens [46, 53].

As consequence of close sequence similarity and identity of Jug r 2 with other vicilins, Barre *et al.* [92] reported the characterization of surface-exposed IgE-binding epitopes on the molecular organization of tri-dimensional models of the vicilin allergens of walnut (Jug r 2), hazelnut (Cor a 11), cashew nut (Ana o 1) and peanut (Ara h 1). When comparing the IgE-binding epitopes of those allergenic vicilins, Jug r 2 was found to be structurally more closely related to Ara h 1, presenting an overall sequence identity of 54% with this peanut allergen [92]. With similar conformational IgE-binding epitopes, cross-reactivity occurrences among vicilins from different plant sources are to be expected.

These proteins exhibit considerable thermal stability, which allows them maintaining their conformations at temperatures below 70–75°C [90]. Still, when submitted to high temperatures, 7S globulins may suffer structural disruptions and covalent modifications with special emphasis for those involved in glycation processes or Maillard rearrangements [90]. Depending on the type of food processing, these alterations are likely to affect the allergenicity of vicilins, which can even be potentially increased. The allergenicity of Jug r 2 was evaluated after treating walnuts with single or combined cooking processes, which included gamma-irradiation (1–25 k Gy), microwave (500 W, 1 and 3 min), roasting (138°C and 160°C–30 min; 168°C and 177°C–12 min), frying (191°C, 1 min), blanching (100°C, 5 and 10 min) and/or autoclaving (121°C, 15 psi, 15 and 30 min) [93]. With this study, it was verified that walnuts were antigenically stable to all the tested procedures as indicated by the stability of the polypeptide profiles. Additionally, the results confirmed that roasting, frying above 191°C or microwave heating had no significant effect on immunoreactivity of walnut. After autoclaving, the immunoreactivity of walnut proteins tested by ELISA was apparently not affected, though results of Western blot analysis showed a decrease on the recognition of 42–45 kDa Jug r 2 proteins.

Jug r 2 is also considered of high clinical importance being classified as a major allergen in walnut [91]. This classification was endorsed to this protein in a study performed by Teuber *et al.* [91], 9 of 15 sera of patients with severe and systemic walnut allergy were reactive to Jug r 2. Presenting high similarity (70%) with the homologue protein in peanut (Ara h 1), which is responsible for the majority of the reported cases of

fatal anaphylaxis [38, 39], Jug r 2 is also expected to induced severe symptoms in walnut-allergic patients. Despite the high similarity between Jug r 2 and Ara h 1, minimal *in vitro* cross-reactivity was demonstrated between peanut and walnut proteins. When testing patients with clear IgE reactivity to Jug r 2, no evidence of high-affinity cross-reactive IgE with Ara h 1 (crude peanut extract) could be observed [91]. Contrarily to the results obtained by Teuber *et al.* [91] using sera from USA patients, in a study performed by Pastorello *et al.* [76] among Italian allergic patients, vicilins were considered as minor allergens as only 9 of 46 allergic patients presented IgE reactivity with vicilin precursor A. In the case of Jug r 3, the majority of the patients were sensitized to different plant foods other than walnut, but the individuals sensitized to vicilins (Jug r 2) were exclusively allergic to walnut. Most of vicilin-sensitized patients had several episodes of anaphylaxis and/or glottis oedema, even after the ingestion of minute quantities of walnut present as a hidden allergen. This finding is in good agreement with the observations described by Teuber *et al.* [91], whose patients had life-threatening reactions. In addition to this, Pastorello *et al.* [76] also verified that sensitization to vicilin can occur through the gastrointestinal route, thus confirming a possible classification as class I food allergen. Like the walnut LTP (Jug r 3), the vicilin (Jug r 2) is also considered a true food allergen because in both cases, these allergens are able to sensitize patients not allergic to pollen, triggering severe and systemic allergic reactions [76].

Jug n 2

An important allergen in black walnut corresponds to the protein Jug n 2, which has been identified as a vicilin from the cupin superfamily. This protein, presenting an estimated molecular weight of 55.7 kDa, is encoded by the *Juglans nigra* vicilin seed storage protein mRNA with 1732 bp, and it is composed of a primary structure of 481 aa (Table 1) [46]. Like for Jug r 2, Jug n 2 also exhibits high sequence identity with other allergenic vicilins, namely 92% with Car i 2 (accession no. ABV49590.1), 46% with Cor a 11 and with Ses i 3 (accession no. AAL86739.1 and AAK15089.1, respectively) and 39% with Pis v 3 (accession no. ABO36677.1) [53]. Regarding Jug n 2 allergen, little information has become available. Ling *et al.* [63] reported the cloning of a protein with 338 aa (Jug n 2) from a sequence of cDNA of black walnut library with 1317 bp. The produced protein evidenced IgE reactivity with sera from walnut-allergic patients. In addition, the cloned protein also exhibited 84% and 83% of sequence homology with pea and tobacco glyceraldehydes, respectively [63]. Jug n 2 has been included in the WHO-IUIS list of

allergens, which only occurs after careful evaluation of the allergen submission, according to the molecular and immunological requirements established for inclusion into the allergen nomenclature that is made by specialized members of the Executive Committee. However, in the case of Jug n 2, this information has not been made available for common researchers [46]. Therefore, further information about molecular characterization and allergenicity assessment of Jug n 2 is needed.

Based on the high homology between Jug n 2 and Jug r 2 allergens, walnut-allergic patients are also expected to experience adverse immunological reactions when ingesting black walnut. However, specific information regarding the clinical presentation of Jug n 2 is also missing, indicating that relevant studies or even case reports should be made available for clinicians, researchers and caretakers in general.

Legumin family (11S globulins)

The other class of functional proteins included in the bicupins relates to the 11S globulins that are also designated as legumins. Mature 11S globulins are nonglycosylated hexameric proteins with a total molecular weight of approximately 360 kDa. The structure of legumins is determined by six monomers interacting non-covalently and displayed in an open ring conformation [48]. Each of the six monomers is initially synthesized as a unique polypeptide, being post-translationally cleaved into two polypeptide chains, one acidic (30–40 kDa) that is linked by a single intermolecular disulphide bond to a basic polypeptide of approximately 20 kDa [90]. The mature and functional 11S globulins (legumins) are rarely glycosylated, in opposition to other bicupins (e.g. vicilins).

Jug r 4

Jug r 4 is classified as a 11S globulin (legumin-type), which is encoded by a nucleotide sequence of *Juglans regia* seed storage protein mRNA with 1524 bp (Table 1) [46]. The cloning of walnut cDNA into a maltose-binding protein (MBP) expression vector pMAL-c2X and transforming into *Escherichia coli* was reported by Teuber et al. [94] and Wallowitz et al. [95]. With the first 23 aa residues predicted to be a signal peptide, the open reading frame was 507 aa in length, accounting for a molecular weight of 58.1 kDa and an isoelectric point of 6.8. The estimated molecular size relates to a single subunit of walnut legumins as these proteins are hexamers (six subunits) [95]. Sequence identity among allergenic legumins from different plant sources is also frequently high. The results from multiple sequence alignments of Jug r 4 with other allergenic legumins revealed 95% identity with Car i 4 from pecan nut

(accession no. ABW86978.1), 72% with Cor a 9 from hazelnut (accession no. AAL73404.1), 57% with Ana o 2 from cashew nut and Pis v 5 from pistachio (accession no. AAN76862.1 and ACB55490.1, respectively), 54% with prunin-2 that is part of Pru du 6 allergen from almond (accession no. ADN39441.1) and 51% with Ber e 2 from Brazil nut (accession no. AA038859.1) [53]. The comparison of these sequences enables suggesting that the majority of the differences among legumin proteins are predominantly located in one of three sections: in the first 34 amino acids (including the signal peptide), in amino acids 210–231, and in the last residues 500–518. However, the verified differences in the region containing the signal peptide (23 aa) are not relevant for the analysis of the mature legumin expressed in walnut because signal peptide is cleaved during translation. According to Wallowitz et al. [95], the alignment of legumins from walnut, cashew, hazelnut and peanut suggests that the major cleavage site separating the protein into acidic (larger) and basic (smaller) subunits is predictably located between residues 315 and 316. The cleavage site containing an Asn-Gly peptide bond seems to be well conserved among a wide variety of plant species. Jug r 4 and several other legumins possess the NGXEET motif: NGLEET is characteristic in Jug r 4 from walnut and in Pru du 6 from almond, NGFEET in Cor a 9 from hazelnut, NGIEET in Ana o 2 from cashew and in Ara h 3 from peanut [96]. Linear IgE-binding epitopes identified in legumins from tree nuts and peanut have been mapped using tri-dimensional models of the Jug r 4 (walnut), Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Ara h 3 (peanut) proteins. The analysis of the conformational organization of these legumins revealed some structural homology on the surface-exposed epitopes that could explain the IgE-binding cross-reactivity observed among tree nut allergens [97]. These evidences support the occurrence of cross-reactivity between the recombinant Jug r 4 and protein extracts from hazelnut (Cor a 9), cashew (Ana o 2) and peanut (Ara h 3) [95]. More recently, Robotham et al. [98] sequenced the four epitopic regions (HS#1 to HS#4) most frequently recognized by patient IgE, terming these binding epitopes as 'hot spots' and mapping the antigenic surfaces onto tri-dimensional models. HS#2 and HS#4 IgE-binding epitopes share primary sequence similarity between Jug r 4 and Cor a 9, (100% for HS#2 and 86% for HS#4) and appear to be structurally related, as evidenced by similarities in the surface topology and charge distribution regions [98]. Epitopic region HS#1 is also similar among Jug r 4 and other legumins, namely Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Gly m 6 (soybean), but not with Ara h 3 (peanut). The majority of the residues are located in the inner part of the structure, indicating that this epitopic region is only

available when the monomeric subunit is denatured and/or fragmented [98].

Like the vicilins, the legumins share a propensity to form large thermally induced aggregates with high tendency to mould heat-set gels and function as emulsifiers [60]. In general, legumins present a high thermal stability responsible for preserving their conformational structures at temperatures below 94°C, and great resistance to proteolysis, which enable to retain their allergenic properties along the gastrointestinal system [90]. Owing to these properties, allergenic legumins can be classified as class I food allergens. The general treatments used in food processing such as heat (roasting, blanching and autoclaving) and radiation (gamma and microwave) are not likely to alter the immunoreactivity of Jug r 4 protein. As demonstrated by Su *et al.* [93], only the combination of gamma-irradiation (25 kGy) and autoclaving for 30 min show a reduction in the recognition of proteins of 45–66 kDa (identified as Jug r 4) by Western blot analysis, but not when tested for immunoreactivity by ELISA [99].

From an immunological and clinical point of view, Jug r 4 is also considered an important major allergen in common walnut. According to a study performed by Teuber *et al.* [94], the sera of 15 individuals from a test population of 23 patients with severe and systemic reactions to walnut evidenced IgE reactivity to the fusion protein containing Jug r 4. This allergen proved to be a major allergen in walnut because more than 65% of the test population was reactant to it. In a different study conducted by Wallowitz *et al.* [95], the sera from 37 patients with histories of potentially life-threatening systemic reactions to walnut, involving bronchospasm, hypotension, or laryngoedema/throat swelling, were tested to verify IgE reactivity with recombinant Jug r 4. According to this report, the sera of 21 individuals evidenced positive IgE reactivity to this allergen (>57%), thus confirming the previous designation of major allergen. Additionally, Jug r 4 displayed significant sequence homology with other allergenic legumins, namely with Cor a 9 (hazelnut), Ana o 2 (cashew nut) and Ara h 3 (peanut), contributing to cross-reactivity occurrences, as already was confirmed *in vitro* for those nuts [95].

Profilins

Profilins assemble a family of small cytosolic molecules (12–15 kDa), presenting highly conserved proteins that share close sequence identities (>75%), even among organisms distantly related [65]. As consequence of the abundant sequence conservation, profilins exhibit highly similar structures and biologic functions [42]. Biochemically, profilins are actin-binding proteins involved in the dynamic turnover and

restructuring of the actin cytoskeleton. They belong to the alpha-beta-class of proteins, evidencing a structure mainly composed of two alpha-helices and five-stranded anti-parallel beta-sheets. Profilins perform an important role in cell-motility through the regulation of actin microfilament polymerization dynamics [42]. The restructuring of the actin cytoskeleton is essential for processes such as the organ development, the wound healing and the hunting down of infectious intruders by cells of the immune system. In plants, these proteins perform an active role in the cytokinesis, the cytoplasmic streaming, the cell elongation and the growth of root hairs and pollen tubes [42, 65, 100]. In addition to actin, profilins have also been described to bind different ligands such as phosphoinositides and poly-L-proline stretches, which suggests their involvement in other biological processes, for example, membrane trafficking and organization, signalling pathways [65, 101]. Due to their wide participation in many essential cellular processes, profilins can be found in most cells of eukaryotic organisms. Consequently, they are also considered as pan-allergens being responsible for several of the observed cases of cross-reactivity between inhalant and food allergens [102] and for the development of class II food allergy [71]. In general, profilins are sensitive to heat denaturation and gastric digestion, suggesting their involvement in allergic reactions that are usually confined to moderate oral symptoms (OAS), as the result of the consumption of raw or poorly processed foods (e.g. fruits) [71]. In contrast to nonpollen-related class I food allergy that is very frequent among young children, class II food allergy is more commonly observed in adults as a consequence of sensitization to inhalant allergens (e.g. pollen) [38].

Jug r 5

Regarding the profilin Jug r 5 identified in walnut, very little information is available. This protein is classified as a food allergen by the ALLERGOME database [47], presenting IgE activity in the *in vitro* nonfunctional test and cross-reactivity with the allergenic profilin Lol p 12 from grass. The route of exposure to this allergen is by ingestion because Jug r 5 is present in walnut seed tissues [47]. Jug r 5 has not yet been included in the WHO-IUIS list of allergens [46], thus its classification as an allergen is rather imprecise. The clinical symptoms related to profilins are commonly considered mild and mainly restricted to the oral cavity (OAS), mostly occurring as the result of the ingestion of raw foods [65]. The profile of profilin sensitization is still unclear, as clinical studies seem to suggest that patients with profilin-specific IgE are frequently asymptomatic or at risk of evolving multiple pollen-related food allergies [103].

With respect to Jug r 5, the pattern of clinical manifestations associated with this walnut profilin is unknown, although it is expected to be similar to symptoms described for other plant profilins.

Walnut detection

Even when following a restrict diet with total avoidance of the offending foods, the sensitized/allergic individuals can still be at risk of suffering abnormal immune episodes as consequence of accidental exposure to hidden allergens in foods owing to incorrect labelling or cross-contaminations during food processing. Therefore, to verify labelling compliance [9, 10], to help the industrial management of food allergens and to ensure consumer's safety, the development of proper and highly sensitive analytical methodologies has attained special emphasis [104].

Presently, the need for adequate methodology has prompted the development of numerous techniques for the evaluation of most allergenic ingredients. Nevertheless, the lack of available testing/reference materials and the absence of official methods for their detection and quantification represent main difficulties in the management of food allergens. In addition, the absence of consensus towards the best methodology for allergen detection is still a matter of extensive debate among researchers. Until now, protein- and DNA-based methods have been considered useful tools for the evaluation of allergenic ingredients in foods. Among the available techniques, the choice of a method lies on specific criteria such as target analyte (proteins vs. DNA), basis of detection (e.g. chemical), cross-reactivity, set-up cost, running cost, the need for expertise knowledge and possibility of multitarget detection [105]. Based on these criteria, protein-based methods as enzyme-linked immunosorbent assays (ELISA) or DNA-based techniques like polymerase chain reaction (PCR) are often preferred. Actually, a reasonable number of methods, either based on proteins or DNA, is available for the detection of walnut as an ingredient or a potential hidden component in foods.

Protein-based methods

Within the protein-based methods, two main groups of techniques can be distinguished: one group based on allergen/antibody interactions such as lateral flow devices (LFD), ELISA, immunosensors and other relevant immunoassays (e.g. immunoblotting); and a second group covering the mass spectrometry (MS) platforms. So far, the methods based on the immunoassays are among the most popular, though recently the MS methods have also conquered a special role in the detection and quantification of allergens in foods.

Lateral flow devices or dipsticks

This type of tests provides qualitative or semi-quantitative information that can be simply read visually. Besides this main advantage, the LFD are of simple and rapid performance, without needing specialized equipment or personnel [106]. Presently, three different LFD can be commercially acquired for the detection of walnut in foods (Table 2). The sensitivity of the LFD ranges from 2 to 10 mg/kg of walnut in foods, allowing the performance of the test in less than 15 min. Therefore, these strips are regularly used by the food industry for allergen control, enabling to answer the need for onsite quick results. However, the LFD present main drawbacks such as the lack of providing quantitative information and the high propensity to false negative results owing to the high degree of food processing and/or to the complexity of the matrix. Consequently, these tests need confirmation by means of other immunological methods of higher sensitivity and precision, namely the ELISA [107].

ELISA

The immunochemical assays such ELISA are by far the most widely used, due to their direct assessment of the allergen and/or marker protein, low set-up cost, moderated running time and no special requirements for expertise knowledge [105]. Additionally, the ELISA tests can provide quantitative information, which is one major requisite in allergen analysis. Resulting from these clear advantages, the immunoassays have been extensively applied to a wide variety of food allergens, in which walnut is included. Currently, some ELISA kits have been commercially available for the detection of walnut in foods (Table 2). They enable the detection of walnut protein down to 0.25–0.35 mg/kg in several food matrices such as cookies, ice-creams or chocolates (Table 2). However, in most kits, the stated performance parameters such as the limit of detection (LOD) can vary according to the type of food matrix. As expected, several ELISA kits present elevated cross-reactivity with other plant species, especially with pecan nut that belongs to the same botanic family (Juglandaceae) of walnut. Cross-reactivity with tree nuts (pistachio, hazelnut, Brazil nut, chestnut, pine nut) and other plant species (quinoa, sesame, buckwheat and soybean) are also referred to be frequent (Table 2).

Besides the commercial kits, noncompetitive sandwich-type ELISA and indirect competitive ELISA have been proposed in the literature to trace walnut allergens or marker proteins in foods (Table 3). Within the methods available so far, ELISA using polyclonal antibodies against the entire fraction of walnut proteins or the allergen Jug r 1 have been proposed for the detection

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

Commercial kits/Brand	Assay type	Cross-reactivity	LOD	LOQ	Estimated time to perform assay
Lateral Flow Walnut (R-Biopharm AG Darmstadt, Germany)	LFD	Pecan nut: 100%	10 mg/kg	–	~15 min (sample preparation)
Walnut Protein Rapid Test (Elution Technologies, Vermont, USA)	LFD	No cross-reactivity observed	2 mg/kg	–	~10 min (applied to extracted sample)
AgraStrip® Walnut (Romer Labs Division Holding GmbH, Austria)	LFD	No available information about the specificity	–	–	~11 min
BioKits Walnut Assay Kit (NEOGEN Corporation, Michigan, USA)	Polyclonal antibody specifically detects walnut proteins, sandwich ELISA (48 wells)	Pecan (2.3%), Quinoa (0.0012%), Pistachio (0.0009%), Hazelnut (0.0005%), Buckwheat (0.00024%)	0.25 mg/kg	2.4–120 mg/kg	~40 min (sample extraction) + ~75 min (incubation time)
AgraQuant® Walnut Assay (Romer Labs Division Holding GmbH, Austria)	Quantitative - Sandwich ELISA	No available information about the specificity	0.35 mg/kg	2–60 mg/kg	~40 min (sample extraction) + ~60 min (incubation time)
Walnut Protein ELISA Kit (Elution Technologies, Vermont, USA)	Sandwich ELISA	No cross-reactivity observed	–	1 mg/kg	~60 min (applied to extracted sample)
Walnut ELISA Kit (Creative Diagnostics, Shirley, New York, USA)	Quantitative - Sandwich ELISA	8/35 species. Pecan nuts (0.8%), chestnut (0.11%), hazelnut (0.02%), soybean, pine nuts, pistachio, sesame and brazil nuts (0.0003%)	0.35 mg/kg	2 mg/kg	~60 min (applied to extracted sample)
Walnut, Food, BioAssay™ ELISA Kit (<i>Juglans regia</i>) (US Biological, Salem, Massachusetts, USA)	Quantitative test ELISA - Sandwich ELISA	No available information about the specificity	0.35 mg/kg	–	~60 min (applied to extracted sample)
DAI Walnut ELISA (Diagnostic Automation/ Cortez Diagnostics, Inc., Calabasas, California, USA)	Quantitative - Sandwich ELISA	8/35 species. Pecan nuts (0.8%), chestnut (0.11%), hazelnut (0.02%), soybean, pine nuts, pistachio, sesame and brazil nuts (0.0003%)	0.35 mg/kg	2 mg/kg	~60 min (applied to extracted sample)
SureFood Allergen Walnut (R-Biopharm AG Darmstadt, Germany)	Real-time PCR (qualitative)	Pecan nut	≤5 DNA copies, ≤0.4 mg/kg	10 DNA copies, 10 mg/kg	~35 min (applied to extracted sample)
NutsKit Real Time - Walnut and Pecan nut DNA detection Real-Time PCR kit (InCura Srl, Casalmaggiore, Cremona, Italy)	Real-time PCR (qualitative)	0/20 plant- and animal-derived foods	1 copy of walnut or pecan nut	–	~35 min (applied to extracted sample)

of walnut in a wide variety of food matrices. Presenting low LOD (<1 mg/kg of walnut protein) and excellent recoveries, all the proposed methods can be applied to determine the presence of walnut as a potentially hidden allergen in processed foods (Table 3). However, the high propensity of these assays to cross-react with other nuts (e.g. pecan nut, Brazil nut, macadamia) and

different plant foods (e.g. mustard, peanut) should always be taken in consideration [108–111].

The ELISA methods are totally dependent on the interaction between the antibodies and the specific proteins (allergens or marker proteins). On the basis of this interaction, the high susceptibility to cross-react with other nontarget proteins can lead to false-positive

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

Method	Antibody (immunization)/target protein	Cross-reactivity	Sensitivity level	Applied food matrices	References
Sandwich ELISA	Rabbit antibodies (raised against walnut 2S albumin protein fraction). Walnut variety - Chandler	2/10 plant-derived foods. Strong cross-reactivity with pecan nut (100 mg/kg), hazelnut (1 mg/kg), brazil nut, almond, pine nut, peanut, cashew nut, pistachio, macadamia and mustard (<0.31 mg/kg which was the LOQ of the calibration curve)	LOQ < 0.31 mg/kg and LOD < 0.16 mg/kg of walnut protein defined by the calibration curve	Model processed foods containing 10 mg/kg of walnut (chicken meatballs, rice porridge, bread, sponge cake, orange juice, jelly, biscuit)	[108]
Sandwich ELISA	Rabbit antibodies (raised against walnut 2S albumin protein fraction). Walnut variety - Chandler (same system as Doi et al. [108])	2/10 plant-derived foods. Strong cross-reactivity with pecan nut (100 mg/kg), hazelnut (1 mg/kg), brazil nut, almond, pine nut, peanut, cashew nut, pistachio, macadamia and mustard (<0.31 mg/kg which was the LOQ of the calibration curve)	LOQ < 0.31 mg/kg and LOD < 0.16 mg/kg of walnut protein defined by the calibration curve	Model processed foods containing 10 mg/kg of walnut (chicken meatballs, rice porridge, bread, sponge cake, orange juice, jelly, biscuit)	[109]
Sandwich ELISA	Polyclonal rabbit and sheep antibodies (raised against several varieties of raw and toasted English walnuts)	5/80 animal- and plant-derived foods and other ingredients. Cross-reactivity with pecan nut (40% of cross-reactivity with pecan for the same concentration), mustard, poppy seeds, hazelnut and mace (1 to 3 mg/kg)	1 mg/kg of walnut in several food matrices	Incurred standards (walnut in chocolate milk 0–100 mg/kg) Spiked samples (chocolates, muffins, cookies and ice-creams)	[110]
Indirect competitive ELISA	Rabbit polyclonal antibodies (raised against recombinant fusion protein Jug r 1)	0/22 plant- and animal-derived food. Considering that IC50 of walnut was 1.94 mg/kg and all the IC50 from other species were > 1000 mg/kg	LOD of 0.22 mg/kg and LOQ of 0.44 mg/kg	Model mixtures of wheat, soybean, rice or corn powder spiked with walnut powder (10 mg/kg)	[111]
LC-ESI-LIT-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (2 Jur r 4 peptideS) (EFQQR and LDALEPTNR)	Not verified	LOD = 55 or 50 mg/kg and LOQ = 180 or 160 mg/kg for LDALEPTNR (Jug r 4) peptide according to the MS ² or MS ³ acquisition mode, respectively	Fortified samples of biscuits spiked with a mix of 5 nuts (peanut, hazelnut, walnut, almond and cashew). Cereal mixes, biscuits (commercial)	[115]
LC-ESI-LIT-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (2 Jur r 4 peptides) (ADIYEEAGR and LDALEPTNR)	1 of the 2 Jug r 4 peptides (LDALEPTNR) in walnut also occurs in Car i 4 in pecan nut	LOD = 0.8 mg/kg and LOQ = 2.6 mg/kg for ADIYEEAGR (Jug r 4) in fortified biscuits. LOD = 5 mg/kg and LOQ = 18 mg/kg for LDALEPTNR (Jug r 4) in fortified dark chocolate	Fortified samples of biscuits or dark chocolates spiked with a mix of 5 nuts (peanut, hazelnut, walnut, almond and cashew). Dark chocolates and biscuits (commercial)	[116]
LC-MS/MS (multiallergen approach)	Not applicable/peptides from walnut (3 Jug r 1 peptides) (DLPNECGISSQR,	Not verified	70 mg/kg of DLPNECGISSQR (Jug r 1) in bread material	Flour and bread spiked with allergen mix (almond, walnut, peanut, hazelnut, soybean, egg and milk)	[117]

(continued)

Table 3 (continued)

Method	Antibody (immunization)/target protein	Cross-reactivity	Sensitivity level	Applied food matrices	References
LC-MS/MS (multiallergen approach)	QCCQLSQMDEQCCEGLR and GEEMEEMVQSAR)	Not verified	70 mg/kg of DLPNECGISSQR (Jug r 1) in bread material	Bread spiked with allergen mix (almond, walnut, peanut, hazelnut, soybean, egg and milk)	[118]
	Not applicable/peptides from walnut (3 Jug r 1 peptides) (DLPNECGISSQR, QCCQLSQMDEQCCEGLR and GEEMEEMVQSAR)				

results. Additionally, the high propensity of target proteins to suffer conformational disruptions of their structures when submitted to harsh conditions, such as those regularly used in food processing (e.g. heat treatments, glycation, pH alterations, formation of Maillard products, fermentation, partial hydrolysis), can consequently lead to false-negative results. Therefore, ELISA results should be interpreted with careful, having in consideration the type of food matrix and processing.

MS platforms

MS technologies allow a range of different types of protein analysis to be undertaken for food allergens, including protein identification, characterization and quantification. The recent advances in modern MS equipment combined with effective bioinformatic tools are currently modernizing the field of protein analysis [105]. MS technologies exhibit several advantages that highlight its potential application for allergen detection, such as the possibility of analysing target analytes with high sensitivity, accuracy, specificity and reproducibility [112]. Additionally, the application of MS platforms for allergen analysis over other technologies presents the benefit of allowing a large degree of freedom in the selection of a target analyte to determine the presence of the allergenic ingredient [113]. Methods based on MS technology are less prone to problems related to cross-reactivity phenomena, which are very frequent in immunoassays (e.g. ELISA), allowing the unequivocal confirmation of the identity of the tested proteins/peptides [114]. Most of MS methods used in the identification of allergens and protein markers are carried out in 'bottom-up' mode, being conducted on digested proteins by the use of proteases such as trypsin [114]. The quantification of the resulting peptides involves their separation using reverse-phase liquid chromatography (LC), often coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS) and multiple reaction monitoring (MRM) [40, 113]. Accordingly, the ability for multiple target analysis in single LC runs together with the high selectivity and rapid adaptability of MS methods reinforce their adequacy for food allergen testing. In spite of all well documented advantages, factors such as the elevated cost of the equipment and the requirement for specialized expertise to maintain MS platforms are likely to constrain the broad application of this technology [105].

Recently, some studies have been reported regarding the development of MS methods for the simultaneous detection of several allergenic foods, including walnut (Table 3). The methods based on LC-MS/MS detection allowed identifying and characterizing walnut peptides in processed food matrices, such as bread, biscuits or chocolates. The reported levels were down to few mg/

kg of walnut proteins, targeting digested peptides of Jug r 1 and Jug r 4, even in complex matrices such as dark chocolate [115–118].

From these reports, it is important to indicate that the application of MS technology to multiple allergen detection and quantification has high potential, though further research is still needed.

DNA-based methods

Although the methods based on the detection of the DNA do not target the allergenic proteins, they are able to target the respective encoding sequences or species-specific markers. The high stability of DNA to pH alterations, partial hydrolysis or thermal treatment, which are processes commonly used by food processing industry, have indicated this molecule as an ideal target for allergen analysis. In addition, the DNA-based methods can be easily implemented in routine analysis and considered, at the same time, as confirmatory tools for the identification of allergenic foods [22, 103]. Therefore, a great number of detection and quantification DNA-based methods have emerged in recent years, demonstrating their potentialities and revolutionizing the field of allergen analysis.

PCR

From the DNA-based methods, the ones relying on polymerase chain reaction (PCR) are among the most commonly used, mostly due to their adequate set-up cost, reasonable running time and moderate requirements for specialized equipment and personnel [105]. In addition to this, PCR methods are less prone to cross-reactivity phenomena as the chosen target sequence can be adapted, being independent on biological effects such as those occurring during antibody production.

In this sense, several PCR assays have been reported in the literature for the specific detection of walnut in processed foods, which are reported in Table 4. In opposition to numerous ELISA and LFD kits, only two commercially available real-time PCR systems could be found for the qualitative analysis of walnut in foods (Table 2). In the case of the SureFood real-time PCR kit, very low LOD and LOQ values (0.4 and 10 mg/kg, respectively) are stated, but as no calibration curve is available, it is not possible to confirm the alleged sensitivity (Table 2). The NutsKit real-time PCR was developed to target both walnut and pecan nut, presenting no cross-reactivity with twenty other foods. In relation to sensitivity, the available information is quite limited. Both real-time PCR kits have restricted specificity because of being unable to discriminate walnut from pecan nut (Table 2).

One of the main advantages attributed to PCR methods concerns their specificity. This is well evidenced in

Table 4, in which the reported methods allow very small or no cross-reactivity to other nontarget food species. With respect to walnut detection, the presented real-time PCR methods were based on the amplification of a region encoding the allergenic proteins Jug r 2 or Jug r 3. Almost all the proposed systems can be virtually applied to a wide variety of processed foods (e.g. cakes, biscuits, sausages), allowing quantification of walnut down to 5–10 mg/kg or few DNA copies (Table 4). These findings highlight the enormous potential of DNA-based methods for the detection and identification of walnut or other allergenic ingredients in highly processed and complex food matrices [119–126].

Genosensors and microarrays

The potential application of biosensors for the detection of allergens in foods has been regarded as an excellent alternative to the above-described protein- and DNA-based techniques [127]. Biosensors are expected to solve several issues concerning simplicity, fastness, reproducibility, low-cost multitarget detection and potential for automation. Therefore, it is predictable that in the near future biosensors could be used at industrial scale for direct and real-time monitoring of allergens along a production line [127]. DNA sensors (genosensors) and DNA chips (microarrays) have emerged as valuable, easy, inexpensive, fast and selective tools with a wide application in very different fields [128]. While most of the proposed biosensors (often called immunosensors) are generally based on the recognition of the antibody/allergen interaction, the genosensors rely on the hybridization of an immobilized DNA probe onto the transducer surface with the target DNA. When the probe hybridizes with the target DNA, a signal is generated being subsequently measured. There are different types of transducers (optical, acoustical, amperometrical or potentiometrical) that are capable of acquiring the signal and further processing it to give a proportional output to the concentration of a specific target. One of the main advantages attributed to this technology corresponds to its massive potential for multitarget analysis. The reduction of reagents, costs and time of analysis, as well as the decrease of possible cross-contaminations, constitute additional benefits commonly indorsed to biosensors (genosensors).

In spite of the high potential for multitarget analysis, very few studies have been performed aiming at developing microarrays and DNA chips for the simultaneous detection of several allergen-encoding sequences in a single assay. In the specific case of walnut detection, only one study based on biochip for Jug r 2 encoding region (91 bp) has been advanced, but no sensitivity level was reported [129]. The limited number of

Table 4. Summary of the reported DNA-based methods for the detection and quantification of walnut in foods

Method	Target gene (NCBI accession number)	Fragment size (bp)	Cross-reactivity	Reference standards or model mixtures (range)	Sensitivity level	Applied food matrices	References
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	91	1/14 plant-derived foods. Cross-reactivity with pecan nut at Ct>35 cycles	Wheat powder spiked with walnut flour (100 000–10 mg/kg) Genomic walnut DNA (12 500–1.25 pg)	10 mg/kg 1.25 pg	No data	[119]
Hexaplex real-time PCR with hydrolysis probe	Jug r 2 (AF066055)	88	1/44 plant- and animal-derived foods. Cross-reactivity with carrot (0.18%)	Boiled sausages spiked with walnut (10 000–32 mg/kg) Rice cookies spiked with walnut (400–0 mg/kg)	100 mg/kg of walnut in boiled sausages 5 mg/kg of walnut in rice cookies	Spreads, chocolates, sauces, parfaits, pasta, sandwiches, lasagne	[120]
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	88	0/23 plant-derived foods	Cookies spiked with walnut (100 000–10 mg/kg)	100 mg/kg of walnut in cookies	Biscuits, wafers, cakes	[121]
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	88	0/23 plant-derived foods (tested by Brezná et al. [121])	Oat flakes spike with nut mix (hazelnut and walnut) (100%–0%)	10%	Cakes	[122]
Real-time PCR using specific hydrolysis probe	Jug r 2 (AF066055)	88	0/23 plant-derived foods (tested by Brezná et al. [121])	Cake filling spiked with walnut (100%–0%)	10%	Nut cakes	[123]
Conventional PCR	matK (AF118027)	120	1/12 plant- and animal-derived foods. Cross-reactivity with pecan nut	No data	No data	Snacks, cookies	[124]
Ligation-dependent probe amplification (multitarget)	Jug r 2 (AF066055)	120	0/48 plant- and animal-derived foods	Walnut cookies were used as matrix	No data	Chocolates, yogurts, cookies, spreads, sausages	[125]
Single-tube nested real-time PCR (real-time PCR coupled with nested PCR)	Jug r 3 (EU780670)	136 99	0/31 plant-derived foods	Batter or sponge cakes spiked with walnut (500 000–1 mg/kg) Batter or sponge cake with 50% walnut serially diluted (100 000–1 pg)	10 mg/kg of walnut in batter or sponge cakes (LOD = LOQ) 1 pg of walnut in batter or sponge cakes (LOD = LOQ)	Sponge cakes	[126]
Optical thin-films biochips (multitarget)	Jug r 2 (AF066055)	91	No data	No data	Sensitivity was assessed with sesame but not with walnut	Bread sticks, wafers, biscuits, cookies, noodles	[129]

genosensors and microarrays applications for allergen analysis, indicates that much effort is still needed for its full development and virtual application on routine analysis, namely at industrial scale.

Immunotherapy-induced tolerance

Food allergy develops as a result of failure or loss of oral tolerance, though naturally acquired tolerance to some allergenic foods may occur mainly in children [130, 131]. Allergies to milk and eggs are quite common among children, who mostly will outgrow their food allergy during childhood. Conversely, in the case of children suffering from allergies to peanut and/or tree nuts, they are more likely to be persistently affected by them throughout adulthood [130]. The true mechanisms involved in naturally acquired tolerance are not yet fully understood, although recent data seem to suggest that in children with persistent egg or milk allergy, the IgE reactivity to linear epitopes is more relevant than with conformational ones. Understanding the mechanisms of natural outgrowth of food allergy may provide a better knowledge on the immune pathways that could be therapeutically targeted in patients with long-life persisting food allergy [130]. Therefore, the search for definitive treatments for food allergy has been centred on strategies able to induce oral tolerance, being the oral immunotherapy (OIT) one of most promising approaches.

At this time, OIT is at preliminary stage, although few clinical trials have already been performed with tolerance as an outcome, mostly targeting egg or milk allergies (see review [131]). It is also important to refer that clinical efficacy of OIT has also been demonstrated in studies using peanut extracts [132, 133]. Based on these studies, OIT seems to present the potential to be applied for different allergenic foods, such as the case of walnut. Still, more extensive and appropriate research is required to provide potential definitive therapies for food-allergic patients [131].

Final remarks

Walnut, along with other tree nuts, has been considered a relevant allergenic food, affecting a small, but rather significant portion of the general population, especially in western countries. In general, clinical manifestations of walnut allergy are frequently severe and systemic potentially life-threatening, leading to anaphylaxis. The most widely consumed walnut species is *Juglans regia*, from which five allergens have been identified and characterized, namely Jug r 1, Jug r 2, Jug r 3, Jug r 4 and Jug r 5. *Juglans nigra*, known as black walnut, is much less consumed than the common walnut, having two allergenic proteins identified, Jug n 1 and Jug n 2.

The current research on the characterization of allergenic components has opened new perspectives in the diagnosis of food allergy. Recently, relevant information on the biochemical classification of walnut allergens has become available, which enabled their correlation with respective clinical symptoms elicited. However, more studies are still needed to better understand the different patterns of sensitization. Whether such patterns of reactivity are the result of multiple independent sensitizations, which are recurrent in atopic patients or derived from true cross-reactivity, it should be questioned and further investigated. From a clinical point of view, in the case of a patient sensitized to multiple foods, *in vivo* and *in vitro* tests should always be performed in the attempt of answering to this question. Additionally, the results of these tests should also be analysed under the perspective of geographic environment, dietary habits and food matrix/processing.

So far, the only effective means of preventing allergic reactions in sensitized individuals still continues to depend on the total avoidance of the offending food. In relation to this, the allergic individuals are highly reliant on proper food labelling when carefully choosing the processed foods that are commercially available. In this context, the control of labelling compliance is much needed, prompting the development of proficient analytical methodology (both protein- and DNA-based techniques) for allergen detection. Until now, no cure is yet available for food-allergic individuals, although some clinical trials, namely OIT, have been advanced in the past years aiming at defining possible therapeutics for the treatment of food allergies. These studies are still at a preliminary stage and in a near future it is expected that forthcoming advances will include a range of important allergenic foods such as walnut and other relevant nuts.

From the addressed issues, it is critical that investigation based on collaborative studies between clinicians and researchers may continue, contributing to establish clinical threshold levels and adequate analytical methods to better manage food allergies.

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Conflict of Interests

The authors declare no conflict of interests.

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