SCIENTIFIC OPINION

Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2,3]

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ABSTRACT

Following a request from the Food Safety Authority of Ireland, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) was asked to deliver a scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. In view of the request, the NDA Panel decided to update its previous opinions relative to food ingredients or substances with known allergenic potential listed in Annex IIIa of 2003/89/EC, as amended. These include cereals containing gluten, milk and dairy products, eggs, nuts, peanuts, soy, fish, crustaceans, molluscs, celery, lupin, sesame, mustard and sulphites. The opinion relates to immunoglobulin (Ig)E- and non-IgE-mediated food allergy, to coeliac disease and to adverse reactions to sulphites in food, and it does not address non-immune-mediated adverse reactions to food. It includes information on the prevalence of food allergy in unselected populations, proteins identified as food allergens, cross-reactivities, the effects of food processing on the allergenicity of foods and ingredients, methods for the detection of allergens and allergenic foods, doses observed to trigger adverse reactions in sensitive individuals and risk assessment methodologies that have been used to derive individual and population thresholds for selected allergenic foods.

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KEY WORDS

food allergy, prevalence, allergens, methods of detection, eliciting dose, thresholds, food labelling

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SUMMARY

Following a request from the Food Safety Authority of Ireland, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) was asked to deliver a scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes.

In view of the request, the NDA Panel decided to update its previous opinions relative to food ingredients or substances with known allergenic potential listed in Annex IIIa of Directive 2003/89/EC, as amended, which include cereals containing gluten, milk and dairy products, eggs, nuts, peanuts, soy, fish, crustaceans, molluscs, celery, lupin, sesame, mustard and sulphites. In this context, EFSA launched a procurement project (CT/EFSA/NDA/2012/02) to review published data on the prevalence of food allergy in Europe and to gather prevalence data on food allergy in the general (unselected) population.

The present opinion relates to immunoglobulin (Ig)E- and non-IgE-mediated food allergy, to coeliac disease and to adverse reactions to sulphites in food, and it does not address non-immune-mediated adverse reactions to food. For each food ingredient or substance listed in Annex IIIa, it includes information on the prevalence of food allergy in unselected populations, on proteins identified as food allergens, on cross-reactivities, on the effects of food processing on the allergenicity of foods and ingredients, on methods for the detection of allergens and allergenic foods, and on doses observed to trigger adverse reactions in sensitive individuals.

Immune-mediated adverse reactions to foods manifest with clinical signs and symptoms of variable severity and duration, which may affect different organs and systems. Anaphylactic reactions to food are IgE mediated and may occur at any age. Non-IgE-mediated food allergy includes a wide range of diseases, including protein-induced enterocolitis and eosinophilic oesophagitis.

A careful family and clinical history are the basis for diagnosis of food allergy. Food diaries, skin prick tests (SPTs), allergen-specific IgE measurements, food elimination diets and food challenges are part of the standard protocol for the diagnosis of food allergy. A positive SPT indicates sensitisation to the tested food, but it is not diagnostic of food allergy. Allergen-specific serum IgE antibodies similarly denote sensitisation to a particular food, but they are not diagnostic without a clinical history or food challenge. The use of atopy patch tests for the diagnosis of food allergy is controversial. Other available tests have no current role in the diagnosis of food allergy. Diagnosis is confirmed by exclusion of the suspected food and the subsequent amelioration of symptoms and by the recurrence of symptoms on re-introduction of the offending food, ideally in double-blind placebo-controlled food challenges, provided that the initial symptoms were not life threatening. The Panel notes that there is a need for standardisation of derived allergens for SPTs. The Panel also notes that guidelines aiming to standardise oral challenge protocols for the diagnosis of food allergy are now available. Dietary avoidance of specific allergenic foods in combination with nutritional advice is the mainstay of management in IgE- and non-IgE-mediated food allergy. Food-allergic individuals may occasionally outgrow their allergy later in life.

The prevalence of food allergies in developed countries is uncertain. The scarcity of studies available for some geographical areas and the use of different methodologies across studies to retrieve prevalence data are the main reasons for this uncertainty. Using food challenges as a criterion for diagnosis, the prevalence of food allergy has been estimated to be around 3 %, when considering data from Europe, the USA and Australia/New Zealand, and about 1 % when considering European studies only, in both adults and children. However, the heterogeneity among the studies used to estimate the prevalence of food allergy was high. There are insufficient objective data to conclude on time trends with respect to the prevalence of food allergy in Europe. About 75 % of allergic reactions among children are due to egg, peanut, cow's milk, fish and various nuts. About 50 % of allergic reactions among adults are due to fruits of the latex group and of the *Rosaceae* family, vegetables of the Apiaceae family, and various nuts and peanuts.

Geographical variation in the prevalence of food allergy is due to differences in in environmental (e.g. pollen exposure or differences in food habits) and individual factors. Sex, age, family history of atopy and the presence of other allergic diseases are among the individual factors considered important in the development of food allergy. Extrapolations of prevalence data on specific food allergies from a single European country to the entire European population are of limited accuracy owing to differences in genetic background, exposure to the offending foods and eating habits.

Owing to the development of proteomics, spectroscopic methods and gene cloning, allergenic proteins can be well characterised. They have been classified into families based on their sequence and three-dimensional (3D) structure. However, although common structural features of proteins and their biological activity have been tentatively related to their immunogenicity, it is not possible to predict the allergenicity of a protein based on these two parameters only. Immunological and clinical data are also required to classify a protein as a food allergen.

Cross-reactivity occurs when IgE antibodies originally triggered against one antigen also bind a different antigen. Not all cross-reactivities identified *in vitro* are of clinical significance, and although most clinical cross-reactions are mediated by IgE antibodies, T cells may also be involved. However, *in vitro* cross-reactivity testing can help understanding allergenicity to multiple foods, as well as improving diagnosis and management of food allergy.

The allergenic activity of a food may decrease, remain unchanged, or even increase by food processing. Considering the multiplicity of the allergenic proteins contained in a whole food, and that different proteins may be differently affected by the same treatment, the impact of food processing on the structural and allergenic properties of allergenic foods/ingredients is difficult to predict. In addition, the extent to which allergenic proteins are modified during food processing depends on the type of process and its conditions, the structure of the proteins, and the composition of the matrix. Although the effects of different (technological and cooking) treatments on the IgE-binding capacity of several allergens have been investigated, less information is available on the effects of processing on clinical reactivity.

The majority of kits commercially available for routine food allergen analysis rely on immunological methods. Enzyme-linked immunosorbent assay (ELISA) methods are the most widely used because they are sensitive and specific for the detection of allergenic proteins and easy to use. However, commercial kits for quantitative analyses employ different extraction buffers and calibration procedures, they differ in the quality of the antibodies used and the results vary among commercial brands and batches. Major limitations include matrix effects, insufficient extraction of the protein, insufficient specificity due to cross-reactions and insufficient reproducibility of results. The use of incurred samples may help to improve the reliability of the method when analysing processed foods.

Mass spectrometry (MS), in combination with techniques such as 2D sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) or chromatography for the preliminary separation of proteins, and using allergen databases for their subsequent identification, is a reliable tool for the detection of known allergens and for the identification of new immunoreactive proteins. MS methods for quantitative analysis based on specific standard peptides or stable isotope labelling are not yet suitable for analyses of large numbers of samples but can confirm results obtained otherwise.

DNA methods allow detection of the allergenic food rather than of the allergenic protein and are complementary to immunological assays. DNA is generally more stable than proteins and thus suitable for analysis of processed foods. The extraction and amplification procedures are well established. Both end-point and real-time polymerase chain reaction (PCR) allow simultaneous multiple analyses. Whenever ELISA kits are not available or not specific for the analysis of a specific allergenic food/ingredient (e.g. celery), DNA analysis becomes the method of choice. Real-time PCR may provide quantitative results and allows multiplexed analysis. Commercial kits are available.

The main problem for the quantification of allergens by immunological or DNA-based methods is the unavailability of certified reference materials (CRMs). Reference materials (not certified) developed by different producers are commercially available for the most important food allergens, but the results obtained may not be comparable. To the Panel's knowledge, a CRM for the detection of food allergens has been developed only for peanuts. For milk and egg, two reference materials are commonly used. Availability of CRMs for the quantification of food allergens is required.

The notion of determining concentrations of allergenic foods/ingredients in foodstuffs below which the majority of sensitised consumers are not at risk of developing severe allergic reactions has attracted much attention from regulatory bodies, consumer associations and industry throughout Europe. To that end, attempts have been made to define a framework for food allergen risk assessment, as it exists for the risk assessment of other food-related hazards (e.g. chemicals, microbiological agents). Three different approaches have been proposed for allergen risk assessment: (i) the traditional risk assessment using the no observed adverse effect level (NOAEL) and uncertainty factors; (ii) the Bench Mark Dose (BMD) and Margin of Exposure (MoE) approach; and (iii) probabilistic models. These approaches may be used to inform different risk management decisions for allergen labelling. The reliability of the risk estimates will depend on the type, quality and amount of data used, to estimate both population thresholds (or threshold distributions) and exposure to the allergenic food/ingredient. The purpose of the risk assessment (e.g. exemptions from labelling, labelling of allergens unintentionally present in food) and the level of risk that may be acceptable (e.g. the fraction of the allergic population that is intended to be protected and to what extent) are risk management decisions, which are outside EFSA's remit.

Coeliac disease is a life-long autoimmune systemic disorder triggered by gluten and similar cereal storage proteins present in wheat, rye and barley. Its prevalence is estimated to be 0.5 to 1 %. A gluten-free diet is the conventional treatment. The limit values of 20 and 100 mg/kg of gluten in "gluten-free" and "very low gluten" foods, respectively, help in managing the diet of most patients with coeliac efficiently.

Labelling of foods containing sulphiting agents in concentrations > 10 mg/kg or 10 mg/L is mandatory in the EU; those levels were based on the limit of detection (LOD) of the analytical methods available at the time. Many very sensitive and reliable methods are now available for analysis of sulphites in foods, with LODs well below 10 mg/kg. However, minimum doses eliciting adverse reactions to sulphites have not been systematically assessed, and the lowest concentration of sulphites able to trigger a reaction in a sensitive person is unknown.

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BACKGROUND AS PROVIDED BY THE FOOD SAFETY AUTHORITY OF IRELAND

A certain proportion of the population (1–3 % of adults and 4–6 % of children)[4] suffer adverse health consequences as result of the consumption of particular foods or food ingredients. Such hypersensitive responses can manifest themselves in various ways, and can be broadly categorised as immune-mediated food allergies or non-immune-mediated food intolerances. The classical food allergy results in a hyper-immune response that is mediated by IgE antibodies, the best known, and potentially most serious of which is peanut allergy. Food intolerances are often more difficult to characterise as they can be caused by non-proteinaceous food components (lactose for example), unlike true allergies which are generally the result of a reaction to one or more individual protein components.

EU food law[5] stipulates that the inclusion of certain allergenic food ingredients in a foodstuff must be indicated on the packaging so that vulnerable consumers are protected from inadvertent consumption. Regulatory authorities across the EU expend considerable resources in sampling and testing foodstuffs to ensure the integrity of food allergen labelling within their jurisdiction. However, effective risk management is hampered by a lack of information on the clinical thresholds applicable to the various allergens as well as variation in risk assessment and management strategies adopted across the EU.

The 2004 opinion of the EFSA Scientific Panel on Dietetic Products, Nutrition and Allergies relating to the evaluation of allergenic foods for labelling purposes[6] is a substantial scientific report that provides details about the main foods and food ingredients that cause allergic or intolerance reactions among EU consumers. The report was the first compiled by EFSA dealing specifically with food allergy and intolerance, and the first at EU level since the Scientific Committee on Food report of 1995.

Though the information within those reports remains valid, a number of developments have occurred, and further information has become available that could be of benefit to risk assessors and risk managers dealing with food allergies and intolerances in the EU. EU-funded research on a multidisciplinary project called EuroPrevall was completed in 2009, and examined “The prevalence cost and basis of food allergy in Europe”. Many EU Member States have developed methods and procedures for the assessment and management of food allergies and intolerances within their own jurisdiction. DNA-based testing methods have been used successfully in the detection of misleading food labelling and food fraud. While the use of DNA-based analytical methods in food allergy testing could bring increased sensitivity and reliability compared to immunological methods such as ELISA, the risk of disproportionate regulatory activity could result in a greater use of precautionary “May Contain….” labels, which would not benefit allergy sufferers.

In conclusion, the EFSA report of 2004 remains a valuable scientific document, but could be enhanced by a review of the scientific and other information that has been generated in the seven years since it was adopted. A considered assessment by EFSA of new scientific information could assist in developing a harmonised approach to protecting vulnerable consumers in the EU.

TERMS OF REFERENCE AS PROVIDED BY THE FOOD SAFETY AUTHORITY OF IRELAND

With the benefit of experience gained since 2004 and based on the allergens listed in the annex of Commission Directive 2007/68/EC except for lactose, as the specific issue of lactose thresholds in

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[4] Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission relating to the evaluation of allergenic foods for labelling purposes (Request No EFSA-Q-2003-016) (adopted on 19 February 2004).

[5] Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. OJ L 308, 25.11.2003, p. 15–18.

[6] Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission relating to the evaluation of allergenic foods for labelling purposes (Request No EFSA-Q-2003-016) (adopted on 19 February 2004).

lactose intolerance and galactosaemia has been already assessed in a recent opinion of EFSA[7], the Food Safety Authority of Ireland requests that EFSA provides a scientific opinion on:

- The prevalence of each allergy in the European Union.
- Recommendations for threshold concentrations of each allergen in food that would provide an acceptable level of protection for at-risk consumers;
- The suitability, or otherwise, of qualitative and quantitative DNA-based tests (PCR) for the detection and quantification of food allergens in comparison with immunological (e.g. ELISA) or other methods.

[7] EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); Scientific Opinion on lactose thresholds in lactose intolerance and galactosaemia. EFSA Journal 2010;8(9):1777, 29 pp. doi:10.2903/j.efsa.2010.1777. Available online: www.efsa.europa.eu/efsajournal.htm

ASSESSMENT

1. Introduction and interpretation of the terms of reference

It is EFSA's role to provide risk managers (European Parliament, European Commission and Member States) with scientific and technical support in order to inform management decisions regarding the adoption and implementation of EU legislation in relation to the labelling of foodstuffs. This includes information to be provided to consumers on allergenic foods and food ingredients that may pose a health risk to sensitive individuals.

Current EU legislation indicates in Annex II of Regulation (EU) No 1169/2011 a list of substances subject to mandatory labelling that can cause allergies or intolerances in sensitive individuals upon oral consumption. Labelling of allergenic foods and ingredients listed in Annex II is mandatory when: (i) they are intentionally added in the manufacturing of foodstuffs; and (ii) they are still present in the final product to be delivered to the consumer. In this regulatory context, it is EFSA's task to provide risk managers with relevant scientific and technical information relative to these substances and their capacity to induce allergic reactions in sensitive individuals.

However, it is not EFSA's task to decide:

- whether certain substances should be added to, or removed from, the list of ingredients subject to mandatory labelling;
- on the labelling of substances listed in Annex II when unintentionally present in foods (precautionary labelling);
- whether allergic reactions induced by these substances by mechanisms other than oral ingestion (e.g. skin contact, inhalation) should be considered for risk management purposes.

The terms of reference (ToRs) specify that, with the benefit of the experience gained since 2004 and based on the allergens listed in the annex (Annex IIIa) of Commission Directive 2007/68/EC (Annex II of Regulation (EU) No 1169/2011), except for lactose, the Food Safety Authority of Ireland requests that EFSA provides a scientific opinion on:

- the prevalence of each allergy in the EU;
- recommendations for threshold concentrations of each allergen in food that would provide an acceptable level of protection for at-risk consumers; and
- the suitability, or otherwise, of qualitative and quantitative DNA-based tests (polymerase chain reaction, PCR) for the detection and quantification of food allergens in comparison with immunological (e.g. enzyme-linked immunosorbent assay, ELISA) or other methods.

In order to address the ToRs, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) decided to update its previous opinions (EFSA, 2004, 2006b, 2006a) relative to food ingredients or substances with known allergenic potential listed in Annex IIIa of Directive 2003/89/EC, as amended, keeping in mind that:

- Prevalence data from the EU-funded multidisciplinary Integrated Project EuroPrevall and from other ongoing research projects relevant to this task will become available only in the next few years. In this context, EFSA launched a procurement project (CT/EFSA/NDA/2012/02) on literature searches and reviews related to the prevalence of food allergy in Europe to gather prevalence data on food allergy in the general (unselected) population (University of Portsmouth, 2013). Details about the literature search and the criteria used to select pertinent studies are depicted in the technical report (University of Portsmouth, 2013).

- The NDA Panel will provide an overview of the current methodologies used for allergen risk assessment as well as information on the aspects that could be taken into account by risk managers when establishing threshold concentrations for allergens in foods for labelling purposes. The NDA Panel will also summarise published eliciting dose levels calculated for populations (or population thresholds) that have been derived for some allergenic foods. It is not EFSA's responsibility to decide which level of protection is "acceptable" for risk managers, consumers and/or other stakeholders, and therefore it is not in the NDA Panel's remit to establish concentrations of allergens in food for labelling purposes.
- The NDA Panel will also address the suitability, or otherwise, of qualitative and quantitative DNA-based tests (PCR) for the detection and quantification of food allergens in comparison with immunological (e.g. ELISA) or other methods, including mass spectrometry. The NDA Panel will provide risk managers with relevant information about:
  - the characteristics of each method available and the current use;
  - the possibilities of combining more than one method for the analysis of allergenic ingredients in foods; and
  - the factors which should be considered when selecting one or the other method for a particular purpose.

Examples of the use of different methods for the detection of a given allergenic food or ingredient in different matrices will be given when available. However, the NDA Panel does not aim to provide an exhaustive list or a compilation of all publications available in this field, nor to decide or recommend the best method or test for the detection or quantification of each particular allergen. The selection of the method or methods for the detection/quantification of allergens in foodstuffs would largely depend on the food targeted for analysis (e.g. food matrix, level and method of processing, quantity and form of the allergenic ingredient expected to be present) and the purpose of the analysis (e.g. screening, quantification).

The NDA Panel wishes to clarify that the present opinion does not aim to be a textbook on food allergy or an exhaustive compilation of the clinical symptoms, diagnostic methods and/or clinical management of food allergy, not to guide choices on infant feeding practices or clinical decisions in the management of food-allergic individuals. However, general information about the above-mentioned aspects is given to risk managers to put into context the clinical implications of management decisions in the labelling of food allergens.

2. Classification of adverse reactions to foods and definition of terms

In this opinion, the terms **allergenic food** and **allergenic ingredient** will be used for substances listed under Annex IIIa, depending on whether they are considered as such or as part of complex foodstuffs, being aware that lactose and sulphites are not food allergens and that gluten may induce both food allergy and coeliac disease. The term **allergen** will be restricted to proteins or peptides responsible for the allergenicity of allergenic foods/ingredients, being aware that certain carbohydrate moieties may also play a role in the allergenicity of foods. **Total protein** refers to the amount of protein within an allergenic food/ingredient, regardless of whether it is allergenic or not, and not to the amount of a specific allergen. **Immunogenicity** denotes the ability to induce a humoral and/or cell-mediated immune response, whereas **antigenicity** refers to the ability to combine specifically with the final products of the immune response, e.g. specific immunoglobulin class E (IgE) antibodies. In this opinion, the term **allergenicity** (i.e. the ability to induce allergy and/or trigger an allergic reaction) will be restricted to the ability to trigger an allergic reaction, and will not refer to the ability to induce sensitisation.

Adverse reactions to food have been classified into different groups on the basis of the pathogenic mechanism (Figure 1). They include immunologically mediated reactions, which may be mediated either by IgE antibodies, by cells (non-IgE-mediated) or both (mixed), and non-immunological

responses (food intolerance), which are dependent on enzyme deficiencies or pharmacological reactions or, in the majority of cases, arise by unknown mechanisms (Ortolani, 1995; Dupont, 2011; Sicherer and Leung, 2011; Vickery et al., 2011; Waserman and Watson, 2011; Muraro et al., 2014a).

Hypersensitivity describes an adverse clinical response in which the exact nature of the underlying pathophysiology is unknown. Occasionally this term is used more broadly to describe all adverse reactions to food, including immunologically mediated diseases and food intolerances. In this opinion, the term "food hypersensitivity" will not be used owing to its ambiguity.

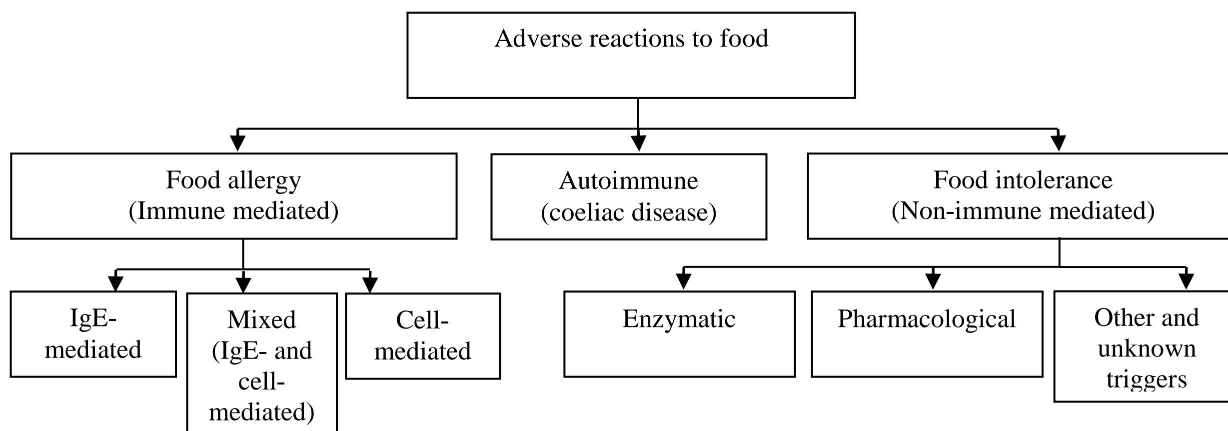

Figure 1: Classification of adverse reactions to food

Food allergy is defined as an adverse health effect arising from a specific immune-mediated response that occurs reproducibly on oral exposure to a given food (Boyce et al., 2011), which can be mediated by food-specific IgE antibodies, by cellular mechanisms or by both (Muraro et al., 2014a).

Immune, IgE-mediated food allergies may result in rapid onset of severe reactions (usually within two hours after oral exposure to a given food) and may manifest with a variety of signs and symptoms that can involve the digestive, respiratory, cardiovascular or cutaneous organ systems (Boyce et al., 2011). The severity of reactions varies from mild (e.g. hives) to severe (e.g. anaphylaxis).

Atopy is a familial tendency to produce IgE antibodies in response to allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis (Johansson et al., 2001). The term "atopic march" has been used to describe the natural history and sequential progression to these atopic disorders.

Immune, non-IgE(cell)-mediated food allergies more commonly affect only the gastrointestinal tract in a subacute or chronic way. They are typically delayed in onset and occur 2 to 48 hours after ingestion of the offending food(s). The primary disorders in this category include food protein-induced enterocolitis, food protein-induced proctitis/proctocolitis and enteropathy, which in a majority of cases resolve before adolescence. Enteropathy resulting from cow's milk is one of the better-understood non-IgE-mediated food allergies. Although eosinophilic gastrointestinal disorders (including eosinophilic oesophagitis and eosinophilic gastroenteropathy) are typically listed under this category, a high number of cases are caused by IgE-mediated responses (Guandalini and Newland, 2011).

Coeliac disease is an autoimmune adverse reaction to food triggered by the ingestion of gluten and related to prolamins found in wheat, barley and rye.

Non-immune-mediated adverse reactions to food (also called food intolerances) encompass disorders such as lactose intolerance (due to lactase non-persistence), other disorders of digestive–absorptive processes, toxic (food poisoning) and pharmacological reactions (also called pseudo-

allergic reactions) due to the release of histamine or tyramine after consumption of specific foods (Guandalini and Newland, 2011).

The present opinion relates to IgE- and non-IgE-mediated food allergy, to coeliac disease and to adverse reactions to sulphites in food, and it does not address non-immune-mediated adverse reactions to food.

3. Clinical symptoms of food allergy

IgE-mediated allergic reactions to food are represented by well-defined clinical features. For an allergic reaction to take place, a two-step process is required. First, the capacity to respond with an allergic reaction when exposed to the particular allergen must be established. This induces the immune system to generate specific IgE antibodies against the allergen. This phase is called the induction phase, or sensitisation. Once an individual has become sensitised to a particular allergen, the individual may develop a symptomatic allergic reaction when exposed again to the allergen in question. This is called the provocation or triggering phase.

Immune-mediated adverse reactions to foods manifest with clinical signs (objective) and symptoms (subjective) of variable severity and duration, which may affect different organs and systems (Table 1).

Table 1: Common clinical features of food allergy

Organ system	Clinical features
Skin	Atopic dermatitis Pruritus Angioedema Urticaria Erythema
Gastrointestinal tract	Oral allergy syndrome Nausea/vomiting Gastro-oesophageal reflux disease Abdominal pain Diarrhoea Enteropathies Infantile colic Constipation Failure to thrive
Respiratory tract	Asthma Rhinitis Cough Stridor
Eyes	Conjunctivitis
Generalised (systemic)	Anaphylaxis (with all its complications, including cardiovascular symptoms and generalised collapse)

The food-allergic nature of some clinical syndromes such as migraine, attention deficit hyperactivity disorder and irritable bowel syndrome is still controversial.

3.1. Skin

3.1.1. Urticaria and angioedema

Urticaria is an intensely itchy rash which results from inflammation and leakage of fluid from the blood into superficial layers of the skin in response to various mediators. Synonyms are "hives" or "nettle rash". Urticaria can be acute (lasting for less than six weeks) or chronic. In childhood, urticaria is more commonly of the acute type. Chronic urticaria seems to be only rarely associated with food allergy (Zuberbier et al., 2004). Angioedema is the presence of fluid in subcutaneous tissues, particularly in the face, and in the submucosa of eyes, lips and sometimes tongue and throat.

Urticaria due to food ingestion generally occurs within hours of ingestion, and often fades within three hours. Initial localised symptoms of itching and burning progress to erythema and urticaria. Immune-(IgE)-mediated contact urticaria to foods is common and may progress to more widespread urticaria, angioedema and eventually anaphylaxis. Rarely, urticaria and angioedema can be induced by exercise soon after eating a food, such as wheat, shellfish, nuts or celery, whereas neither the food nor the exercise alone causes any reaction.

3.1.2. Atopic dermatitis

Atopic dermatitis is an extremely pruritic form of chronic inflammatory skin disease usually presenting in early infancy and sometimes persisting in adulthood. Atopic dermatitis represents the first clinical allergic manifestation in children who later develop asthma and, subsequently, allergic rhinitis. This progression is often named atopic march (Spergel and Paller, 2003). Patients with atopic dermatitis usually have elevated specific IgE levels, a positive skin prick test (SPT) for several allergens and a genetic predisposition (i.e. if one parent is atopic there is a 20–40 % probability of a child developing this condition and, if both parents are atopic, a 50–80 % probability). Epidemiological studies are identifying genes involved in atopic predisposition (Walley et al., 2001; Weidinger et al., 2008; Genuneit et al., 2009). For example, filaggrin gene defects have recently been identified as a major risk factor for the development of atopic dermatitis. These skin barrier defects increase the risk of early-onset, severe and persistent forms of atopic dermatitis and concomitant asthma (Marenholz et al., 2006; Worth and Sheikh, 2010).

Other characteristic features of atopic dermatitis are ichthyosis, keratosis pilaris, white dermographism, atopic folds, orbital darkening, anterior sub-capsular cataracts and keratoconus. Acute atopic dermatitis is an acute rash represented by an erythematous, papulovesicular eruption. Chronic dermatitis is characterised by lichenification, excoriation and dyschromic lesions.

In young infants, atopic dermatitis may be difficult to distinguish from seborrhoeic dermatitis. The acute rash is typical of the first (infancy) stage up to two years of age. This eczematous lesion is highly pruritic and usually involves both cheeks and the extensor part of the extremities. Lesions of the scalp and wheal formation may also be associated with this stage. The second (childhood) stage, from 2 to 12 years, is characterised by papular lesions and a rash that occur in the flexural areas, such as the antecubital and popliteal ones, hands and feet (Rudikoff and Lebwohl, 1998). The third (adulthood) stage is characterised by diffuse lichenification in facial areas such as the periorbital and perioral areas. Chronic lesions and remission periods may characterise the life of older atopic patients. Atopic dermatitis can be divided into two distinct variants: the extrinsic, allergic form, which occurs with sensitisation towards foods or aeroallergens and elevated levels of total IgE antibodies; and the intrinsic, non-allergic variant, with low levels of IgE antibodies, in which no sensitisation to foods or aeroallergens can be detected.

The diagnosis of atopic dermatitis is based on well-accepted international criteria, and it takes into consideration different clinical and laboratory parameters, such as the kind of skin manifestation and distribution, age of onset, frequency of relapses, association with other atopic diseases, total serum IgE, specific IgE and blood eosinophilia, among others. On this basis, it is also possible to distinguish between the intrinsic and the extrinsic forms of atopic dermatitis. The standard for diagnosis of

immune-mediated reactions to food in chronic atopic dermatitis is a double-blind placebo-controlled food challenge (DBPCFC) (Fleischer, 2008) complemented by a reliable scoring system such as SCORAD (Hanifin and Rajka, 1980; Dermatitis, 1993).

Several clinical studies have addressed the role of food allergy in atopic dermatitis demonstrating the significant effect of food elimination on the improvement of the lesions (Niggemann et al., 1999; Burks, 2003; Greenhawt, 2010). Egg allergy is the most frequent trigger of severe atopic dermatitis in children (Sampson, 1997; Heine, 2006), and eggs, together with milk, peanut, soy and wheat, account for about 90 % of food allergy in children with atopic dermatitis. The underlying role of food allergy in the development of atopic dermatitis is more evident in young patients with severe disease. Patients who are allergic to peanuts, tree nuts, fish and shellfish are less likely to outgrow their food-related atopic dermatitis (Skolnick et al., 2001).

3.2. Gastrointestinal tract

Adverse reactions affecting the gastrointestinal tract range from mild oral discomfort after allergen exposure to severe diarrhoea and failure to thrive. Any part of the gastrointestinal tract can be involved and the clinical features may occur alone or together as part of a syndrome. Whereas the oral allergy syndrome is the consequence of IgE-mediated immune reactions, the remaining gastrointestinal symptoms described in this section are mostly mixed.

3.2.1. Oral allergy syndrome

Oral allergy syndrome (OAS) is an IgE-mediated immediate-type allergic reaction characterised by symptoms within several minutes of contact with food, involving the mouth and the pharynx (Amlot et al., 1987; Ortolani et al., 1988). The direct contact of the offending food triggers oral and pharyngeal itching, oral papule or blisters, lip irritation and swelling, labial angioedema, and glottis oedema. In some instances, these symptoms are followed by a more complex clinical picture involving several organs and may lead to life-threatening reactions such as anaphylactic shock (Ortolani et al., 1993). Indeed, OAS can be classified into four grades depending on the extent to which other organs are involved and on whether systemic reactions occur. Local oral symptoms are most commonly experienced, while the more severe forms are rare (Ballmer-Weber et al., 2000; Rodríguez et al., 2000; Ballmer-Weber et al., 2001). OAS-induced reactions may rarely spread to cause extra-intestinal symptoms or anaphylaxis (Webber and England, 2010).

OAS's underlying pathophysiology may play a role in the clinical presentation and outcome, depending on whether the cross-reactive protein is a heat-labile pathogenesis-related protein 10 (PR-10), a partially labile profilin or a relatively heat-stable lipid transfer protein (LTP). OAS is frequently associated with selectively labile allergens contained in fresh fruits and vegetables, so that standard diagnostic procedures must include exposure to fresh fruits and raw vegetables. Oral symptoms are less frequent in patients allergic to foods of animal origin such as milk, eggs, fish and shrimp (Amlot et al., 1987; Helbling et al., 1999; Schafer et al., 2001; Sugita et al., 2007).

3.2.2. Vomiting and gastro-oesophageal reflux disease

Vomiting is a common feature of allergic reactions to food (Hill et al., 1984; Heine, 2006). It may result from dysmotility induced by inflammation of the oesophagus and stomach mucosa. The inflammatory response may cause bleeding, with blood in the vomit. Gastro-oesophageal reflux disease can occur as an adverse reaction to food, particularly in children, with or without development of eosinophilic oesophagitis (Moon and Kleinman, 1995; Ireland-Jenkin et al., 2008; Dalby et al., 2010).

3.2.3. Diarrhoea and enteropathies

The passage of frequent loose stools can result from impaired absorption of nutrients and water, from intestinal secretion of fluid as part of an inflammatory response, or from a combination of both. Food protein-induced enterocolitis syndrome (FPIES) is a severe systemic reaction to food proteins,

typically occurring within four hours of food ingestion and mainly in young infants, but it can also occur in older children and adults (Nowak-Wegrzyn and Muraro, 2009).

In infancy and childhood, adverse reactions to food proteins may cause severe diarrhoea ultimately leading to failure to thrive (Savilahti, 2000; Walker-Smith and Walker, 2003).

The major feature of enteropathies is a loss of the normal structure of the intestinal mucosa, which reduces its mucosal digestive and absorptive function (Kuitunen et al., 1975; Walker-Smith, 1992; Vighi et al., 2008). In young children, transient enteropathies to cow's milk, soya, eggs and other foods may occur. Enteropathy in the context of coeliac disease is discussed in section 13.

Allergic eosinophilic gastroenteropathy is a rare disease, which comprises a spectrum of conditions characterised by eosinophilic inflammation of the gastrointestinal wall. They predominantly affect infants and young children but may occur at any age (Kelly, 2000). Any part of the gastrointestinal tract can be affected and the symptoms and signs reflect the site and extent of the damage. Loss of blood and exudation of serum into the intestinal lumen may result. Involvement of the stomach or oesophagus may present with vomiting. Damage to the small intestine and colon can cause significant loss of endogenous protein and nutrients, as well as impaired digestion and absorption (Maloney and Nowak-Wegrzyn, 2007; Oh and Chetty, 2008).

The causes and mechanisms of these conditions are not well understood (Lieberman and Chehade, 2012). Some cases are associated with atopic clinical features and food-specific IgE and SPTs to milk allergens, but others do not have these features (Moon and Kleinman, 1995; Bischoff, 2010).

3.2.4. Infantile colic

Infantile colic affects approximately 7 to 20 % of babies (Lucassen et al., 2001). Its aetiology is unknown and is likely to be multifactorial. Some cases could be attributed to adverse reactions to foods, such as cow's milk or proteins excreted in maternal breast milk (Drug and Therapeutics Bulletin, 2013)

3.2.5. Constipation

Up to 10 % of children with cow's milk allergy may suffer from constipation. Constipation due to other food items has been described (Kiefte-de Jong et al., 2010). The underlying mechanisms and exact diagnostic criteria of allergy-related gastrointestinal motility disorders have not been established (Iacono et al., 1998; Heine, 2008; El-Hodhod et al., 2010).

3.3. Respiratory tract

3.3.1. Asthma

Asthma is a reversible obstruction of the small airways associated with constriction of the airways, mucus production and inflammation. Asthma may occur as a manifestation of a food-allergic reaction. It may sometimes be the dominating symptom, but it is often associated with eczema, urticaria, pollen–food allergy syndrome (i.e. food allergy following sensitisation to inhaled allergens) or gastrointestinal symptoms. Asthmatic symptoms may constitute an important part of a generalised anaphylactic reaction. Deaths from anaphylactic reactions are more often caused by respiratory problems than by hypotension and circulatory failure. Furthermore, asthmatics who are also food-allergic are at a higher risk of developing the most severe anaphylactic reactions to food.

The foods triggering allergic asthma are similar to the general allergic prevalence pattern observed in the community. Comorbidities related to environmental allergens need to be considered when evaluating individuals with a history of food-related asthma (Rancé and Dutau, 2002). Most reactions to sulphites are characterised by bronchospasm, occasionally severe, which can occur within minutes of ingestion of sulphite-containing foods or beverages.

3.3.2. Heiner syndrome

Heiner syndrome is a rare pulmonary hypersensitivity syndrome, in which individuals are likely to be cow's milk sensitive, which affects primarily young children and is characterised by pulmonary haemosiderosis, diarrhoea, anaemia and poor growth (Moissidis et al., 2005).

3.3.3. Laryngeal oedema

Laryngeal oedema, swelling of the mucosa of the larynx, is often seen as part of an anaphylactic reaction to food and may lead to airway obstruction and, in the worst case, to respiratory arrest (Summers et al., 2008). Symptoms of laryngeal oedema include inspiratory dyspnoea, hoarse voice/aphonia and dysphagia.

3.3.4. Rhinitis

Rhinitis is manifested as an inflammation of the nasal mucosa, which gets swollen and itchy. The condition is often accompanied by clear watery nasal secretion and by nasal obstruction. Allergic rhinitis has also been reported as a symptom of food allergy, although less frequently than asthmatic symptoms (Oehling et al., 1992). Symptoms suggestive of rhinitis were reported in a number of pollen-allergic infants with cow's milk and egg allergy (Balatsouras et al., 2011). Symptoms of rhinitis have also been reported to occur in response to food challenges (Pelikan and Pelikan-Filipek, 1987).

3.4. Eyes

The main form of allergic reaction in the eyes is conjunctivitis, in which the surface of the eyes and the inner side of the eyelids become red, swollen and itchy. Conjunctivitis and rhinitis often, but not always, accompany each other, and conjunctivitis tends to occur less frequently than rhinitis. Conjunctivitis in pollen-sensitised individuals has been reported in connection with the intake of specific food items, although less frequently than asthmatic symptoms (Oehling et al., 1992; Kurosaka et al., 2011).

3.5. Generalised symptoms—anaphylaxis

Anaphylaxis is an acute, potentially life-threatening and sometimes fatal condition which involves the cardiovascular system, the respiratory tract, the mouth, the pharynx and the skin, singly or in combination (Yunginger et al., 1988; Bock and Atkins, 1990; Sampson, 2006). Major cell types involved through the secretion of vasoactive mediators are eosinophils, mast cells and basophils. Reactions can be triggered by both IgE and IgG (IgG1 > IgG4) antibodies, depending on the cell type involved (Tsujimura et al., 2008).

The initial symptoms often involve the skin or the oropharynx. Symptoms in the mouth region include oedema, tingling and pruritus of the lips, oral mucosa and pharynx. Skin symptoms may be urticaria or more diffuse erythema, angioedema and pruritus. Respiratory symptoms include bronchospasm, cough, stridor, dyspnoea and wheezing, and may be mistaken as worsening of pre-existing asthma. Oedema of the larynx induces cough, and difficulties with talking, breathing and swallowing. Respiratory function may be severely compromised. Anaphylactic shock may consist of cardiovascular collapse and a marked drop in blood pressure, cardiac arrhythmia and, in the worst case, cardiac arrest. In some cases, the initial manifestation of an anaphylactic reaction may be loss of consciousness. The symptoms, their sequence and their severity may vary from one episode to the other and from one individual to another. In fatal food-induced anaphylaxis, initial symptoms commonly develop within 3 to 30 minutes and severe respiratory symptoms within 20 to 150 minutes of exposure (Sampson and James, 1992; Pumphrey and Gowland, 2007). Some reactions may, however, show a bi-phasic course and be mild at their start (Stewart and Ewan, 1996). Exercise-triggered, food-induced anaphylactic reactions may occur several hours after food intake. Asthmatic subjects who are also food-allergic are at a higher risk of developing the most severe anaphylactic reactions to food (Gonzalez-Perez et al., 2010; Calvani et al., 2011).

The diagnosis of anaphylaxis in retrospective studies has been difficult owing to the wide spectrum of clinical presentations. The criteria proposed by the US National Institute of Allergy and Infectious Diseases and the Food Allergy and Anaphylaxis Network are useful for the diagnosis of anaphylaxis in the emergency department (Sampson, 2006; Campbell et al., 2012).

A novel IgE-mediated reaction to a mammalian oligosaccharide epitope, galactose-alpha-1,3-galactose (alpha-gal), has been described in adult patients in association with delayed-onset anaphylaxis, angioedema and urticaria three to six hours after ingestion of mammalian meat (e.g. beef, pork, lamb). The symptoms can be severe and may require adrenaline injections and care in emergency departments (Commins et al., 2009). Tick bites appear to be the route of sensitisation. Patients with specific IgE antibodies to alpha-gal continue to emerge, particularly among children (Kennedy et al., 2013).

3.6. Conclusion

Immune-mediated adverse reactions to foods manifest with clinical signs and symptoms of variable severity and duration, which may affect different organs and systems. Anaphylactic reactions to food are IgE mediated and may occur at any age. Non-IgE-mediated food allergy includes a wide range of diseases, including protein-induced enterocolitis and eosinophilic oesophagitis.

4. Diagnosis of food allergy

4.1. Clinical diagnosis

The diagnosis of immunological adverse reactions to food and food ingredients depends on clinical insight, suspicion and acumen in interpreting the history and clinical examination of the patient. Diagnosis of food allergy is often difficult because of the variable and subjective nature of the symptoms and the lack of objective clinical signs (Boyce et al., 2011; Dupont, 2011; National Institute for Health and Clinical Excellence, 2011). Guidelines and protocols for the clinical diagnosis of food allergy have been published (Boyce et al., 2011; Dupont, 2011; National Institute for Health and Clinical Excellence, 2011; Muraro et al., 2014a).

The patient's history, and particularly the temporal relationship between exposure and reaction, is key for diagnosis. A family history of atopy will increase the suspicion of immune-mediated adverse reactions to food. However, the lack of a family history does not exclude the diagnosis of a food allergy or an allergic cause of clinical symptoms. Investigation of any patient with clinical suspicion of anaphylaxis of unknown cause for possible underlying food allergy is recommended. Important information in relation to the causal role of a foodstuff in the development of symptoms can be derived from the resolution of such symptoms when the offending foodstuff is eliminated from the diet.

4.2. Specific diagnostic tests

Diagnostic procedures for allergic disease of the gastrointestinal tract in childhood have been detailed by several professional bodies and expert reports (Wershil et al., 2002; Bachert and van Cauwenberge, 2003; Lieberman and Sicherer, 2010; Burks et al., 2011; National Institute for Health and Clinical Excellence, 2011; Caubet and Sampson, 2012).

Tests commonly used for the diagnosis of food allergies are described below.

4.2.1. Food challenges

The diagnosis of IgE-mediated and other immunologically mediated adverse reactions to food can only be confirmed by exclusion of the suspected food and the subsequent amelioration of symptoms, and by the recurrence of symptoms on re-introduction of the offending food.

The offending food can be given in open challenges (subjects are aware of being challenged with the offending food, no use of placebo), in single-blind placebo-controlled challenges (SBPCFCs; subjects are unaware of whether the offending food or a placebo is given), or in double-blind placebo-controlled food challenges (DBPCFCs; both subjects and investigators are unaware of whether the offending food or a placebo is given). Results from open-label food challenges (OFCs) are more difficult to interpret than results from SBPCFCs (i.e. difficult to attribute delayed or subjective symptoms to the ingestion of the offending food in the absence of placebo). DBPCFCs are costly, time consuming and difficult to perform. OFCs are sometimes rejected by patients or their parents and by health professionals, as there is a risk of severe reactions in highly sensitised individuals unless appropriate measures are taken. However, DBPCFCs are the gold standard because all subjective bias is removed.

Guidelines for the diagnosis of food allergy and consensus papers aiming for the standardisation of oral challenge protocols have been recently published in Europe (Muraro et al., 2014a) and the USA (Sampson et al., 2012).

4.2.2. Measurement of specific serum IgE antibodies

Allergen-specific serum IgE antibodies denote sensitisation to a particular food, but they do not provide information about the occurrence or the severity of allergic reactions following oral exposure to that food (Soares-Weiser et al., 2014). The radioallergosorbent test (RAST) is being increasingly replaced by quantitative immunochemical tests for the determination of food-specific serum IgE antibodies. The clinical sensitivity and specificity of these tests for the diagnosis of food allergy vary according to the conditions in which they are used. Depending on the incriminated food, high levels of specific IgE antibodies are a good indication to prevent oral provocation tests in highly sensitised patients (Rancé et al., 2002; Bernard et al., 2003; Caubet and Sampson, 2012). However, up to 40 % of individuals with significant allergen-specific IgE levels may not experience any clinical symptoms when challenged with this allergen (Boyce et al., 2011).

In order to confirm the specificity of the binding of serum IgE to the test food allergen, RAST tests are sometimes complemented by inhibition studies in which the IgE-binding capacity is inhibited by various competitors that are related to the incriminated food. The lack of standardisation of RAST tests for the determination of antibodies to dietary antigens and the lack of discrimination between high and low affinity antibodies have made the quantitative evaluation and the comparison of different studies difficult.

ImmunocapISAC is a blood test based on microchip technology to detect specific IgE antibodies to food and airborne allergens. It allows simultaneous measurement of specific antibodies to multiple allergen components in a single test and may allow analysis of sensitisation patterns more likely to be associated with recovery or persistence of allergic sensitisation. There is, however, the risk of over-diagnosis and misinterpretation of the complex results of such tests (Skamstrup Hansen and Poulsen, 2010; Melioli et al., 2011).

The Panel notes that there is a need for optimisation of antibody-based diagnostic tests to facilitate both the interpretation of published studies and patient management (Muraro et al., 2014a).

4.2.3. Skin prick tests

In cases of suspected IgE-mediated immunological reactions to food, an SPT may be performed. A small amount of an allergen in solution is placed on the skin and then introduced into the epidermis by gently pricking the skin surface. A positive reaction is manifested as the development of a wheal, the diameter of which can be measured to grade the reaction. The diagnostic accuracy and sensitivity of an SPT in suspected food allergies varies according to the possible offending food and is slightly higher than measuring allergen-specific IgE. A positive SPT indicates sensitisation to the tested food, but it is not diagnostic of food allergy (Soares-Weiser et al., 2014). Negative reactions have a 95 % predictive value to exclude IgE-mediated reactions. However, positive tests have only a 50 to 60 % positive

predictive value (Costa et al., 2011), although strong reactions to certain allergens indicate a higher likelihood of an allergic reaction. SPTs are usually performed on the upper back or volar surface of the forearm. Skin locations may vary in their reactivity and eczematous areas should be avoided (Cox et al., 2008).

In subjects with suspected OAS, fresh food SPTs typically have the highest sensitivity (Fernandez-Rivas et al., 2008; Webber and England, 2010). The presentation of allergens within the food matrix during an SPT challenge has to be carefully considered, since it may have a marked effect on the reactions experienced after allergen ingestion (Grimshaw et al., 2003).

4.2.4. Labial and conjunctival challenges

Labial and conjunctival food allergen challenges for diagnostic purposes have been performed mostly in children (Rancé and Dutau, 1997; Krane Kvenshagen et al., 2010). However, these tests have not been included into the routine diagnostic work-up owing to lack of standardisation, varying clinical readouts, and the absence of validation against prospective DBPCFC studies.

4.2.5. Atopy patch tests

The atopy patch test identifies allergens, which may induce a non-IgE-mediated (delayed hypersensitivity) reaction. There are published guidelines for the performance of atopy patch test (Turjanmaa et al., 2006). It involves the application of the allergen under an occlusive dressing for 48 hours onto a non-affected part of the patient's skin, and the results are read 20 minutes and 24 hours after removal of the occlusive dressing. The test has been proposed for patients affected by atopic dermatitis and gastrointestinal food allergy (Liacouras et al., 2011). Confirmation of the result by food elimination and subsequent food challenge is needed. However, the specificity and sensitivity of atopy patch tests are still a matter of debate.

4.2.6. Tests of respiratory function

Tests of respiratory function are useful where respiratory signs and symptoms are present in immunologically mediated adverse reactions to food. Such tests may include those for assessing narrowing of the airways and/or inflammation (bronchopulmonary provocation) (Pierce et al., 2005; Beydon et al., 2007), but they are not specific to food allergy.

4.2.7. Other tests in immune-mediated adverse reactions to food

Measurements of IgG and IgG subclass antibodies against food antigens in serum have no role in the diagnosis of food allergy and should not be the basis for exclusion of particular foods from the diet (Hamilton, 2010).

Flow cytometric studies of peripheral blood mononuclear cells and IgE in faecal extracts have been proposed as screening tools to identify groups of potentially food-allergic patients, but their usefulness in the diagnosis of food allergy in the individual remains to be demonstrated (Beyer and Teuber, 2005; Lock and Unsworth, 2011). The cellular basophil activation test (BAT) (e.g. expression of basophil activation markers such as CD63 and CD203c detected by flow cytometry) has also been proposed for screening, although available technologies may be optimised and better standardised (Sicherer and Sampson, 2013).

The measurement of IgE against specific components of allergens during components-resolved allergy diagnosis (Vieira et al., 2012) is not yet able to discriminate reliably between sensitisation and clinically relevant food allergy (Ebo et al., 2010b; Soares-Weiser et al., 2014).

4.3. Conclusion

A careful family and clinical history is the basis for diagnosis of food allergy. Food diaries, SPTs, allergen-specific IgE measurements, food elimination diets and food challenges are part of the standard protocol for the diagnosis of food allergy. A positive SPT indicates sensitisation to the tested

food, but it is not diagnostic of food allergy. Allergen-specific serum IgE antibodies denote sensitisation to a particular food, but they are not diagnostic without a clinical history or food challenge. The use of atopy patch tests for the diagnosis of food allergy is controversial. Other available tests have no current role in the diagnosis of food allergy. Diagnosis is confirmed by exclusion of the suspected food and the subsequent amelioration of symptoms, and by the recurrence of symptoms on re-introduction of the offending food, ideally in DBPCFCs, provided that the initial symptoms were not life threatening. The Panel notes that there is a need for standardisation of derived allergens for SPTs. The Panel also notes that guidelines aiming for the standardisation oral challenge protocols for the diagnosis of food allergy are now available.

5. Management of food allergy

5.1. Allergen avoidance

The mainstay of dietary management of food allergies is the exclusion of the offending allergenic food from the diet and the avoidance of inadvertent exposure under uncontrolled conditions of intake, i.e. travel, restaurant menus, unlabelled food sources (Eigenmann et al., 2008; Lack, 2008; Boyce et al., 2011; Burks et al., 2011). Mothers of exclusively breastfed food-allergic infants (i.e. with a clinical diagnosis of food allergy) are also advised to eliminate the offending foods from their diet, since breast milk may contain the allergen in amounts able to trigger an adverse reaction in their infants and maintain the underlying disease process (Isolauri et al., 1999; Koletzko et al., 2012).

5.2. Immunological approaches for the management of food allergies

5.2.1. Specific oral tolerance induction

Systemic oral tolerance induction (SOTI) to proteins has been recognised for a long time and frequently demonstrated in biological experiments (Niggemann et al., 2006). The underlying mechanisms relating to oral tolerance induction and desensitisation procedures are still a matter of scientific investigation (Eigenmann et al., 2008). Advantages of SOTI could be an increased individual threshold dose for the offending food and thus a reduction in the risk of experiencing severe allergic reactions after inadvertent ingestion of the allergenic food.

SOTI studies with the objective of increasing the minimum dose of an allergenic food eliciting an allergic reaction or even allowing a normal intake of that food have been performed in children allergic to peanut, milk or egg (Staden et al., 2007; Jones et al., 2009; Burks et al., 2012b). A number of study participants were able to increase their minimum eliciting dose after completing the DBPCFC protocol. Since it is unclear whether these therapies lead to immunological tolerance induction with continued allergen exposure or are a variation on (rush) desensitisation protocols, children were advised to continue the intake of the allergenic food at different time intervals. In one of these studies (Staden et al., 2007), 64 % of the treatment group had a good or at least partial response to SOTI while on treatment. Food challenges performed two months off treatment revealed that only 36 % continued to be tolerant to the allergenic food, a percentage comparable to that achieved in untreated control subjects.

Desensitisation strategies involve rush desensitisation (Itoh et al., 2010) and administration of the food allergens after heating or denaturation. In individuals with egg allergy, egg allergens have been administered in cake or as boiled or scrambled eggs, egg powder, or separated in egg white and yolk (Burks et al., 2012b). The long-term efficacy, safety and cost-effectiveness of SOTI requires further assessment (Fisher et al., 2011).

The Panel notes that SOTI is not yet recommended in routine practice as a means of inducing tolerance in children with IgE-mediated food allergy (de Silva et al., 2014).

5.2.2. Sublingual immunotherapy

Sublingual immunotherapy (SLIT) has been mainly applied to the treatment of allergic diseases triggered by environmental allergens (Larenas-Linnemann, 2009), and only rarely to food allergy (de Boissieu and Dupont, 2006). A combination of SOTI and SLIT in egg-allergic children has been reported (Keet et al., 2012). This study suggests that SLIT is less effective in the treatment of egg allergy than SOTI but has potentially less serious side effects.

5.2.3. Immunological approaches under clinical development

A number of allergen-specific and allergen non-specific immunotherapeutic approaches have reached the clinical trial stages (Nowak-Wegrzyn and Sampson, 2011). These include epicutaneous immunotherapy (Dupont et al., 2010) on the allergen-specific side, whereas anti-IgE therapy (Wang et al., 2010), Chinese herbal therapy (Srivastava et al., 2009) and anti-cytokine therapy (Straumann et al., 2010) are allergen non-specific.

5.3. Conclusion

Dietary avoidance of specific allergenic foods in combination with nutritional advice is the mainstay of management in IgE- and non-IgE-mediated food allergy. Close monitoring of growth of infants and children with food allergy is advised, as well as re-evaluation of food allergy at regular intervals to avoid unnecessary dietary restrictions. Regular pharmacological treatment of food-allergic conditions is generally not recommended.

6. Epidemiology of food allergy

6.1. Methodological considerations

Numerous publications reporting on the prevalence of food allergy are available. However, the reliability of the estimates and how these reflect the true prevalence of food allergy in the general population depend on the criteria used for the diagnosis of food allergy and on the selection of the study population. Differences in sample selection and diagnostic criteria may hamper the comparability of results among studies, as well as conclusions on time trends.

The majority of studies rely on self-reported adverse reactions to food to calculate the prevalence of food allergy (Rona et al., 2007; University of Portsmouth, 2013; Nwaru et al., 2014). They are easy to perform (generally based on questionnaires) and may include high numbers of subjects from the general (unselected) population. Such studies overestimate the prevalence of food allergy and do not differentiate between food allergy and non-immune reactions to food. However, they give an indication of the proportion of subjects who may follow dietary restrictions to avoid (and experience anxiety towards) the consumption of the “offending” food, regardless of whether they have food allergy or not (Soller et al., 2012).

Positive SPTs and/or IgE-binding (sensitisation) to the offending food in subjects with self-reported adverse reactions may strengthen the suspicion of food allergy, particularly if combined with a convincing history of food allergy and diagnosis by a physician. Still, owing to the poor positive predictive value of these tests, studies using these diagnostic criteria only overestimate the prevalence of food allergy and are usually conducted in selected population subgroups (subjects with self-reported food allergy or clinical diagnosis of food allergy), in which the prevalence of food allergy is expected to be higher than in the general population.

Positive DBPCFCs are highly reliable for the diagnosis of food allergy. Nonetheless, studies using DBPCFCs for diagnosis have been generally conducted in selected subjects with a high suspicion of food allergy, exclude highly sensitised subjects, are difficult to perform, and are generally of small sample size. OFCs are easier to perform, but the link between food ingestion and delayed or subjective symptoms is difficult to demonstrate in the absence of placebo.

An accurate estimation of the incidence and prevalence of immune-mediated adverse reactions to foods and their time trends has also been hampered by serious discrepancies in the way the International Classification of Diseases (ICD) coding has been used to characterise and classify food-allergic reactions across Europe (WHO, 1975)WHO, 2011[8]) and by the transition from ICD-9 to ICD-10.

The Panel considers that population-based studies with a step-wise diagnostic approach and confirmation of food allergy using DBPCFCs would be required to assess the prevalence of food allergies, but such studies have not been regularly conducted so far.

In this opinion, only prevalence data for the general population or for age-specific subgroups within the general (unselected) population, rather than data obtained in subjects selected based on their disease risk or disease condition, will be considered whenever available. Data obtained in European countries will be presented first. Prevalence data in Western countries such as the USA, Canada and Australia–New Zealand are considered more relevant for Europe than data obtained in Asian or African countries, and only data from the former will be considered for time trends.

6.2. Prevalence

The prevalence of food allergies in developed countries is uncertain. The scarcity of studies available for some geographical areas and the use of different methodologies across studies to retrieve prevalence data are the main reasons for this uncertainty.

The discrepancy between prevalence of perceived and confirmed food allergy among children and adults has been reported in several studies (Zuberbier et al., 2004; Osterballe et al., 2005; Pereira et al., 2005; Venter et al., 2008; Pyrhonen et al., 2009) and two meta-analyses (Rona et al., 2007; Nwaru et al., 2014).

The first meta-analysis (Rona et al., 2007) provided separate analysis for the prevalence of food allergy for five allergens (milk, eggs, peanut, fish and shellfish) stratified by age group (in children and adults), considering data from Europe, the USA, and Australia/New Zealand. The pooled prevalence of self-reported food allergy to any of these five foods (approximate figures, given in graphic form only) was 12 % (95 % confidence interval (CI): 9–14 %) and 13 % (95 % CI: 10–15 %)) for adults and children, respectively. However, pooled results were lower (3 %; 95 % CI: 2–4 %)) when the diagnosis of food allergy was based on oral food challenges for adults and children combined. The lifetime and point prevalence of self-reported food allergy in Europe have been recently estimated to be 17.3 % (95 % CI: 17.0–17.6 %)) and 5.9 % (95 % CI: 5.7–6.1 %)), respectively (Nwaru et al., 2014). In the second meta-analysis, the point prevalence was higher among children (6.9%; 95 % CI: 6.6–7.2 %)) than among adults (5.9 %; 95 % CI: 5.7–6.1 %)). However, when the diagnosis of food allergy was confirmed by a food challenge, the prevalence of food allergy in Europe was estimated to be only 0.9 % (95 % CI: 0.8–1.1 %), in both adults and children. Prevalence of both self-reported and oral challenge-confirmed food allergy was lower in Southern Europe than in Northern and Western Europe, but the number of studies available from this region was small. Only studies on self-reported food allergy were available from Eastern Europe, where the self-reported prevalence was highest. In both meta-analyses (Rona et al., 2007; Nwaru et al., 2014), the heterogeneity among the studies used to estimate the prevalence of food allergy was high.

Several studies indicate that 75 % of allergic reactions among children are due to a limited number of foods, namely egg, peanut, cow's milk, fish and various nuts. Among adults, fruits of the latex group (e.g. kiwi, banana), fruits of the *Rosaceae* family (e.g. apples, pears, prunes), vegetables of the *Apiaceae* family (e.g. carrot, celery), and various nuts and peanuts (Kanny et al., 2001; Sastre, 2010; Dupont, 2011; Gadermaier Gabriele et al., 2011) are responsible for 50 % of allergic reactions.

[8] http://www.who.int/classifications/icd/en/

6.3. Time trends

There is evidence that the prevalence of atopy has increased over the last decades (Linneberg et al., 2000; Strannegard and Strannegard, 2001; Kosunen et al., 2002). With respect to food allergy, hospital admissions in the UK rose from 5 to 26 per million in adults from 1991 to 2004 and from 16 to 107 per million in children during the same period (Gupta et al., 2003; Gupta et al., 2007). However, at the beginning of the 1990s, awareness of food allergy in the medical community was not as widespread as it was in the 2000s, and these trends can be explained by changes in perception and diagnostic practices over time. Increase in public awareness of food allergy with broader media attention should also be considered when interpreting these results.

Owing to the lack of repeated cross-sectional studies over time conducted with comparable methodologies, there are no objective data to conclude on time trends with respect to the prevalence of food allergy in Europe.

6.4. Severe reactions/anaphylaxis

Data on the prevalence of food anaphylaxis are to be taken with caution owing to the lack of a universally accepted definition and the risk of misclassification, mostly because of selection bias based on hospital presentation.

From national mortality registers, anaphylaxis fatalities from all causes were estimated to be 0.33 deaths per year per million in the UK between 1992 and 2003 (n = 202). The cause of fatal episodes of anaphylaxis was reported to be “food or possible food” in 31 % of cases (n = 63), which corresponds to approximately 0.1 deaths per year and per million (Pumphrey, 2004). Tree nuts and peanuts contributed to 50 % of fatal food anaphylaxis in this study. A registry kept in the USA recorded 31 individuals who died of food-induced anaphylaxis between 2001 and 2006. Subjects ranged from 5 to 50 years of age. Peanuts accounted for 17 deaths, tree nuts for eight, milk for four and shrimps for two. All subjects for whom there are data had asthma (Bock et al., 2007). A total of 197 anaphylactic reactions (defined as severe systemic allergic reactions with concomitant pulmonary and/or cardiovascular symptoms) were registered between 2006 and 2009 in the anaphylaxis registry of German-speaking countries in children and adolescents (Hompes et al., 2011). Food allergens accounted for 58 % of cases. Legumes (n = 36), and in particular peanuts (n = 26), were the most frequent food allergens causing severe allergic reactions, followed by tree nuts (n = 29), cow’s milk (n = 11) and hen’s egg (n = 8).

The prevalence of asthma in Europe varies from about 10 to 20 % according to the International Study of Asthma and Allergies in Childhood (ISAAC) programme (Pearce et al., 2007). Some studies suggest that about 2 % of adults (Ozol and Mete, 2008) and about 6 to 8 % of children (Oehling and Cagnani, 1980; Novembre et al., 1988) with asthma may show an asthmatic reaction on food challenge. Food allergy has been found to be a major risk factor for severe asthma and life-threatening asthma episodes (Liu AH et al., 2010). Asthma is also present in nearly all people who have fatal anaphylactic reactions, and severe asthma is a common manifestation of food allergy (Pumphrey, 2004; Bock et al., 2007).

6.5. Conclusion

The prevalence of food allergies in developed countries is uncertain. The scarcity of studies available for some geographical areas and the use of different methodologies across studies to retrieve prevalence data are the main reasons for this uncertainty. Using food challenges as a criterion for diagnosis, the prevalence of food allergy has been estimated to be around 3 % when considering data from Europe, the USA and Australia–New Zealand, and about 1 % when considering European studies only, both in adults and in children. However, the heterogeneity among the studies used to estimate the prevalence of food allergy was high. There are insufficient objective data to conclude on time trends with respect to the prevalence of food allergy in Europe. About 75 % of allergic reactions among children are due to egg, peanut, cow’s milk, fish and various nuts. About 50 % of allergic reactions

among adults are due to fruits of the latex group and of the *Rosaceae* family, vegetables of the *Apiaceae* family and various tree nuts and peanuts.

7. Influence of environmental and individual factors in the distribution of food allergies

The occurrence of allergies in general, and of food allergies in particular, requires the susceptibility of the host and the exposure to the allergen. Geographical variations in the prevalence of food allergy are driven by genetic factors and further modified by regional or local factors, such as pollen exposure or differences in food habits. Although little is known about the variability of genetic susceptibility among populations or the factors that may modify allergic responses, extrapolation of prevalence data on specific food allergies from a single European country to the entire European population may be limited by differences in exposure to the offending foods and eating habits. Inter-country differences in reporting adverse reactions to foods have also been noted and probably attributed to cultural differences (Woods et al., 2001). Although several environmental and individual factors have been proposed as potential modifiers of the risk of developing food allergy, there is inconsistency across studies regarding the factors investigated and the results obtained (Nwaru et al., 2014). Examples of environmental and individual factors, which have been proposed to influence the distribution of food allergies, are briefly discussed below.

7.1. Environmental factors

7.1.1. Food consumption

Some foods are more allergenic than others, i.e. they have a greater intrinsic capacity to induce allergic sensitisation and elicit allergic reactions in the general population. Examples of highly allergenic foods are milk, egg, fish and other seafood, peanuts and other nuts, soy, sesame seeds and celery. Other foods, e.g. potatoes, induce allergy more rarely, in spite of high levels of consumption. The amount of allergen consumed is considered an important determinant of food allergy, which in turn depends on the amount of a given allergenic food that is consumed on a regular basis (eating habits).

7.1.1.1. Allergenic proteins in foods

Common proteins that are present in large quantities in a food will have a greater probability of becoming allergens than proteins that are present in small quantities. Storage proteins of many nuts and seeds are an example. These proteins may account for half the weight of the seed or nut. The amount of some allergens in a food will depend on plant variety and growing conditions, and this may contribute to geographical variation in some food allergies, and to variation in the allergenicity of a given plant product (Codina et al., 2003).

7.1.1.2. Eating habits

In a geographic area where a certain food is commonly consumed, the risk of allergy to that food will generally be greater than in areas where that particular food is more rarely eaten. If a food is commonly eaten, not only will the induction of allergy to that food be more likely, but also allergic reactions will be more frequently triggered. The individual dose–response relationship between the consumption of a specific food and the development of sensitisation/allergy to that food is unknown. The individual dose–response relationship appears to be dependent on genetic and other individual factors.

Despite uniformisation of diets, notably in Western countries, regional differences in the type and amount of food allergies are clear. For example, prevalence rates of peanut allergy are higher in North America and the UK than in Mediterranean countries. This is illustrated by the fact that the prevalence of peanut allergy is 10-fold higher in Jewish children living in the UK than in Jewish children living in Israel (Du Toit et al., 2008). Such differences in prevalence may be due to differences in the level of allergen exposure or food processing rather than to differences in genetic background (Lack, 2012).

7.1.1.3. Introduction of food and breastfeeding

Best practices in relation to maternal diet, breastfeeding, and time of introduction of solid foods in order to decrease the risk of atopic diseases in infants at risk (i.e. with at least one (in Europe) or at least two (in the USA) first-degree family member being allergic) have been a matter of debate (Greer et al., 2008).

There is no evidence that maternal dietary restrictions during pregnancy play a significant role in preventing atopic disease (asthma, allergic rhinitis, food allergies or eczema) in infants (Kramer and Kakuma, 2012). Exclusive breastfeeding for at least four months is associated with a decrease of the incidence of eczema and cow's milk allergy in the first two years of life compared with feeding intact cow's milk-based formulas in at-risk infants (Zeiger, 2003; Greer et al., 2008; Boyce et al., 2011).

In infants at risk who are not breastfed, early dietary intervention in the first four months of life with cow's milk protein hydrolysates (extensively hydrolysed casein or partially hydrolysed whey formulas) appears to reduce allergic manifestations significantly (Osborn and Sinn, 2006; Szajewska and Horvath, 2010). Most studies showing a preventative effect were mainly on atopic dermatitis (Szajewska and Horvath, 2010) but also on food allergy and early wheezing (Zeiger, 2003). A 10-year follow-up of the German Infant Nutritional Intervention (GINI) Study showed that feeding a partially hydrolysed whey or an extensively hydrolysed casein formula may decrease the cumulative incidence of eczema but not of asthma, allergic rhinitis or sensitisation to common food allergens or aeroallergens in children at risk up to the age of 10 years compared with feeding an intact cow's milk-based or extensively hydrolysed whey formulas (von Berg et al., 2013). In this context, it is of note that no EU regulatory definition of the level of protein hydrolysation in formulas is available and that the extent to which a formula is declared to be hydrolysed does not imply an effect on the risk of developing an allergy. The Panel considers that clinical studies are necessary to demonstrate the potential of each particular hydrolysed formula to prevent the occurrence of short- and long-term clinical manifestations of food allergy in infants at risk who are not breastfed.

Current evidence does not support feeding with a hydrolysed formula for the prevention of allergy compared with exclusive breastfeeding (Osborn and Sinn, 2006). Amino acid-based formulas, and intact or hydrolysed soy or rice formulas, have not been shown to prevent allergic diseases in intervention studies.

It has been suggested that early complementary feeding (before four months) may reduce allergic sensitisation in children with a parental history of asthma or allergy (Joseph et al., 2011), and that dietary manipulation might affect the risk of developing food allergy and atopic manifestations such as asthma or eczema in infants at risk (Kumar et al., 2010). However, the evidence is insufficient to recommend the introduction of complementary feeding before four months for that purpose. In addition, there is no convincing scientific evidence that avoidance or delayed introduction of foods beyond four to six months reduces the risk of allergies in infants at risk (Greer et al., 2008; Muraro et al., 2014b). Concerns have been raised by the National Institute for Allergy and Infectious Diseases (NIAID) in relation to delaying the introduction of certain foods, which could result in inadequate nutritional intake, growth deficits and feeding problems (Boyce et al., 2011).

7.1.2. Food processing and preparation

Processing and preparing food may increase or decrease its allergenicity. The same raw product may be processed and prepared in different ways according to local traditions and socioeconomic setting. Also, the food matrix may influence the likelihood of inducing an allergic reaction, its severity and/or the time of the reaction after food ingestion (food matrix effect).

7.1.3. Other environmental factors modulating allergic reactions to food

The "hygiene hypothesis" suggests that the lack of early exposure to microorganisms increases susceptibility to atopic diseases by modulating the development of the immune system (Prescott et al.,

2010; Gourbeyre et al., 2011). However, while certain infections are suggested to be protective, other studies do not support this hypothesis, and some parasites (e.g. *Ascaris suum*) and respiratory viral infections have been associated with an increased risk of developing food allergy (Ben-Shoshan et al., 2012).

7.2. Individual factors

Sex, age, family history of atopy and the presence of other allergic diseases are among the individual factors considered important in the development of food allergy (Nwaru et al., 2014).

7.2.1. Genetic background

A family history of food allergy is a major risk factor for the development of food allergy. For example, having a sibling with peanut allergy increases the risk of developing peanut allergy by five (Hourihane et al., 1996).

A limited number of reports describe a significant association between specific food allergies and specific human leucocyte antigen (HLA) types. Since some HLA types show distinct geographical variation, such variation could, in principle, contribute to the geographical variation in food allergy. However, the extent to which HLA types determine clinical allergic reactions to particular foods is uncertain. For example, HLA-DRB1, -DQB1 and -DPB1 have been associated with an increased frequency of peanut allergy (Boehncke et al., 1998; Howell et al., 1998). However, none of such associations were statistically significant after adjustment for multiple testing (Hong et al., 2009), and a genotypic association between the HLA class II alleles and peanut allergy in a cohort of sibling pairs discordant for peanut allergy could not be established (Shreffler et al., 2006).

Mutations in the profilaggrin gene resulting in loss of function of fillagrin, an epidermal protein with a role in the skin barrier function, have been identified as a risk factor for developing allergic sensitisation, atopic eczema, and allergic rhinitis, as well as asthma in individuals with atopic eczema (van den Oord and Sheikh, 2009). Fewer studies have reported on the relationship between filaggrin loss-of-function (FLG-LOF) mutations and risk of food allergy (Brown et al., 2011). It has been suggested that FLG-LOF mutations could modulate the risk of food allergy through early sensitisation to food owing to the impairment of the skin function barrier (Filipiak-Pittroff et al., 2011; Venkataraman et al., 2014).

7.2.2. Age and sex

The overall occurrence of food allergy changes with age (Osterballe et al., 2005; Pereira et al., 2005; Venter et al., 2006a; Venter et al., 2006b; Venter et al., 2008; Zuidmeer et al., 2008; Osterballe et al., 2009) and so do the specific allergies (Kagan et al., 2003; Rona et al., 2007; Zuidmeer et al., 2008). Egg and milk allergy are quite common among infants but are often outgrown in early childhood. Conversely, shellfish allergy is more common among adults than among children, while peanut allergy is more common among children than among adults. The age dependency of food allergy is partly due to the so-called “atopic march” and can also be in part explained by exposure factors. Milk consumption is high for small children, while shellfish is consumed more commonly by schoolchildren and adults.

To what extent sex may determine the individual susceptibility to food allergy has not been systematically investigated. In adults, food allergy is somewhat more common in adult women (Schafer et al., 2001; Zuberbier et al., 2004). Of the first 250 cases reported to the Norwegian National Reporting System and Register of Severe Allergic Reactions to Food, the female to male ratio was about 3:2 (Løvik and Namork, 2004) in adults. The putative gender difference could be due to physiological differences or to differences in healthcare-seeking behaviour. There is also little information on sex differences in food allergy in children, among whom food allergy seems to be more common in males (Ben-Shoshan et al., 2012).

7.2.3. Socioeconomic factors

Most (Metsala et al., 2010; Gupta et al., 2011), but not all (Victorino and Gauthier, 2009; Liu AH et al., 2010), studies suggest an increased rate of food allergy in higher socioeconomic populations, but evidence on that association is still controversial. The direct association between parental socioeconomic status and food allergy observed in children may be explained by the fact that mothers of high socioeconomic status consult a physician more often than mothers of lower socioeconomic status.

The geographic remoteness from cities (rural areas) characterised by difficulties in accessing primary and specialist medical care, and sometimes the significant cost of transport, could also explain why socioeconomic advantage and residence in major cities may be considered as risk factors for childhood food allergy (Mullins et al., 2010). However, it remains possible that the association of a decreased risk of food allergy with a low socioeconomic status could be explained by confounding factors, such as mode of infant feeding and environmental conditions.

7.2.4. Ethnicity

Differences in the prevalence of food allergy among ethnic groups could be due to genetic differences (e.g. in the HLA system), different food habits and possibly different prevalences of food allergy in the country of origin for immigrants. Information on the prevalence of food allergy in different ethnic groups is scarce. It appears that immigrants from less developed countries generally present fewer atopic diseases at the time they migrate to European countries. However, they gradually adapt to the new environment and, over a decade, they become more similar to the people who grew up in the country of destination (Kalyoncu and Stalenheim, 1992). In the European Community Respiratory Health Survey (Tobias et al., 2001), immigrants as a group had similar levels of atopy as non-migrant Europeans.

7.2.5. Other individual factors

Physical exercise, alcohol, antibiotics, gastric acidity inhibitors and non-steroidal anti-inflammatory drugs (NSAIDs) may increase the likelihood of food-allergic reactions (Sicherer and Sampson, 2013).

7.3. Conclusion

The occurrence of food allergies requires susceptibility of the host and exposure to the allergen. Geographical variation in the prevalence of food allergy is due to differences in environmental (e.g. pollen exposure or differences in food habits) and individual factors. Sex, age, family history of atopy and the presence of other allergic diseases are among the individual factors considered important in the development of food allergy. Extrapolations of prevalence data on specific food allergies from a single European country to the entire European population are of limited accuracy owing to differences in genetic background, exposure to the offending foods and eating habits.

8. Characterisation of food allergens

8.1. Introduction and nomenclature

Allergenicity of a given complex food might not be due to a single protein component but to different proteins that constitute the allergen repertoire of the food. The combination of food science and medical science allows definition of the clinically relevant food allergens contained in different foods. Several food allergens have been isolated, purified and characterised.

A systematic International Union of Immunological Societies (IUIS) Allergen Nomenclature has been established and adopted by WHO (King et al., 1994). The official site (http://www.allergen.org) lists all recognised allergens and isoforms and is regularly updated. By convention, allergens in the systematic IUIS nomenclature are designated by the first three letters of the genus, the first letter of the species name according to the Linnaean taxonomic system and an arabic number reflecting the chronological order in which the allergen was identified and characterised (e.g. Bos domesticus 4)

(Chapman et al., 2007). The nomenclature also defines isoallergens and variants. Isoallergens are allergens from a single species, which share similar molecular size, identical biological function, and more than 67 % of the amino acid sequence (sequence identity). Isoallergens are denoted by the addition of two numeral suffixes to the allergen name (e.g. Ara h 1.01). A variant is an allergen that shows a limited number of amino acid substitutions in the isoallergen structure and is denoted by the addition of another two numbers to the allergen name (e.g. Ara h 1.0101). A gene encoding for a specific allergen is denoted in italics (e.g. *Ara h 1*). Natural allergens may be denoted by the prefix “n” to distinguish them from recombinant allergens, which are indicated by the prefix “r” (e.g. nPru p 3/rPru p 3). The insertion of a synthetic peptide in an allergen structure is indicated with “s”, with the particular peptide residue indicated in parentheses after the allergen name (e.g. sBet v 1.0101 (100–120)).

The WHO/IUIS Allergen Nomenclature database uses the terms “major” and “minor” for allergens, depending on whether more or less than 50 % of the allergic patients tested show allergen-specific IgE-binding to this allergen in a given test system, respectively. These terms do not refer to the ability of the allergen to trigger clinical allergic reactions or to their severity, i.e. clinical reactions may be similar whether they are triggered by major or minor allergens.

A number of allergen databases that differ with respect to the number of molecules listed as allergens and to the type of information displayed have been reviewed (Brusic et al., 2003) (Gendel and Jenkins, 2006; Mari et al., 2006; Schein et al., 2007; Gendel, 2009). The Allergome database provides regular updates on allergens from publications in the scientific literature (http://www.allergome.org/). Not only allergenic molecules are reported but also allergenic sources, organisms and IgE-binding molecules, either causing clinical allergic reactions or not. The Protein family (Pfam) database (http://pfam.sanger.ac.uk) assigns sequences of clinically proven food allergens to protein families (Jenkins et al., 2005). AllFam merges the Allergome allergens database with data on the Pfam database (Radauer and Breiteneder, 2007) and contains all allergens with known sequences that can be assigned to at least one Pfam family (http://www.meduniwien.ac.at/allergens/allfam/). The Structural Database of Allergenic Proteins (SDAP) provides detailed structural data on allergens in the IUIS Nomenclature, including sequence information, Protein Databank files (PDB-files) and computational tools to analyse IgE epitopes (http://fermi.utmb.edu/). The Food Allergen Research and Resource Program (FARRP) is focused on food allergens, providing sequence similarity searches (http://www.farrp.org/), while the PROTALL, which includes only allergens derived from plants and clinical data (SPTs and provocation tests), has developed into the InformAll Database dedicated to all food allergens (http://foodallergens.ifr.ac.uk). The SWISS-PROT (http://www.genscript.com/) and the National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov/) databases include protein and nucleotide sequence information not restricted to allergens.

In this opinion, only food allergens listed in the IUIS database as of December 2013 will be mentioned and discussed in the sections dedicated to specific allergenic foods, unless otherwise specified.

8.2. General considerations on the structure of food allergens

The allergenicity of a protein is due to the IgE-binding epitopes that are widespread within the protein molecule. Epitope mapping is the characterisation of all epitopes in an allergen molecule. Similar to allergens, not all epitopes in a protein are recognised by all patients allergic to that protein: some epitopes are immuno-dominant, while others are recognised by only a few patients. Two types of epitope have been described depending on their structure: conformational epitopes, which are associated with the secondary and tertiary structure of the protein, and linear/sequential epitopes, formed by a continuous sequence of amino acid residues in the protein chain. Once the protein is denatured, conformational epitopes are generally modified or destroyed, whereas linear epitopes are maintained. The clinical significance of epitopes may depend on their structure and location within the molecule. For example, short linear IgE-binding epitopes located in hydrophobic parts of allergenic proteins could be used as markers of a persistent food allergy, i.e. to milk and to peanut (Chatchatee et al., 2001).

Although there are no common structural features which allow predicting the allergenic potential of a protein, food allergens generally belong to protein families that have conserved structural features in relation to their biological activity, have a globular compact structure stabilised by hydrogen and disulphide bonds and are often glycosylated, stable to processing and resistant to proteolysis by digestive enzymes.

8.3. Classification of food allergens based on their structural properties

Owing to the development of proteomics, spectroscopic methods and gene cloning, proteins have been classified into families on the basis of their sequence and three-dimensional (3D) structure. The structural features and potential allergenicity of the most important allergen protein families have been reviewed (Hoffmann-Sommergruber and Mills, 2009).

The biological activity of a protein is related to its structure. Some allergens bind ligands, such as metal ions, lipids and steroids. Others interact with bacterial or fungal membranes inducing a leakage, thus protecting plants from microbial pathogens. Some enzymes are allergens, such as lysozyme, cysteine proteases, transferrins and arginine kinases. While in some cases allergenicity is strictly related to the biological activity (e.g. proteolytic activity), in other cases it is not.

The Panel notes that, although common structural features of proteins and biological activity have been tentatively related to their immunogenicity, it is not possible to predict the allergenicity of a protein on the basis of only these two parameters (Breiteneder and Mills, 2005).

8.3.1. Allergens of plant origin

Both Pfam and AllFam databases classify plant allergens into four main families on the basis of sequence homology, conserved 3D structures and function: the prolamin superfamily; the cupin superfamily; profilins; and the Bet v 1 superfamily.

The prolamin superfamily contains the largest number of plant food allergens: 2S seed storage albumins; cereal seed storage proteins; cereal α-amylase/trypsin inhibitors; and non-specific lipid transfer proteins (nsLTPs). Prolamins were originally defined on the basis of their water/alcohol solubility and of their content of proline and glutamine. Prolamins are characterised by a high content of sulphur-containing amino acid residues and often consist of bundles of four α-helices stabilised by disulphide bonds, involving eight well-conserved cysteine residues. The major role of 2S albumins is to provide proteins to the developing seed. They also have a defensive role against pathogenic fungi. Major allergens in tree nuts, sesame and mustard seeds belong to this family. Cereal seed storage proteins are characterised by a high content of proline and glutamine. Cereal α-amylase and protease inhibitors induce a certain resistance in plant tissues to insect pests and include allergens present in wheat, barley, rice and maize (Pastorello et al., 2002b). The lipid transfer protein family comprises low-molecular-weight monomeric proteins (around 7–9 kDa) involved in the synthesis of cutin, and thus have a protective role in the plant, and particularly in the fruit. They have a very compact and stable tertiary structure constituted by the association of α-helices and loops stabilised by eight disulphide bonds, which define a central cavity containing a lipid-binding site. Binding with hydrophobic ligands also contributes to the stabilisation of the molecule. Lipid transfer proteins are frequent and potentially severe allergens: they are one of the numerous defence protein families (also called pathogenesis-related proteins) that are responsible for most of the severe allergic reactions to fruits from the *Rosaceae* family.

The cupin superfamily includes the major globulin storage proteins, which are the cause of most allergic reactions to legumes and nuts. The name comes from their common architecture, consisting of six-stranded β-sheets associated with α-helices which form a β-barrel cavity (Latin *cupa*, barrel) with a binding site for a hydrophobic ligand (Breiteneder and Ebner, 2000). Subgroups in the cupin superfamily have been defined depending on the number of cupin domains present in the protein. Monocupins comprise the majority of cupin proteins, can be monomeric, dimeric or oligomeric, and most are enzymes (e.g. dioxygenases). Germin and germin-like proteins (GLPs) are oligomeric

monocupins ubiquitous in plants (e.g. wheat and barley). They have a disc-shape homohexameric structure organised as trimers of dimers. The globulin fractions of seed storage proteins, which can be extracted with saline solutions, are two-domain cupins. According to their sedimentation coefficient determined by ultra centrifugation, globulins are divided in a smaller fraction, i.e. 7S/8S globulins (called vicilins), and a bigger fraction, i.e. 11S globulins (called legumins). 7S/8S globulins are generally trimers with a molecular weight of 50 to 60 kDa. Post-translational modifications such as glycosylation often occur. 11S globulins consist of six subunits with a molecular weight of around 60 kDa and are rarely glycosylated. Each subunit consists of a non-covalent association of two polypeptide chains. 7S and 11S globulins have a relatively low sequence identity but a common 3D conformation. Globulins are clinically relevant allergens in peanuts, soybeans, lentils, walnuts, hazelnuts and sesame seeds.

Profilins are cytosolic proteins of 12 to 15 kDa exclusively found in flowering plants, such as peanut (Ara h 5), apple (Mal d 4) and celery (Api g 4). They are folded in a compact globular structure of an antiparallel β-sheet enclosed by α-helices on both sides. The high sequence conservation and the even higher 3D structure similarity account for the strong serological cross-reactivity with other plant foods, pollens and *Hevea latex*, which may be of variable clinical significance.

The Bet v 1 superfamily comprises eight families, among which are the "pathogenesis-related proteins 10" (PR 10), the major latex proteins. These allergens are homologous to the major birch pollen allergen Bet v 1 and are present in fruits of the Rosaceae family (e.g. apple, cherry, apricot, and pear) and Apiaceae vegetables (e.g. celery, carrot). They are polypeptides of 154–160 amino acids with high sequence similarity. The Bet v 1 homologous proteins contain a GXGXXG or a GXG motif, responsible for binding of the phosphate group of oligonucleotides, and share a characteristic fold formed by seven β-sheets surrounding a long C-terminal helix and two additional short helices connecting two β-sheets and forming a large y-shaped hydrophobic cavity able to bind sterols, as observed in structures obtained by X-ray crystallography. Because of their sequence and 3D similarities, the Bet v 1-related proteins cross-react with allergens present in birch pollen, in particular with Bet v 1, sometimes inducing severe allergic reactions.

8.3.2. Allergens of animal origin

Food allergens of animal origin, less numerous than allergens of plant origin, are classified into three main structurally related families: tropomyosins; EF-hand proteins; and caseins.

Tropomyosins are a family of closely related proteins present in muscle and other cells with a regulatory role in muscle contraction. They contain a seven-amino acid repeat (heptad), with most isoforms having a series of 40 continuous heptads. These proteins form a parallel α-helical coiled-coil dimeric structure, which then binds head to tail to form a cable winding around the helix. Tropomyosins, which are clinically relevant as food allergens, are present in molluscs and crustaceans.

The EF-hand proteins present a helix–loop–helix motif characterised by a sequence of usually 12 amino acid residues, which form a loop flanked on both sides by a 12-residue α-helical domain. This loop is capable of coordinating calcium or magnesium ions with different geometries. The same motif is present in a large family of calcium-binding proteins, such as parvalbumins, which have three EF-hand motifs, two of which are capable of binding calcium. Loss of calcium by thermal treatment induces major conformational changes in the protein, with loss of conformational epitopes. However, the remaining IgE-binding epitopes are sufficient to trigger allergic reactions in fish-allergic subjects (Lewit-Bentley and Rety, 2000).

Caseins are mammalian proteins present in milk that bind calcium ions through the phosphoserine or phosphothreonine residues of αS1-, αS2- and β-casein, forming nanoclusters in which amorphous calcium phosphate is included, stabilised by κ-casein. Nanoclusters aggregate (ca. 1 000 molecules) to form macrostructures, corresponding to the milk micelles.

8.4. Stability of food allergens

An important characteristic shared by the majority of allergens is stability, defined as the capacity to maintain their native 3D structure upon thermal, chemical or enzymatic (proteases) treatment. No single structural motif can account for the stability of a protein (Breiteneder and Mills, 2005). However, structural features clearly related to stability include the β-barrel structure of cupins and the presence of intra- and intermolecular disulphide bonds, which constrain the molecule in a rigid scaffold not easily disrupted which can be eventually reformed in a different position following the treatment. This type of covalent bond is found in the prolamin superfamily and in thaumatin-like proteins (TLPs).

Although a number of allergens share a compact globular shape with a well-defined 3D structure, others contain large regions of disordered structures. Such proteins are constituted by polypeptidic chains with different secondary structures in equilibrium with each other, resembling unfolded or partially unfolded proteins, and are called rheomorphic. On account of their flexibility, they are more susceptible to hydrolysis by proteases, but do not undergo conformational changes and their epitopes remain exposed even after thermal treatments. Caseins and the seed storage prolamins belong to this group.

Glycosylated allergenic proteins appear to be more resistant to proteolysis. N-glycosylation, which usually occurs on asparagine residues in a specific three amino acid sequence (asparagine–any amino acid–serine or threonine), can have a significant stabilising effect on a protein, as in the case of the 7S globulin of pea. Hydroxyproline, serine and threonine can also be O-glycosylated, contributing to the 3D structure of the protein. Glycosylation plays a role in inducing cross-reactivity between pollen and plant allergens.

Under physiological conditions or following industrial treatments, food allergens with repetitive structures form non-covalent aggregates, which are particularly stable to heat. Tropomyosin allergens from shellfish and seed storage proteins belong to this category. Protein aggregates in foods may be more allergenic than monomeric proteins owing to the higher number of IgE epitopes they contain.

8.5. Resistance of food allergens to *in vitro* digestion

The digestibility of allergens *in vitro* has been studied either to provide a biochemical measure of their physicochemical stability under non-physiological conditions or to investigate the role of digestion on their allergenic potential under simulated physiological conditions.

The *in vitro* simulated gastric fluid (SGF) pepsin resistance test shows that there is a certain correlation between resistance to proteolysis and allergenic properties of food allergens (Astwood et al., 1996) (FAO/WHO, 2001; Codex Alimentarius Commission, 2003), although a cause–effect relationship cannot be established owing to the variability of results obtained from digestibility studies performed under different testing conditions (e.g. pH, protein–enzyme ratio, purity of the protein). Thus, resistance to proteolysis cannot be used as a parameter to predict the allergenicity of a protein, whereas the SGF test is commonly accepted as a way of establishing the chemical stability of the protein and for structure determination. A "simulated intestinal fluid" test with the use of trypsin and chymotrypsin as duodenal digestion enzymes is also available. A sequential treatment with SGF and the "simulated intestinal fluid" has been proposed to simulate the entire transit of food in the gastrointestinal tract (Mouécoucou et al., 2004).

The digestibility of a protein depends on its structure but also on the food matrix in which it is contained, which may hamper or favour the accessibility of digestive enzymes to the protein. Thus, more sophisticated static models aiming to mimic physiological conditions as closely as possible by the addition of other substances present *in vivo* which may affect digestibility, e.g. biosurfactants such as phosphatidyl choline and bile salts or food ingredients such as lipids and carbohydrates, have been developed. A standardised digestion protocol with and without the addition of surfactants has been tested in a multi-laboratory trial in the course of the EuroPrevall EU-funded Project (Mandalari et al.,

2009), showing consistency between laboratories for two allergenic milk proteins, β-casein and β-lactoglobulin.

Dynamic *in vitro* digestion models, which take into account other factors affecting digestibility, such as mastication, gastrointestinal transit and peristalsis, have also been developed. The effects of different *in vitro* models on the stability of food allergens to digestion have been reviewed (Moreno, 2007).

The Panel notes that *in vitro* digestion tests should be combined with immunological assays in order to understand the interaction of peptides derived from proteolysis (or their aggregates) with the immune system.

8.6. Physicochemical characterisation of food allergens

8.6.1. Extraction, isolation and purification

The first step in the structure characterisation of a food allergen is the extraction of the protein from the food matrix in suitable amounts to allow the characterisation of the structure by spectrometric and spectroscopic methods, as well as the verification of its IgE-binding capacity. Cloning techniques can be used to obtain sufficient amounts of protein, as long as the identity of the recombinant product with the native protein is confirmed. Extraction methods vary according to the nature of allergens, e.g. incubation of the raw material in a buffer at different pH plus centrifugation, extraction protocols to eliminate concurrent extraction of contaminants (phenols), use of salting out methods and defatting procedures to eliminate undesired sugars and lipids.

Purification of the protein is obtained by monodimensional electrophoretic separation on SDS-PAGE or by chromatographic methods, using different columns according to the nature of the protein. Ion-exchange chromatography as well as preparative reversed-phase high-performance liquid chromatography (RP-HPLC) are efficiently used according to the inherent polar/apolar character of the protein. Size exclusion chromatography (SEC), also called gel filtration, allows the exclusion of contaminants and other proteins on the basis of their different size or molecular weight relative to a porous matrix (swollen gel) with pores of a particular size (mesh). Affinity chromatography is a very specific tool for protein purification, which relies on the affinity of a ligand (e.g. specific antibody) immobilised in the column for the particular allergen, but requires previous knowledge about the nature of the allergen to be purified.

Methods for the isolation and purification of food allergens have been described in detail elsewhere (Pastorello and Trambaioli, 2001a).

8.6.2. Allergen identification: sequencing and mass spectrometry

Sequencing and/or physicochemical methods are used to verify the identity of the purified allergen (Harrer et al., 2010). The unequivocal identification of a known protein may be achieved by combining Edman degradation data on the amino acid sequence of the N-terminal region with the exact molecular mass of the entire molecule obtained by mass spectrometry (MS) or high-resolution MS (HR-MS).

The molecular characterisation of an allergen starts with the determination of its molecular weight by MS. Such methods allow the detection and unambiguous identification of food allergens because of their specificity and sensitivity (Monaci and Visconti, 2009). Two strategies are available for the identification of proteins by MS: the “bottom-up” and the “top-down” strategies.

8.6.2.1 The “bottom-up” strategy

The “bottom-up” strategy can be conducted following two workflows: (i) the proteins are first fractionated and separated, and the single protein is digested with one or more proteolytic enzymes in gel, in solution or in a column, then the peptides obtained are analysed by MS; (ii) protein digestion is

performed without any prefractionation/separation, peptides are separated by multidimensional chromatography (shotgun proteomics) and identified by tandem mass spectrometry (MS/MS). Although the shotgun approach is quite simple, it requires highly sensitive and efficient separation of the complex peptide mixture generated, and can lead to incorrect identification.

One route to protein identification is based on the mass measurement of a number of peptides that are unique for the protein, whose masses are used as a fingerprint of the original protein (peptide mass fingerprinting (PMF)). The other route is based on the information obtained from fragmentation of one or more of these peptides by MS/MS (peptide fragment fingerprinting (PFF)). While mono-stage MS usually performs PMF, MS/MS is mostly used for peptide sequencing, since it provides detailed structural features of peptides, which may be inferred from the resulting fragments. In order to identify the allergenic protein, the peptide spectra are scanned against specific protein-sequence databases using statistical tools.

Different instruments are available to perform MS/MS: quadrupole (Q), tandem time of flight (TOF^2), ion trap (IT) and Fourier transform ion-cyclotron resonance (FT-ICR). These MS instruments may be coupled to different ion sources: matrix-assisted laser desorption ionisation (MALDI) or surface-enhanced laser desorption ionisation (SELDI), which are traditionally coupled with TOF analysers and are able to measure masses of intact proteins or peptides, while electron spray ionisation (ESI), which is mostly coupled to ion traps (ITs) and triple quadrupoles (QqQs), generates fragment-ion spectra from selected precursor ions. These MS configurations are most used for allergen identification (Monaci and Visconti, 2009).

8.6.2.2. The "top-down" strategy

In the "top-down" strategy, the intact protein is ionised and directly fragmented in the mass spectrometer, without preliminary digestion. Subsequent high-resolution mass measurement of the relevant ions produced allows sequence-specific information for database searching and protein identification to be obtained. MALDI-TOF analysis is currently applied in the "top-down" strategy for the identification of intact proteins, and in particular of small proteins (Monaci and Visconti, 2009). The efficient fragmentation of intact proteins can be achieved by the MALDI-TOF^2 technology and FT-ICR, as well as by IT and linear ion trap (LTQ)-Orbitrap, which grants large ion capacity, large dynamic range, high mass accuracy and high resolution. MALDI-TOF-MS technology was used to identify and characterise several allergenic proteins from hazelnut (Lauer et al., 2008), cow's milk (Natale et al., 2004) and soybean (Krishnan et al., 2009), among other allergenic foods.

Details on the use of MS for the qualitative/quantitative determination of food allergens are given in section 11.1.3.

8.6.3. Identification of epitopes

Identification of epitopes is important for the characterisation of food allergens. Sequential epitopes may be identified by an ELISA system with the use of patient sera (PEP-SCAN). Conformational epitopes are best characterised by the phage display technique, in which libraries of randomised short peptides are fused to the coat proteins of filamentous phages and examined with sera of allergic patients. Another method consists of replacing each amino acid, one by one, with a different amino acid in the IgE-binding epitope or by inducing mutations in the IgE-binding epitope with amino acid substitution or deletion.

8.6.4. Three-dimensional structure

Knowledge of the 3D structure of an allergen is needed to gain information on the surface of the protein, to provide evidence for the epitopes and to evaluate potential cross-reactivities. The 3D structure of an allergenic protein in the solid state may be assessed by X-ray crystallography, and in solution by nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD). In most cases the allergen was obtained by recombinant techniques because of the amount of protein needed to perform crystallisation trials or NMR studies (ca. 5–20 mg).

X-ray crystallography operates by diffraction of monochromatic X-rays by protein crystals. From the diffraction pattern, the electron density map of the molecule is converted by a Fourier transform algorithm and the protein sequence is fitted into atomic coordinates. Crystal structures of some food allergens have been elucidated (e.g. peanut allergens Ara h 1, Ara h 2, Ara h 6, wheat profilin Tri a 12, peach Pru p 3, celery Api g 1, cherry Pru av 1) and can help in understanding cross-reactivity.

NMR spectroscopy allows the study of the structure of a protein allergen in solution (also in water) under similar conditions to those present in food matrices. The most useful magnetic nuclei for that purpose are those of ^{1}H, ^{13}C, ^{15}N and ^{31}P, which can be used alone (homonuclear NMR) or in combination (heteronuclear NMR). High-resolution spectrometers (600–1 100 MHz) are required. The monodimensional homonuclear (1D) 1H-NMR provides information about structured and non-structured parts of the protein (Alessandri et al., 2012). Bidimensional (2D) NMR allows characterisation of the conformation and is suitable for small proteins and peptides. Tridimensional (3D) NMR can be used to determine the structure of larger proteins, but requires an isotopically (^{13}C and ^{15}N) labelled protein and a high concentration of the protein. The nuclear Overhauser enhancement (NOE) allows determination of the inter-proton distances and provides a 3D model. In addition, NMR can give information on the dynamics of the protein (e.g. the flexibility of the protein in a disordered structure) by measuring the relaxation times T_1 and T_2. While protein structures obtained in the solid state are static, those obtained in solution by NMR are dynamic and also depend on the interactions with the solvent.

CD is a valuable tool to investigate the secondary and tertiary structure of proteins, which has also been used to characterise allergens. The method is based on the different absorption of the polarised light by chiral and by achiral molecules immersed in a rigid chiral environment. The chiral centre in proteins is represented by the Cα atom of the amino acid adjacent to the peptide bonds, which absorb below 240 nm (far ultraviolet light) with maxima and minima related to the conformations of the angle bonds of the adjacent groups. α-Helix, β-sheet, β-turn and random coil structures show characteristic patterns of positive and negative bands. Other chromophores such as aromatic amino acid side chains and disulphide bonds allow information on the tertiary structure of the protein to be obtained.

Fourier transform infrared (FTIR) spectroscopy can confirm the folding of the protein and allow useful comparison with known allergens and monitor eventual conformational changes or aggregation following cooking or technological treatments. Indeed a secondary structure change can be visualised by a shift of the frequency and/or intensity of the longitudinal amide I vibration, and transitions from α-helices and intra- to inter-chain β-sheets can account for protein aggregation. Aggregation of the protein can also be detected by atomic force microscopy and fluorescence.

8.7. Immunological characterisation of food allergens

Characterisation of food allergens requires immunological data. Immunological tests include IgE-binding assays, such as ELISA. The CAP system, a fluoroenzyme immune assay, which is completely automated, is most currently used on account of its high sensitivity and specificity, as is the SDS-PAGE followed by immunoblotting with IgE-containing human sera. Functional immunological tests, such as the basophil activation test (BAT), have been used for the characterisation of food allergens with inconsistent and variable results. These methods cannot replace other immunological tests.

The Panel notes that the IgE-binding capacity of a protein is related to its antigenicity (i.e. the ability to combine specifically with the final products of the immune response, e.g. specific IgE), and not necessarily to its allergenicity upon ingestion (i.e. the ability to trigger immune-mediated clinical reactions).

8.8. Conclusion

Following the development of proteomics, spectroscopic methods and gene cloning, allergenic proteins can be well characterised. They have been classified into families on the basis of their sequence and 3D structure. However, although common structural features of proteins and their

biological activity have been tentatively related to their immunogenicity, it is not possible to predict the allergenicity of a protein on the basis of these two parameters only. Immunological and clinical data are required to classify a protein as a food allergen.

9. Cross-reactivities

Cross-reactivity occurs when IgE antibodies originally triggered against one antigen also bind a different antigen. Not all cross-reactivities identified *in vitro* are of clinical significance, and although most clinical cross-reactions are mediated by IgE antibodies, T cells may also be involved (Bohle et al., 2003). However, *in vitro* cross-reactivity testing can help in understanding allergic reactions to multiple foods, as well as improving the diagnosis and management of food allergy.

Cross-reactions occur among proteins with high-sequence homology and/or with similar 3D structure or common epitopes. Both linear and conformational epitopes may induce cross-reactivity. The Structural Database of Allergenic Proteins (SDAP) (http://www.fermi.utmb.eu/SDAP) can be used to predict the likelihood of cross-reactivity among proteins on the basis of sequence homology and the presence or absence of particular amino acids in non-contiguous positions.

In order to assess the potential IgE cross-reactivity of a protein with a known allergen, an identity > 35 % over an 80-amino acid window is currently recommended for further testing. Sequences are then compared with an allergen database using alignment tools, such as Fast All (FASTA) (Pearson and Lipman, 1988) or the Basic Local Alignment Search Algorithm (BLAST) (Altschul et al., 1990). Computational methods for assessing potential cross-reactivity among proteins have been reported and discussed in detail (EFSA, 2010).

Examples of highly cross-reacting allergen groups are the profilins and the LTPs, both generally regarded as panallergens (Bonds et al., 2008). Panallergens are defined as homologous molecules that originate from a multitude of organisms and cause IgE cross-reactivity between evolutionarily unrelated species (Hauser et al., 2010).

Food allergy may occur following sensitisation to inhaled allergens, such as pollen. An example is the so called “pollen–food allergy syndrome”, which usually manifests as OAS, although systemic symptoms may occur. Many patients allergic to birch pollen become allergic to apples, hazelnuts, celery and carrots. These patients have specific IgE antibodies to Bet v 1 or Bet v 2 (profilin), the major birch pollen allergens.

Non-specific LTP (nsLTPs) have been identified in most plant-derived foods and in pollen from several plants. Sensitisation to nsLTPs is characterised by geographic differences. While in the Mediterranean countries allergy to *Rosaceae* fruits is mostly associated with sensitisation to nsLTPs, in Northern Europe it is more often associated with sensitisation to birch pollen (Bet v 1). However, the co-presence of specific cross-reacting antibodies in patients’ serum does not indicate which came first: the pollen allergy or the food allergy (Hauser et al., 2010). Cross-reactions are also observed between pollen of *Compositae* (mugwort) and celery.

Other examples of cross-reactivity include those between latex and fruits, dust mite and shrimp tropomyosin, and mould and spinach. All foods belonging to the latex group (e.g. chestnut, walnut, kiwi, banana, avocado) have defence proteins (chitinases) with a common “hevein” domain that is present in the latex prohevein and accounts for most cross-reactivities.

IgE antibodies interact *in vitro* with N-glycans, i.e. carbohydrate moieties linked to proteins (asparagine). α(1–3)-fucose and β(1–2)-xylose are considered the major cross-reactive carbohydrate determinants (CCDs) in plants (Andersson and Lidholm, 2003). N-glycans may be shared by pollen, plants and insects, but these are different from N-glycans present in mammalian proteins. There is evidence that N-glycans may contribute to the allergenic potential of some foods (e.g. celery) (Bublin et al., 2003). However, the biological role of CCDs in triggering clinical symptoms is a matter for debate (Jin et al., 2008).

In vitro techniques do not allow us to distinguish between dual sensitisation (i.e. synthesis of IgE against proteins in two different foods) and cross-reactivity (i.e. synthesis of IgE against proteins in one food, which also bind proteins in a second food). In cross-reactions to several foods, different allergens may be causing cross-reactivity between different pairs of foods, as observed with snail, mite and shrimp (van Ree et al., 1996b). Moreover, profilins from certain species have been shown to induce cross-reactive IgE antibodies (birch, celery and latex), whereas others induce species-specific IgE (Radauer and Breiteneder, 2006).

9.1. Conclusion

Cross-reactivity occurs when IgE antibodies originally triggered against one antigen also bind a different antigen. Not all cross-reactivities identified *in vitro* are of clinical significance, and although most clinical cross-reactions are mediated by IgE antibodies, T cells may also be involved. However, *in vitro* cross-reactivity testing can help in understanding allergic reactions to multiple foods, as well as in improving the diagnosis and management of food allergy.

10. Effects of food processing on allergenicity

Food and food ingredients undergo different treatments to improve their palatability, to inactivate pathogenic microorganisms and/or to destroy toxins. Food is processed at home, in restaurants and institutional settings and by the food industry.

The structure and chemical properties of proteins are influenced by food-processing techniques. Major modifications include protein unfolding and aggregation, proteolysis, glycosylation and glycation, solubility and pH effects, and networking to gel formation, which may alter its allergenic potential (Paschke, 2009). The extent to which proteins are modified during food processing depends upon the process conditions, the nature of the protein and the composition of the matrix.

The allergenic activity of a complex food may be decreased, remain unchanged, or even be increased by food processing. Considering the multiplicity of the allergenic structures contained in a whole food and that different proteins may be differently affected by the same treatment, the impact of food processing on the structural and allergenic properties of food allergens is difficult to predict (Mills et al., 2009). In addition, the effects of processing on the IgE-binding capacity of allergens do not necessarily predict the allergenicity of the modified food in the allergic patient population.

This section provides an overview of the most common methods of food processing and their effects on the allergenic potential of foods. Most studies available report on the IgE-binding capacity of processed foods rather than on their allergenicity, whereas systematic investigations on the effects of food processing on allergenicity under controlled conditions are scarce. The specific alterations induced by processing on foods/ingredients included in Annex IIIa of Directive 2003/89/EC (as amended) are reported in the dedicated sections.

10.1. Thermal processing

Significant alterations in protein structure occur during heat treatments. The nature and extent of such changes depend on the temperature and duration of the thermal processing, as well as on the intrinsic characteristics of the protein and the physicochemical conditions of its environment (e.g. pH, matrix composition). Typically, loss of tertiary structure is followed by (possibly reversible) unfolding, loss of secondary structure (55–70 °C), cleavage of disulphide bonds (70–80 °C), formation of new intra-/inter-molecular interactions, rearrangements of disulphide bonds (80–90 °C) and formation of aggregates (90–100 °C) (Davis and Williams, 1998). These modifications reflect a progressive passage to a disorganised structure with denaturation of the protein that adopts an unfolded, random-coil conformation. Other chemical modifications of the protein may also occur at high temperatures (100–125 °C and higher), e.g. formation of covalent bonds between the lysine residues and other constituents of the food matrix, leading to various adducts. In thermal treatments, the conformational epitopes responsible for allergenicity are generally destroyed, whereas the linear epitopes may be maintained and others, hidden in the native conformation, may become exposed. In addition, thermal

processing can generate new immunologically reactive structures (neoallergens), among which are the advanced glycation end-products (AGEs) produced by the Maillard reaction of amino groups of proteins with sugars (Mills et al., 2009). Thermal processing can also destroy existing epitopes by cleavage of the protein (Davis and Williams, 1998).

The structure of the protein strongly influences its stability and hence its modification upon heating. Roasting (i.e. 140 °C for 40 minutes) reduces the allergenicity of Cor a 1.04 (a major hazelnut allergen) and of hazelnuts by approximately 100-fold (Hansen et al., 2003). In contrast, IgE-binding to Ara h 1 (a major peanut allergen) increases approximately by 90-fold in roasted vs. raw peanuts (Maleki et al., 2000). The explanation is that Cor a 1 belongs to the Bet v 1 superfamily of plant food allergens, which are generally thermolabile, whereas Ara h 1, a seed storage globulin, may form trimers upon roasting.

An opposite effect of wet vs. dry heating on the allergenic potential of Ara h 1 and Ara h 2 has been reported (Mondoulet et al., 2005). Whereas roasting (dry heating at high temperature) increases the allergenic potential of peanuts, boiling (wet heating at lower temperature, < 100 °C) and frying (wet heating at high temperature, 120 °C) decrease it. Dietary and cooking habits may thus explain in part the geographical differences observed in the prevalence of peanut allergy, i.e. peanut allergy seems to be rare in countries where peanuts are eaten boiled or fried (e.g. China) compared with Western countries where peanuts are mostly eaten roasted (Beyer et al., 2001).

Allergens belonging to the prolamin superfamily have an inherently stable structure characterised by the presence of several cysteine residues forming three or four intramolecular disulphide bridges, which induce a constrained folded structure. These proteins are particularly stable to thermal and chemical treatments. Allergens exhibiting such stability are the Brazil nut allergen, Ber e 1, the sesame allergen, Ses i 1, and the nsLTP from apple, Mal d 3. Other examples of "heat-stable" allergens are milk proteins and the prolamin seed storage proteins from wheat, which form gluten. The type of thermal treatment (e.g. autoclaving, blanching, microwave heating, dry roasting) does not seem to decrease the allergenicity of stable proteins, such as those present in cashew nuts (Venkatachalam et al., 2008).

Nevertheless, the consequences of thermal treatment on allergenicity are generally unpredictable. Some allergenic foods are described as heat stable (e.g. milk, egg, fish, peanuts and products thereof), while others are considered partially stable (e.g. soybean, cereals, celery, tree nuts and their products) or heat-labile (fruits of the *Rosaceae* family and carrots) (Besler et al., 2001).

10.2. Hydrolysis

10.2.1. Enzymatic hydrolysis

Enzymatic hydrolysis is the most common process used industrially to reduce the allergenicity of a protein. For example, proteases to reduce the allergenic potential of soybean and actinase to reduce the allergenicity of rice, as well as trypsin and chymotrypsin, are used for producing hydrolysed infant formulas.

The type and degree of hydrolysis depend on the primary structure of the protein, but also on its secondary/tertiary structure and on post-translational modifications (e.g. glycosylation). The sites of hydrolysis depend on the specificity of the proteolytic enzymes utilised, whereas the degree of hydrolysis is related to the working conditions. Most proteolytic treatments generate partial hydrolysis, so that not all epitopes are destroyed (Asero et al., 2000). Moreover, proteolysis can destroy some epitopes, but it can also unmask linear epitopes that were buried into the 3D native structure and/or located in hydrophobic domains of the protein, becoming available for IgE binding. Some peptides resulting from partial hydrolysis are still allergenic because they contain the epitope and/or may form allergenic aggregates. For example, treatments of hazelnut with trypsin or elastase, of soybeans with proteases and of wheat with bromelain decrease the likelihood of triggering allergic reactions in

sensitised individuals consuming these foods, whereas enzyme-mediated proteolysis does not destroy IgE binding epitopes in peanut and peach (Paschke, 2009).

10.2.2. Chemical hydrolysis

Chemical hydrolysis under acid or alkaline conditions has seldom been used in industrial processes, mostly in combination with heat and high hydrostatic pressure (HHP) treatments. Wheat protein hydrolysates produced by either enzymatic or acid treatments are commercially available. The latter were shown to contain peptides with lower molecular weight than the former and to be less antigenic (Akiyama et al., 2006). Furthermore, chemically hydrolysed salmon had a reduced or abolished IgE-binding capacity (Sletten et al., 2010). The clinical significance of these findings has to be established.

Deamidation is an industrial way of modifying the protein structure for increasing solubility by chemical hydrolysis. Gluten proteins are deamidated to enhance their solubility and technological applications. Severe allergic reactions have been reported after the consumption of food products containing deamidated gluten in subjects tolerant to wheat (Denery-Papini et al., 2012). The sera of these patients displayed IgE binding to deamidated γ- and ω_2-gliadins and deamidated total gliadins, generally at high concentrations.

10.3. Fermentation

A decreased IgE-binding capacity of β-lactoglobulin was observed in fermented milk and yogurt (Ehn et al., 2004). In these highly pasteurised products, the protein is partially hydrolysed by the enzymatic activity of the starter culture, which may destroy some epitopes. IgE binding may also be prevented by the protein gel structure and other aggregates. Fermentation with lactic acid bacteria (*Lactobacillus helveticus* and *Streptococcus thermophilus*) also decreased the IgE-binding capacity of α-lactalbumin and β-lactoglobulin in skim milk (Bu et al., 2010). No clinical data on the effects of milk fermentation on allergenicity are available.

Fermentation of soy and products thereof with bacteria and yeast (e.g. *Lactobacillus plantarum*, *Bifidobacterium lactis*, *Saccharomyces cerevisiae*) generally reduces the IgE-binding capacity of soy allergens (up to 89 %). All the commercial soy-containing products tested (e.g. yogurt, miso, tempeh) show very low immunoreactivity (Song et al., 2008). However, allergenicity was retained in a soy sauce, a fermented product containing both wheat and soy (Hefle et al., 2005).

10.4. High-pressure processing

HHP processing is a non-thermal technology, which allows homogeneity of treatment throughout the food product on account of the fact that the applied pressure is uniformly distributed within the HHP chamber, regardless of the size and shape of the product. It affects only non-covalent bonds, such as the hydrogen, ionic and hydrophobic bonds, thus exerting a substantial impact on the tertiary and quaternary structures of the protein, inducing denaturation and conformational changes.

HHP shows a variety of effects on food allergens depending on the protein structure, the pressure applied (100–400 MPa in general), the temperature and the duration of the treatment. High-pressure treatments may reduce the allergenicity of a protein by different mechanisms: by protein denaturation, by induction of conformational changes (thus destroying conformational epitopes), by making linear epitopes more accessible to digestive enzymes and by allergen removal (extraction into the medium).

In HHP-treated rice grains in distilled water, the reduced amount of allergenic proteins in rice was attributed to the release of these proteins from the grains into the aqueous solution (Estrada-Girón et al., 2005). In soy sprouts obtained from HHP-treated seeds, the reduced immunoreactivity was explained by a higher availability of the HHP-treated proteins for enzymatic hydrolysis during germination (Peñas et al., 2011). In contrast, Kleber et al. (2007) found that antigenicity of β-lactoglobulin in whey protein isolate increased with an increase of pressure (200–600 MPa), temperature (30–68 °C) and duration of the treatment (10–30 minutes). This effect may be due to unfolding of the protein, with exposure of epitopes previously buried within the protein structure

(Mills and Mackie, 2008). The potential utility of HHP processing in reducing the allergenicity of foods has been reviewed recently (Huang et al., 2014).

10.5. Methods for the production of oils

Methods for the extraction of oils from seeds/fruits/fish affect the presence of proteins in the final product and, thus, their eventual allergenicity. Cold-pressed extraction, thermal-pressed extraction and extraction with different solvents have a different impact on the amount of proteins present in oil. The crude oil can be refined following different subsequent steps: degumming, neutralising, bleaching and deodorising, each step potentially reducing the amount of protein in the final product.

Few data exist on the effect of different methods for oil refining on total residual protein content. Crude oils may contain 100 times more proteins than refined oils (Crevel et al., 2000). However, the reported protein content in crude oils varies substantially depending on the method used for protein determination. The Panel notes that the protein content of refined oils, and hence their allergenicity, strongly depends on the type of processing and the degree of refinement of the oil.

10.6. Preservation

Methods commonly used to preserve safety, nutritional value and organoleptic properties of foods include control of pH, salting, smoking and addition of spices and antioxidants. Little is known about the effects of long storage of preserved food products on allergenicity.

10.6.1. Effect of pH

The effect of pH on immunoreactivity has been studied in protein extracts from unprocessed and processed foods, where changes in the solubility of proteins resulting from the process-induced modifications must be considered. Denaturation of the protein may induce loss of conformational epitopes, increased accessibility of previously hidden epitopes, or the burying of previously exposed epitopes by unfolding/refolding/aggregation of the protein. A combined effect of pH changes and heating can induce partial hydrolysis of the protein, eventually destroying linear epitopes and decreasing allergenicity.

Cashew nut allergens Ana o 1, Ana o 2 and Ana o 3 were examined over a range of pH (1–13) in relation to different processes (autoclaving, blanching, microwave heating and dry roasting, and γ-irradiation). The three allergens were stable over the tested pH range in any process, except at the extreme pHs 1 and 13 (Venkatachalam et al., 2008). The IgE-binding capacity of Ara h 1, Ara h 2 and Ara h 3 was reduced after treatment at pH 1 with acetic acid or commercial vinegar (Kim et al., 2012).

10.6.2. Other preservation treatments

Some preservation methods have been used to reduce the allergenicity of foods, but only data in relation to their effects on the IgE-binding capacity of allergens are available at present. Pulsed ultraviolet light (PUV) treatment reduced the IgE-binding capacity of peanut extracts and liquid peanut butter (Chung SY et al., 2008), while γ-irradiation had no effect. Ultrasound treatments have been reported to reduce the IgE-binding capacity of shrimp proteins (Li et al., 2006). Ultrafiltration was also used to reduce the IgE-binding capacity of peach juice and nectar by partially removing the offending proteins (Brenna et al., 2000).

10.7. Multiple treatments

Whereas any single treatment is unlikely to effectively reduce or abolish the allergenicity of a food, the IgE-binding capacity of proteins could be extensively reduced by combining two or more treatments, although the effect of treatment combination on the allergenic potential of a food is again unpredictable.

The IgE-binding capacity of eggs and products thereof can be decreased about 100 times by combining enzymatic and thermal treatments (Hildebrandt et al., 2008). Also the susceptibility of

ovalbumin to proteolysis by pepsin was increased by the simultaneous application of HHP and enzymatic treatments, leading to lower IgE-binding capacity of the hydrolysates (López-Exposito et al., 2008). However, despite the absence of intact protein, hydrolysates maintained IgE- and IgG-binding capacity on account of the formation of long hydrophobic peptides, which retained sequential epitopes. In contrast, the IgE-binding capacity of almond, cashew nut and walnut proteins remained stable after γ-irradiation (1–25 kGy) even when combined with common thermal processing methods, including autoclaving, dry roasting, blanching, oil roasting and microwave heating (Su et al., 2004).

10.8. Conclusion

The allergenic activity of a complex food may be decreased, remain unchanged, or even be increased by food processing. Considering the multiplicity of the allergenic structures contained in a whole food and that different proteins may be differently affected by the same treatment, the impact of food processing on the structural and allergenic properties of allergenic foods/ingredients is difficult to predict. In addition, the extent to which allergenic proteins are modified during food processing depends on the type of process and its conditions, the structure of the proteins and the composition of the matrix. Although the effects of different (technological and cooking) treatments on the IgE-binding capacity of several allergens have been investigated, less information is available on the effects of processing on clinical reactivity.

11. Methods for the detection of allergens and allergenic ingredients in food

Reliable methods for the detection and quantification of food allergens are necessary in order to ensure compliance with food labelling legislation. Different approaches have been designed to detect the presence of allergenic ingredients in food products, depending on the allergen to be detected, the food matrix and the technological treatments applied, so that no single method fits all purposes.

The choice of the method requires first the identification and selection of the target analytes. Several methods target a specific allergenic protein or a number of allergenic proteins present in the food (direct analysis), whereas others target the DNA as a marker of the allergenic ingredient (indirect analysis). Several reviews on qualitative and quantitative methods for the analysis of food allergens are available (Poms et al., 2004a; van Hengel, 2007; Kirsch et al., 2009; Monaci and Visconti, 2010; Sancho and Mills, 2010).

Analysis of proteins is commonly performed by immunological (notably ELISA) and physicochemical methods (in particular MS). Analysis of DNA is based on the amplification of specific DNA fragments by means of the PCR and on the use of specific primers, which identify the sequence of the food ingredient to be amplified. PCR methods are commonly used for detecting the presence of allergenic ingredients in foods (Table 2).

Table 2: Commonly used methods for food allergen analysis

Analysis of proteins		Analysis of DNA
Physicochemical methods	**Immunological methods**	**PCR-mediated methods**
(1DE/2DE) SDS-PAGE	ELISA	End-point PCR
HPLC	Immunoblotting	Real-time PCR
Capillary electrophoresis	RIE	PCR-ELISA
Mass spectrometry	LFD	DNA biosensors
	Dipsticks	DNA microarrays
	Dot-blot	
	Protein biosensors	
	Protein microarrays	

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LFD, lateral flow devices; PCR, polymerase chain reaction; RIE, rocket immuno-electrophoresis; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Any given analytical method needs a well-defined reference material and a reliable method of recovery. The usual criteria of sensitivity, specificity, accuracy and precision (repeatability, reproducibility) have to be fulfilled. Cross-reactivity, matrix effects and food processing should also be considered (Poms and Anklam, 2004). When investigating the effects of food processing on allergen detection, incurred samples (i.e. to which the allergen of interest has been added before processing) are preferred to spiked samples (i.e. to which the allergen of interest has been added after processing).

11.1. Detection of allergens without previous separation of proteins

The extraction procedure (using solvents and buffers) is a critical initial step in food allergen analysis, which strongly impacts recovery and performance of the subsequent detection system. Extractability of the proteins depends on their isoelectric point and their polar/apolar nature, the pH and the temperature of the extraction solvent, the presence of eventual interferents in the food, the food matrix and the production process, so that there is no universal solvent/buffer suitable to extract all proteins. The use of a reducing agent (e.g. mercaptoethanol) or of a mixture of chaotropic agents (thiourea/urea) and detergents (e.g. CHAPS), though improving solubility, may induce denaturation of the protein and hamper its detection by antibodies.

11.1.1. Immunological methods

Immunological methods utilise antibodies for the recognition of specific allergenic proteins and can be performed either directly on a protein mixture or with previous separation of the proteins. The former methods include ELISA, lateral flow devices (LFDs), dipsticks, rocket immuno-electrophoresis (RIE), dot-immunoblotting (dot-blot), protein microarrays and protein biosensors, which are rapid screening methods. The latter methods involve preliminary separation by mono- or bidimensional gel electrophoresis (1DE or 2DE SDS-PAGE), HPLC or capillary electrophoresis (CE) of the proteins, followed by immunoblotting.

11.1.1.1. Enzyme-linked immunosorbent assay

The most frequently used allergen detection technique is ELISA, which allows the detection of known allergens by using specific antibodies. "Sandwich" and "competitive inhibition ELISA" are commercially available, with both direct and indirect detection.

In sandwich ELISA, the antigen present in the food sample is captured by a specific antibody immobilised on a solid surface forming an Ab–Ag complex. The complex reacts with a second analyte-specific antibody that is conjugated to an enzyme, forming a "sandwich", and the enzyme reacts with a specific substrate developing a colour. The concentration of the Ab–Ag complex, measured by the absorbance of the coloured product, is directly proportional to the amount of allergen present in the sample (direct detection).

In competitive ELISAs, the antigen bound to the solid phase competes with the food antigen present in the sample for binding to an analyte-specific antibody. If no second enzyme-labelled, analyte-specific antibody is available, an enzyme-labelled species-specific antibody is used (indirect detection). The absorption of the coloured product formed after addition of the substrate is inversely proportional to the concentration of the analyte.

ELISA methods have been automated allowing high throughput and routine analysis with a limited amount of reagents. They are fast and relatively easy to handle. The recovery and selectivity depend on the protein extraction solvent, which may affect the Ag–Ab binding. The sensitivity is generally good, with a limit of detection/limit of quantification (LOD/LOQ) in the range of 0.1 to 1.5 mg/kg depending on the allergenic ingredient and food matrix. An overview of the most recently published

ELISA methods for most allergenic foods/ingredients in Annex IIIa of Directive 2003/89/EC, as amended, is available (Monaci and Visconti, 2010). The results are reproducible for the same ELISA kit and food matrix, whereas results from different kits may diverge depending on the specificity of the antibody and the reference material (RM) or calibrator used. Antibodies present in the kit may cross-react with other proteins or other food matrix components, leading to false-positive results. Monoclonal antibodies are directed to a single epitope of a particular allergen and are very selective. Polyclonal antibodies recognise multiple epitopes and are more tolerant to slight protein modifications and thus preferred for testing allergens eventually modified by technological processes. Among the ELISA kits commercially available for the detection of food allergens (Schubert-Ullrich et al., 2009), only a few have been validated by the Association of Analytical Communities (AOAC) International (Mermelstein, 2008).

Very important for the quantification of allergens is the availability of certified reference materials (CRMs). Reference materials developed by different producers are commercially available for most major food allergens. However, the analytical results obtained using these reference (but not certified) materials may differ depending on the type of allergen/antibody and the procedures used to obtain them. To the Panel’s knowledge, only a peanut test material has been produced by the Institute for Reference Materials and Measurements (IRMM), IRMM-481, containing five different varieties of peanuts. For egg detection, egg powder from the National Institute of Standards and Technology (NIST) (NIST RM-8445) and for milk NIST fat-free milk powder (NIST RM-1549) are available, though not certified.

Normalisation of results obtained by different ELISA kits has been proposed by applying experimental Food Analysis Performance Assessment Scheme (FAPAS) proficiency tests. It was shown that a standardised calibrant (e.g. a matrix-matched standard) can be successfully used to normalise the data set from different allergen ELISA kits (Sykes et al., 2012). More recently, a dessert matrix incurred with pasteurised egg white or skimmed milk powder was produced and evaluated in a multilaboratory trial as a promising quality control material for food allergen analysis (Johnson et al., 2014). Availability of quality control materials based on incurred samples would greatly improve the reliability of ELISA methods for allergen analysis in processed foods.

ELISA has been combined with other techniques, such as inductively coupled plasma mass spectrometry (ICP-MS), in order to increase the sensitivity and the precision of the assay (Careri Maria et al., 2007). In ELISA-ICP-MS, the secondary antibody is labelled with a stable isotope (europium), so that the Ag–Ab1–Ab2 complex can be quantified by MS. The method was able to detect small amounts of peanuts (down to approximately 2 mg/kg) in a cereal-based matrix.

11.1.1.2. Lateral flow devices and dipsticks

LFDs and dipsticks are simplified versions of ELISAs for which sandwich and competitive formats are also available. In LFDs, the sample flows along a polyvinylidene difluoride (PVDF) membrane by capillarity to reach a line where the antibody has been adsorbed, giving rise to a coloured Ab–Ag complex. Dipsticks are based on the same principle but do not have a mobile phase moving up the strip. A number of LFDs and dipsticks are commercially available for most allergenic foods/ingredients listed in Annex IIIa of Directive 2003/89/EC (Schubert-Ullrich et al., 2009). They are inexpensive, quick, portable and easy to use, but are only qualitative or semi-quantitative (LOD ca. 1 mg/kg) and suffer from all the limitations described for ELISA (e.g. matrix interference, inter- and intra-assay variations, batch-to-batch variations, etc.). They are mainly used for a preliminary screening.

11.1.1.3. Rocket immuno-electrophoresis

In RIE, antibodies are incorporated in the gel covering an electrophoresis plate. The protein extract is allowed to flow onto the plate where the proteins are separated according to their electrophoretic mobility. Precipitation of the Ag–Ab complex occurs from the beginning of migration, so that the

allergen detected appears as a rocket shape. The method is semi-quantitative and not suitable for routine analysis (LOD = 2.5–30 mg/kg) (Besler et al., 2002).

11.1.1.4. Dot-immunoblotting

This technique is a simplified version of the Western blotting in which proteins are not separated. The sample extract is spotted on a nitrocellulose or PVDF (polyvinylidine fluoride) membrane as a dot and incubated with an enzyme-labelled allergen-specific antibody. Upon binding with the target antigen, a coloured spot is observed after the enzyme–substrate interaction. Dot-blot is a qualitative/semi-quantitative test (2.5 mg/kg for peanut) suitable for preliminary screening purposes (Blais and Phillippe, 2000).

11.1.1.5. Protein microarrays

Immunoassays for allergen testing, in particular microarrays, allow a large number of allergens to be tested simultaneously for diagnostic purposes. The most common protein microarrays for food allergens are the antibody microarrays, where antibodies raised against known allergens are immobilised by microprinting or microstructuring processes to form a patterned surface on the chip (e.g. a glass slide or a well array), allowing the recognition and quantification of allergens in food samples in the microlitre to nanolitre range (Seidel and Niessner, 2008). Detection can be achieved by different techniques and data analysis is performed with software for image processing. Analytical flow-through microarray platforms for quantification of allergens by sandwich ELISA have also been developed. The platform consists of a fluidic system for sample introduction, a reagent supply, a flow cell, a microarray and a detection system.

"Multiplexed" format immunoassays allow simultaneous detection of several analytes with smaller amounts of sample and reagents and lower cost with respect to conventional single-analyte immunoassays. However, allergens present in the food sample have not been separated previously and may have a different accessibility to antibodies.

11.1.1.6. Protein biosensors

Biosensors are based on an integrated receptor–transducer device able to provide semi-quantitative or quantitative signals and provide a novel approach for allergen detection. The receptor is generally an antibody raised against an allergenic protein, which is immobilised on a sensor chip surface. The recognition event is converted by a transducer into a signal, which is detected by various physicochemical techniques, e.g. surface plasmon resonance (SPR), resonance enhanced absorption (REA), electrochemical impedance (EI) (Monaci and Visconti, 2010). Several biosensors for allergenic proteins have been reported to be quite sensitive (LOD/LOQ 0.5–2 mg/kg) (van Hengel, 2007). An optical biosensor was able to detect proteins from milk, egg, hazelnut, peanut, shellfish and sesame down to 1–12.5 mg/kg in food samples (Yman et al., 2006). Biosensors are quick, easy to use and suitable for automation, and thus they can be implemented as screening methods along the food chain.

11.1.1.7. Expression of the results on food allergens obtained by immunological methods

The European standard EN 15633-1 (CEN, 2009), relative to the research on food allergens by immunological methods, requires that results are expressed as total amount of allergenic ingredient per kilogram of food (mg/kg), or as total amount of protein per kilogram of food, using an appropriate factor that allows conversion to the amount of allergenic ingredient per kilogram of food. However, conversion factors are difficult to calculate when the composition of the food is not well known and the amount of protein per kilogram of food may change according to the origin of the ingredient and following technological treatments or cooking. LODs and LOQs are mostly expressed in the literature as milligrams of allergenic protein per kilogram of food or as mg/L in the case of liquid foods or asng/mL of buffer solution.

11.1.2. Detection of allergens with previous separation of protein

Separation of proteins is a key point for the detection of allergens. The most commonly used protein separation method is SDS-PAGE in 1DE or 2DE. 1D-SDS-PAGE separates the proteins according to their molecular mass relative to the migration of standard protein markers (Pastorello and Trambaioli, 2001a). 2DE is the combination of two electrophoretic techniques based on isoelectric focusing (IEF), which separates the proteins according to their isoelectric point followed by SDS-PAGE (De Angelis et al., 2010).

Once the proteins of a food extract have been separated, they are transferred onto a membrane of nitrocellulose or of a hydrophobic polymer (e.g. PVDF) by blotting (or printing). The detection of the immobilised allergens is performed by incubation with an antibody solution, usually a human allergic individual serum or with antibodies raised against the allergens in animals. The antibodies specific to the immobilised allergens are then detected by incubation of the blot with an enzyme-labelled second antibody, followed by the addition of the enzyme substrate. In order to confirm the identity of each immunoreactive protein, the spot obtained by 2DE is excised from the gel, digested by proteolytic enzymes (e.g. trypsin) into peptides, and subjected to MS analysis.

2D-SDS-PAGE is an extremely powerful tool for separating proteins with similar molecular mass; it is most important for research studies, but it suffers from significant drawbacks. Solubilisation of the proteins with chaotropic agents and detergents may modify the isoelectric point (pI) and immuno-detectability. There can be spots overlapping/too many spots (low dynamic range). A concentrated antibody solution, usually human serum (individual sera) or antibodies raised against animals, is necessary. Gel-to-gel variations may be relevant. There is a need for confirmation and identification of the protein off-line (by MS). A skilful operator is required and it is labour intensive and time consuming. It has also some limitations for hydrophobic and alkaline proteins. It is not quantitative and not suitable for routine analysis.

Improvements in sensitivity, accuracy and precision have been obtained with the introduction of 2D fluorescence-based difference gel electrophoresis (2D-DIGE), which eliminates gel-to-gel variation in protein migration, hindering computer-assisted comparison of spot patterns, thus allowing resolution and identification of spots corresponding to isoforms or to process modified allergens (Chassaigne et al., 2009).

Other separation techniques are also available for the separation of proteins, such as liquid chromatography (LC) (Heick et al., 2011a), CE, and field-flow fractionation (Reschiglian and Moon, 2008). LC is advantageous owing to its separation power, ease of automation and routine coupling with various detection techniques, in particular MS. In conclusion, LC techniques, despite their lower resolution power relative to 2D-SDS-PAGE, show a higher dynamic range and are suitable for automation. In connection with MS, they are becoming the methods of choice for the identification and quantification of proteins.

11.1.3. Detection of allergens by mass spectrometry

Mass spectrometry is useful not only to identify allergens (see section 8), but also to detect and quantify them in foods. MS is currently used mostly as a confirmatory method, although it is increasingly being applied for allergen quantification. A review of quantitative methods for food allergen analysis, and in particular MS methods, is available (Kirsch et al., 2009). Hyphenated methods, which couple separation techniques and MS, allow the identification and quantification of allergens in complex food mixtures even at trace level. These methods require the use of external or internal standards, and they are based on the comparison between the MS signal intensities of the analyte and those of the standard.

11.1.3.1. Quantification at the protein level

Quantification of the intact protein may be obtained by directly spraying the protein solution using electrospray. MS spectra are obtained consisting of a series of peaks corresponding to the charge state

distribution of the protein. The first quantitative experiment on whey proteins was able to determine the concentrations of α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A in a commercial whey drink on the base of an external calibration curve (0.01–1 mg/mL) (Huber and Premstaller, 1999). The selected ion monitoring mode (SIM) was used to measure the most abundant ions.

The same cow's milk proteins were detected and quantified as intact proteins in mixed fruit juice samples (Monaci and van Hengel, 2008). Proteins were first extracted by a solid phase extraction and separated by LC. Two different acquisition modes were used and compared: full scan (FS) and multiple ion monitoring (MIM) modes, which proved to be more selective. With the latter the most abundant specific masses, corresponding to different protonated states of the same protein were recorded. External standards were used with a matrix-matched calibration curve. The LOD and LOQ were estimated at 1 and 4 µg/mL respectively.

Lysozyme, previously extracted from cheese using immunocapture with magnetic particles covered with the specific antibody, was detected at a level of 5 mg/kg with a method based on MALDI-TOF (Schneider Nadine et al., 2010).

Although the quantification of intact proteins gives good results, it suffers from several limitations, such as ion suppression and the superposition of numerous peaks in the mass spectra in the presence of different proteins. It can be useful for analysing protein modifications, but its analytical throughput is still limited, though it has been improved using linear ion trap–Fourier transform–ion cyclotron resonance (LTQ-FT-ICR).

11.1.3.2. Quantification at the peptide level

In order to achieve the quantification of allergens at the level of peptides, a preliminary enzymatic digestion of the allergen is needed. Different methods can be used (Kirsch et al., 2009) involving tagging by light (^{12}C) and heavy (^{13}C-labelled) tags, using isotopically labelled synthetic peptides or the so called "label-free quantitative methods".

Tagging methods

Tagging methods, which are used mainly for relative quantification, are also suitable for absolute quantification. Tagging methods incorporate isotopically labelled chemical moieties into the samples, and the tags are particularly designed to react with a specific amino acid, such as cysteine or lysine. As an example, the isotope coded protein label (ICPL) method implies differential labelling of the free amino groups of a protein with isotope-encoded (e.g. heavy, D or ^{13}C) or isotope-free (e.g. light, H or ^{12}C) tags. Thus, identical peptides (one modified with the heavy and the other with the light ICPL label) differ in mass and consequently appear as doublets in the MS spectra. The ratios of the peak intensities for these labelled peptide pairs provide relative quantitative information about the amount of their parent proteins in the original sample. α-Lactalbumin, β-lactoglobulin and α-casein have been quantified using a tagging method together with MALDI-TOF/TOF (Schmidt et al., 2005).

Isotopically labelled synthetic peptide method

This method uses an isotopically labelled synthetic peptide as a reference analyte for absolute quantification. It is the method of choice when the protein is known, provided that attention is paid to critical steps, such as the selection of the peptide, the digestion of the protein and the choice of the MS configuration.

In the so-called "absolute quantification (AQUA) method" (Gerber et al., 2003), the reference peptide incorporates ^{13}C and ^{15}N isotopes into one of its amino acids, obtaining a mass difference with respect to the endogenous peptide. The concentration of the endogenous peptide is calculated from its ratio to the isotopically labelled peptide. The selected peptide must be unique to the protein of interest and quantitatively obtained by complete enzymatic digestion of the native protein, since the concentration of the protein is deduced from the concentration of the endogenous peptide. The MS method most suitable for this analysis is the triple quadrupole in the multiple reaction monitoring mode (MRM).

Whereas several variants of this method have been described, only a few are dedicated to allergenic proteins. This method has been used, for example, for the detection and quantification of Ara h 1, Ara h 2 and Ara h 3/4 from peanut (Shefcheck et al., 2006; Careri M. et al., 2007) and of casein from cow's milk (Weber et al., 2006)in complex food matrices.

Label-free method

Quantification with the label-free method relies either on the so-called "spectral counting" or on the ion signal intensity, based on external or internal standards. In the former, the intensity is estimated by the number of times a MS/MS transition of a peptide belonging to the quantified protein is chosen (Choi et al., 2008). The latter is based on the classic principle of the internal standard, based on the addition of a known amount of a reference sample of the authentic protein (Bantscheff et al., 2012). For example, the simultaneous detection and quantification of allergenic proteins from five foods (cashew nut, hazelnut, almond, walnut and peanut) was obtained by monitoring five biomarker peptides by LC-LIT-MS/MS (Bignardi et al., 2010). To overcome the matrix effect, matrix-matched calibration curves were built up.

The advantage of the quantification at the peptide level ("bottom-up" approach) is the low LOD, because peptides are more efficiently separated than proteins and can be detected by the more sensitive MS instruments. However, a limitation is the necessity of using a preliminary enzymatic digestion process, which might be non-exhaustive, thus hampering the quantification of the protein. Depending on the extraction method, digestion process, separation method and MS technique used, the LODs are in the order of 1–5 mg/kg, although LODs of 0.2 mg/kg and LOQs of 0.5 mg/kg have been obtained in some cases (see sections on specific allergenic foods). The LODs and LOQs obtained by MS are generally expressed in the literature as milligrams of the allergenic protein per kilogram of food, but often as pg/mg of food or as ng/mL. In few cases, absolute values are given (in picagrams of the allergenic protein).

11.2. Detection of allergenic ingredients by DNA analysis

Indirect methods based on the detection of specific DNA sequences for the allergenic protein or the allergenic food of interest may be used whenever direct methods for the detection of allergenic proteins fail (e.g. foods containing low amounts of protein and processed foods with extensive modification of native proteins). DNA methods for allergen detection can be considered complementary to the methods for analysing proteins, in particular ELISA. Their applicability to several allergenic foods has been extensively reviewed (Poms et al., 2004; Demmel et al., 2008; Scaravelli et al., 2008; Monaci and Visconti, 2010).

DNA extraction from lipophilic matrices (e.g. fats and oils) with low amounts of DNA and from complex matrices containing surfactants and emulsifiers is sometimes difficult and may lead to false-negative results. DNA extraction methods include precipitation (e.g. with cetyltrimethylammonium bromide (CTAB)), resin binding methods and magnetic particles (Pafundo et al., 2011).

11.2.1. Polymerase chain reaction

DNA methods are based on the amplification of specific DNA fragments by means of PCR which, by using specific oligonucleotides serving as primers, amplifies only the DNA originating from the offending food. Some foods may contain compounds that are PCR inhibitors, such as polyphenols, so that it is necessary to carry out a preliminary extraction of these compounds before DNA amplification. False-positive results owing to improper choice of primers or similarity of the sequence to be amplified with other species are less frequent. DNA may be amplified by end-point PCR, which is qualitative (i.e. it detects the presence of a specific DNA sequence), or by real-time PCR, which is quantitative, provided that an adequate reference material is used. The targeted DNA sequences are not necessarily located in the genes encoding for the allergenic protein, and thus the analysis detects genomic DNA of the offending ingredient but does not necessarily indicate the presence of the protein, which is responsible for the allergic response. Several PCR kits are commercially available.

Restriction site analysis, DNA sequencing, or hybridisation with probes based on oligonucleotides or their peptide analogues peptide nucleic acids (PNAs) (Rossi et al., 2006) can be used to confirm the detection of the correct amplified sequence (amplicon).

In end-point PCR, after amplification, gel electrophoresis either as slab or as CE is used for routine separation of DNA. Compared with conventional slab gel electrophoresis, CE is more sensitive and provides correct size information with improved resolution. Instrumentation with chip-like multichannel CE is available for this purpose. HPLC analysis can also be performed (Germini et al., 2005).

Real-time PCR requires more expensive equipment, but allows amplification and detection of DNA in "real time". In the most common set up of real-time PCR, the DNA is amplified in the presence of a specific oligonucleotide probe carrying a reporter dye and a quencher dye at the two extremities of the strand. The probe hybridises to the single-strand DNA in the region limited by the two primers to be amplified. As the quencher and the reporter are relatively close, fluorescence is suppressed. During amplification, the polymerase (a 5′-exonuclease) cleaves the probe, displacing reporter and quencher from the new copy strands, so that fluorescence is switched on. The number of cycles required to increase fluorescence above a standard predefined line correlates with the amount of the PCR product.

The quantitative analysis of DNA by PCR (qPCR) depends on the availability of reference materials and on the knowledge of the genomic sequences. Standard materials/calibrants are provided together with the commercially available kits or are produced "in house". No CRMs are available.

DNA methods available for the detection of several allergenic foods/ingredients and their LODs (0.1–100 mg/kg) have been reviewed (Monaci and Visconti, 2010). With few exceptions where the correlation between the amount of DNA and the amount of allergenic protein present in a food has been determined (Scaravelli et al., 2009), the DNA measured cannot be directly correlated to the amount of the allergenic protein but rather to the amount of the allergenic ingredient in foods.

11.2.1.1. Multiplex PCR methods

Multiplex PCR methods based on different approaches have been developed for the simultaneous determination of several allergens. These systems save time and resources but must be carefully designed and validated. In general, the amplification of a specific single sequence, both in end-point PCR and in real-time PCR, is considered more reliable on account of the higher specificity.

A duplex real-time PCR assay for the simultaneous detection of sesame and hazelnuts in spiked food down to 0.005 % of both sesame and hazelnut has been developed (Schoringhumer et al., 2009), as well as a qualitative duplex real-time PCR method for the simultaneous detection of lupin and soy mitochondrial DNA with a LOD of 2.5 mg/kg in processed food (Gomez Galan et al., 2011). The simultaneous detection of hazelnuts and peanuts down to 50 pg DNA has been obtained after PCR amplification on a peptide nucleic acid (PNA) microarray (Rossi et al., 2006).

Two tetraplex qPCRs were developed for the simultaneous detection of eight allergenic foods (peanut, hazelnut, celery, soy, egg, milk, almond and sesame), with specificity and sensitivity in the range of 0.01 % (Köppel et al., 2010). Two quantitative hexaplex real-time PCR systems for the detection and quantification of 12 allergenic ingredients in foods became available thereafter. The first system simultaneously determines DNA of cashew, peanut, hazelnut, celery, soy and mustard, whereas the second determines DNA of milk, egg, almonds, sesame, pistachio and walnut (Köppel et al., 2012). The two tests showed good specificity and a LOD of at least 0.1 % for all allergenic ingredients in mixed foods. Quantification on a weight-to-weight basis was not possible in the absence of reference materials. However, the two multiple PCR systems are suitable as screening tools in routine analysis.

Another six-plex qPCR able to detect cashew, hazelnut, peanut, walnut, almond and sesame has been developed (Pafundo et al., 2010). The LOD of the template DNA is 5 pg for almond, peanut and hazelnut and 0.5 pg for cashew, walnut and sesame.

A multiplex ligation-dependent probe amplification (MLPA) method for the detection of different nuts (peanut, cashew, pecan, pistachio, hazelnut, macadamia nut, almond, walnut and Brazil nut) and sesame has been described (Ehlert et al., 2009). The technique does not amplify the target sequences, but rather the products resulting from the ligation of bipartite hybridisation probes. Ligation-mediated amplification offers many advantages over traditional qPCR in terms of specificity and reproducibility and may be extended to further targets of interest. The method is specific and sensitive, allowing the simultaneous detection of nuts and sesame seeds in the lower mg/kg range. The LOD for single allergenic ingredients in different food matrices was 5 mg/kg. Quantification was not possible, owing to the lack of appropriate reference materials.

A quantitative 10-plex competitive MLPA method for the detection of eight allergenic ingredients (sesame, soy, hazelnut, peanut, lupin, gluten, mustard and celery) with an internal positive control (IPC) is available (Mustorp et al., 2011). Amplicons were easily separated by CE. The sensitivity is high: the LODs varied from approximately 5 to 400 gene copies depending on the allergenic ingredient. For spiked foods the LODs were of the same order of magnitude or higher than those obtained with qPCR.

Optical thin-film biochips for multiplex visible detection of eight allergenic ingredients (celery, almond, oat, sesame, mustard, lupin, walnut and hazelnut) in foods have been developed on the basis of two tetraplex PCR systems (Wang W et al., 2011). The PCR fragment targets are recognised by the biochip by enzymatic conversion of the nucleic acid hybrids to molecular thin films. The mass contributed by the thin film alters the interference pattern of light on the biochip surface, giving rise to a visible colour change on the chip surface. The absolute LOD was measured only for sesame (0.5 pg DNA); the practical LOD for sesame concentration in a blended mixture was 0.001 %, the lowest value observed so far.

A DNA microarray on a digital versatile disk (DVD) has been developed for the simultaneous detection of hazelnut, peanut and soybean in food (Tortajada-Genaro et al., 2012). The method is versatile, specific and sensitive, with a LOD of 1 µg/g, and it is particularly suitable for screening.

11.2.1.2. PCR-ELISA

PCR-ELISA is a combination of the highly specific DNA methodology and ELISA. A specific DNA segment of an allergenic food is amplified and then hybridised to an oligonucleotide probe labelled with a specific protein. The protein is recognised by a specific antibody carrying an enzyme, which in the presence of a substrate develops a colour. The concentration of DNA is proportional to the absorbance of the coloured solution (LOD 10 mg/kg) (Holzhauser et al., 2002). Although some kits are available on the market, this method is complex and seldom used.

11.2.2. DNA microarrays

The main feature of the microarray technology is the simultaneous detection of multiple analytes in one sample. Microarrays based on oligonucleotides (Bettazzi et al., 2008) or their analogues complementary to the DNA of several allergens have been developed (Rossi et al., 2006), but few are commercially available. The specific probes are immobilised on a solid surface by different techniques and recognise the complementary fluorescently labelled PCR amplicons. The resulting fluorescent spots are read with a fluorescence scanner at the proper wavelength. The method is qualitative and useful for a rapid screening.

11.2.3. DNA biosensors

DNA-based biosensors are in rapid development. An electrochemical DNA sensor was developed for peanut allergen Ara h 1 detection with a LOD of $0.35.10^{-15}$ M. A surface plasmon resonance imaging (SPRI)-based biosensor using an immobilised PNA probe as receptor was able to detect non-amplified genomic soy DNA down to 41.10^{-21} M (D'Agata et al., 2010). DNA biosensors are used for research rather than for routine analyses.

11.2.4. Expression of the results on allergenic ingredients obtained by DNA analysis

The European Standard EN 15634-1:2009 relative to the detection of allergenic ingredients in foodstuffs by molecular biology methods based on DNA analysis establishes that LODs/LOQs must be expressed as the number of copies of DNA equivalent to a total quantity of the allergenic ingredient per kilogram of food (mg/kg). The equivalence should be based on reference materials certified by the EU, but these are not available at present. In most cases, standard materials have been produced in different laboratories independently by means of genomic DNA purified and quantified "*in situ*". The more recent European Standard EN 15842 (CEN, 2010) provides general considerations on the validation of the methods of the detection (immunochemical, DNA analysis and chromatography) of allergens and allergenic ingredients in foodstuffs. LODs are expressed in the literature in different ways: as number of copies of DNA, as absolute pg of DNA detected, as pg DNA/mg of food, or as percentage of the allergenic ingredient in food (% w/w) when the value is referred to a spiked or incurred preparation. When using biosensors, the LOD is expressed as molarity (M).

11.3. Detection of allergens and allergenic ingredients in processed foods

Technological processes and cooking generally affect the structure of the proteins, whereas DNA is more resistant to technological treatments (i.e. DNA is cleaved only at high temperatures or at acidic pH). Food processing also modifies the food matrix (e.g. disruption of structure and cells, gelification, generation of new intermolecular interactions among the components, aggregated/disaggregated assemblies and other supramolecular structures). Thus the extractability of the protein/DNA from processed food may be either easier or more difficult than that from raw materials.

As for proteins, reduced detectability of allergens from processed foods can be due to either poor extraction efficiency or to reduced accessibility to the epitopes in the immunological assay. Several buffers and extraction modes have been considered (including the use of reductive agents, such as mercaptoethanol), depending on the nature of the protein (hydrophilic/lipophilic) and of the matrix (Chassaigne et al., 2007). Ultrasonic and microwave extraction increased protein homogenisation and allergen extractability from a soybean meal and from roasted almonds (Albillos et al., 2011). The performance of available extraction kits for DNA analysis strongly depends on the food matrix (Pafundo et al., 2011).

Food processing may hamper the detectability of food allergens by immunological analysis. For example, the milk protein β-lactoglobulin is a globular compact protein, which is thermo-labile and resistant to degradation by digestive proteases. Heat treatments induce denaturation of the protein (i.e. loss of its 3D and 2D structure), which becomes undetectable by most antibodies raised against the native form and thus by immunological methods. However, the denatured β-lactoglobulin keeps most of its allergenic potential because linear epitopes are recognised by IgE of allergic patients (Negroni et al., 1998). Moreover, heat treatment followed by sharp cooling (i.e. tempering) decreased the detectability of casein and β-lactoglobulin by commercial ELISA kits in a dark chocolate matrix, whereas it did not affect the detectability of peanut and egg (Khuda et al., 2012a).

The effect of heat treatment on the detection of peanut was investigated using three real-time PCR methods and two ELISA kits (Scaravelli et al., 2009). A comparison was made between the two methods on both peanut kernels and peanut-containing cookies baked under different conditions. A detrimental effect of the processing temperature/time on the detection of peanut was observed with either method. The performance of both methods was similar. The same trend was observed with roasted peanuts. In this case, the variability was higher between the two ELISA kits, which targeted two different proteins (Ara h 1/Ara h 2), than among the three PCR methods.

Whenever possible, the use of incurred samples would be preferable to the use of spiked samples to assess the effects of food processing on food allergens (Monaci and Visconti, 2010).

11.4. Conclusion

Screening (qualitative), quantitative and confirmatory methods are available for the detection of food allergens.

The majority of kits commercially available for routine food allergen analysis rely on immunological methods. ELISA methods are the most widely used because they are sensitive and specific for the detection of allergenic proteins and easy to use. However, commercial kits for quantitative analyses use different extraction buffers and calibration procedures, they differ in the quality of the antibodies used and the results vary among commercial brands and batches. Major limitations include matrix effects, insufficient extraction of the proteins, insufficient specificity owing to cross-reactions and insufficient reproducibility of results. The use of incurred samples may help to improve the reliability of the method when analysing processed foods.

Mass spectrometry, in combination with techniques such as 2D-SDS-PAGE or chromatography for the preliminary separation of the proteins and with allergen databases for their subsequent identification, is a reliable tool for the detection of known allergens and for the identification of new immunoreactive proteins. MS methods for quantitative analysis based on specific standard peptides or stable isotope labelling are not yet suitable for analyses of large numbers of samples, but they can confirm results obtained otherwise.

DNA methods allow detection of the allergenic food rather than of the allergenic protein and are complementary to immunological assays. DNA is generally more stable than proteins and thus suitable for analysis of processed foods. The extraction and amplification procedures are well established. Both end-point and real-time PCR allow simultaneous multiple analyses. Whenever ELISA kits are not available or not specific (e.g. celery), DNA analysis becomes the method of choice. Real-time PCR may provide quantitative results and allows multiplexed analysis. Commercial kits are available.

The main problem for the quantification of allergens by immunological or DNA-based methods is the unavailability of CRMs. Reference materials (not certified) developed by different producers are commercially available for most major food allergens, but the results obtained with different kits may not be comparable. To the Panel’s knowledge, a CRM for the detection of food allergens by immunological methods has been developed only for peanuts (IRMM-481). For milk and egg, two reference materials are commonly used, NIST RM-1549 and NIST RM-8445, respectively. CRM for immunological and DNA-based methods are needed.

12. Allergen risk assessment and determination of “thresholds” for allergenic foods/ingredients

12.1. Introduction and terminology

The notion of determining concentrations of allergenic foods/ingredients in foodstuffs below which the majority of sensitised consumers are not at risk of developing severe allergic reactions has attracted much attention from regulatory bodies, consumer associations and industry throughout Europe.

To that end, attempts have been made to define a framework for food allergen risk assessment, as it exists for the risk assessment of other food-related hazards (e.g. chemicals, microbiological agents). Specific terms and definitions currently used in the context of allergen risk assessment, and how they relate to terms and definitions used in traditional risk assessment of other hazards in food, are briefly summarised below (Crevel et al., 2007b; Crevel et al., 2014).

The **no observed adverse effect level (NOAEL)** is the highest tested dose of an allergenic food that does not trigger an adverse reaction in an allergic individual. The terms **lowest observed adverse effect level (LOAEL)** and **minimum eliciting dose (MED)** have been used to describe individual allergen exposure levels below which an allergic individual is unlikely to react. The MED used for

allergenic foods is similar to the LOAEL used for chemicals (Spanjersberg et al., 2007; Blom et al., 2013). The true (rather than tested) individual MED for an allergenic food, which is the **individual (elicitation) threshold**, lies between the NOAEL and the MED, by definition. The terms individual threshold and MED, which are occasionally used interchangeably, have different meanings.

An **objective allergic reaction** is characterised by at least one sign that could be discernible to a clinical observer (e.g. vomiting, urticaria, rash, angioedema). A **subjective allergic reaction** is defined as the occurrence of symptoms (e.g. abdominal pain, headaches, tingling sensation in the throat) that is not discernible to a clinical observer.

A **minimum observed eliciting dose (MOED)** is defined as an individual's lowest level of exposure at which an objective allergic reaction has occurred and below which an objective adverse effect is not expected in that individual. Subjective allergic symptoms, such as abdominal pain, headaches, tingling sensation in the throat and similar could occur at lower dose levels (FDA, 2006; Taylor et al., 2009a). MED is the lowest tested dose of an allergen triggering an allergic reaction in an individual, whether objective or subjective. In this context, the term allergic reaction is restricted to IgE-mediated adverse effects occurring usually within two hours after administration of the offending allergenic food.

The **Bench Mark Dose** (BMD) is the dose of an allergen likely to trigger an allergic reaction in a given percentage of the allergic population. The **BMD lower limit** (BMDL) is the lower 95 % CI of the BMD. The term **eliciting dose** (ED*p*) denotes the dose of an allergen at which a percentage *p* of the allergic population is likely to react. ED_{10} is equivalent to the BMD_{10}.

Population threshold is the dose of an allergen at which no individual of the allergic population is likely to react (e.g. NOAEL for a population) or at which a defined percentage of the allergic population is likely to react. The BMD and ED*p* can be considered population thresholds.

In risk assessment for chemicals, a **reference dose** is the daily dose that is likely to be without adverse effects even if continued exposure occurs over a lifetime. In allergen risk assessment, **reference dose** refers to the amount of an allergen that, when consumed on a single occasion or within a short time frame, is unlikely to trigger an allergic reaction in the majority of the allergic population, and thus incorporates a judgement about the level of risk that is considered acceptable. **Action levels** denote concentrations (amount of an allergenic food/ingredient, usually expressed as amount of protein, per amount of food) which may be used for labelling purposes (i.e. equivalent to the concept expressed in the ToRs as "threshold concentrations of each allergen in food that would provide an acceptable level of protection for at-risk consumers"). **Action levels** also incorporate a judgement about the level of risk that is considered acceptable.

The Panel notes that, whereas the derivation of individual and population thresholds is a matter of scientific judgement, the setting of **reference doses** and **action levels** requires, in addition, risk management decisions which are outside EFSA's remit. These include, but are not limited to, the purpose for which **action levels** may be used (e.g. exemptions from labelling, labelling of allergens unintentionally present in food) and the level of risk which may be considered acceptable at an individual and/or at a population level (e.g. the fraction of the allergic population that is aimed to be protected, and to what extent).

12.2. Determination of thresholds for an individual

Standardised DBPCFCs are the standard for analysis of individual threshold levels of an allergenic food in food-allergic patients (Sicherer et al., 2000b; Bahna, 2003), with the exception of infants and children for which blinding is not considered necessary. However, the selection of subjects, the doses of allergen tested and the interpretation of the results vary from investigator to investigator. Variability is particularly related to the scoring of the patient's signs (objective) and symptoms (subjective) and their severity. In addition, clinicians tend to exclude from challenge studies those patients likely to have the most severe reactions based on the individual's history (Taylor et al., 2002). DBPCFCs do

not consider food-allergic patients in the general population who manage their allergy outside the clinical settings.

Doses of the allergenic food/ingredient reported to trigger adverse reactions in controlled studies range from micrograms to milligrams, and sometimes grams (Wensing Marjolein et al., 2002). It is not always stated whether NOAELs or MEDs refer to discrete or cumulative doses, or whether the doses reported relate to the administered allergenic protein equivalent or to the allergenic food/ingredient. In some studies, the allergenic food is not administered in the form that it is usually eaten (e.g. freeze dried, introduced as flour or modified in other ways) (Hourihane et al., 1997). Although such food preparations are necessary to fulfil strict DBPCFC criteria, they may affect the LOAELs and MEDs for a particular allergenic food that are derived from that specific study (Grimshaw et al., 2003).

Variables affecting the determination of individual MEDs in DBPCFCs are listed in Table 3.

Table 3: Variables affecting minimum eliciting dose levels

Variables
Severity of the allergic condition
Signs and/or symptoms used as the clinical read-out system (subjective vs. objective reactions and their associated severity)
Administration protocols, challenge conditions and food preparations
Raw versus processed food
Food matrix and allergen content of challenge foods
Total amount of administered dose and time frame
Reproducibility (false positives and negatives)
Co-factors (for example exercise, alcohol, medication)
Patient population (geographical distribution of underlying sensitisation rates for cross-reacting allergens; genetic background)

Most DBPCFCs conducted in food-allergic patients have been designed for diagnostic purposes rather than for establishing individual thresholds for an allergen (Hourihane et al., 1997; Wensing M. et al., 2002; Moneret-Vautrin and Kanny, 2004). In some cases, the gap between the NOAEL and the MED can be considerable, depending on the dose intervals used, and these studies do not provide a scientific basis for setting a NOAEL or for recommending acceptable levels of intake of the allergenic food/ingredient for an individual (Morisset et al., 2003b). In some other cases, either the MED (e.g. allergic individuals not reacting to the higher tested dose) or the NOAEL (e.g. allergic individuals reacting to the first tested dose) cannot be established.

Guidelines for the standardisation of DBPCFCs and for the design and reporting of DBPCFCs for threshold-finding purposes, which generally require lower starting doses of the allergen and wider dose ranges, have been published to increase the comparability of the results among studies (Bindslev-Jensen et al., 2004; Taylor et al., 2004; Sampson et al., 2012).

Minimum (observed) eliciting doses for individuals reported in oral challenge studies for specific allergenic foods/ingredients will be addressed in the remaining sections of this opinion, clearly specifying whether they refer to objective reactions, subjective reactions or both. Doses will be reported as in the original publications, i.e. as amount of food or as milligrams of protein, and conversion factors will not be used to transform one into the other, unless specified by the authors.

12.3. Determination of thresholds for a population

Two different approaches have been proposed to derive thresholds for allergenic food/ingredients at a population level (Madsen et al., 2009; Crevel et al., 2014): (i) the NOAEL (or LOAEL); and (ii) the BMD approach.

12.3.1. The NOAEL/LOAEL approach

In traditional toxicological risk assessment, experimental studies testing different doses of a substance are generally used to determine the NOAEL or the LOAEL, and then uncertainty factors (often between 100 and 1 000) are applied to account for extrapolation from animals to humans and to account for inter-individual human variation (Calabrese and Baldwin, 1994; Pelekis et al., 2003; Madsen et al., 2009). The NOAEL or LOAEL is thus a point estimate resulting from the worst-case value.

In food allergy, the use of human data to derive the NOAEL or the LOAEL for a population (DBPCFCs) avoids the need for uncertainty factors to account for inter-species variability. On the other hand, the highest level of exposure to allergenic foods/ingredients that may not trigger adverse allergic reactions (or the lowest level of exposure at which reactions are observed) varies widely from subject to subject and, within a subject overtime, it is very low for some food-allergic patients and may be difficult to establish for the allergic population as a whole (Madsen et al., 2009; Crevel et al., 2014).

12.3.2. The Bench Mark Dose approach

The BMD approach has been used in toxicology safety assessment (EFSA, 2009). Rather than using a single data point from a single study, all experimental data available are fitted in a distribution by means of different mathematical models. The BMD may then be defined as the dose that induces an adverse event in a given percentage of the tested sample, e.g. 10 % (BMD_{10}). The dose used for further calculations (point of departure) is usually the lower 95 % CI of the BMD, or BMD lower limit (BMDL). It is important that the BMD lies within (or very close to) the experimental data points to make the model less sensitive to the choice of the mathematical model to fit data.

In food allergy, statistical dose-distribution modelling of individual thresholds (NOAELs and MEDs obtained in DBPCFCs) allow the calculation of BMDs, BMDLs and eliciting doses (usually ED_{01}, ED_{05} or ED_{10}) (Madsen et al., 2009; Crevel et al., 2014). However, many DBPCFCs do not allow establishment of either the MED or the NOAEL for some individuals (see section 12.2). A statistical methodology, the interval censoring survival analysis (ICSA), has been applied to determine individual thresholds, taking into account these uncertainties (Taylor et al., 2009a). If the highest challenge dose is the NOAEL, the LOAEL is set to infinity and subjects are right censored; if the lowest challenge dose (first dose tested) is the LOAEL, the NOAEL is set to zero and subjects are left censored. The reliability of the BMD and ED_p estimates depends on the type, quality, and amount of data used, particularly to describe the lower end of the threshold distribution (e.g. on the amount of left-censored individuals), and on the extent to which the sample used to derive the distribution is representative of the overall allergic population.

ED_p for allergenic foods (in milligrams of protein) listed in Annex IIIa have been estimated in several publications using data from DBPCFCs and the ICSA to calculate individual thresholds (see Appendix A).

Ballmer-Weber et al. (2007) derived ED_{01} for soy based on cumulative threshold doses obtained from 30 children participating in a threshold-finding DBPCFC. Two threshold distributions were used: one for objective reactions and one for subjective reactions. The ED_{01} for subjective reactions was more than 10 times lower than the ED_{01} for objective reactions. Blom et al. (2013) calculated ED_{01}, ED_{05}, and ED_{10} for cow's milk, hen's egg, peanut, hazelnut, and cashew nut based on discrete threshold doses using two threshold distributions: one for objective reactions and one for any reaction (objective and subjective, whichever happened first). Individual threshold data were obtained from DBPCFCs conducted in children routinely tested to diagnose food allergy. Data available for soy (n = 10) and walnut (n = 13) were deemed insufficient to derive threshold distributions. ED_p for any reaction were, on average, two to six times lower than for objective reactions.

ED*p* (expressed as milligrams of peanut and not as milligrams of protein, not included in Appendix A) were calculated by the same authors for peanut using two different datasets (Taylor et al., 2009b; Taylor et al., 2010). In the first study, data were obtained from 185 subjects participating in DBPCFCs for diagnostic purposes, because they were enrolled in immunotherapy trials, or for threshold-finding purposes. ED_{10} were calculated based on cumulative threshold doses using threshold distributions for any reaction. An insufficient number of data points were available to make ED_{01} or ED_{05} estimates with adequate confidence. ED_{10} of 17.6 mg (95 % CI: 9.19, 33.7 mg), 17.0 mg (95 % CI: 8.10, 35.8 mg) and 14.6 mg (95 % CI: 5.97–35.5 mg) were estimated using the log-normal, log-logistic and Weibull distribution models, respectively, with data from the three types of DBPCFCs. Using the log-normal probability distribution model, significantly higher ED_{10} were estimated from immunotherapy trials (65.5 mg; 95 % CI: 18.7–229 mg) than from diagnostic series (18.0 mg; 95 % CI: 5.8–55.8 mg) or threshold studies (11.9 mg; 95 % CI: 4.8–29.8 mg). In the second study (Taylor et al., 2010), data from diagnostic DBPCFCs of a series of 286 peanut-allergic individuals collected over 14 years in the same clinical setting were used. ED_{05} (7.3 mg, 95 % CI: 5.2–10.4 mg) and ED_{10} (14.4 mg, 95 % CI: 10.7–19.6 mg) were based on cumulative threshold doses using threshold distributions for objective reactions. When data were analysed on the basis of the patient’s history, the threshold distribution and ED_{10} of patients with histories of more severe reactions did not differ significantly from the threshold distributions from patients with histories of less severe reactions.

Eller et al. (2012) calculated ED_{05}, and ED_{10} for cow’s milk, hen’s egg, peanut and hazelnut, based on continuous threshold doses using log-normal threshold distributions for objective reactions. Individual threshold data were obtained from DBPCFCs conducted in adults and children (48 % of subjects under four years of age) routinely tested to diagnose food allergy. There were first-dose responders for all four allergenic foods, and starting challenge doses were relatively high compared with other studies (5 mg egg protein, 1 mg hazelnut and peanut protein, and 5 mL of milk). More than half of the patients underwent OFC instead of DBPCFCs to increase compliance. The severity of reactions (graded from 1 to 5) correlated with age (older subjects had more severe reactions), and type of food tested (patients challenged with peanut had significantly more severe reactions), but not with threshold dose, sex or type of study design (OFC vs. DBPCFC).

A comprehensive assessment of ED*p* has been undertaken by (Remington, 2013), who considered data from the above-mentioned studies, with the exception of Eller et al. (2012), and from other published and unpublished DBPCFCs conducted in adults and children for diagnostic purposes, in immunotherapy trials or studies specifically designed to assess individual thresholds. ED_{01}, ED_{05} and ED_{10} were estimated for 11 allergenic foods (hen’s egg, cow’s milk, peanut, hazelnut, cashew nut, soy, wheat, mustard, lupin, sesame and shrimp) using log-normal, log-logistic and Weibull threshold distribution models for objective reactions (see Appendix A). Estimates were obtained for both discrete and cumulative doses. No individual threshold data were found below the predicted ED_{30} for celery or fish, and thus estimating lower ED*p* by extrapolation was deemed inappropriate.

Sensitivity analyses were conducted to assess whether the type of allergen, age, gender, patient population, geographical region, and the testing material (i.e. food matrix used for the oral challenges) had an effect on the estimated ED*p* . Mustard and egg were the most potent allergenic foods, followed by peanut and milk, whereas soy and shrimp were the least potent allergens. Only data for peanut and hazelnut allowed sensitivity analysis by age (as expected, data on cow’s milk and egg were mostly in children, as these allergies are generally outgrown). ED_{05} and ED_{10} were comparable for peanut in adults and children but different for hazelnut (ED_{05} estimates 1.2 mg and 4.0 mg hazelnut protein, respectively). Only data for peanut and milk allowed analyses by geographical region. Significantly lower ED_{05} and ED_{10} estimates for peanut were calculated from studies conducted in the UK compared with other regions (USA, France, the Netherlands). It is unclear whether this difference was owing to differences in patient selection, rather than to geographical differences in patients’ reactivity. Similar ED_{05} and ED_{10} were estimated for Italy and the Netherlands for milk, which were significantly lower than those estimated for Australia, where initial oral challenges started at higher doses of milk protein. The use of pulverised peanut versus peanut flour, or the use of liquid milk versus non-fat dry milk in oral challenges did not affect the lower end of the threshold distribution, whereas more data were

considered necessary for a meaningful assessment of differences among raw, boiled or fried, and baked eggs (Remington, 2013; Allen et al., 2014).

Only recently, ED_{10} were estimated for milk egg, peanut, hazelnut, celery, fish and shrimp using oral challenge data from the EuroPrevall project using standardised, low-dose DBPCFCs specifically designed to assess individual thresholds (Defernez et al., 2013). Cumulative dose distributions of individual thresholds considering either LOAELs or the ICSA method were calculated using the log-normal, log-logistic and Weibull distribution models. Dose distributions were modelled using objective reactions (signs), subjective reactions (symptoms) or any reaction (whichever came first). Whereas the mathematical model used did not affect the ED_{10} significantly, these were generally lower using the ICSA method than the LOAEL (threshold which triggers a reaction versus the lower dose at which a reaction is observed) and significantly lower using subjective reactions or any reactions compared with objective reactions only. ED_{10} estimated from objective reactions using the ICSA method for the above-mentioned allergens are shown in Appendix A.

A simulation study was performed to investigate the effects of sample size, distribution model (log-normal, log-logistic and Weibull) and four dosing schemes on the estimation of the ED_{01}, ED_{05}, ED_{10} and ED_{50} (Klein Entink et al., 2014). Peanut, egg and soy were selected for the simulation for having different potency. The bias and accuracy of estimation under those test conditions improved with each step in sample size ranging from n = 20 to n = 60, suggesting that n = 60 or larger would be needed for obtaining stable estimates of threshold distributions for the above-mentioned allergenic foods. Compared with the EuroPrevall normal dosing scheme, all other dosing schemes reduced the accuracy in the calculation of ED*p*, particularly for small sample sizes. However, adaptations of the EuroPrevall normal dosing scheme may be needed for less potent allergens (e.g. soy).

12.4. Exposure assessment

The exposure of interest for allergen risk assessment is the amount of an allergenic food/ingredient consumed by subjects allergic to that allergenic food/ingredient in a single meal. Data from food consumption surveys conducted in the general population or specific population subgroups (e.g. children, adolescents, elderly) are available from most European countries. These surveys, however, have not been designed to specifically address this question (i.e. information on the food-allergic status of participants is usually lacking; food consumption data are often reported on a daily basis and not per meal or eating occasion). In addition, food composition tables ignore the distribution of specific allergens present by cross-contact in the general food supply, which is at present unknown (Crevel et al., 2014).

The Panel notes that food consumption surveys should be adapted to retrieve data on food consumption patterns in food-allergic subjects and to investigate how these relate to the general, non-food-allergic population.

12.5. Risk assessment approaches for the derivation of reference doses and action levels

Three different approaches have been proposed for allergen risk assessment: (i) the traditional risk assessment using the NOAEL and uncertainty factors; (ii) the BMD and Margin of Exposure (MoE) approach; and (iii) probabilistic models. These approaches may be used to inform different risk management decisions for allergen labelling. Advantages and disadvantages of each approach in allergen risk assessment have been extensively reviewed elsewhere (Madsen et al., 2009; Crevel et al., 2014).

12.5.1. The traditional risk assessment using the NOAEL and uncertainty factors

This approach makes little assumptions and accepts a low level of risk. It applies the NOAEL/LOAEL (a single data point from a single study) plus uncertainty factors (e.g. to account for inter-individual variability), the worst-case exposure estimate, and the most sensitive adverse health effect to derive **reference doses** for a given allergenic food/ingredient. These may lead to **action levels** that are below the limits of detection of current assays for some allergens. Advantages and disadvantages of this

approach have been reviewed in detail elsewhere (FDA, 2006; Madsen et al., 2009; Crevel et al., 2014).

12.5.2. The Bench Mark Dose and Margin of Exposure approach

The Margin of Exposure (MoE) is the $BMDL_{10}$ of the individual threshold distribution for an allergenic food/ingredient divided by an estimation of exposure to such an allergenic food/ingredient. The higher the MoE, the lower the likelihood of an allergic reaction in the allergic population. The MoE is highly dependent on the exposure estimate selected for the assessment (e.g. maximum exposure anticipated, 95th percentile of the entire population, 95th percentile of the allergic population, 95th percentile of consumers only, etc.). An example of how the BMD and MoE approach could be used to determine action levels for peanut in foodstuffs has been published (Madsen et al., 2009).

ED*p* can be used to derive **reference doses** by defining the level of risk that may be acceptable (i.e. the percentage of the allergic population that will be protected). Reference doses based on ED*p* estimates by Remington (2013) have been derived by industry for the voluntary labelling of allergens unintentionally present in foods (Taylor et al., 2014). The ED_{01} (for peanut and milk), a combination of the ED_{01} and the 95 % lower CIs of the ED_{05} (for egg and hazelnut), or the 95 % lower CIs of the ED_{05} (for soy, wheat, cashew, mustard, lupin, sesame and shrimp) were used to derive reference doses. The choice depended on how robust (based on the amount and quality of data available) the estimates of the low ED*p* for each allergenic food were found to be. The lowest reference doses proposed were for mustard (0.05 mg) and egg (0.03 mg), followed by milk and hazelnut (0.1 mg) and by peanuts and sesame seeds (0.2 mg). Higher reference doses were proposed for soy and wheat (1 mg), cashew (2 mg) and lupin (4 mg), and the highest for shrimp (10 mg) (Allen et al., 2014; Taylor et al., 2014). **Action levels** calculated from reference doses need to consider the amount of a foodstuff that is consumed per meal, the concentration of the allergenic food/ingredient in the foodstuff, and co-consumption with other foodstuffs also containing the allergenic food/ingredient.

12.5.3. Probabilistic models

A probabilistic approach for food allergy risk assessment has been proposed (Spanjersberg et al., 2007; Madsen et al., 2009). This approach estimates the probability distribution of intake of an allergenic food (e.g. peanut) in a given population (from presence and concentration of the allergenic food in a foodstuff, the likelihood that an allergic person consumes the food and the amount of the food consumed per meal) and the threshold probability distribution for that allergenic food (e.g. peanut) in the same population (from individual MEDs reported in, or calculated from, DBPCFCs). By comparing the probability threshold distribution with the probability distribution of consumption of the allergenic food, the probability of an allergic reaction occurring upon exposure to an allergenic foodstuff is predicted.

Since input variables can be modified independently, the probabilistic risk assessment methodology can be used to estimate the proportion of an allergic population that may suffer from a reaction owing to the presence of a certain level of allergen in a food product, and also to predict the impact of decreasing the concentration of allergens in food products or for predicting the analytical sensitivity needed for a certain level of protection. However, in the absence of actual data on the incidence of allergic reactions in the population under the conditions defined in the probabilistic models, formal validation of these models is lacking at present (Madsen et al., 2009).

Sensitivity analyses have been conducted to determine how changes in input variables (both the MED and the exposure components) affect the output (Kruizinga et al., 2008). Both the location of the distribution of the MED and the proportion of the population consuming a food have a large influence on the number of allergic reactions predicted using this risk assessment model for allergens, whereas the statistical model used to fit the threshold (MED) distribution, the portion size of the food and using more “severe” reactions as the basis of the MED had a relatively minor influence under the conditions tested. The probabilistic model has been used to assess the impact of the presence of milk proteins in dark chocolate, following a report of an allergic reaction in a consumer (Spanjersberg et al., 2010).

12.6. Conclusion

Different approaches have been proposed for allergen risk assessment, which may be used to inform risk management decisions for allergen labelling. The reliability of the risk estimates will depend on the type, quality and amount of data used, to estimate both population thresholds (or threshold distributions) and exposure to the allergenic food/ingredient. The purpose of the risk assessment (e.g. exemptions from labelling, labelling of allergens unintentionally present in food) and the level of risk that may be acceptable (e.g. the fraction of the allergic population that it aims to protect and to what extent) are risk management decisions, which are outside EFSA's remit.

13. Coeliac disease

13.1. Background

Coeliac disease is an autoimmune systemic disorder triggered by gluten in genetically susceptible individuals. It is a life-long disease with permanent gluten intolerance and is characterised by the presence of a variable combination of gluten-dependent clinical manifestations, coeliac disease-specific antibodies, HLA-DQ2 or HLA-DQ8 haplotype, and a small intestinal mucosal lesion (enteropathy) (Marsh, 1992; Collin et al., 1994; Fasano and Catassi, 2001; Tack et al., 2010; Husby et al., 2012; Ludvigsson et al., 2013).

Coeliac disease is strongly associated with HLA-DQ2 and DQ8 (Marsh, 1992; Sollid, 2002; Schuppan et al., 2009). Gluten peptides are presented by DQ2- and DQ8-positive antigen-presenting cells to immunocompetent cells of the small intestinal lamina propria. Tissue transglutaminase, which has also been identified as the important autoantigen in coeliac disease, is released and it may potentiate antigen presentation by deamidating or cross-linking gluten peptides (Schuppan et al., 2009). As a result, T-cell activation, cytokine production, mucosal inflammation and destruction evolve. As a secondary event, production of humoral antibodies to the autoantigen transglutaminase and to gluten/gliadin peptides occurs. Immune pathophysiology of coeliac disease involves innate and adaptive immunity. The mechanisms are different from IgE-mediated food allergy. As a consequence, the time course and clinical manifestations of the reactions are different.

Coeliac disease has a wide range of clinical presentations in all age groups. The most severe cases may present with diarrhoea and cachexia; less severely affected patients may present with malabsorption resulting in weight loss and, in children, failure to thrive. However, the disease may present with more insidious symptoms. In some childhood cases, impaired weight or height gain, or delayed puberty, may be the only clinical evidence of illness. In adults, infertility, osteoporosis, iron deficiency or other deficiency syndromes may be the only clinical manifestation. Clinical manifestations and enteropathy are responsive to elimination of the trigger by following a gluten-free diet, except for a few cases of refractory coeliac disease (Meresse et al., 2009; Tack et al., 2010).

The diagnosis of coeliac disease relies on a combination of typical symptoms and a small intestinal biopsy indicating enteropathy, which responds to a gluten-free diet, on the demonstration of autoantibodies against endomysium and tissue transglutaminase as well as those against deamidated gliadin peptides, and on the demonstration of the DQ2/DQ8 haplotype (Volta and Villanacci, 2011; Husby et al., 2012). Coeliac disease can also be diagnosed in the absence of symptoms.

Coeliac disease can be associated with other autoimmune diseases, with type 1 diabetes mellitus and with IgA deficiency. First-degree relatives of patients with coeliac disease have a 10 % risk of developing coeliac disease themselves. Underestimation and unawareness of the diagnosis is still common.

In some patients in whom coeliac disease had been excluded, non-coeliac gluten sensitivity has been suggested (Biesiekierski et al., 2011). However, this entity has not been well defined, and there are no reliable and accepted diagnostic criteria.

13.2. Epidemiology

13.2.1. Prevalence

Worldwide, the prevalence of coeliac disease based on the clinical diagnosis, classic gastrointestinal symptoms and enteropathy formerly appeared to be about 1:3 000 (Fasano and Catassi, 2001). However, this classic picture, with abdominal distension, steatorrhoea and deficient growth, has become rare. Oligosymptomatic forms (patients with anaemia, retarded puberty, dental anomalies, oral ulcers, infertility, abdominal pain, constipation, short stature, arthritis, and neurological and psychiatric complaints) have become more predominant (Collin et al., 1999; Husby et al., 2012; Ludvigsson et al., 2013). Considering the classic picture, oligosymptomatic and even asymptomatic forms together, the overall prevalence is estimated to be as high as 0.5 to 1 % in Europe and the Western world (Fasano and Catassi, 2001; Rubio-Tapia et al., 2009; Virta et al., 2009).

13.3. Proteins identified to trigger coeliac disease

Gluten is defined as the rubbery dough-forming protein that remains when wheat flour is washed to remove starch (Stern et al., 2001; Wieser and Koehler, 2008). Gluten is characterised by a unique high content of glutamin and proline. It consists of glutenin and gliadin, two seed storage proteins, which have been shown to trigger the pathophysiological and clinical features of coeliac disease. Glutenin is a high-molecular-weight protein fraction insoluble in alcohol. The alcohol-soluble gliadins contain mainly monomeric low-molecular-weight proteins. Gliadins belong to a group of plant storage proteins rich in proline (prolamins). Gliadins contain repetitive peptide units such as QPQPFPPQQPYP and PQQPFPQ. These repetitive peptide units are contained in α, γ, ω subtypes of gliadin, which have all been shown to elicit the disease. A 33-mer peptide (LQLQPSTQPQLPYPQPQLPYPQPQLPYPQPQPF) was the first primary initiator of the inflammatory response in coeliac disease identified (Shan et al., 2002).

Wheat, rye and barley contain coeliac-active prolamins, whereas maize, rice, millet and sorghum do not. Oats contain low amounts of the prolamin-type avenin. Wheat, rye and barley have been established as triggering coeliac disease, whereas maize, rice and buckwheat were found not to be harmful. The toxicity of oats has been questioned. Uncontaminated oats seem to be safe in the vast majority of patients of all ages (Janatuinen et al., 2002; Lundin et al., 2003; Holm et al., 2006; Kemppainen et al., 2007), although oat cultivars may show variable toxicity depending on the presence of specific peptide sequences with higher or lower immunogenicity (Comino et al., 2011; Real et al., 2012). Yet oats are commonly contaminated with other cereals containing gluten (mostly barley).

Different gluten peptides have been shown to elicit coeliac effects, both at the intestinal epithelia and at the immunocompetent cells level (van de Wal et al., 1998; Anderson et al., 2000; Shan et al., 2002; Vader et al., 2002; Koning et al., 2005; Bodd et al., 2012). At the level of T-cell reactivity, stimulatory gluten peptides have been identified up to the molecular level. There is a diverse repertoire of gluten peptides eliciting a coeliac response, including immuno-dominant T-cell stimulatory peptides rich in proline residues (Arentz-Hansen et al., 2002; Vader et al., 2002; Molberg et al., 2005).

13.4. Possible effects of food processing on coeliac “toxicity” of gluten

Food processing generally does not affect coeliac “toxicity” of gluten. For example, partial hydrolysis and enzymatic peptic-tryptic degradation of gluten do not affect coeliac-triggering properties, since the important peptide units are unaffected. Heat treatment (baked products) does not change coeliac “toxicity” either, whereas complete acid hydrolysis abolishes toxicity.

All food technology processes affect extractability and detectability of gluten, which are important in any attempts to measure gluten quantitatively in food (Stern et al., 2001; Hischenhuber et al., 2006).

13.5. Detection of gluten in food

The existing methods for the detection of gluten in foods have been recently reviewed (Haraszi et al., 2011; Diaz-Amigo and Popping, 2012).

13.5.1. Immunological methods

13.5.1.1. ELISA

A number of rapid and sensitive monoclonal and polyclonal sandwich ELISA kits are commercially available for gluten analysis. However, the results obtained with such kits are often not comparable, since they target different gluten components and differ in antibody specificity, extraction conditions and matrix effects (Immer and Haas Lauterbach, 2010; van Eckert et al., 2010; Diaz-Amigo and Popping, 2013).

The Association of Analytical Communities and the Codex Alimentarius Commission (Codex Alimentarius Commission, 2006, 2008) have endorsed two different sandwich ELISAs for gluten analysis in foods, which are reported to be suitable to quantify native and heat-treated gluten. The former, based on the 401/21 mAb (Skerritt and Hill, 1991), mainly binds to ω-gliadin and glutenin subunits and is now obsolete. The latter, based on the mAbs R5 (Valdes et al., 2003), recognises the epitope QQPFP occurring repeatedly in α-/β-, γ- and ω-gliadin fractions which are conserved in wheat, barley (hordeins) and rye (secalins) varieties. The method uses a "cocktail extraction method", i.e. a disaggregating agent (guanidine hydrochloride) and a reducing agent (2-mercaptoethanol) in combination (García et al., 2005) that is able to solubilise gluten aggregates and has an acceptable repeatability and low LOD (1.5 mg/kg). However, both methods present limitations in quantifying barley proteins in gluten-free foods, such as oats, which is often contaminated with barley (Kanerva et al., 2006). The ELISA kit based on ω-gliadin underestimates the amount of barley prolamin, whereas the R5 antibody overestimates it. In the latter case, the problem may be overcome by using a hordein standard. A detailed comparison between the two methods has been published (Thompson and Mendez, 2008).

Another antibody, G12 mAB, raised against the toxic 33-mer from α-gliadin as a detection antibody together with the antibody A1 as a capture antibody, has been applied in both sandwich and competitive ELISAs with high sensitivity. The LOD for gliadin was 0.6 mg/kg in the sandwich format and 0.44 mg/kg in the competitive format. Thus, the LOD for gluten in the competitive format (obtained by multiplying prolamin concentrations by 2) was < 1 mg/kg. The method can be applied to both native and partially hydrolysed cereals (Moron et al., 2008). In a collaborative study, it has been shown that the G12 sandwich ELISA method can quantify gluten in foods with a LOD of 4.3 mg gluten/kg of food with good to sufficient precision in the 20 to 100 mg/kg range and also in complex matrices (e.g. chocolate cake) (Don et al., 2014).

Deamidation affects the accuracy of detection and quantification by immunoassays. In a model system (gluten treated with 0.1 M HCl at 100 °C for 2 hours), deamidation decreased the recognition of the antibody R5 by 600 times when analysed by the sandwich method and 125 times by the competitive format, while it completely abolished recognition by the ω-gliadin-based antibody (Kanerva et al., 2011). A new monoclonal antibody mAb PQQ3B4 binding both gliadins and glutenin subfractions was selected, able to detect either native or modified prolamins to similar degrees from wheat and other cereals and proposed as a promising candidate for improved gluten quantification (Tranquet et al., 2012).

For gluten analysis in hydrolysed products (such as syrup and beer), competitive ELISA systems are more suitable than sandwich ELISAs because they allow detection of smaller peptide fragments (Haraszi et al., 2011). Some commercial assays, based on different antibodies, are available. A competitive ELISA based on the mAb R5 in combination with an efficient and compatible extraction (UPEX) solution is able to detect gluten in heat-treated and hydrolysed foods with a LOD of 0.36 µg/L and a LOQ of 1.22 µg/L for gliadins, respectively (Mena et al., 2012). For the same assay, a calibrator

with a 1:1 mixture of hydrolysed gliadin from the Prolamin Working Group (PWG) and purified prolamins from rye and barley has also been used (Haas-Lauterbach et al., 2012).

PWG gliadin is a well-characterised material that has been proposed (and generally accepted) as a reference material for gluten (van Eckert et al., 2006), although it is specific for gliadin only and has some limitations for gluten analysis (Diaz-Amigo and Popping, 2013).

13.5.1.2. Lateral flow devices and dipsticks

LFDs and dipsticks for rapid and sensitive qualitative detection of gluten are commercially available. An LFD which utilises the R5 mAB is able to recognise prolamins in wheat, rye and barley with a LOD of 2.5 mg/kg (Immer and Haas Lauterbach, 2010).

13.5.1.3. Biosensors

A number of biosensors for detecting gliadin contamination in gluten-free foods have been developed but are not yet commercially available. Two electrochemical biosensors have been described (Nassef et al., 2008; Nassef et al., 2009). One uses an antibody raised against the immunodominant epitope of gliadin with a LOD of 5.5 µg/L. The second is based on the adsorption of anti-gliadin Fab fragments on gold surfaces. Detection of gliadin was evaluated by impedance (LOD = 0.42 mg/L) and amperometry (LOD = 3.29 µg/L).

A quartz crystal microbalance biosensor incorporating gold nanoparticles was able to detect gliadin with a LOD of 8 µg/kg (Chu et al., 2012). Another biosensor used anti-gliadin antibody-conjugated immunomagnetic beads as the capture reagent to extract gliadin from food and fluorescence-dye-loaded immunoliposomal nanovesicles to form a fluorescent sandwich complex (Chu and Wen, 2013). The polyclonal antibody showed a slight cross-reactivity with barley and rye. The LOD for gliadin was 0.6 mg/L, consistent with those obtained with the mAbs R5-based sandwich ELISA kit.

13.5.2. Mass spectrometry

Although MS has widely contributed to assess the structure of gluten, its use for the quantitative determination of gluten in food faces several challenges. Gluten proteins are not soluble in the salt buffers typically used for protein characterisation and have few sites for tryptic hydrolysis. However, by using LC-MS/MS it was possible to detect and quantify relevant wheat gluten peptides in food products with a LOD of 1 to 30 µg/kg and a LOQ of 10 to 100 µg/kg (Sealey-Voyksner et al., 2010).

Gluten proteins in red wines fined with gluten, in which gluten could not be identified by immunological methods, were isolated by precipitation with potassium dodecyl sulphate and analysed by LC-MS/MS. Wheat gluten proteins were detected down to 1 g/hL of commercial wheat gluten (Simonato et al., 2011). An LC-ESI-MS/MS method was applied (Weber D et al., 2009) to detect and identify gluten in beer from different sources. In contrast with the most common ELISAs, this MS method allowed discrimination between wheat and barley proteins.

By using a proteomic approach, the prolamin proteins present in purified hordeins, wort and beer were characterised and their relative amounts were quantified (Colgrave et al., 2012). MRM-MS was used as a robust and sensitive methodology to detect gluten hordein in beer. A comparison between ELISA and MS methods in the MRM mode for measuring hordein in beer (Tanner et al., 2013) showed that MS was more reliable than ELISA, as ELISA recognises only some epitopes of hordeins, whereas MS measures peptides that are specific and unique, allowing quantification of hordein isoforms. Several beers manufactured with barley were found to contain unforeseen wheat proteins.

Although MS methods are more reliable than ELISAs for gluten analysis, they are mostly used as confirmatory methods at present and are more suitable for regulatory agencies and research laboratories than for screening and routine analysis.

13.5.3. DNA-based methods

Several end-point and real-time PCR methods allow verification of the absence of wheat, barley and rye DNA in "gluten-free" products. A quantitative competitive PCR system using gel electrophoresis for the simultaneous detection of wheat, barley and rye in gluten-free food based on amplification of a non-coding region of the chloroplast *trnL* gene does not discriminate among the three cereals (Dahinden et al., 2001). The absolute LOD was 20 pg of DNA for wheat and 2 pg of DNA for rye and barley. A PCR method targeting the glutenin gene to detect wheat DNA in a number of raw and heat-processed foods had a LOD of 21.5 pg of DNA (Debnath et al., 2009). A real-time PCR method for the specific detection of wheat, rye, barley and oats could discriminate among the four cereals, but no quantitative results were provided (Sandberg et al., 2003).

Another real-time PCR method for qualitative and quantitative detection of the rye (*Secale cereale* and *Triticosecale*) content in raw materials and processed foods was developed using a SYBR Green detection system and a TaqMan® fluorogenic probe (Terzi et al., 2004). It was possible to detect 1 % of rye in a rye–rice model mixture. The SYBR Green detection method was judged to be more precise than the TaqMan.

A sensitive qPCR system employing the fluorescent dye SYBR Green for wheat contamination in gluten-free food had a LOQ of 20 pg DNA/mg (Mujico et al., 2011). This DNA-based method was more sensitive than the mAbs R5-based sandwich ELISA kit, able to detect wheat below the LOQ of the ELISA (< 1.5 mg/kg).

The performances of ELISA and PCR methods for the determination of gluten in different foods were evaluated by proficiency testing (Scharf et al., 2013). Although test kit-specific differences were observed for the ELISA kits, both ELISA and PCR methods showed reliable results for the determination of gluten and wheat in food.

13.6. "Gluten-free" food products and diets

A gluten-free diet excluding wheat, rye, barley and oats (because oats is commonly contaminated by other grains containing gluten) is the conventional cornerstone treatment for the management of coeliac disease. Owing to individual variation and the clinical heterogeneity of patients with coeliac disease, it is difficult to find an acceptable value for trace amounts of gluten in foods that could be tolerated by the majority of coeliac patients (Stern et al., 2001; Hischenhuber et al., 2006).

The usual daily intake of gluten is 15–20 g in the adult European population. In patients with coeliac disease any effort has to be undertaken to exclude gluten/gliadin from the diet. Early studies have shown that 100 mg of gliadin per day were able to induce coeliac-specific histological lesions in children (Catassi et al., 1993). After a long-term dietary survey using naturally gluten-free products and also wheat starch-based products with reduced gluten content (Collin et al., 2004), a level of 100 mg/kg gluten was proposed for the dietary management of patients with coeliac disease. The daily consumption of flour, which differs between European countries (range 10–500 g), needs to be considered.

In a low-dose DBPCFC trial carried out in Italy (Catassi et al., 2007), where 0, 10 or 50 mg of gluten were given to coeliac patients daily for 90 days, 50 mg gluten per day produced measurable damage to the small intestinal mucosa. To keep gluten intake < 50 mg/day, which was considered to be safe for most patients with coeliac disease, a limit of 20 mg/kg gluten in foods was proposed. This figure took into account high regional consumption of wheat substitutes, thus allowing a "safe margin" according to the dietary habits of patients (Catassi et al., 2007).

Based on these observations, Codex Alimentarius adopted in 2008 a revised codex standard for foods for special dietary uses addressed to persons intolerant to gluten (Codex Alimentarius Commission, 2008). "Gluten-free" foods were defined as dietary foods consisting of or made only from one or more ingredients that do not contain wheat, rye, barley or oats, and in which the gluten content does not

exceed 20 mg/kg of the food as sold or distributed to the consumer. In addition, the standard defined foods specially processed to reduce the gluten content to a level > 20 to 100 mg/kg as foods consisting of one or more ingredients from wheat, rye, barley, oats or their crossbred varieties that have been specially processed to reduce the gluten content to these levels.

Based on this, the European Commission has issued a Regulation[9] concerning the composition and labelling of foods suitable for people intolerant to gluten, in which the terms "gluten-free" (not exceeding 20 mg/kg) and "very low gluten" (not exceeding 100 mg/kg) are set. It came into force on 1 January 2012.

Although the labelling of "gluten-free" and "very low gluten" foods is helpful for the dietary management of most patients with coeliac disease, questions regarding low-dose and long-term gluten sensitivity, and the testing and toxicity of glutenin and gluten hydrolysates, remain open. New standards, new methods of detection of gluten-related proteins in foods and new therapies for coeliac disease are likely to be developed in the future (FDA, 2006; Donnelly et al., 2011; Sollid and Khosla, 2011).

13.7. Conclusion

Coeliac disease is a life-long autoimmune systemic disorder triggered by gluten and similar cereal storage proteins present in wheat, rye and barley. Its prevalence is estimated to be 0.5 to 1 %. Coeliac disease is under-diagnosed owing to its various clinical manifestations. Diagnosis relies on a combination of typical symptoms, the presence of enteropathy responding to a gluten-free diet, and demonstration of coeliac-specific antibodies and of the HLA DQ2/DQ8 haplotype. A gluten-free diet excluding wheat, rye, barley and oats (because oats are commonly contaminated with other grains) is the conventional treatment for the management of coeliac disease. Methods for gluten analysis are available for the control of "gluten-free" products. ELISA methods, which are most frequently applied, present some analytical drawbacks. MS has not yet been used for quantification of gluten, whereas DNA-based methods are useful tools to detect eventual contaminations. The limit values of 20 and 100 mg/kg of gluten in "gluten-free" and "very low gluten" foods, respectively, help in managing the diet of most patients with coeliac disease efficiently.

14. Allergy to cereals containing gluten

14.1. Background

The term "cereal" indicates any kind of plant-producing grains, which are milled in order to obtain edible flour. It follows that "cereals" do not belong to a single botanical family, though the majority are from the grass family, namely *Poaceae* or *Gramineae* (wheat, spelt wheat, rye, barley, oats, rice, maize, millet, sorghum, teff). Some belong to the family *Polygonaceae* (buckwheat) and others to the family *Amaranthaceae* (quinoa).

From the grass family, rice (*Oryza sativa*) (Yamakawa et al., 2001), maize (*Zea mays*) (Pastorello et al., 2000; Scibilia et al., 2006; Weichel et al., 2006), millet (*Panicum milliaceum*) (Bohle et al., 2003), sorghum (*Sorghum bicolor*) and teff (*Eragrostis tef*) are non-gluten-containing cereals that may induce food allergy. From the non-grass family, buckwheat (*Fagopyrum esculentum*) (Heffler et al., 2011) and quinoa (*Chenopodium quinoa*) are plants used as cereals.

Only some cereals (wheat, spelt wheat, rye, barley, oats, Khorasan wheat or their hybridised strains) contain gluten, which is defined as the rubbery dough-forming protein that remains when wheat flour is washed to remove starch. Gluten consists of the seed storage proteins glutenin and gliadin (see section 13). Only cereals containing gluten have been included in Annex IIIa of Directive 2000/13/EC. Nevertheless, allergies to cereals are elicited not only by gluten proteins but also by other proteins

[9] Commission Regulation (EC) No 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten, OJ L 16, 21.1.2009, p. 3–5.

present in gluten-containing and gluten-free cereals. The focus of this chapter is restricted to cereals containing gluten, following Annex IIIa.

Cereals are a major source of food in all parts of the world and account for 72 % of the protein in the human diet. World production of all cereal grains is about 1 600 billion tonnes annually. Wheat is the leading cereal grain, representing about one-third of the world cereal production, followed by rice and maize. Nearly two-thirds of the wheat produced is used for food. Wheat is consumed in different forms, all of which involve some degree of processing: products such as breakfast cereals are obtained from the whole kernel, but most of the wheat is milled into flour for baking. About 6 % of wheat undergoes industrial processing into gluten and starch, which are used in food as protein enrichment and as thickening agents, respectively.

IgE-mediated allergic reactions to cereals were first described as occupational diseases caused by the inhalation of cereal flour by bakers or millers (“baker’s asthma”). Cereals can also induce immediate or delayed clinical reactions after ingestion (food allergy). The severity of symptoms varies from mild to severe (Armentia et al., 2002; Scibilia et al., 2006; Tatham and Shewry, 2008). Oral allergy syndrome, urticaria, flare-up of atopic dermatitis, respiratory and gastrointestinal symptoms, eosinophilic gastroenteropathy, non-coeliac gluten sensitivity and even anaphylaxis, which can also be induced by exercise, have been described in relation to cereal ingestion. The gluten-containing cereals described as causing IgE-mediated reactions are wheat, rye, barley and oats.

14.2. Epidemiology

14.2.1. Prevalence

14.2.1.1. Europe

Population-based studies investigating the prevalence of cereal allergies in unselected populations are scarce (Table 4). The most commonly mentioned allergies are related to wheat and, to a lesser extent allergies related to barley, rye and oats (University of Portsmouth, 2013). It is sometimes difficult to differentiate between the prevalence of cereal allergy (IgE-mediated and non-IgE-mediated) and the prevalence of cereal-related intolerances (including gluten sensitivity). In some cases, clinical symptoms of food allergy to cereals can be similar to those observed in food intolerance, particularly in children, and studies relying only on questionnaire-based methods are generally not a reliable way of differentiating between food allergy and food intolerance in relation to gluten-containing cereals.

Table 4: Estimated prevalence (%) of allergy to gluten-containing cereals in unselected European populations by type of cereal, age group and method of diagnosis

	All Cereals	Wheat	Barley	Rye	Oats
All ages					
Self-reported	–	0.9	–	–	–
Clinical history and sensitisation	–	0.9	1.7	0.9	1
Young children (≤ 3 years)					
Self-reported	0.2–2.3	0.8–2.1	1.3–1.8	–	–
Sensitisation	–	0–0.2 (SPT)	–	–	–
Clinician diagnosed	0.9–1.1	0.3–2.4	1.3–2	–	–
Clinical history and sensitisation	0	–	–	–	–
Clinical history and FC	–	0–0.4	–	–	–
Children/adolescents (> 3–17 years)					
Self-reported	1.5	0.2–1.5	1.8	–	–
Sensitisation	–	0.3–1.2 (SPT) 4 (IgE)	–	–	–
Clinician diagnosed	2	0.3–3.4	2.7	–	–

	All Cereals	Wheat	Barley	Rye	Oats
Clinical history and sensitisation	–	1.3		–	–
Clinical history and FC	–	–	–	–	–
Adults/elderly (≥ 18 years)					
Self-reported	–	0.8	–	–	–
Sensitisation	–	2.8–13.9 (SPT) 2.8–5.5 (IgE)	–	7.3–11.1 (SPT) 0–2.8 (IgE)	–
Clinical history and FC	–	0	–	–	–

FC, food challenge; IgE, allergen-specific IgE; SPT, skin prick test.

Cereals

Three studies evaluated self-reported allergy to grains or cereals in Europe, but only one (Pyrhonen et al., 2009) detailed the cereals consumed (oats, maize, rice, millet and buckwheat). No data were available in adult populations. The estimated prevalence of cereal allergy ranged from 0.2 % at 18 months in Norway (Eggesbo et al., 1999) to 2.3 % at one year in Finland (Pyrhonen et al., 2009). Based on a clinician's diagnosis, the estimated prevalence of cereal allergy was 1.1 % at one year, 0.9 % at two years and 2 % at three and four years in Finland (Pyrhonen et al., 2009). These two studies examined IgE- and non-IgE-mediated cereal allergies. Self-reported allergy to cereals was estimated at 1.2 % in young children in Sweden (Kristjansson et al., 1999). However, when the diagnosis was based on clinical history of food allergy and positive SPTs, this figure was zero.

Wheat

Several studies assessed the prevalence of self-reported wheat allergy (IgE- and non-IgE-mediated). In young children (≤ 3 years), prevalence ranged from 0.8 % in Sweden (Ostblom et al., 2008b) to 2.1 % in Finland (Pyrhonen et al., 2009). The lowest prevalence (0.2 %) of self-reported wheat allergy was found in a group of 7- to 13-year-olds in Greece (Zannikos et al., 2008). For the same age category (> 3–17 years), the highest prevalence was reported in France at 1.5 % (Touraine et al., 2002). Clinician-diagnosed wheat allergy was assessed in two studies, in which 0.3 % of one- and eight-year-olds in Sweden (Ostblom et al., 2008a) and 2.4 % and 3.4 % of two- and four-year-olds in Finland (Pyrhonen et al., 2009), respectively, were diagnosed with wheat allergy by a clinician.

Sensitisation determined via positive SPT was zero for one- and three-year-olds in the UK (Venter et al., 2008), and 13.9 % in adults in Hungary (Bakos et al., 2006). The prevalence of wheat allergy based on a positive SPT and clinical history was estimated to be 0.9 % for all ages combined in Germany (Zuberbier et al., 2004). Based on positive IgE levels and clinical history, only one study (Ostblom et al., 2008a) reported a prevalence of 1.3 % among four-year-olds in Sweden.

Using a combination of history and SPT and/or OFC and DBPCFC, the prevalence of wheat allergy was 0.4 % in one-year-olds, 0.3 % in two-year-olds, 0.2 % in three-year-olds and 0.3 % in six-year-olds in the UK (Venter et al., 2006a; Venter et al., 2008). Based on clinical history and positive OFC/DBPCFC, no cases of confirmed wheat allergy were found in children under three years old (n = 486), three years old (n = 111), and over three years old (n = 301), or in adults (n = 936), in Denmark (Osterballe et al., 2005).

Rye, barley and oats

In Finnish children, the prevalence of IgE- and non-IgE-mediated clinician-diagnosed allergy to barley and rye was 1.9 % in children one to four years of age (Pyrhonen et al., 2009). Sensitisation rates based on positive SPT and IgE levels to rye were between zero and 11.1 % in adults in Hungary (Bakos 2006). In a German population combining all ages, the prevalence of allergy was estimated to be 2.2 %, 1.2 %, and 1.2 % for barley, rye and oats, respectively, based on a clinical history and positive SPTs (i.e. IgE-mediated allergy only) (Zuberbier et al., 2004). There are no studies available using food challenges to confirm diagnosis.

14.2.1.2. Outside Europe

Sensitisation rates to wheat in adults have been estimated to be 2.2 % in Australia based on positive SPTs (Woods et al., 2002) and 1.4 % in Japan based on serum IgE levels (Morita et al., 2012). When sensitisation rates were combined with clinical history, the prevalence of wheat allergies was estimated to be zero in Australia and 0.2 % in Japan in the same studies. In the USA the prevalence of wheat allergy in under-three-year-olds was 0.2 % when using food challenges (Bock, 1987) considering both IgE- and non-IgE-mediated allergy.

14.2.2. Natural history

Wheat allergy resolves frequently by adolescence (Keet et al., 2009; Kotaniemi-Syrjanen et al., 2010).

In Finland, 28 children diagnosed with wheat allergy (median age 21 months) were tested annually by OFC (Kotaniemi-Syrjanen et al., 2010). Wheat was tolerated by 59 % of the children by the age of four years, by 69 % by the age of six, by 76 % by the age of eight, by 84 % at the age of 10 and by 96 % by the age of 16 years. Sensitisation to gliadin (SPT wheal size at least 5 mm) was significantly associated with a slower achievement of tolerance and an increased risk of asthma.

In the USA, 103 children (median age 19 months) with a symptomatic reaction to wheat and a positive IgE test result were studied. Resolution of wheat allergy was determined on food challenge results. Resolution rates were 29 % by four years, 45 % by six, 56 % by eight, 62 % by 10, 65 % by 12 and 70 % by 14 years. In this referral population, higher wheat IgE levels were associated with an increased risk of persistence. However, 20 % of children with wheat IgE level > 100 kU/L outgrew their wheat allergy (Keet et al., 2009).

No data are available on the natural history of food allergy in relation to other gluten-containing cereals.

14.2.3. Time trends

Two studies on the prevalence of self-reported wheat allergy were conducted in Finland in 1980 and 2001 using similar methodology. Both IgE- and non-IgE-wheat allergies were considered. At one year of age, self-reported allergy to wheat was estimated at 1 % in 1980 (Kajosaari, 1982) and at 2.1 % in 2001 (Pyrhonen et al., 2009). At two years of age, self-reported allergy to wheat was 1 % in 1980 (Kajosaari, 1982) and 2 % in 2001 (Pyrhonen et al., 2009). These two studies relied on questionnaire-based methods, and thus they do not allow conclusions to be reached about time trends of wheat allergy.

14.2.4. Severe reactions/anaphylaxis

Wheat may trigger severe anaphylactic reactions in children with wheat allergy (Cianferoni and Muraro, 2012). In Japan, wheat is often reported among the top three foods responsible for food-induced anaphylaxis. Wheat also appears to be the third trigger of food-induced anaphylaxis in children after milk and eggs (Imamura et al., 2008). In Europe, anaphylaxis to wheat seems to be less frequent than in Asia (Panesar et al., 2013).

Wheat has also been reported to be an important triggering factor for food-dependent exercise-induced anaphylaxis (FDEIA) (Morita et al., 2007) and is considered the most frequent cause of FDEIA in Japan (Aihara et al., 2001).

14.2.5. Factors affecting the prevalence of cereal allergy

In children, IgE-mediated wheat allergy is associated with birch pollen sensitisation and the development of allergic rhinoconjunctivitis later in childhood (Kotaniemi-Syrjanen et al., 2010). The timing of initial exposure to cereal grains and family history may also modify the risk of wheat allergy (Poole et al., 2006).

Some cereal-allergic subjects develop symptoms only if they exercise within a few hours after cereal ingestion. This condition usually results in anaphylactic reactions and is denoted as wheat-dependent exercise-induced anaphylaxis (WDEIA). In adults and adolescents, anaphylactic reactions to wheat are most often food-dependent exercise-induced anaphylaxis.

14.3. Identified allergens

14.3.1. Cereals containing gluten

Cereals containing gluten (wheat, rye, barley and, to a lesser extent, oats) are neighbours in the grass family and show similarities in chemical composition, functional properties and allergenic potential of their seed storage proteins (Battais et al., 2008; Tatham and Shewry, 2008). Identified allergens (IUIS database) are shown in Table 5.

Table 5: Allergens in cereals containing gluten

Common name/scientific name	Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)] (kDa)
Wheat *Triticum aestivum*	Tri a 12	Profilin	Profilin	14
	Tri a 14	ns-LTP 1	Prolamin	9
	Tri a 18	agglutinin isolectin 1	Hevein-like domain	–
	Tri a 19	ω-5-Gliadin	Prolamin	65
	Tri a 25	Thioredoxin	–	–
	Tri a 26	HMW glutenin	Prolamin	88
	Tri a 36	LMW glutenin GluB3-23	Prolamin	40
	Tri a 37	α-Purothionin	–	12
Rye *Secale cereale*	Sec c 20	γ-Secalin	–	70
Barley *Hordeum vulgare*	Hor v 12	Profilin	Profilin	14
	Hor v 15	α-Amylase inhibitor BMAI-1 precursor	Prolamin	14.5
	Hor v 16	α-Amylase	–	–
	Hor v 17	β-Amylase	–	–
	Hor v 20	γ-Hordein 3	–	34
Oats [(b)] *Avena sativa*	NA	Avenin	–	–

(a): Molecular weight (SDS-PAGE).
(b): Not in the IUIS database.
HMW, high molecular weight; LMW, low molecular weight; NA, not assigned.

Wheat is the prominent cereal causing allergies in humans. Wheat grain proteins are traditionally divided into four classes on the basis of their solubility (Osborne): water-soluble albumins (15 % of the total); salt-soluble globulins (5 %); and 70 % ethanol-soluble prolamins (seed storage proteins rich in prolin and glutamin), which include gliadins (40 %) and acid- or alkali-soluble glutenins (40 %). Glutenins and gliadins are the constituents of gluten, which is responsible for the baking quality of wheat flour. A more recent classification of wheat storage proteins is based on molecular characteristics rather than on solubility: high-molecular-weight (HMW) prolamins, corresponding to HMW glutenin subunits (about 88 kDa); sulphur-poor prolamins, corresponding to ω-gliadins (65 kDa); and sulphur-rich prolamins, comprising low-molecular-weight (LMW) glutenin subunits, α-, β- and γ-gliadins (31–45 kDa).

The allergenic potential of cereal proteins was first demonstrated in wheat flour. Gliadin, the antigenic protein of wheat also triggering coeliac disease, was identified as the major allergen involved in WDEIA of adults and in immediate allergy to ingested wheat in children (Palosuo et al., 1999;

Varjonen et al., 2000; Palosuo et al., 2001b; Battais et al., 2003; Battais et al., 2005; Denery-Papini et al., 2011). In these clinical studies, the 65 kDa ω-gliadin, a 40 kDa α-gliadin, γ-gliadins and also LMW and HMW glutenin subunits have shown clinical allergenic potential and IgE-binding using patients' sera. Non-gluten proteins from wheat, such as an α-amylase/trypsin inhibitor and LTPs, cupins and profilins, have been identified as important B-cell epitopes in wheat allergy (Sander et al., 2011).

Typical primary sequences found in gliadins triggering wheat allergy are QQIPQQQ and related sequences (Matsuo et al., 2004); PQQPFP, QQQFPGQQQQ and similar peptides from gliadin and glutenin (Denery-Papini et al., 2011). These sequences are similar to those triggering coeliac disease.

Tissue transglutaminase, an intestinal enzyme locally activated during exercise, is able to cross-link ω-5-gliadin-derived peptides causing a marked increase in IgE binding, which may explain the role of this gliadin in WDEIA (Palosuo et al., 2003).

14.3.2. Other proteins from wheat, rye and barley

A non-gluten wheat protein fraction involved in baker's asthma contains water-/salt-soluble albumins and globulins. To this group belong the α-amylase inhibitors. The same group of non-gluten proteins has been recognised by sera from patients selected on the basis of history and positive SPT or IgE binding, and by sera from patients undergoing oral open or double-blind challenges (Pastorello et al., 2007; Battais et al., 2008).

Other cereal allergens have been described that are not included in the IUIS database. Two proteins of 20 kDa and 47 kDa, respectively, were identified as specific food allergens of wheat. These proteins were recognised by sera from patients with oral sensitisation to wheat confirmed by DBPCFC and no evidence of grass pollen allergy according to history or SPT results. Grass-sensitised patients, on the contrary, did not have IgE antibodies that bound to these fractions (Jones et al., 1995).

Wheat 15 kDa α-amylase inhibitor, the major allergen in baker's asthma, is able to sensitise not only by inhalation but also via the gastrointestinal route, as suggested by the IgE binding of sera from five atopic children with positive DBPCFC for wheat (James et al., 1997). Armentia et al. (Armentia et al., 2002) confirmed the finding that cereal allergens (wheat, barley and rye) were able to sensitise by inhalation or by ingestion. Similar proteins were involved in both routes (e.g. 11–16 kDa α-amylase inhibitor), judged by detection of cereal IgE-binding components (SDS-PAGE). Clinical significant reactivity was observed in children and adults in this study. Water-insoluble allergens (gluten, prolamins) were not investigated.

An allergen of the same molecular weight as wheat α-amylase inhibitor (16 kDa), recognised by the sera of atopic patients positive for wheat CAP-RAST and open challenge, has been identified (Simonato et al., 2001b). That allergen was bound to a lesser extent by sera from non-atopic patients who had negative CAP-RAST for wheat despite a positive open challenge. These patients recognised some proteins of the gluten fraction, such as the 42 kDa protein. α-Amylase inhibitor was confirmed to be the most important wheat allergen in WDEIA. Other important allergens were LTPs and LMW glutenin subunits (Pastorello et al., 2007; Bouchez-Mahiout et al., 2010). LMW glutenins behaved as independent allergens, partly sharing common epitopes with ω-5-gliadins. Primary sequences of non-gluten wheat allergens were dissimilar from gluten peptides (e.g. QARSQSDRQS for LTP1) (Denery-Papini et al., 2011).

Durum wheat (*Triticum durum*), einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*), spelt wheat (*Triticum spelta*) and Khorasan wheat (*Triticum turgidum* subsp. *polonicum*) are other wheat species sharing antigenic potential with bread wheat (*Triticum aestivum*). The same applies to hybrids, such as triticale. Rye and barley also share epitopes with wheat (Armentia et al., 2002).

14.4. Cross-reactivities

Since almost all cereals belong to the *Gramineae* family, a high degree of IgE cross-reactivity exists between allergens from cereal seeds and allergens from grass pollen (Sutton et al., 1982; Walsh et al., 1987; Sander et al., 1997; Palosuo et al., 2001a). Only two wheat-specific allergens (20 and 47 kDa) did not cross-react with homologous grass pollen allergens (Jones et al., 1995). *In vitro* cross-reactivity has little clinical significance, since few grass pollen-allergic patients also have food allergy to cereals. The route of sensitisation may explain differences in clinical reactivity to the same allergens. For example, “baker’s asthma” is a disease in which cereal allergens cause symptoms only when inhaled and not when ingested.

As to the cross-reactivity among different cereal grains, cross-reaction between a 65 kDa gliadin, a 70 kDa secalin from rye and a γ-3-hordein from barley was demonstrated in WDEIA (Palosuo et al., 2001a). Although food allergy to cereals is often due to monosensitisation (Jones et al., 1995), 20 % of patients with cereal allergy demonstrate clinical reactivity (DBPCFC) to more than one cereal grain.

14.5. Effects of food processing on allergenicity

Wheat is usually consumed after heat treatment and allergenicity survives thermal treatment (baking, cooking). In addition, complex fermentation processes, hydrolysis and deamidation are used for processing of cereals (Battais et al., 2008; Tatham and Shewry, 2008).

In one study (Simonato et al., 2001a) on the effect of bread baking on the *in vitro* digestibility and antigenicity of wheat proteins, the wheat allergen α-amylase inhibitor was shown to be destroyed by heating, whereas prolamins were thermostable. The *in vitro* digestibility of bread crumb and crust was much lower than that of unheated dough, probably due to the formation of aggregates, cross-linking and Maillard products, which were inaccessible to the proteolytic enzymes. However, the IgE-binding capacity remained unaltered in bread crumbs and even increased in the crust, demonstrating the maintenance or the formation of new epitopes. Deamidation of gliadin was shown to reduce IgE binding (Kumagai et al., 2007).

Proteolytic enzymatic treatments, as well as microwave heating, were shown to decrease immunoreactivity of wheat flour but also to affect its rheological properties, making it unsuitable for product development (Susanna and Prabhasankar, 2011).

Immunoreactivity of wheat bread made from wheat flour fermented with lactobacilli was shown to be reduced by 20 to 60 %, depending on the antibody used for detection (Leszczyńska et al., 2012).

Anaphylaxis can also be elicited by wheat isolates used in the bakery and meat industry after treatment by acid, heat and enzymes (Leduc et al., 2003; Pelkonen et al., 2011). In WDEIA, reactivity to hydrolysed wheat, barley and rye proteins was high (Snégaroff et al., 2006).

14.6. Detection of allergens and allergenic ingredients in food

The detection of cereal allergens in food is more complicated than the detection of gluten owing to the multiplicity of cereal proteins involved. Analytical techniques that have been applied to characterise wheat proteins include 1D and 2D electrophoresis (Mamone et al., 2005), HPLC (Wieser et al., 1998), CE (Di Luccia et al., 2009) and MS (Weber D et al., 2009). PCR-based methods allow detection of the genomic DNA of the cereal, and occasionally the gene encoding for a specific allergen (Zeltner et al., 2009). Integration of different technologies is advised to overcome the methodological difficulties intrinsic to cereal-based materials. The gluten proteins gliadin and glutenin, which are also implicated in wheat allergy, can be detected with the same methods used for gluten (section 13.5). Specific methods for the detection of non-gluten cereal allergens are scarce.

14.6.1. Immunological methods

1D- and 2D-PAGE coupled to Western blotting, ELISA with monoclonal or polyclonal antibodies, and LFDs have been used for detecting wheat allergens. ELISA kits and LFDs targeting different proteins, mainly wheat gliadin, are commercially available (Immer and Haas Lauterbach, 2010; Diaz-Amigo and Popping, 2012, 2013). The two sandwich ELISAs developed for gluten analysis in foods may be used to detect wheat, barley and rye allergenic proteins in foods. The ELISA kit based on the mAb R5 (Valdes et al., 2003) actually detects prolamins from wheat, barley and rye, the accuracy depending on the food matrix, whereas the ELISA kit based on a mAb 401/21 (Skerritt and Hill, 1991) reacts mainly with HMW glutenin and ω-5-gliadin.

A double antibody sandwich ELISA (DAS-ELISA), based on anti-gliadin IgE as capture antibody and a biotinylated monoclonal antibody as detecting antibody, was developed for detection of gliadin in foods (Gujral et al., 2012). The method was able to detect gliadin in wheat, barley and rye with a LOD of 4 ng/mL gliadin in buffer, equivalent to 0.8 mg/kg in foods. The PWG gliadin proposed as reference material for gluten (van Eckert et al., 2010) can also be used for quantifying prolamins in gluten-containing cereals.

14.6.2. Mass spectrometry

MALDI-TOF and ESI-MS have been used to characterise cereal proteins for quality assessment, tracking of commercial frauds or searching technological properties, but also for identifying allergens. MALDI-TOF has been applied to detect cereal proteins through the accurate measurement of molecular weight, while liquid chromatography coupled to ESI-MS (LC-ESI-MS) allows identification of the proteins. Although a number of cereal allergenic proteins have been characterised using MS methods (Tatham and Shewry, 2008; Cunsolo et al., 2012), the quantification of cereal allergens by MS remains, however, a challenging task.

Wheat gliadins (α-/β-, γ-, ω-gliadin) and HMW glutenin were identified by MALDI-TOF MS and nano-ESI-MS/MS with preliminary 2DE and chymotryptic digestion (Mamone et al., 2005). MALDI-TOF/TOF MS allowed identification of serpin, α-amylase inhibitor, γ-gliadin and LMW glutenin in wheat after extraction of total wheat flour proteins, separation by 2DE and tryptic digestion (Akagawa et al., 2007).

Salt-soluble wheat extracts were separated by 1DE- and 2DE-PAGE and immunoblotting using sera of patients with allergy to ingested wheat. Proteins recognised by IgE separated on 2DE were analysed by MALDI-TOF and Q-TOF and those separated on 1DE were analysed by LCQ^{DECA} nLC-MS/MS IT. Wheat α-amylase inhibitors, β-amylase and profilin, as well as barley α-amylase/trypsin inhibitor precursor and β-amylase, were identified (Sotkovsky et al., 2008).

LMW and HMW glutenins, α- and γ-gliadins, and β-amylase can be detected by both MALDI-TOF MS (previous 2DE) and nanoLC-MS/MS (Mamone et al., 2009). HMW glutenin subunits were detected by MALDI TOF MS and RP-HPLC/nESI-MS/MS in durum wheat (Muccilli et al., 2011; Lagrain et al., 2013). A heterotetrameric α-amylase inhibitor (ETI) was detected by MALDI-TOF MS in hulled emmer wheat as an assembly of proteins highly similar to that found in durum wheat (Capocchi et al., 2013).

The beer proteome has been extensively investigated mainly for characterisation and quality control purposes. In these proteomic studies, several cereal allergenic proteins were detected. Different strategies were adopted for protein separation prior to MS analysis. Among 30 proteins from *Hordeum vulgare*, α-amylase inhibitors, γ-hordein and a globulin from *Triticum aestivum* were identified by using isoelectric focusing in solution followed by 2DE (Konecna et al., 2012). In another study, the ProteoMiner-like fractionation step was incorporated with combinatorial peptide ligand libraries designed for beer proteins before gel electrophoresis (Fasoli et al., 2010). γ-Hordein 3 and a fragment of the α-amylase inhibitor were among the 20 proteins identified in beer. A gel-free shotgun proteome analysis of beer was performed with preliminary separation of proteins from polypeptides by size

exclusion chromatography (Picariello et al., 2012). Protein mixtures were reduced and alkylated, and the tryptic digests were analysed by μHPLC/ESI-MS/MS. The allergenic γ-hordein 3 and α-amylase inhibitor were found to be present along with peptides derived from α-amylase inhibitor and β-amylase. A number of hordein-derived peptides that encrypt gluten-like sequence motifs were also detected.

14.6.3. DNA-based methods

A number of real-time PCR systems for the detection and quantification of DNA of gluten-containing cereals have been described and several kits are commercially available. Two independent real-time PCR assays based on TaqMan probes targeting γ-hordein and acetyl-CoA carboxylase sequences and suitable for the identification and quantification of barley and wheat have been described (Hernandez et al., 2005). The absolute LOD for both wheat and barley was one genome copy and the absolute LOQ was 10 genome copies, corresponding to approximately 50 and 150 pg of template DNA for wheat and barley, respectively. The methods were applied successfully to highly processed solid foods (bread, cakes, biscuits) but were not suitable to detect barley and wheat in beer, refined oils or soluble extracts of cereals owing to the paucity of DNA present in these products. Another real-time PCR assay based on TaqMan probes allowed detection of 2.5 mg/kg of wheat in vegetal food matrices and 5 mg/kg of wheat in meat products (Zeltner et al., 2009). A more sensitive qPCR system employing the fluorescent dye SYBR Green was developed to detect wheat contamination in gluten-free foods with a LOQ of 20 pg DNA/mg of food (Mujico et al., 2011).

Specific detection and quantification of common wheat-derived DNA was also achieved by a real-time PCR assay targeting the *ALMT1* gene, an aluminium-activated malate transporter (Vautrin and Zhang, 2007). The absolute LOD and LOQ were 2 and 20 haploid genome copies of common wheat, respectively. A duplex real-time PCR for detection and quantitation of wheat- and barley-derived DNA targeting the gene *PKABA1* used minor groove-binding probes to distinguish between the two cereals (Ronning et al., 2006). The assay was specific and allowed simultaneous detection of wheat and barley in food samples with absolute LODs of 5 PFUs (corresponding to 1.8 DNA copies) for wheat and 10 PFUs (1.8–16 DNA copies) for barley.

14.7. Minimum (observed) eliciting doses

Oral challenges with different doses of wheat have been performed in clinical studies. However, only a few patients with a convincing history of wheat anaphylaxis have been challenged orally (Hischenhuber et al., 2006). In children with atopic dermatitis, 20 % showed a positive DBPCFC for wheat with doses ranging from 0.4 to 10 g of food (Sicherer et al., 2000b). In another study of 38 children and 41 adults with wheat allergy, 2.5 % of children reacted to doses less than 10 mg of wheat flour (single-blind challenge). A MOED was not given by the authors (Moneret-Vautrin et al., 2003).

In a study conducted in Japan (Ito et al., 2008), 35 children sensitised to wheat underwent an OFC with noodles containing wheat flour (2.6 %) in stepwise increasing amounts (0.1, 1, 2, 5, 10 and 20–50 g). Twenty-one patients reacted to the challenge. Children with a convincing history of wheat allergy were not challenged because of their high risk for anaphylaxis. One subject had a severe allergic reaction to 2.6 mg of wheat protein (MOED, first dose tested), whereas two reacted to 26 mg.

Adult subjects with suspected wheat allergy (convincing clinical history) were recruited in Italy (n = 24) and Denmark (n = 3) and underwent a DBPCFC with wheat flour (Scibilia et al., 2006). A minimum starting dose of 100 mg raw wheat flour was administered, followed by 500 mg, then 1 g, and 1.5 g; the last dose was then doubled (3 g, 6 g, 12 g) until symptoms were reported or signs were observed, or until the entire test meal was eaten. The cumulative dose schedule was 100 mg, 600 mg, 1.6 g, 3.1 g, 6.1 g, 12.1 g, and 25 g. Doses were administered at 20-minute intervals. The same dose schedule was used for cooked wheat DBPCFC in patients who had a positive result to raw wheat. The MED was 100 mg of raw (three patients, first dose tested) and cooked (two patients) wheat flour.

The lowest reported MED/MOED in paediatric patients undergoing OFCs with wheat flour is 2.6 mg of wheat protein (first dose tested). The lowest reported MED/MOED in adult patients undergoing DBPCFC is 100 mg of wheat flour. However, doses of wheat triggering allergic reactions in sensitive individuals may be lower because patients with a history of severe allergic reactions have been excluded from the challenge studies available, where participants already reacted to the first dose tested.

14.8. Conclusion

IgE-mediated allergy to cereals is caused both by inhalation of cereal flour (baker's asthma) and by ingestion of cereal-based products (food allergy). Cereal-induced IgE-mediated food allergy is well documented. DBPCFC studies have been performed confirming that cereals are able to elicit anaphylactic reactions. Wheat is the gluten-containing cereal most often reported to induce cereal allergy, compared with barley, rye, and oats. Prevalence of wheat allergy based on clinical history and positive food challenges is as low as 0.4 % in young children. Wheat allergy frequently resolves during adolescence. Cereal seed storage proteins (gluten), but also non-gluten components, such as albumins and globulins, α-amylase inhibitor and lipid transfer proteins, are clinically relevant allergens. Immunological methods used for the detection of gluten may be used for the detection of cereal glutenin and gliadin. Specific and sensitive PCR methods are available. MS methods have been extensively utilised for the identification of the allergenic proteins, but no limits of detection have been provided. The effect of food processing on allergenicity, including heat resistance of single allergens, is conflicting. The lowest reported MED/MOED in paediatric patients undergoing OFCs with wheat flour is 2.6 mg of wheat protein. The lowest reported MED/MOED in adult patients undergoing DBPCFC is 100 mg of wheat flour. However, doses of wheat triggering allergic reactions in sensitive individuals may be lower because patients with a history of severe allergic reactions have been excluded from the challenge studies available, where participants already reacted to the first dose tested.

15. Allergy to milk and dairy products

15.1. Background

Milk is a liquid substance secreted by the mammary glands of females of all mammal species to support their offsprings' nutritional needs. Milk and dairy products are a source of proteins, fat, minerals and vitamins and play a key role in human nutrition (Darewicz et al., 2011).

Milk allergy is an adverse immunological response to milk proteins of different mammalian species, particularly cow, goat and ewe, seen mainly in children. It can be broadly divided into IgE- and non-IgE-mediated disease, or mixed, involving other immunoglobulins, immune complexes and/or cell-mediated mechanisms. These differ in clinical presentation, diagnostic testing, and prognosis (Berni Canani et al., 2008). IgE-mediated reactions are characterised by an acute onset of symptoms generally within two hours of ingestion of or exposure to milk protein-containing food. IgE-mediated reactions to food typically involve the skin, gastrointestinal tract and respiratory tract and may also include systemic reactions (anaphylactic shock). Non-IgE-mediated immunological reactions (e.g. cell-mediated) include food-protein-induced enterocolitis, proctocolitis and enteropathy syndromes. These conditions primarily affect infants or young children, who present with gastrointestinal symptoms, such as vomiting, abdominal cramps, diarrhoea and occasionally blood in the stools, often associated with failure to thrive or poor weight gain. Examples of food allergy comorbidities with mixed IgE- and non-IgE-mediated causes include eosinophilic oesophagitis and atopic dermatitis (Burks et al., 2012a).

15.2. Epidemiology

15.2.1. Prevalence

15.2.1.1. Europe

Forty studies have assessed the prevalence of cow's milk allergy (CMA) in Europe. The studies were from Denmark, Estonia, France, Finland, Germany, Greenland, Hungary, Iceland, Ireland, Italy, Norway, Portugal, Spain, Sweden, the Netherlands, Turkey and the UK. Data were published between the years 1982 and 2012 and included all age groups (University of Portsmouth, 2013).

Self-reported prevalence of CMA in young children (≤ 3 years) ranged from 2 % in Finland (Kajosaari, 1982) to 7.5 % in Norway (Eggesbo et al., 1999) at one year, from 4 % (Ostblom et al., 2008a) to 6.8 % (Pyrhonen et al., 2009) at two years, and from 1.3 % (Kilgallen and Gibney, 1996) to 5.9 % (Pyrhonen et al., 2009) at three years of age. Self-reported prevalence of CMA at one year was 4.5 % in Sweden (Ostblom et al., 2008a) and 5.3 % in Ireland, where reported adverse reactions to dairy products were 4 to 4.7 % at the same age (Kilgallen and Gibney, 1996). The highest self-reported prevalence was 10.8 % in Iceland at 18 months of age (Kristjansson et al., 1999). Studies in the same population subgroup (young children up to 3 years) that used other questionnaire-based methods (e.g. diagnosis by a physician) reported similar figures. In older children, the prevalence of clinician-diagnosed CMA was reported to be 1.8 % at eight years in Sweden (Ostblom et al., 2008b). In adults, prevalence based on self-reported diagnosis of food allergy ranged between 1.8 % (Schafer et al., 2001) in Germany and 3.3 % (Osterballe et al., 2009) in Denmark. Self-reported prevalence in the UK at all ages combined was 2.7 % (Young et al., 1994).

In young children, the prevalence of positive SPTs to cow's milk proteins (CMPs) was between zero in Estonia (Julge et al., 2001) and 0.9 % in Norway (Ro et al., 2012), whereas in older children it ranged from 0.2 % at seven years in the UK (Roberts et al., 2005) to 3.9 % at five to six years in Germany (Schafer et al., 1999). Higher sensitisation rates were generally observed in adults, ranging from 2.3 % in Germany (Schafer et al., 1999) to 14.7 % in Hungary (Bakos et al., 2006). Based on serum-specific IgE, sensitisation rates were between 4.8 % (Ro et al., 2012) and 25.8 % (Julge et al., 2001) in young children and between 8 % (Ostblom et al., 2008b) and 23.2 % (Julge et al., 2001) in older children, with lower rates generally reported for adults, ranging from 1 % in Finland (Isolauri et al., 2004) to 13.9 % in Hungary (Bakos et al., 2006).

The prevalence of CMA was generally lower when sensitisation tests were combined with a clinical history. Prevalence based on clinical history plus a positive SPT was 0.3 % and 0.6 % in Iceland and Sweden at 18 months, respectively (Kristjansson et al., 1999), 0.4 % at six to nine years in Turkey (Orhan et al., 2009) and 0.1 % in the overall population combining all ages (Zuberbier et al., 2004). The prevalence of CMA based on a clinical history plus a positive serum-specific IgE was only reported in Turkey (0.2 %) in a category of age between 8 and 18 months (Kucukosmanoglu et al., 2008) and in Sweden (1.8 %) at four years of age (Ostblom et al., 2008a).

Few studies used food challenges to confirm diagnosis of allergy to CMP. Based on OFC, the prevalence of CMA in Denmark was reported to be 1 % at one year, 0.5 % at two years, 0.3 % at three years and 0.2 % at five and 10 years (Host et al., 2002). Similar figures were reported for the same country some years later (Eller, 2009): 0.4 % at six months of life, 1.1 % at 18 months, 0.7 % at three years, and zero cases at six years. A prevalence of 0.3 % at 8 to 18 months was found in Turkey (Kucukosmanoglu et al., 2008), whereas higher rates (4.7 %) were reported in Finland in children < 34 months (Saarinen et al., 1999). When the diagnosis was based on DBPCFC, prevalence ranged between 0.1 % in Turkey (six to nine years) and 0.6 % (three years) in Denmark (Osterballe et al., 2005). Values for adults were within that range (0.3 %) in the only study available (Osterballe et al., 2005).

15.2.1.2. Outside Europe

In Western countries outside Europe, the prevalence of self-reported CMA in children up to 18 months ranged from 2.2 % in Canada (Soller et al., 2012) and 6.1 % in Australia (Osborne et al., 2011) to 13.1 % in the USA (Bock, 1987). Similar figures were reported for adults in these countries: 1.9 % in Canada (Soller, 2012), 1.9–4.8 % in Australia (Woods et al., 2002), and 10.5 % in the USA (Greenhawt et al., 2009).

Using a method that combined history of CMA, SPT and food challenges to determine a diagnosis of "probable or confirmed" CMA (both IgE- and non-IgE-mediated), prevalence rates of CMA in the USA were much lower (Bock, 1987): about 5 % in one-year-old children, 0.2 % at two years, and zero at three years. In the same country, prevalence of CMA was reported to be 0.3 % (Liu AH et al., 2010) in young children and adolescents, 0.4 % in the overall population combining all ages (Liu AH et al., 2010) and 1.4 % in adults (Vierk et al., 2007) for IgE-mediated reactions only.

15.2.2. Natural history

CMA can develop from the neonatal period and peaks during the first year of life, tending to remit in childhood. Reaction to CMPs occurred at an average of 1.67 ± 1.67 days after initial exposure (Elizur et al., 2012).

In the 1990s, a Danish birth cohort study reported that more than 50 % of children outgrow their CMA at one year of age. However, subsequent studies have reported a longer duration of CMA, with tolerance developing in half of cases within the two years following diagnosis (Elizur et al., 2012). Referral studies indicate that 80 % of patients achieve tolerance within three to four years. A prospective study conducted in the USA showed that CMA resolved in 154 (52.6 %) subjects at a median age of 63 months in a cohort of 293 children aged 3 to 15 months at baseline (Wood et al., 2013).

Children with delayed reactions became tolerant faster than those with immediate reactions. Children with higher risk of persistence had respiratory symptoms at onset, severe atopic dermatitis, sensitisation to linear epitopes of CMA, and sensitisation to multiple foods and to respiratory allergens. A larger wheal diameter at SPT with fresh milk, elevated levels of specific IgE (especially to casein), and antibody binding to other ingested and inhaled allergens have been associated with longer duration of CMA (Skripak et al., 2007; Fiocchi et al., 2008). Low milk-specific IgE levels correlate with earlier onset of tolerance and a 99 % reduction in specific IgE concentrations for more than 12 months translates into a 94 % likelihood of achieving tolerance to cow's milk protein within that period (Shek et al., 2004). Tolerance of CMP correlates with reduced concentrations of specific IgE and IgG1.

15.2.3. Time trends

There are no data on time trends regarding CMA in Europe.

The prevalence of challenge-proven CMA in China was 1.6 % and 3.5 % in 1999 and 2009, respectively, among children from birth up to two years (Hu et al., 2010). Up to one year of age, the prevalence was 1.3 % in the same country (Chen et al., 2011).

15.2.4. Severe reactions/anaphylaxis

Patients with CMA develop gastrointestinal symptoms in 32 to 60 % of cases, skin symptoms in 5 to 90 %, and anaphylaxis in 0.8 to 9 % of cases. This frequency of anaphylaxis is the main concern in many CMA studies. CMA has been reported to be responsible for up to 42 % of hospital admissions because of food-induced anaphylaxis in childhood (Berni Canani et al., 2012) and up to 13 % of fatal food induced anaphylaxis (Bock et al., 2007).

15.2.5. Factors affecting prevalence of milk allergy

CMA is the most frequent milk allergy, and it is often the first step of the allergic march.

In a review, nearly one-third of children with atopic dermatitis were diagnosed with CMA after an elimination diet and OFC, and about 40 to 50 % of children less than a year of age with CMA also had atopic dermatitis (Fiocchi et al., 2010). The maintenance of tolerance in atopic patients is associated with persistently elevated milk-specific IgG4 antibody concentrations (Ruiter et al., 2007). In a prospective cohort study (Wood et al., 2013), low milk-specific IgE level (< 2 kU/L), SPT size (< 5 mm) and severity of atopic dermatitis were the baseline characteristics of patients with CMA, which were most predictive of resolution of their allergy. A smaller eliciting dose at OFC also correlates with the duration of CMA.

15.3. Identified allergens

Milk from different ruminant species (e.g. cow, buffalo, sheep, goat), and also human milk, contains similar proteins regarding its structural, functional and biological properties and its composition changes during lactation.

CMPs are very heterogeneous regarding structure and function, and this heterogeneity is further increased by genetic polymorphisms or post-translational modifications (e.g. phosphorylation, glycosylation), which may affect their IgE-binding capacity and their allergenicity (Malik et al., 1988; Bernard et al., 2000).

Cow's milk contains about 30 to 35 g of proteins per litre. The action of chymosin (rennin), or the acidification of the milk to pH 4.6, allows two fractions to be obtained: lactoserum (whey) and coagulum (casein), which contain about 20 % and 80 % of the CMPs, respectively. Cow's milk allergens are listed in Table 6.

Whey contains essentially globular proteins, mostly β-lactoglobulin (BLG) (whose homologue is not present in human milk), α-lactalbumin (ALA), and lactoferrin (LF), which are synthesised in the mammary gland, while other proteins, such as bovine serum albumin (BSA) or immunoglobulins come from the blood. In the coagulum, the casein (CAS) fraction comprises four proteins coded by different genes carried on the same chromosome: αS1-, αS2-, β-, and κ-caseins. Owing to the great variability observed in human IgE response, no single protein or protein structure accounts for a major part of milk allergenicity. Studies on large populations of allergic patients show that most are sensitised to BLG, CAS, ALA, BSA, LF, and immunoglobulins (EFSA, 2004). Polysensitisation to several proteins is observed in about 75 % of patients with CMA (Goldman et al., 1963b; Goldman et al., 1963a; Restani et al., 1995; Docena et al., 1996; Wal, 2002). CAS and BLG, as well as ALA, are major allergens, and sensitisations to these proteins are closely linked. However, all CMPs appear to be allergenic. About 35 to 50 % of milk-allergic patients are sensitised to CMP present in very low quantities, such as BSA, immunoglobulins, and especially LF (Fiocchi et al., 2010) and, occasionally, only these CMPs (e.g. lactoferrin) are responsible for the clinical symptoms observed. Sensitivity to BSA appears to be independent of other CMPs (Wal et al., 1995).

In IgE-mediated allergy, circulating antibodies recognise specific molecular regions on the antigen surface (epitopes), which are classified according to their specific amino acid sequence (sequential or linear epitopes) or the folding and conformation of their protein chains (conformational epitopes). Subjects with transient milk allergy produce IgE antibodies primarily directed at conformational epitopes (dependent on the protein tertiary structure), whereas those with persistent allergy also produce IgE antibodies against sequential epitopes, which are heat stable (Cooke and Sampson, 1997; Chatchatee et al., 2001; Vila et al., 2001; Busse et al., 2002; Jarvinen et al., 2002). Greater IgE epitope diversity and higher IgE affinity are associated with more severe milk allergy (Wang et al., 2010).

Table 6: Cow's (*Bos domesticus*) milk allergens

Allergen	Biochemical name	Concentration (g/L)	Molecular weight [(b)]	pI [(c)]
Whey proteins		~ 5.0		
Bos d 4	α-Lactalbumin	1–1.5	14.2	4.8
Bos d 5	β-Lactoglobulin	3–4	18.3	5.3
Bos d 6	Bovine serum albumin	0.1–0.4	67.0	4.9–5.1
Bos d 7	Immunoglobulin	0.6–1.0	160.0	–
Bos d lactoferrin	Lactoferrin[1]	0.09	80.0	8.7
Caseins		~ 30		
Bos d 8		20–30		
Bos d 9	α_{s1}-Casein	12–15	23.6	4.9–5.0
Bos d 10	α_{s2}-Casein	3–4	25.2	5.2–5.4
Bos d 11	β-Casein	9–11	24.0	5.1–5.4
NA	γ_1-Casein [(a)]		20.6	5.5
NA	γ_2-Casein [(a)]	1–2	11.8	6.4
NA	γ_3-Casein [(a)]		11.6	5.8
Bos d 12	κ-Casein	3–4	19.0	5.4–5.6

(a): www.allergome.org
(b): Molecular weight (SDS-PAGE).
(c): Isoelectric point.
NA, not assigned.

15.3.1. Whey allergens

Whey allergens include ALA, BLG, BSA, bovine immunoglobulins and lactoferrin.

ALA (Bos d 4) is a monomeric globular protein of 123 amino acid residues with four disulphide bridges and a molecular weight of 14.2 kDa. It is a regulatory component of the enzymatic system of galactosyl transferase responsible for the synthesis of lactose. It possesses a high-affinity binding site for calcium, and this binding stabilises its secondary structure. The complete amino acid sequence of bovine ALA shows extensive homology with hen's egg white lysozyme and also with human ALA (EFSA, 2004). The role of ALA in milk allergy is controversial and prevalence data across studies vary between zero and 80 % of patients reacting to this protein (Besler et al., 2002).

BLG (Bos d 5), the most abundant cow's milk whey protein, occurs naturally in the form of a 36-kDa dimer in many other species but is not present in human milk. Each subunit is a polypeptide of 162 amino acid residues, and the molecule contains two disulphide bridges and one free cysteine. This structure is responsible for the main physicochemical properties and for the interaction with casein during heat treatments. BLG is relatively resistant to acid and enzymatic hydrolysis. The tertiary structure of BLG is known. It belongs to the lipocalin family and is considered a retinol-binding protein. Lipocalins have a high allergenic potential, and several allergens of animal origin belong to this family. They share a well-conserved sequence homology in their N-terminus moiety, where tryptophan at position 19 is always present. Crystallographic studies revealed a very similar folding, called β-barrel structure, with the same arrangements of eight (or 10) antiparallel β-sheets (EFSA, 2004). There are two main isoforms of this protein in cow's milk, the genetic variants A and B, which differ only by two point mutations at amino acids 64 and 118. The prevalence of allergic subjects reacting to this protein is between 13 and 76 % (Restani et al., 2009).

BSA (Bos d 6) can bind water, fatty acids, hormones, bilirubin and drugs, as well as calcium, potassium and sodium. Its main function is the regulation of the colloidal osmotic pressure in blood (Fiocchi et al., 1995). The tertiary structure of BSA is stable, and its 3D conformation is well documented. The protein contains three homologous domains (I to III) and consists of nine loops linked by 17 covalent disulphide bridges. Most of the disulphide bonds are well protected in the core of the protein and are not easily accessible to the solvent. BSA is involved in other allergies such as beef. It correlates with the clinical features of lip oedema, urticaria, cough and rhinitis. It accounts for

between zero and 88 % of sensitisation events, while clinical symptoms occur in up to 20 % of patients (Martelli et al., 2002). Bovine immunoglobulins (Bos d 7) seldom trigger clinical symptoms in CMA.

15.3.2. Casein allergens

The four casein allergens are collectively known as Bos d 8. Each individual casein (αS1-, β-, αS2- and κ-casein) represents a well-defined chemical compound but they cross-link to form ordered aggregates (micelles) that are in suspension in lactoserum. The proportion of different caseins in micelles is relatively constant (ca. 37 %, 37 %, 13 % and 13 %, respectively) but their distribution within the micelles is not uniform. The micelles have a central hydrophobic part and a peripheral hydrophilic layer in which major sites of phosphorylation-containing phosphoserine residues are present, responsible for the calcium-binding and -transfer properties of caseins. αS1-, αS2-, β-, and κ-casein have little primary structure homology and their functional properties also differ (e.g. αS1-, αS2- and β-casein appear to be calcium sensitive, while κ-casein is not). However, the four caseins display common features, which differ from other CMPs. Another group, the γ-caseins, are present in very low quantities in milk and are by-products of β-casein proteolysis.

Caseins are phosphorylated proteins with a loose and highly hydrated tertiary structure, which are not significantly affected by severe heat treatments but are susceptible to proteinases and exopeptidases. Multi-sensitisations to the different caseins most often occur in patients sensitised to the whole casein fraction (Bernard, 1999). The composite allergen Bos d 8 exhibits limited sequential homology. In spite of this, polysensitisation of many casein fractions is usually observed, perhaps due to cross-sensitisation through some common or closely related epitopes. Sensitisation is particularly frequent against α-casein (100 %) and κ-casein (91.7 %) (Restani et al., 1995). Several studies have identified αS1-casein as a major allergen inducing strong immediate or delayed allergic reactions (Ruiter et al., 2006). αS1-Casein was found to contain both conformational and sequential IgE epitopes (Schulmeister et al., 2009).

15.4. Cross-reactivities

The sequence similarity (expressed in percentages) between milk proteins from different mammalian species is shown in Table 7. The greatest homology is between cow's, sheep's and goat's milk proteins as *Bos* (oxen), *Ovis* (sheep) and *Capra* (goat) are genera belonging to the *Bovidae* family of ruminants. The proteins in their milks consequently have less structural similarity with those from the *Suidae* (pig), *Equidae* (horse and donkey), and *Camelidae* (camel and dromedary) families and also with those in human milk. It is noteworthy that the milks of camels and dromedaries (as well as human milk) do not contain BLG.

Owing to high-sequence homology, there are frequent cross-reactions between milk proteins from different species. Clinical reactions to milk from different species are similar. Sequence homology in caseins ranges between 80 and > 90 %, so high IgE cross-reactivity between ewe's, goat's and cow's milk casein occurs in most patients with CMA (Dean et al., 1993; Spuergin et al., 1997; Bernard, 1999; Restani et al., 1999). However, the IgE response may also be species specific, with clinical manifestations occurring after consumption of ewe's and/or goat's cheese but not of cow's milk or other dairy products (Wuthrich and Johansson, 1995). In an individual with CMA, the risk of an allergic reaction to goat's milk is up to 92 %, to donkey's up to 17 % and to horse's up to 4 % (Sicherer, 2001; Jarvinen and Chatchatee, 2009).

Allergy to ewe's milk can also evolve into allergy to cow's milk. Mare's and donkey's milks have sometimes proved useful to some patients, but uncertainties remain about chemical composition and hygienic control. The same considerations apply to *Camelidae* (camel and dromedaries) milks, which could represent an alternative to cow's milk for allergic subjects because of their low sequence homology with cow's milk and the absence of BLG, provided that problems related to availability and technological processing to avoid new sensitisation are adequately addressed (Restani et al., 2002).

Adverse reactions to soy have been reported in milk-allergic patients fed with soy-based formulas as cow's milk substitutes. A 30-kDa glycinin-like protein from soybean that cross-reacts with cow's milk casein has been isolated and partially sequenced (Rozenfeld et al., 2002).

Table 7: Sequence homology between mammalian milk proteins (in percentage, relative to cow's milk proteins)

Protein	Goat	Ewe	Buffalo	Sow	Mare	Donkey	Dromedary	Human
ALA	95.1	97.2	99.3	74.6	72.4	71.5	69.7	73.9
BLG	94.4	93.9	96.7	63.9	59.4	56.9	Absent	Absent
BSA	–	92.4	–	79.9	74.5	74.1	–	76.6
αs_1-CAS	87.9	88.3	–	47.2	–	–	42.9	32.4
αs_2-CAS	88.3	89.2	–	62.8	–	–	58.3	–
β-CAS	91.1	92.0	97.8	67.0	60.5	–	69.2	56.5
κ-CAS	84.9	84.9	92.6	54.3	57.4	–	58.4	53.2

–, allergen is not present in the Swiss-Prot DataBank; ALA, α-lactalbumin; BLG, β-lactoglobulin; BSA, bovine serum albumin; CAS, casein.

15.5. Effects of food processing on allergenicity

The structure and properties of CMPs and the structure and location of their IgE-binding epitopes, and particularly of linear epitopes, need to be considered while interpreting the impact of food processing on milk allergenicity.

Milk may undergo extensive processing (e.g. thermal treatment), also by novel processes such as high-pressure treatment, extrusion or ultrasound, which can significantly alter the structural characteristics of milk allergens and thereby increase or attenuate their antigenic potential (Maleki and Hurlburt, 2004).

15.5.1. Heat treatment

Cow's milk is usually marketed after it has been subjected to a technological process, usually pasteurisation, which reduces potential pathogen load (70–80 °C for 15–20 seconds). Ultra-high temperature (UHT) processing with flash heating (at 135–145 °C for 0.5–4 seconds) and evaporation for the production of powdered infant formula (dry blending or wet mixing–spray drying process) have a minor or no effect on the allergenic potential of CMP.

Comparative studies have shown no difference in antigenicity between raw and heated milks (Werfel et al., 1997). However, in some cases, the aggregation of new protein polymers capable of binding specific IgE has been demonstrated. After boiling BSA at 100 °C for 10 minutes, dimeric, trimeric and higher polymeric forms increased and all maintained their IgE-binding properties (Restani et al., 2004). The persistence of allergenicity in heat-treated milk is clinically confirmed by the fact that, in some children, CMA develops after the ingestion of heat-treated milk. Domestic heating processes can only modify conformational epitopes, which might lose their binding capacity to a specific IgE antibody, while sequential epitopes maintain their allergenic potential even after heating (Sampson, 2004). CMPs contain both types of epitopes. Even though a slight reduction of antigenicity can be observed in whey proteins, which experience a limited unfolding of their globular structure upon heating, insignificant alterations in binding properties are reported with caseins, which have mainly linear and thermostable epitopes.

Vigorous heating (such as that used for certain sterilisation processes; 121 °C for 20 minutes), but also the less drastic pasteurisation process, could increase milk allergenicity by enhancing uptake of peptides by Peyer's patches in the intestine (Roth-Walter et al., 2008). Furthermore, CMPs can be oxidised during industrial treatment, resulting in the formation of modified/oxidised amino acid residues, particularly in BLG, which may be responsible for the development of new immunologically reactive structures.

The effects of heat treatment on the antigenicity of ALA and BLG in whey protein isolate (WPI) were studied via *in vitro* competitive ELISA inhibition tests with rabbit serum (Bu et al., 2009). The antigenicity of ALA and BLG increased with increasing temperature from 50 to 90 °C. However, the antigenicity of both proteins decreased remarkably above 90 °C. When treated at 120 °C for 20 minutes, the antigenicity of ALA decreased by 25 % compared with the untreated sample.

Boiling milk for 10 minutes reduces the SPT response in patients who react to BSA and BLG, whereas wheal diameter remains the same in those sensitised to caseins (Norgaard et al., 1996).

One study evaluated whether patients with CMA could tolerate extensively heated (baked) milk products in 100 children undergoing food challenges with heated milk (Nowak-Wegrzyn and Fiocchi, 2009). Sixty-eight children (68 %) tolerated the extensively heated milk, 23 reacted to the heated milk, and nine tolerated both the heated and the unheated milk. Heated milk-tolerant subjects had significantly smaller SPT wheals, lower milk-specific and casein-specific IgE, and lower IgE/IgG4 ratios to casein and BLG compared with the heated milk-reactive subjects.

15.5.2. Enzymatic hydrolysis

Enzymatic hydrolysis of CMPs reduces their allergenicity. However, specific IgE from patients with CMA may recognise enzymatic digestion products of whey proteins (i.e. BLG and ALA) or CAS (Fiocchi et al., 2010). Attempts have been made to classify formulas into partial and extensively hydrolysed products according to the degree of protein fragmentation, but there is no agreement on the criteria on which to base this classification.

15.5.3. Fermentation

Lactic acid bacteria (LAB) have a complex proteolytic system consisting of proteinases, peptidases and peptide transport systems that contribute to milk protein degradation during fermentation (Bertrand-Harb et al., 2003; El-Ghaish S. et al., 2011). Specific LAB strains have proteolytic activity against some antigenic proteins, such as ALA, BLG, αS1-casein and β-casein, and may decrease their IgE-binding capacity (Tzvetkova et al., 2007; El-Ghaish Shady et al., 2011). However, the degradation of antigenic proteins by LAB does not always lead to significant changes in their IgE-binding capacity (Ehn et al., 2005; Kleber et al., 2006), and reductions in protein IgE-binding capacity do not always correlate with less allergenicity (Jedrychowski, 1999).

The evidence available indicates that the extent to which milk proteins are hydrolysed and the peptide pattern generated may depend on the LAB strain used and on fermentation conditions. It also indicates that proteolytic degradation of antigenic proteins is not always associated with reductions in their IgE-binding properties and their allergenicity, as some epitopes may be broken down in the process, while others previously buried may become accessible.

15.5.4. Combined treatments

Hydrolysed infant formulas are produced from caseins or whey proteins by a combination of heat treatments and enzymatic hydrolysis (Restani et al., 2006). Allergenicity of milk may be decreased by enzymatic treatment with proteases followed by ultrafiltration, which removes the remaining HMW peptides and the residual protein.

Another attempt to reduce allergenicity involves the use of proteolysis combined with high pressure. Different authors have shown increased fragmentation of BLG if proteolysis occurs after or during the application of high pressure (Peñas E. et al., 2006). The partial ineffectiveness of proteolysis under ordinary atmospheric conditions may be due to the inability of enzymes to reach epitopes that are less exposed. However, thermal denaturation can also induce the formation of aggregates with greater resistance to hydrolytic attack, as is the case with BLG (Restani et al., 2006).

Small clinical studies have reported controversial results with hydrolysed formulas, depending on the enzymes used and on the degree of hydrolysis. The incidence of reported adverse effects in allergic

infants fed partially or extensively hydrolysed milk (either casein or whey) formulas in tertiary care centres range around 45 to 65 % and 3 to 6 %, respectively (Giampietro et al., 2001; Caffarelli et al., 2002; Fiocchi et al., 2010).

15.6. Detection of allergens and allergenic ingredients in food

Several analytical methods have been developed to determine the presence of milk and of milk-derived allergens in foods. Immunological methods, in particular ELISA, are commonly used, which may provide semi-quantitative/quantitative results. The milk powder NIST SRM 1549 has been used as reference material. DNA-based methods consisting of the PCR amplification of oligonucleotidic sequences specific for the allergenic ingredient are rarely used for the detection of milk traces in food products owing to the relatively low DNA content (Tregoat and van Hengel, 2010). Separation techniques, such as 2DE, CE and HPLC, eventually coupled to mass spectrometry, are also successfully used.

15.6.1. ELISA

Numerous competitive and sandwich ELISA kits for the detection of milk-derived allergens are commercially available, with sensitivity down to 1 mg/kg (Poms et al., 2004a). Some kits detect BSA, casein and BLG separately, whereas others detect whole milk or whey proteins with LODs between 0.1 and 5 mg/kg. Monoclonal and more suitable polyclonal antibodies have been used against either BLG or casein.

An inter-laboratory study was performed in order to validate an ELISA kit based on a rabbit polyclonal antibody for the quantitative determination of BLG in foods (Stumr et al., 2009). The LOD was 0.07 mg BLG/kg and the LOQ was 0.22 mg BLG/kg.

A dessert matrix incurred with different amounts of milk protein (as skimmed milk powder) was evaluated as a quality control material for allergen analysis in a multi-laboratory study (Johnson et al., 2014). Analyses were performed with five ELISA kits based on casein, five kits based on β-lactoglobulin and one based on total milk. Allergen levels were calculated by using calibration curves and reporting units were converted into mg/kg of skimmed milk powder protein. In general, ELISA kits based on casein were more accurate, while all kits detected milk protein at the 3 mg/kg level. When considering the ISO criteria, only one kit based on casein accurately determined milk protein at 6 and 15 mg/kg against the target value. This study confirms the variability among different commercially available ELISA kits in their ability to quantify the amount of milk protein in complex foods and the need for CRMs, and possibly incurred CRMs.

LFDs and dipsticks, which are used for rapid screening, are commercially available. The former detect casein and whey residues in food products down to 0.12 mg/kg (Schubert-Ullrich et al., 2009).

Antibodies are also used in combination with biosensors and other detection technologies. SPR was used as a label-free technology for the simultaneous quantification of α-, β- and κ-casein in raw and heat-treated dairy products (Dupont and Muller-Renaud, 2006). The LODs were of 870, 85 and 470 ng/mL, respectively. A resonance-enhanced absorption (REA) biosensor with a direct immunoassay on a chip, in which the read-out antibody was labelled with monodispersed colloid gold clusters, was used for detecting BLG in processed milk (Hohensinner et al., 2007). A very good sensitivity (LOD of 10 ng/L) for casein was obtained with a localised surface plasmon resonance immunosensor based on a gold-capped nanoparticle substrate on which anti-casein antibodies were immobilised (Minh Hiep et al., 2007).

In several foods, e.g. fermented dairy products, linear epitopes can be hydrolysed while retaining their allergenic potential. Epitopes released from the parent proteins tend to be underestimated or to escape the most commonly utilised sandwich ELISA-based tests (de Luis et al., 2007). In similar cases, competitive ELISA tests can be successful in detecting as low as 5 mg/kg of "hidden" milk-derived peptides in complex foods (Monaci et al., 2006).

15.6.2. Capillary electrophoresis and mass spectrometry

CE with a laser-induced fluorescence detector has been efficiently used for the detection of ALA, BLG and BSA (Veledo et al., 2005).

MS has been used for identifying and characterising CMPs and as confirmatory method to support ELISA results. The so-called "bottom-up" approach, which involves the enzymatic (tryptic) digestion of the protein, followed by LC-MS/MS, is generally used. The method is not suitable for quantitative determinations, unless the digestion step is perfectly reproducible and a standard marker peptide is available. A confirmatory method based on LC/selected reaction monitoring (SRM)-MS/MS was developed and validated for the quantification of milk traces in foods (Lutter et al., 2011). Tryptic peptides of BLG and β-, αS2-, and κ-casein were selected as markers for quantification. Internal standard peptides containing isotopically labelled amino acids were used for quantification. LOD values were 0.2 to 0.5 mg/kg.

A similar procedure was followed for the determination of α- and β-casein, ALA and BLG in foods (Ansari et al., 2011). After tryptic digestion of the four proteins, several peptides were identified by LC-MS/MS. Seven of these peptides were synthesised and used for calibration of the LC-MS/MS system. The peptides were determined down to 1 ng/mL in food samples.

Whey proteins (ALA and BLG A and B) were detected and quantified as intact proteins in mixed fruit juices by the HPLC-QpQ-MS method (Monaci et al., 2011). The method is based on the detection of selected fragment ions used as markers. Proteins were first extracted by solid phase extraction and separated by HPLC. The multiple ion monitoring (MIM) mode proved to be more selective than the full scan mode. External standards were used with a matrix-matched calibration curve. The LOD and the LOQ were estimated at 1 and 4 µg/mL, respectively.

15.6.3. Detection of CMPs in wine

Several reports regard the detection of CMPs used as fining agents in wine by ELISA. A sandwich ELISA was used for the detection of residual casein in wine with a LOD of 8 ng α-casein/mL wine (Rolland et al., 2008). α- and β-caseins in fined wines were detected at 0.2 µg/mL (Weber P et al., 2009). A commercial ELISA kit for the detection of caseinates (LOD of 0.28 mg/kg; LOQ of 0.76 mg/kg) has been validated in an inter-laboratory trial (Restani et al., 2012).

MS has also been used for the detection of residual CMPs in wine. An LC/high-resolution (HR) MS method has been developed for the analysis of milk proteins in incurred cookies and white wine spiked with milk powder and caseinate, respectively (Monaci et al., 2011). The method is based on the identification of peptides in the tryptic digest of proteins using HPLC coupled to MS using the Orbitrap analyser. On account of the high mass accuracy and resolution provided by the Orbitrap, it was possible to identify four peptides as markers of casein using accurate values of the mass/charge ratio (m/z) of their ions. LODs ranged from 39 to 51 µg/mL, and referred to the amount of protein initially added to the wine. The HR-MS-based method has been further developed to detect simultaneously milk and egg proteins in wine by using isotopically labelled (^{15}N-valine-containing) peptides of ovalbumin and αS1-casein. LODs were in the range of 0.4 and 1.1 µg/mL. One study (Tolin et al., 2012b) revealed the presence of residual milk allergens in commercial wines by LC-MS/MS.

15.7. Minimum (observed) eliciting doses

There are several reports documenting severe allergic reactions to very low amounts of CMP. CMPs (including BLG) are excreted through breast milk and have been reported to induce severe allergic reactions in breastfed infants at concentrations of about 5 ng/mL (from 0.5 to 50 ng/mL) in breast milk (Axelsson et al., 1986; Machtinger and Moss, 1986; Host and Samuelsson, 1988; Sorva et al., 1994). Fatal anaphylaxis occurred after ingestion of a meal sausage containing an amount of cow's milk equivalent to 60 mg of casein (Kjelkevik et al., 1997). Frozen desserts containing trace amounts of

whey proteins (9 μg/mL) triggered anaphylaxis in a three-year-old boy (Laoprasert et al., 1998). A fatal reaction has been documented with inhaled milk proteins in a dairy shop (Barbi et al., 2004).

Data are also available from DBPCFCs in subjects with CMA. Most studies have been conducted exclusively in children (Hill et al., 1984; Host and Samuelsson, 1988; Baehler et al., 1996; Patriarca et al., 2002; Fiocchi et al., 2003b; Devenney et al., 2006; Flinterman et al., 2006b; Morisset et al., 2007; Staden et al., 2007; Longo et al., 2008; Skripak et al., 2008; Caminiti et al., 2009; Orhan et al., 2009), few in children and adults (Morisset et al., 2003b; Lam et al., 2008), and only one in adults (Norgaard and Bindslev-Jensen, 1992). Studies were variable in size, challenge protocol and type of food tested. The total number of patients showing objective reactions during the DBPCFC in a given study ranged from two to 60. The lowest MOEDs also varied widely among studies, ranging from 3.3 to 1 815 mg of total protein (Remington, 2013).

More recent studies in children with CMA report similar results. In a population of 633 children referred consecutively to a tertiary centre in Germany for the evaluation of suspected CMA through an OFC, 10 % experienced reactions at the first dose (0.1 mL of milk, equivalent to 3 mg of proteins). Of these, 4 % experienced a severe reaction (Rolinck-Werninghaus et al., 2012). In another study conducted in the Netherlands, 38 (33 %) of 224 consecutive children undergoing a DBPCFC owing to suspected CMA reacted to the challenge. In three children, an objective reaction was observed after the first dose (18 mg of total protein), whereas more than 50 % of subjects reacted at doses > 100 mg of total protein (Dambacher et al., 2013).

In a tertiary centre in the Netherlands, 93 children were challenged through a DBPCFC with CMPs at doses of 0.2 mg (mucosal), 1.8 mg (dose 1), and five additional doses up to 1 750 mg of protein (cumulative dose 2 190 mg). Both objective and subjective reactions were recorded. 6 % of children reacted to the first dose of 0.2 mg of protein and 9 % to the first oral dose of 1.8 mg of protein (Blom et al., 2013). In children with IgE-mediated CMA, MEDs for subjective reaction have been reported to be, on average, two to six times lower than for objective reactions (Blom et al., 2013).

Concerns have been raised about the possibility of children with CMA reacting to lactose following incidents after inhalation of lactose-containing drugs, possibly because of contamination with CMPs. However, no single case of an adverse reaction to lactose ingestion has been reported among children with CMA, and a prospective study on the allergenicity of whey-derived lactose investigated by serology and DBPCFC did not document such reactions (Fiocchi et al., 2003a). Indeed, some products intended for use by milk-allergic children may contain lactose (Fiocchi et al., 2010), and elimination of lactose from the diet of children with CMA is not warranted.

15.8. Conclusion

CMPs are common triggers of allergic reactions to food in children. Most CMPs, even those present at low concentrations, are potential food allergens. The prevalence of CMA in unselected European populations, using food challenges to confirm the diagnosis, has been estimated to be around 1 % in children and 0.5 % in adults. Heat treatments can decrease or increase the allegenicity of CMPs depending on the temperature and duration of the treatment. Fermentation and hydrolytic processes may decrease allergenicity depending on the microorganisms used and the reaction conditions. ELISA and MS techniques are available for the detection of CMPs in food products. Data available from case reports or DBPCFCs do not allow the derivation of a level of exposure that could be safe for most milk-allergic consumers, since the amount of CMPs that may trigger allergic reactions in sensitive individuals varies widely. The lowest reported MOED in milk-allergic patients undergoing DBPCFC was 200 μg of milk protein. Since this was the first dose tested, allergic reactions to lower doses cannot be excluded.

16. Allergy to eggs

16.1. Background

Female animals of many species, including birds, reptiles, amphibians and fish, lay eggs, but hen's eggs are most frequently consumed by man. Egg products are used widely by the food industry. Properties such as binding, emulsification, coagulation and adhesion are important for the production of a large number of food products, such as dairy products, confectionery, beverages, ready prepared meals, cakes, icings, custard fillings and frozen bakery products.

In Western countries, egg allergy is one of the most frequent allergies in childhood together with milk and peanut allergy (Eggesbo et al., 2001; Nwaru and Sheikh, 2012). Clinical symptoms include anaphylactic, immediate (IgE-mediated) and delayed immunological reactions that can affect all organ systems of the body. The skin and the gastrointestinal and respiratory tracts are typically involved.

16.2. Epidemiology

16.2.1. Prevalence

16.2.1.1. Europe

The prevalence of egg allergy in unselected European populations has been assessed in 17 countries (35 studies), including Denmark, Estonia, Finland, France, Germany, Greece, Greenland, Hungary, Iceland, Ireland, Italy, Norway, Portugal, Spain, Sweden, Turkey and the UK. Studies were published between 1980 and 2012 (University of Portsmouth, 2013)

Self-reported prevalence of egg allergy at one year ranged from 1.5 % in Norway (Eggesbo et al., 1999) to 6 % in Finland (Kajosaari, 1982). At two years, the range was between 3 % (Eggesbo et al., 1999; Ostblom et al., 2008b), 2008) and 7 % (Kajosaari, 1982) and between 2 % (Kilgallen and Gibney, 1996) and 9 % (Kajosaari, 1982) at three years of age. When the diagnosis was made by a physician, the prevalence at one year of age ranged from 1.9 % in Italy (Frongia and Bellamo, 2005) and Finland (Pyrhonen et al., 2009) to 2.6 % in Sweden (Ostblom et al., 2008b). In older children (> 6 years), the self-reported prevalence of egg allergy ranged between 1 % (Kajosaari, 1982) and 2.1 % (Zannikos et al., 2008), except in two studies conducted in Turkey in 2010 (Mustafayev et al., 2012)and Spain in 2000 (Martínez-Gimeno et al., 2000), where the prevalence was 5.6 % and 13 %, respectively. In adults, the range was between 0.4 % in Germany (Schafer et al., 2001) and 2 % in Turkey (Gelincik et al., 2008).

Some studies report on sensitisation rates assessed by the SPT and/or specific IgE levels. In young children (up to three years), the prevalence of positive SPTs to eggs ranged from 1.4 % (Venter et al., 2008) in the UK to 5.2 % (Julge et al., 2001) in Estonia. Lower sensitisation rates were reported for older children, ranging from zero (Julge et al., 2001; Ronchetti et al., 2008) to 2.8 % (Schafer et al., 1999) (Ro et al., 2012), and, for adults, between 0.4 % and 1.9 % (Schafer et al., 2001), except in Hungary, where 7.3 to 11.1 % of adult subjects were sensitised to egg yolk as assessed by positive SPT (Bakos et al., 2006). Higher sensitisation rates were observed when specific IgE levels were used for diagnosis. In younger children, sensitisation rates were between 4.2 % and 20.6 % (Julge et al., 2001), whereas in older children ranged between 0.4 % (Krause et al., 2002) and 22.7 % (Julge et al., 2001). In adults the figures were zero to egg yolk (Bakos et al., 2006) and 2.8 % to egg white.

Prevalence of egg allergy was generally lower when sensitisation tests were combined with clinical history. When the SPT was used, prevalence was 1.5 % in 18-month-olds in Sweden (Kristjansson et al., 1999), 0.1 % in adults in Turkey (Gelincik et al., 2008) and 0.2 % in the overall German population combining all ages (Zuberbier et al., 2004). When serum-specific IgE was used, the highest prevalence was observed in Sweden (0.6 %) (Ostblom et al., 2008a) and the lowest in Turkey (0.1 %) (Gelincik et al., 2008). Studies performed in eight European centres are in keeping with these sensitisation rates (Burney et al., 2013).

Studies providing data about the prevalence of egg allergy based on clinical history and confirmed by food challenges (OFC or DBPCFC) are limited. In Denmark (Eller et al., 2009), prevalence of egg allergy was estimated to be 0.2 % at six months of age, 2.6 % at 18 months and 2.3 % at three years, decreasing to 0.6 % at six years. Lower rates (1.6 %) were reported in the same country at three years of age using DBPCFC instead of OFC. In other countries, prevalence of egg allergy among children at six years of age based on OFC was 0.1 % in Turkey (Orhan et al., 2009) and 1 % in Finland (Kajosaari, 1982).

In adults, challenge-proven egg allergy data come from two studies, both reporting 0.1 % prevalence in Denmark (Osterballe et al., 2005) and Turkey (Gelincik et al., 2008). The same prevalence rate was reported in Germany for all ages combined based on clinical history and DBPCFC (Zuberbier et al., 2004).

16.2.1.2. Outside Europe

In an Australian study (Osborne et al., 2011) among 2 079 children, 11 to 15 months old, the prevalence of any sensitisation to raw egg white was 16.5 %. The prevalence of challenge-proven raw egg allergy was 8.9 %, 80.3 % of which could tolerate baked egg. These figures are much higher than those reported in Europe, the reason(s) for which are unclear.

The prevalence of challenge-proven egg allergy in China was 2.9 % and 5 % in 1999 and 2009, respectively, among children from birth up to two years (Chen et al., 2011). A rate of 2.5 % was also reported in another study (Hu et al., 2010) conducted in the same country.

16.2.2. Natural history

Egg allergy is frequently outgrown in later life. Resolution rates vary among studies, probably owing to differences in patient selection and methods used to assess egg allergy. In a retrospective chart review in North America, ~ 40 % and ~ 70 % of egg-allergic children with clear clinical history of an IgE-mediated allergic reaction to egg ingestion or egg-specific IgE > 2 kU/L had developed tolerance to concentrated egg at 10 and 16 years of age, respectively (Savage et al., 2007). In Spain, 50 % of 42 children with egg allergy developed tolerance at around four years of age and only 26 % remained allergic at five years (Montesinos et al., 2010), whereas egg allergy tended to resolve in 55 % of 58 egg-allergic children in the first six years of life (Boyano-Martinez et al., 2002). A high level of egg-specific IgE was correlated to egg allergy persistence (Savage et al., 2007; Caubet et al., 2011). In an Australian study of 130 challenge-proven egg-allergic children, egg allergy resolved by two years in 66 %. The resolution of egg allergy was lower in children with baked egg allergy at one year of age than in children with baked egg tolerance (13 % vs. 56 %, respectively) (Peters et al., 2013).

16.2.3. Time trends

Based on self-reported diagnosis of egg allergy, two cross-sectional studies were carried out in 1980/2001 and in 1995/2005 in Finland and the UK, respectively. In both cases methodologies used and age groups studies were similar, and thus it is possible to compare prevalence rates over time.

In Finland (Kajosaari, 1982; Pyrhonen et al., 2009), prevalence of egg allergy reported by parents of young children was higher in 1980 than in 2001 at all ages. Prevalence of self-reported egg allergy in 1980 and 2001 was 6 % and 2.7 %, respectively, in one-year-olds, 7 % and 4 %, respectively, in two-year-olds, and 9 % and 3.6 %, respectively, in three-year-olds. Conversely, self-reported prevalence of egg allergy increased from 0.7 % to 3 % between 1995 and 2005 in the UK in 15-year-old adolescents (Emmett et al., 1999; Pereira et al., 2005).

Owing to the high risk of bias of questionnaire-based methods for the diagnosis of food allergy and based on the available data, the Panel notes that there is no evidence for a change in the prevalence of egg allergy in Europe over the timeframe assessed.

16.2.4. Severe reactions/anaphylaxis

Severe life-threatening events and fatal anaphylaxis to egg in children are less common than to peanut or milk. Over the 15-year period between 1990 and 2005, 6 series of food-related anaphylaxis in children from 4 different countries (UK, USA, Sweden and Germany) have been published, recording 31 deaths and 132 life-threatening reactions (Sampson et al., 1992; Allen et al., 2007; Pumphrey and Gowland, 2007). The triggers of fatal reactions were egg 7 %, milk 17 %, peanut 48 % and peanut or tree nut 62 %. Both fatalities to egg occurred in young children (3 months and 2 years).

16.2.5. Factors affecting prevalence of egg allergy

The onset of egg sensitisation is related to the introduction of eggs into the diet, although there seems to be other routes (prenatally through the placenta, skin, and respiratory route by inhalation).

Although egg allergy is among the most common food allergy in infants and young children, environmental risk factors specific for egg allergy remain largely unknown. Egg sensitisation at one year of age is predictive of asthma in later life (Kulig et al., 1998; Tariq et al., 2000). IgE antibodies against egg proteins are associated with a higher risk of developing asthma at the age of three years (Nickel et al., 1997). A high level of egg-specific IgE was correlated with egg allergy persistence. It is unclear whether continued exposure to cooked eggs induces immunological changes associated with tolerance induction in egg-allergic children (Lemon-Mule et al., 2008), or whether the introduction of heated/baked egg into the infants' diet from four to six months of age may modify the development of egg allergy and sensitisation (Fleischer et al., 2013).

16.3. Identified allergens

Major allergens of the eggs of hens (*Gallus domesticus*) are known, characterised and classified as Gal d 1–6 by the IUIS (Table 8). However, major allergen sources are still unassigned and their relevance in human egg allergy is still unknown (Mine and Zhang, 2002; Amo et al., 2010).

Table 8: Hen's (*Gallus domesticus*) egg allergens

Fraction	Allergen	Biochemical name	Concentration (%)	Molecular weight [(a)]
Egg white	Gal d 1	Ovomucoid	11	28
	Gal d 2	Ovalbumin	54	44
	Gal d 3	Ovotransferrin	13	78
	Gal d 4	Lysozyme C	3.5	14
Egg yolk	Gal d 5	Serum albumin (α-livetin)	48	69
	Gal d 6	YGP42	–	35 [(b)]

(a): Molecular weight (SDS-PAGE).
(b): kDa.

Clinically relevant egg allergens have been identified both in the egg white and the egg yolk fractions. Based on SPT, RAST assays and CRIE, the most common egg allergens are ovomucoid, ovalbumin and lysozyme (Mine and Yang, 2008).

Ovomucoid (Gal d 1), one of the major egg allergens for clinical reactions (Bernhisel-Broadbent et al., 1994), is a highly glycosylated protein containing 186 amino acids which exhibits trypsin inhibitory activity. The molecule consists of three structurally independent domains, has nine intramolecular disulphide bridges, and displays 20 to 25 % of carbohydrates entities (Kato et al., 1987). IgE specific to Gal d 1 in hen's egg white appears to be a risk factor for persistent egg allergy and indicates that neither raw nor heated egg is likely to be tolerated (Caubet et al., 2011).

Ovalbumin (Gal d 2) is a phosphoglycoprotein constituting 54 % of egg white's total protein content. Its complete sequence of 385 amino acids has been determined (Nisbet et al., 1981). Ovotransferrin (Gal d 3) displays an N domain and a C domain, belongs to the transferrin protein family and has iron-

scavenging properties (Li-Chan and Nakai, 1989). Lysozyme (Gal d 4) is a glycosidase containing four disulphide bonds with bacteriolytic activity. It is used in the food industry to maintain product quality and reduce the incidence of spoilage.

Serum albumin (α-livetin; Gal d 5) is involved in the bird-egg syndrome and sensitisation is most likely to occur via inhalation (Jacobsen et al., 2008). It consists of 589 amino acid residues and is homologous to mammalian serum albumins (47 and 44 % identity to human and bovine albumins, respectively). The protein has one potential glycosylation site and 35 cysteine residues.

Gal d 6 is the newly identified yolk glycoprotein YGP42, a fragment of VTG-1, which has been described in monosensitised egg-allergic patients (Amo et al., 2010). It is heat resistant but sensitive to pepsin digestion. The VTG-derived proteins are the major yolk components. Cleavage of VTG-1 and VTG-2 produces apolipovitellins and phosvitins, which are components of the water-insoluble yolk granular lipoproteins. The C-terminal part of VTGs gives rise to yolk glycoproteins YGP40 and YGP42, which are major components of the yolk plasma (Mann and Mann, 2008).

IgE antibodies of egg-allergic children directed against conformational structures seem to indicate an earlier recovery from disease compared with those children who have developed IgE antibodies against linear epitopes (Jarvinen et al., 2007; Leonard et al., 2012).

16.4. Cross-reactivities

Clinical cross-reactivities of primary egg-allergic individuals are generally restricted to other avian eggs, although primary sensitisations to duck and goose eggs without sensitisation to hen's eggs have been reported (Añíbarro et al., 2000). Hen's egg white immunologically cross-reacts with egg white from turkey, duck, goose and seagull (Langeland, 1983). The level of cross-reactivity is related to the extent of sequence homology of the shared protein, and a homology of around 50 % is required in most instances to allow IgE binding and trigger adverse reactions (Ferreira et al., 2004). All egg whites contain moieties able to bind human IgE antibodies of patients with allergy to hen's egg white. Several cross-reacting proteins in egg white were also detected in egg yolks and to some extent in chicken sera and meat. Individuals who react to chicken meat are generally sensitised to chicken serum albumin. Occasionally, patients with allergies to chicken and other avian meats are able to eat eggs without symptoms (Cahen et al., 1998; Añíbarro et al., 2000). The probability of cross-reactions is likely to be affected by interspecies relationships (Kelso et al., 1999) and possibly by different chicken breeds (Egger et al., 2011).

16.4.1. Bird-egg syndrome

Patients with allergy to egg yolk may also present respiratory symptoms caused by bird exposure at home (Szepfalusi et al., 1994; Quirce et al., 2001). The identified cross-reacting allergens include α-livetin (Gal d 5), which is partially heat-labile. Incubation of pooled sera from patients with bird-egg syndrome with bird feather extracts led to complete blocking of IgE binding to allergens in egg yolk and bird feather extracts. Serum from patients with egg white allergy did not react with allergens in egg yolk or bird feather extract (Szepfalusi et al., 1994).

16.4.2. Bird's nest allergy

Anaphylaxis after ingestion of edible nests of *Collocalia* species, used in Chinese cuisine (Ou et al., 2001), has been reported. Immunochemical characterisation of a putative 66 kDa allergen revealed homology with the egg white allergen ovoinhibitor, a serine protease inhibitor (Goh et al., 2000; Goh et al., 2001).

16.5. Possible effects of food processing on allergenicity and derived products

A number of studies have explored the effects of different food-processing methods, such as heat treatments, enzymatic proteolysis, irradiation or high-pressure treatments, on the allergenicity of egg in food products (Mine and Yang, 2008).

16.5.1. Thermal processing

Thermal processing is often undertaken to enhance flavour, consistency and microbiological safety, rather than to reduce allergenicity. When egg white is subjected to heat, its globular proteins change in structure and conformation. Ovalbumin, the most abundant protein in egg white, unfolds completely when heated in a solution of pH 10 (Van Kleef, 1986). The unfolded, randomly coiled ovalbumin molecules are mainly cross-linked via covalent disulphide cross-links.

Effects of heating (and chemical denaturation procedures) on the IgE-binding capacity (Mine and Zhang, 2002; Manzocco and Nicoli, 2012; Shin et al., 2013) and allergenicity (Koplin et al., 2010; Burks et al., 2012b) of major egg allergens have been described. Many egg-allergic individuals react to cooked and raw eggs (Langeland, 1982a, 1982b). However, some individuals react only to raw eggs and tolerate cooked eggs (Kemp, 2007; Burks et al., 2012b). These individuals often exhibit lower egg-specific IgE levels (Boyano Martinez et al., 2001).

Heating and freeze drying can reduce the allergenicity of egg for some patients (Urisu et al., 1997; Nowak-Wegrzyn and Fiocchi, 2009), but this process does not reliably prevent IgE binding or clinical reactions, probably because the major allergen ovomucoid is heat stable.

16.5.2. Enzymatic treatments

Enzymatic proteolysis may reduce the allergenicity of egg allergens by targeting sequential epitopes (Wal, 2003). During enzymatic hydrolysis, the functional properties of egg proteins, such as foaming and gelling, are usually lost. The IgE-binding capacity of egg was reduced by applying a combination of thermal treatments and enzymatic hydrolysis, while maintaining flavour and texturising properties (Hildebrandt et al., 2008).

16.5.3. γ-Irradiation

Radiation technology has been explored in a number of studies for the modification of egg allergens (Seo et al., 2007). Treatment doses up to 3 kGy are applied to ensure a bacteriological quality for liquid, frozen or dehydrated egg white preparations. γ-Irradiation $>$ 10 kGy may alter the structure of ovalbumin and decrease its IgE-binding capacity. γ-Irradiation in combination with heat treatment may reduce the IgE-binding properties of ovomucoid (Kim et al., 2002).

16.5.4. Egg-derived products used in food processing

Egg lysozyme (E1105) is used by the food industry as a bactericide to prevent the growth of anaerobic bacteria and in the preparation of medications (Fremont et al., 1997). Egg lysozyme in medications, including vaccines, has been reported to trigger adverse reactions in egg-allergic individuals at doses in the milligram and microgram range (Ledesma Benitez et al., 2007; Perez-Calderon et al., 2007; Artesani et al., 2008).

Egg lecithin, commonly used as an emulsifier (E322), is increasingly being replaced by soy lecithin (Gultekin and Doguc, 2013). The possibility of residual allergenicity in food products manufactured using egg lecithin has been reported in a DBPCFC (Palm et al., 1999)..

Egg white is commonly used in the clarification of wines throughout the world.

16.6. Detection of allergens and allergenic ingredients in food

16.6.1. Immunological methods

16.6.1.1. ELISA

The most frequent methods used for egg allergen analyses are based on ELISA techniques preferably targeting ovalbumin and ovomucoid.

An ELISA based on polyclonal antibodies specific to whole egg proteins (Yeung et al., 2000) with a LOD of 0.2 mg/kg and a sandwich ELISA which used ovalbumin and dehydrated egg white solids as antigens with a LOD of 1 mg/kg (Hefle et al., 2001) have been developed and applied to numerous foods. Two more sensitive indirect competitive ELISAs are now available for the detection of both native and denatured ovomucoid in hen's egg white with LODs of 0.041 ng/mL (Li et al., 2008) and 30 ng/mL (Dong-Hwa et al., 2010) in processed foods.

Many ELISA kits with variable performance and inherent limitations are commercially available (Schubert-Ullrich et al., 2009; Shoji, 2009). ELISA kits may target total egg protein, egg white proteins, ovomucoid, ovalbumin or ovomucoid and ovalbumin together, with LODs from 0.08 to 0.6 mg/kg and LOQs between 0.3 and 1 mg/kg. False-positive and false-negative results can be expected as a consequence of several factors, such as matrix effects or cross-reactivity between ovalbumin and other avian eggs, such as pheasant, goose, duck or quail, probably owing to the high sequence homology.

Most important are the effects of thermal processes on the detectability of egg allergens, on account of the reduced recognition of the modified native protein by antibodies and/or the decreased solubility of the proteins. Three commercial ELISA kits were evaluated and found to be highly affected by heat treatments (Fu et al., 2010). The underestimation was attributed to changes in the immunoreactivity of residual proteins rather than to differences in the amount of protein extracted. The effects of processing on the accuracy and precision of five ELISA commercial kits were tested for the simultaneous presence of peanut, egg and milk in incurred dark chocolate (Khuda et al., 2012a) and sugar cookies (Khuda et al., 2012b). The effect on accuracy and precision of ELISA kits was found to depend more on the heating conditions than on the type of matrix. Tempering (46 °C for 4 hours) had no significant effect on the detection of egg in chocolate, whereas baking (190 °C for 25–30 minutes) negatively affected the recovery and variability of egg proteins in sugar cookies when using all five ELISA kits. Similar results were obtained in baked cookies incurred in a non-wheat flour matrix using two commercial ELISA test kits and flow cytometry as detection methods (Gomaa and Boye, 2013). No recoveries were obtained for egg proteins under some thermal treatments.

The solubilisation of egg allergenic proteins is a critical issue to be tackled when using immunoassays. The addition of sodium dodecyl sulphate and 2-mercaptoethanol to the extraction buffer greatly improved the extraction of proteins from raw eggs, boiled eggs and fried noodles, as 2-mercaptoethanol acts as a reducing agent cleaving the disulphide bonds and sodium dodecyl sulphate acts as surfactant (Watanabe et al., 2005). An ELISA for egg proteins employing this extraction method used an anti-sodium dodecyl sulphate ovalbumin antibody rather than an anti-native ovalbumin antibody to allow recognition of the denatured protein. The method was applied to incurred processed samples allowing high recoveries and was validated in a collaborative study (Matsuda et al., 2006).

The reference material NIST RM-8445 (spray-dried whole egg) for allergen detection is available. Another egg reference material NIST RM-8415 (egg powder), used for nutritional studies, was found unsuitable for allergen detection immunoassays owing to the low solubility of the proteins. Several commercial ELISA kits were evaluated for the analysis of egg spiked with NIST RM 8445 in wheat flour (raw) and egg-containing cookies (Diaz-Amigo, 2010). An incurred reference material for the analysis of egg allergens in baked foods is also available (Dumont et al., 2010).

A dessert matrix incurred with different amounts of egg protein from pasteurised egg white was evaluated as a quality control material for allergen analysis in a multilaboratory trial (Johnson et al., 2014). Analyses were performed with five commercial ELISA kits. Estimation of egg protein concentrations varied among the different kits. Only one kit was able to detect the target level of the incurred egg protein in the dessert matrix, which gave the exact concentration of the incurred allergen only at the 3 mg/kg level.

16.6.1.2. Lateral flow devices and dipsticks

LFDs which provide fast qualitative data are commercially available, with LODs from 0.5 to 5 mg/kg. A dipstick assay based on a non-competitive ELISA format, where the antibody was directly spotted on a nitrocellulose membrane and the detection performed with an antibody coupled to peroxidase, was also developed, with a LOD of > 20 µg/kg egg proteins in food (Baumgartner et al., 2002).

16.6.1.3. Biosensors

Several optical-based biosensors have been described for the detection of ovalbumin. Polymer brush-modified cap-shaped gold nanoparticles have been used as sensing elements using localised SPR with a LOD of 100 nM (Anraku et al., 2007). The target of this sensor being sugars, specificity for ovalbumin needs to be demonstrated.

An optical REA-based immunochip sensor in direct and sandwich assay formats using antibodies functionalised with gold nanoparticles has been proposed as a rapid colorimetric method for detecting ovalbumin and ovomucoid in foods (Maier et al., 2008). The biosensor gave reproducible and selective results with a LOD of 1 ng/mL, enabling high-throughput screening.

An optical planar waveguide array platform has also been developed for the detection of multiple allergens, including ovalbumin, using fluorescence sandwich immunoassays with a LOD of 25 pg/mL in buffer and of 1.3 ng/mL (13 ng/g) in pasta (Shriver-Lake et al., 2004).

A label-free voltammetric immunosensor, based on the ovalbumin antibody immobilised on carboxyphenyl-modified graphene has been used for the detection of ovalbumin in the concentration range between 1 pg/mL and 0.5 mg/mL with a LOD of 0.83 pg/mL in phosphate-buffered saline (PBS) (Eissa et al., 2013).

16.6.2. Mass spectrometry

An LC-ESI-MS/MS method was compared with commercial ELISA kits for the detection of ovalbumin in egg white, whole egg and incurred (with egg white powder) pasta before and after heating (Azarnia et al., 2013). Protein extraction was performed with the buffers recommended by the ELISA kits' producers. Several peptides were selected following tryptic digestion of the protein, none of which was detected by MS in cooked samples or by the ELISA kits in all incurred pasta samples. This shows that both MS and ELISA methods are affected by matrix, processing and extraction conditions.

A multi-method for the detection of seven allergenic foods (egg, milk, soy, hazelnut, peanut, walnut and almond) based on LC-QpQ-MS/MS implied extraction of the allergenic proteins from the food matrix (incurred reference bread material baked with a standard recipe), digestion with trypsin and selection of the marker peptides. Peptides were separated by HPLC and analysed in the MRM mode, with a LOD of 50 µg/g (Heick et al., 2011a). This method was found to be superior for the detection of egg allergens to the commercial ELISA kits (Heick et al., 2011b).

16.6.3. Detection of lysozyme in dairy products

An indirect inhibition ELISA for the specific detection of lysozyme in hen's egg white with a LOD of 0.264 µg/mL (Vidal et al., 2005) and a competitive ELISA to quantify the amount of lysozyme in cheese using a commercially available monoclonal antibody (Schneider N. et al., 2010), with a LOD of 2.73 ng/mL, have been described.

A rapid chemoluminescent immunoassay based on bacterial magnetic particles conjugated to an antibody in a fully automated system was also used to detect lysozyme with a LOD of 10 ng/mL (Sato et al., 2001), as well as an online coupled capillary isotachophoresis capillary zone electrophoresis (CITP-CZE) method, which allowed a good separation, with a LOD of 0.25 µg/mL and a LOQ of 1 µg/mL (Kvasnička, 2003).

Lysozyme is used in cheese to prevent blowing by *Clostridium tyrobutyricum.* Lysozyme was efficiently detected and quantified in milk and cheese (LOQ 0.8 mg/kg) by using a RP-HPLC in connection with a fluorescence detector (Pellegrino and Tirelli, 2000), whereas a commercial ELISA kit was unsuitable for the detection of lysozyme in cheese on account of the low recovery owing to the interaction between lysozyme and other proteins in cheese and of matrix interferences during the immunological reactions (Kerkaert et al., 2010).

Lysozyme was found to remain unaltered during ripening (up to 24 months) of a hard-type cheese by using SELDI-TOF/MS (Dragoni et al., 2011). Another method which combines immunocapture purification and MALDI-TOF-MS analysis was also developed for the detection of lysozyme in cheese samples (Schneider Nadine et al., 2010), with a LOD of 5 mg/kg lysozyme in cheese.

16.6.4. Detection of egg products in wine

Egg white proteins are used as fining agents in wines. Lysozyme may also be added to wines as a stabiliser for its antimicrobial activity. By applying a competitive ELISA to laboratory-fined wines, lysozyme was detected in the range of approximately 0.01–0.06 mg/L and dried egg white at 0.2 mg/L (Weber et al., 2007). A specific sandwich ELISA was established using commercially available monoclonal and polyclonal antibodies for ovalbumin detection in wine with a LOD of 1 mg/L (Rolland et al., 2008). The method was applied to a panel of commercially available bottled wines, where egg residues were detected in two red wines fined with whole eggs.

In order to overcome the poor sensitivity of immunological methods in a matrix rich with interfering substances such as wine, a direct LC-MS/MS (nano-HPLC/ESI-Q-TOF) method for detecting residual egg proteins (ovalbumin, ovomucoid and lysozyme) in red wine fined with a commercial egg white preparation was developed (Tolin et al., 2012a). On the basis of three peptides taken as markers, it was possible to unequivocally detect the presence of egg white in wines treated with the minimum dose commonly adopted for red wine fining (5 g/hL). When the method was applied to a panel of commercial red wines, the presence of egg proteins was demonstrated in some, with an estimated minimum residual concentration of ovalbumin of about 0.1 µg/L (Tolin et al., 2012b).

In order to achieve quantitative measurements of egg (ovalbumin and lysozyme) and milk (casein) proteins in white wine by MS, a method based on HR-MS has been described (Monaci et al., 2013). The method implies previous ultrafiltration of wine, tryptic digestion of the dialysed wine extracts and LC/HR-MS. Tryptic peptides were selected as quantitative markers of the allergenic proteins. Analyses were performed on wines fined with either caseinate or egg white powder at concentrations of 0.25 and 10 mg/L, respectively. LODs were 0.4 and 1.1 mg/L, respectively.

16.6.5. DNA-based methods

The limitations of DNA-based techniques for the detection of egg in foods relate to the low content of DNA in eggs and to the fact that egg DNA cannot be distinguished from chicken DNA, which may lead to misinterpretation of the data, obtained when analysing complex food mixtures.

Two tetraplex qPCR were developed for the simultaneous detection of eight allergenic foods, including egg, with specificity and sensitivity in the range of 0.01 % (Köppel et al., 2010). Two quantitative hexaplex real-time PCR systems for the detection and quantification of 12 allergenic ingredients (including eggs) in foods became available thereafter (Köppel et al., 2012). The two tests showed good specificity and sensitivity (LOD of at least 0.01 % for all allergenic ingredients) in mixed foods. The inherent sensitivity was lower for eggs owing to the low amount of DNA present. However, the two multiple PCR systems are suitable as screening tools in routine analysis.

16.7. Minimum (observed) eliciting doses

Some egg-allergic patients react to small (µg) amounts of egg (Wuthrich, 2000; Wuthrich and Ballmer-Weber, 2001).

A number of studies performed for different purposes (i.e. diagnostic, threshold-finding and immunotherapy trials) have reported on MED/MOED following food challenges mostly in children (Atkins et al., 1985; Caffarelli et al., 1995; Eggesbo et al., 2001; Knight et al., 2006; Staden et al., 2007; Benhamou et al., 2008; Orhan et al., 2009; Blom et al., 2013), but also in adults and children combined (Norgaard and Bindslev-Jensen, 1992; Morisset et al., 2003b) and in adults only (Unsel et al., 2007). Studies vary in size, in the challenge protocol used and in the type of food preparation tested (Taylor et al., 2014). The total number of patients showing an objective reaction during the challenge in a given study ranged from 1 to 53. The lowest MOEDs also varied widely among studies, ranging from 0.21 to 583 mg of total egg protein (Blom et al., 2013; Remington, 2013). Raw egg white, whole raw egg and whole cooked (boiled, fried or baked) egg were tested in these studies. Doses (as milligrams of total protein) of raw egg white eliciting allergic reactions were significantly lower than doses of both raw and cooked whole egg, possibly owing to the higher proportion of egg allergens in egg white protein.

Hefle et al. (2003) used spray-dried whole egg to determine the individual threshold doses in 39 egg-allergic individuals. Most subjects showed no objective reactions to a cumulative dose of 330 µg spray-dried whole egg (150 µg egg protein), whereas one subject reacted to the first dose of 30 µg spray-dried whole egg (14 µg egg protein).

Threshold-finding DBPCFCs with raw hen's egg were conducted in 20 children with IgE-mediated, challenge-confirmed hen's egg allergy undergoing desensitisation therapy (Meglio et al., 2013). Oral, liquid doses of 0.05 g, 0.1 g, 0.3 g, 0.6 g, 1.3 g, 2.5 g, 6.3 g and 14 g of egg white protein were given every 15 minutes. The test was terminated when either signs (objective) or symptoms (subjective) arose. Five children reacted to the first dose tested.

Minimum doses reported to elicit objective reactions in egg-allergic individuals are variable depending on the study population, challenge protocol and food matrix tested. The lowest reported MOEDs in egg-allergic patients undergoing food challenges of 14 µg of egg protein could be even lower considering that the individual already reacted to the first challenge dose tested.

16.8. Conclusion

Egg proteins are frequent triggers of allergic reactions. Prevalence of challenge-proven egg allergy in unselected populations is about 1.5 to 2.5 % in young children (< 3 years), whereas lower prevalence rates have been reported in older children and adults (from 0.1 % to 1 %). A number of egg allergens have been identified and characterised. Most egg-allergic individuals exhibit IgE binding to sequential epitopes of egg white. However, both egg white- and egg yolk-derived proteins have been described to trigger clinical allergic reactions. Heat denaturation and other food-processing treatments do not reliably reduce the allergenicity of egg. A number of methods of detection are available, based on ELISA, MS and PCR technologies. Specific methods for the detection of lysozyme in dairy products and of egg products in wine based on these technologies have also been developed. MEDs of ingested egg proteins reported to trigger objective reactions in clinical studies range from few micrograms to milligrams. Most egg-allergic individuals are likely to react to raw egg proteins at the low milligram level.

17. Allergy to nuts

17.1. Background

Nuts include a wide variety of fruits or seeds of various species contained within a hard shell. These species do not form a taxonomic group. Almonds are not nuts, but are included in this section because they are specifically mentioned in Annex IIIa. Nuts are consumed in many forms, varying from raw seeds to roasted snacks. The intake of tree nuts, peanuts and unspecified nuts consumed in the EU was 2.23 g/day for the entire population. The mean intake of total nuts varied ~ eight-fold from northern to southern Europe, ranging from 0.61 g/day in Sweden to 4.83 g/day in Spain. Walnuts, almonds, pistachios and hazelnuts are the tree nuts most consumed in Europe (Jenab et al., 2006). Nuts are

known to trigger a wide range of allergic manifestations in sensitive individuals, ranging from OAS to anaphylaxis.

Hazelnut belongs to the family *Betulaceae*, a group of plants whose pollen is often responsible for respiratory symptoms. Brazil nut belongs not to the subclass of *Rosidae*, like hazelnut, English walnut, black walnut, almond, cashew nut, macadamia nut and Queensland nut, but to the subclass of *Asteridae*, like sesame seeds and *Apiaceae* (carrot and celery). Pecan nut (*Carya illinoinensis*) is closely related to walnut and belongs to the same family of *Juglandaceae*. The *Rosaceae* family includes almonds (*Prunus dulcis* or *Prunus amygdalus*), but also apple, pear and *Prunoideae* fruits (peach, apricot, plum and cherry). Cashew nut (*Anacardium occidentale*), pistachio (*Pistacia vera*) and mango belong to the same *Anacardiaceae* family. Chestnut (*Castanea sativa*) belongs to the *Fagaceae* family, together with trees such as oak and beech.

17.2. Epidemiology

17.2.1. Prevalence

17.2.1.1. Europe

Data on the prevalence of tree nut allergy in unselected European populations by type of nut, age group and method of diagnosis are depicted in Table 9. Prevalence data come from a number of different European countries (i.e. Finland, Germany, Greenland, Hungary, Iceland, Norway, Spain, Sweden, the Netherlands, Turkey and the UK), but geographical comparisons are difficult to make because country-based studies differ in the type of nut, age group and method of diagnosis investigated.

Table 9: Estimated prevalence of tree nut and almond allergy in unselected European populations by type of nut, age group and method of diagnosis

	Tree nuts (unspecified)	Hazelnut	Walnut	Almond	Cashew nut	Brazil nut	Pistachio	Pecan nut
All ages								
Self-reported	1.7 %	–	–	–	–	–	–	–
Sensitisation	–	23 %		–	–	–	–	–
Clinical history and sensitisation	–	4.5 %	1.4 %	–	–	–	–	–
Clinical history and FC	–	2.2 %	1 %	–	–	–	–	–
Young children (≤ 3 years)								
Self-reported	0–2 %	–	–	0 %	–	–	–	–
Sensitisation	–	0.2 %	–	0.3 %	0.2 %	0.3 %	–	–
Clinician diagnosed	0.3–0.4 %	–	–	–	–	–	–	–
Clinical history and sensitisation	0 %	–	–	0 %	–	–	–	–
Children/adolescents (> 3–17 years)								
Self-reported	1.3–6.9 %	0.3–1.5 %	0.1–1.2 %	3.8 %	–	–	0.8 %	–
Sensitisation	–	0.1–0.4 %	0.1–4.5 %	0.5 %	0.4 %	0.5 %	–	0.2 %
Clinical history and sensitisation	–	0.1 %	–	–	0.1 %	–	–	–
Clinical history and FC	–	0–0.1 %	0–0.4 %	–	–	–	–	–
Adults/elderly (≥ 18 years)								
Self-reported	0.1 %	–	–	–	–	–	–	–
Sensitisation	–	0–11.3 %	3.7 %	0 %	–	–	–	–
Clinical history and sensitisation	–	0 %	–	–	–	–	–	–

	Tree nuts (unspecified)	Hazelnut	Walnut	Almond	Cashew nut	Brazil nut	Pistachio	Pecan nut
Clinical history and FC	–	0 %	0 %	–	–	–	–	–

FC, food challenge.

The prevalence of self-reported allergy to any nut was 1.7 % in the general population (UK) (Young et al., 1994), ranging from 0.1 % in adults (Turkey) (Orhan et al., 2009), to 1.3 % (the Netherlands) (Brugman et al., 1998) and 6.9 % (Spain) (Martínez-Gimeno et al., 2000) in children.

The prevalence of self-reported hazelnut allergy ranged from 0.3 to 1.5 % in children (Turkey) (Orhan et al., 2009; Mustafayev et al., 2012). No data are available for other population subgroups or the general population. Data on sensitisation rates are more abundant and somehow contradictory. Although sensitisation rates based on positive SPT have been reported to be as high as 23 % in the general German population (Zuberbier et al., 2004), lower rates have been observed in German (11.3 %) (Schafer et al., 2001) and Hungarian adults (2.8–3.6 % by SPT; 0–9.2 % by specific IgE) (Bakos et al., 2006), whereas sensitisation rates in children and adolescents were very low in Turkey and the UK (0.1–0.4 %). Prevalence of hazelnut allergy based on positive clinical history plus sensitisation (4.5 %) and on clinical history plus food challenge (2.2 %) was again higher in the German general population than in four-year-old children in the UK (0.1 %; history plus SPT; (Tariq et al., 1996) or in any specific population subgroup in Turkey, where the prevalence of hazelnut allergy using food challenges was close to zero (Gelincik et al., 2008; Orhan et al., 2009; Mustafayev et al., 2012).

Some of the studies above have also assessed the prevalence of walnut allergy, which appears to be even lower than the prevalence of allergy to hazelnuts. As for hazelnuts, prevalence of self-reported allergy is low (0.1–1.2 %), but data are only available for Turkish children. Sensitisation rates based on positive SPT were 3.7 % in Hungarian adults and 0.1–4.5 % in Turkish children. Prevalence of walnut allergy based on positive clinical history plus sensitisation (1.4 %) and on clinical history plus food challenge (1 %) in the German general population was again higher than in any specific population subgroup in Turkey, where the prevalence of walnut allergy using food challenges was zero to 0.4 %.

Prevalence data from unselected European populations regarding almond allergy are available almost exclusively for children and are limited to self-reported allergy and sensitisation rates, with or without clinical history. Whatever the method used, prevalence was low in both adults and children. Self-reported allergy ranged from 0 % in young children (Iceland and Sweden) (Kristjansson et al., 1999) to 3.8 % in older children (Sweden) (Ostblom et al., 2008a), whereas sensitisation rates ranged from 0.3 to 0.5 % (UK), respectively (Roberts et al., 2005; Venter et al., 2008).

Prevalence data for tree nuts other than hazelnut and walnut are scarce and almost limited to sensitisation rates (positive SPT and/or specific IgE) in children. Sensitisation rates (positive SPT) to cashew nut were 0.2 % in young children (UK) (Venter et al., 2006b) and 0.4 % in older children (UK) (Roberts et al., 2005), whereas prevalence of cashew nut allergy in older children based on clinical history and positive SPT was only 0.1 % (Turkey) (Tariq et al., 1996). Sensitisation rates to Brazil nut in young and older children (0.3 and 0.5 %, respectively) and sensitisation rates to pecan nuts in older children (0.2 %) were similar to those for cashew nut and were assessed in the same studies. The only available data for pistachio referred to self-reported allergy in older children (0.8 %; Turkey) (Mustafayev et al., 2012).

17.2.1.2. Outside Europe

Most studies on the prevalence of nut allergy in unselected populations conducted outside Europe (mainly in the USA and Canada) address allergy to unspecified nuts. Prevalence of self-reported allergy to tree nuts in Canada was 1.2 % in the general population and 1.1 % in adults (Canada) (Ben-

Shoshan et al., 2010). Lower rates (0.6 %) were observed in Australian adults (Woods et al., 1998). In children, prevalence of self-reported allergy was generally higher, ranging from 1.7 % in Canada (Ben-Shoshan et al., 2010) to 4.7 % in Singapore (Shek et al., 2010).

Data on prevalence of tree nut allergy based on clinical history or diagnosis by a clinician was quite consistent across countries, ranging from 0.2 to 0.4 % in children and from 0.9 to 1.6 % in adults in the USA (Sicherer et al., 1999; Sicherer et al., 2003; Sicherer et al., 2010).

Only one study reported on prevalence of allergy to a particular tree nut. Based on clinical history and specific IgE concentrations, 2.2 % of six- to eight-year-old Taiwanese children were allergic to pistachio (Wan and Chiu, 2012).

No prevalence studies were available in unselected, non-EU populations where allergy to particular tree nuts has been confirmed by challenge studies.

17.2.2. Natural history

Little is known about the natural history of nut allergy. Patients with diagnosed nut allergy are generally advised to avoid eating nuts for the rest of their lives, but some patients outgrow their allergy.

The proportion of subjects who outgrow tree nut allergy was evaluated using nut challenges in a group of children with a history of acute allergic reactions to nuts and evidence of nut-specific IgE or positive nut-specific IgE level and no history of nuts ingestion (Fleischer et al., 2005). The authors concluded that 8.9 % of children acquired oral tolerance (nine out of 101 with a history of prior nut reactivity), but this level of resolution of nut allergy may be underestimated. It was also observed in the same study that none of the patients whose nut allergy resolved had a history of reacting to more than two different nuts, so patients with allergy to multiple nuts may be less likely to outgrow allergy.

17.2.3. Time trends

A random-calling telephone survey conducted across the USA in 1997, 2002 and 2008 observed an increase in self-reported tree nut allergy in children (0.2 %, 0.5 % and 1.1 %, respectively) (Sicherer et al., 1999; Sicherer et al., 2003; Sicherer et al., 2010). Prevalence rates of hospital admissions for food-induced anaphylaxis in Australia increased by 350 % between 1994 and 2005, mostly in children below four years of age (11 cases per 100 000 population in 2005) and mostly due to peanut and tree nut anaphylaxis (39 % of all cases of anaphylaxis), whereas the increase in the frequency of admissions was more modest in older age groups and in relation to other allergies (Liew et al., 2009).

A study on self-reported allergy to nuts (unspecified) conducted in Finland in 1980 (Kajosaari, 1982) showed a 2 % prevalence at one year of age, zero at two years, and 2 % at three years. A similar study conducted in 2001 (Pyrhonen et al., 2009) found a 0.8 % prevalence at one year of age, 2 % at two years, and 1.4 % at three years.

Data from studies using OFCs on time trends for nut allergy are lacking.

17.2.4. Severe reactions/anaphylaxis

In comparison with other foods, allergic reactions to nuts seem to be particularly severe and are characterised by multi-systemic or respiratory symptoms. It has been estimated that nuts represent the triggering factor for about one-third to one-quarter of all anaphylactic reactions attributed to food consumption (Cianferoni and Muraro, 2012; Huang et al., 2012).

Registers of anaphylaxis deaths have been kept since 1992 in both the UK and the USA. Out of the 37 food-induced fatalities reported from 1992 to 2000 in the UK, 10 were attributable to peanut, five to walnut and 10 to other non-specified nuts (Pumphrey, 2000). The next UK survey (up to 2006) reported 48 additional deaths, of which nine were attributed to nuts (Pumphrey and Gowland, 2007).

In the USA, 32 fatal cases were registered in the first period (up to 2000), 20 caused by peanut, three by walnut, two by Brazil nut, two by pecan nut, one by pistachio and two by unspecified nuts (Bock et al., 2001). In the next period (2001–2006), 31 additional cases were identified, eight of which were associated with tree nut consumption (Bock et al., 2007).

An epidemiological study on the cases of anaphylaxis requiring emergency treatment in the north-west of England reported 23 cases of reactions to nuts out of 172 total cases of anaphylaxis (Pumphrey and Stanworth, 1996). Another study reported 14 cases of severe food-allergic reactions to nuts in children, especially cashew nut (seven cases), out of 55 severe non-fatal reactions recorded in the UK and Ireland from 1998 to 2000 (Macdougall et al., 2002).

17.2.5. Factors affecting prevalence of nut allergy

Allergy to one type of nut is a risk factor for developing allergy to other types of nuts. Multiple sensitisations, assessed as a positive SPT, were found in 19 % of sensitised children at the age of two years and 86 % of children at 5 to 14 years. Similarly, clinical reaction to multiple nuts was found in 2 % of children at two years and 47 % at 14 years (Clark and Ewan, 2003). Retrospective analysis of 201 patients with peanut allergy showed that at the time of peanut allergy diagnosis, almost one-third of patients were sensitised to one or more tree nuts and became sensitised to an increasing number of tree nuts with advancing age (Fleischer, 2007).

One randomised placebo-controlled double-blind study showed the prolonged effect of sublingual immunotherapy with a standardised hazelnut extract on clinical symptoms of hazelnut allergy (Enrique et al., 2008). However, these results were not confirmed in other studies (van Hoffen et al., 2011).

17.3. Identified allergens

The majority of nut allergens are seed storage proteins, such as vicilins, legumins and 2S albumins. Other nut allergens are PR proteins (chitinases, Bet v 1 homologues and LTPs) and structural proteins (profilins and oleosins). Profilins are panallergens (present in pollens, nuts, seeds, fresh fruit and other vegetables). Additional proteins have recently emerged as allergens in tree nuts, including manganese superoxide dismutase (MnSOD), 60S acidic ribosomal protein P2, and cytosolic small heat shock protein.

17.3.1. Hazelnut

Hazelnut (pollen and non-pollen-related) allergens are shown in Table 10.

Table 10: Hazelnut (*Corylus avellana*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Cor a 1	PR-10	Bet v 1	17
Cor a 2	Profilin	Profilin	14
Cor a 8	ns-LTP 1	Prolamin	9
Cor a 9	11S Globulin (legumin-like)	Cupin	40
Cor a 11	7S Globulin (vicilin-like)	Cupin	48
Cor a 12	Oleosin	Oleosin	17 [(b)]
Cor a 13	Oleosin	Oleosin	14–16 [(b)]
Cor a 14	2S albumin	Prolamin	15–16 [(b)]

(a): Molecular weight (SDS-PAGE).
(b): kDa.

The hazelnut (*Corylus avellana*) allergen first identified (Cor a 1) bound IgE from 63 out of 65 patients with DBPCFC-confirmed OAS to hazelnut (Ortolani et al., 2000; Pastorello et al., 2002a). Cor a 1 and Cor a 2 are homologues of the major birch pollen allergen Bet v 1 (Hirschwehr et al.,

1992). Cor a 2 shows high amino acid sequence identity (77–91 %) with other plant profilins and similar tertiary structure (Radauer et al., 2006), and has been described as a food allergen in seven of 17 hazelnut-allergic patients with concomitant birch pollen allergy (Lüttkopf D et al., 2002).

Pollen-unrelated hazelnut allergy was initially described in adults from a Mediterranean region who were sensitised to the LTP Cor a 8 (Schocker et al., 2000). It was demonstrated that the primary sensitiser to Cor a 8 is the protein Pru p 3 from peach (*Prunus persica*) (Hartz et al., 2010). The limited sensitisation potential of Cor a 8 seems to be explained by the rapid lysosomal degradation during antigen processing and the lack of dominant T-cell epitopes (Schulten et al., 2011).

Cor a 14 represents a second member of the prolamin superfamily that is associated with hazelnut allergy (Pastorello et al., 2002a; Garino et al., 2010). Cor a 14 bound IgE from 5 out of 15 patients (Garino et al., 2010). Clinical, demographic and epidemiological data on sensitisation to Cor a 14 remain limited.

Severe forms of hazelnut allergy are related to two allergens of the cupin superfamily: Cor a 9 (11S legumin) and Cor a 11 (7S vicilin). Reactivity to Cor a 9 was demonstrated in 12 out of 14 patients with systemic reactions (Beyer K. et al., 2002) and in four out of seven patients with severe hazelnut allergy (Hansen et al., 2009). Sensitisation to Cor a 9 can appear in very young infants prior to tree pollen sensitisation and independently from sensitisation to its homologues in legumes such as soy or peanut (Verweij et al., 2011). IgE binding to Cor a 11 was found in 31 of 65 adult patients with hazelnut OAS (Lauer et al., 2004). Sensitisation to Cor a 11 was seen in 12 of 32 of children with systemic reactions to hazelnut and only in one out of eight adult patients (Verweij et al., 2012).

The role of Cor a 12 and Cor a 13, two oleosins identified as hazelnut allergens, remain to be established (Akkerdaas et al., 2006).

17.3.2. Walnut

Walnut allergens are listed in Table 11. Only Jug r 1, Jug r 2 and Jug r 3 are reported in the IUIS database.

Table 11: Walnut (*Juglans regia*) allergens

Scientific name (common name)	Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Juglans regia (English walnut)	Jug r 1	2S Albumin	Prolamin	14
	Jug r 2	Vicilin	Cupin	44
	Jug r 3	ns-LTP 1	Prolamin	9
	Jug r 4	11S Globulin	Cupin	58
Juglans nigra (Black walnut)	Jug n 1	2S Albumin	Prolamin	15
	Jug n 2	Vicilin	Cupin	56

(a): Molecular weight (SDS-PAGE).

The first allergen identified in English walnut (*Juglans regia*) was Jug r 1, a protein belonging to the 2S albumin family with its subunits joined by disulphide bridges (Teuber et al., 1998). The recombinant walnut 2S albumin was found to be a major allergen, as it was recognised by 12 of 16 patients (75 %). This protein is similar to allergens present in Brazil nut, castor bean, cottonseed and mustard seed (Robotham et al., 2002).

Teuber et al. (1999) identified a second major allergen in walnut, a recombinant protein belonging to the vicilin-like protein family (Jug r 2). Jug r 2 bound IgE in sera from 9 out of 15 walnut-allergic patients (60 %). Despite its high amino acid sequence identity (70 %) with peanut vicilin Ara h 1, this allergen does not cross-react with homologous peanut proteins (Teuber et al., 1999).

Two other major walnut allergens are Jug r 3, a lipid-transfer protein, and Jug r 4, an 11S legumin-like globulin. Jug r 3 and Jug r 4 bound IgE in sera from 78 % (Pastorello et al., 2004) and from 57 to 65 % of walnut-allergic patients, respectively (Roux et al., 2003; Wallowitz M et al., 2006). Jug r 1 and Jug r 3 appear to be the most potent allergens of walnut (Rangsithienchai et al., 2013).

The information about black walnut (*Juglans nigra*) allergens is much more limited. Two recombinant allergens were identified: Jug n 1, a 2S seed storage albumin; and Jug n 2, a vicilin seed storage protein. The Jug n 1 and 2 were found to be 96 % and 97 % identical to Jug r 1 and 2, respectively.

17.3.3. Almond

Almond (*Prunus dulcis*) allergens include Pru du 3, Pru du 4, Pru du 5, and Pru du 6 (Table 12).

Pru du 6, the most fully described almond allergen, is amandin, a legumin that forms 65 to 75 % of the extractable proteins in almonds (Sathe et al., 2002). Pru du 6 is hexameric, and each polypeptide comprises a large acidic α-chain and a small basic β-chain. The two chains are linked by a disulphide bond. Two isoforms have been identified: Pru du 6.01 and Pru du 6.02. Pru du 6.01 was recognised by 50 % and Pru du 6.02 by 28 % of almond-allergic patients (Willison et al., 2011).

Pru du 3 consists of three isoallergens (Chen et al., 2008). It is usually accumulated in the outer epidermal layers of plant organs and is thought to be responsible for the stronger allergenicity of the peels in comparison to the inner layers of almonds (Costa et al., 2012b). Pru du 4 has ≥ 90 % sequence identity with profilins from a variety of plant sources, including apple, cherry, peach, orange and melon, and is cross-reactive to ryegrass pollen profilins (Rodríguez-Perez et al., 2003; Tawde et al., 2006).

Pru du 5 is a 60S acidic ribosomal protein (Abolhassani and Roux, 2009). The biological function of this protein is based on the successive addition of amino acid residues to a polypeptide chain during protein biosynthesis. It exhibits 81 % identity and 94 % homology with the protein ARP60S from tomato, which may indicate possible cross-reactivity between them (López-Matas et al., 2011).

Four other potential allergens have been identified: Pru du 1, Pru du 2, Pru du 2S albumin, and Pru du γ-conglutin (Costa et al., 2012b).

Table 12: Almond (*Prunus dulcis*) allergens

Allergen	Family	Superfamily/family	Molecular weight [(a)]
Pru du 3	ns-LTP 1	Prolamin	9
Pru du 4	Profilin	Profilin	14
Pru du 5	60s Acidic ribosomal prot. P2		10
Pru du 6	Amandin, 11S globulin (legumin-like protein)	Cupin	ca. 360

(a): Molecular weight (SDS-PAGE).

17.3.4. Cashew nut

Allergens from cashew (*Anacardium occidentale*) are listed in Table 13 and include three major allergens classified as seed storage proteins: Ana o 1, a 7S vicilin-like protein with homotrimer subunits (Wang et al., 2002); Ana o 2, an 11S globulin member of the legumin family (Wang et al., 2003); and Ana o 3, a 2S albumin (Robotham et al., 2005). Ana o 1, Ana o 2, and Ana o 3 have been recognised by the serum of 50 % (Wang et al., 2002), 62 % (Wang et al., 2003), and 81 % (Robotham et al., 2005) of patients with an allergy to cashew nut, respectively. The molecular structure of Ana o 2 closely resembles that of soybean Gy2 glycinin (Robotham et al., 2010). Ana o 3 is highly homologous to the walnut allergen Jug r 1 (Wang et al., 2003).

Table 13: Cashew nut (*Anacardium occidentale*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Ana o 1	Vicilin-like protein	Cupin	50
Ana o 2	Legumin-like protein	Cupin	55
Ana o 3	2S Albumin	Prolamin	14

(a): Molecular weight (SDS-PAGE).

17.3.5. Brazil nut

Brazil nut allergens are listed in Table 14.

Table 14: Brazil nut (*Bertholletia excelsa*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Ber e 1	2S Albumin (sulphur-rich)	Prolamin	9
Ber e 2	11S Globulin	Cupin	29

(a): Molecular weight (SDS-PAGE).

Ber e 1, the first major allergen identified in Brazil nut (*Bertholletia excelsa*), is a sulphur-rich 2S albumin seed storage protein. Its antigenicity was tested using sera from nine patients showing allergic reactions after ingestion of Brazil nut. Eight out of nine showed IgE-binding to the 2S albumin (Nordlee et al., 1996). A subsequent study confirmed that Ber e 1 was the major allergen of Brazil nut, as it was recognised by all 11 patients with documented history of anaphylactic shock or laryngeal oedema after ingestion of the nut (Pastorello et al., 1998). Ber e 1 does not trigger an allergenic response on its own, as other components of the lipid fraction are required (Mirotti et al., 2013).

Another recognised Brazil nut allergen is Ber e 2, a 11S globulin legumin-like protein that showed IgE-binding in 12 out of 27 Brazil nut-sensitised patients (Beyer K. et al., 2002).

17.3.6. Pecan nut

There are two pecan (*Carya illinoinensis*) allergens listed in the IUIS database (Table 15).

Table 15: Pecan nut (*Carya illinoinensis*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight (kDa)
Car i 1	2S Albumin	Prolamin	16
Car i 4	Legumin	Pupin	55.4 [(a)]

(a): Subunit of hexameric protein.

Car i 1 is a 2S albumin seed storage protein consisting of two subunits connected by a disulphide bond. IgE-binding to Car i 1 was shown in 22 out of 28 sera from patients with convincing histories of allergic reactions to pecan nut (Sharma et al., 2011b). In turn, Car i 4 is a hexameric legumin 11S seed storage protein. Each monomer consists of basic and acidic subunits linked by disulphide bonds. Car i 4 was bound by serum IgE from 16 out of 28 subjects allergic to pecan (Sharma et al., 2011a).

17.3.7. Pistachio

Pistachio (*Pistacia vera*) allergens are listed in Table 16.

Table 16: Pistachio (*Pistacia vera*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Pis v 1	2S Albumin	Prolamin	7
Pis v 2	11S Globulin subunit	Cupin	32
Pis v 3	Vicilin	Cupin	55
Pis v 4	MnSOD	–	25.7
Pis v 5	11S Globulin subunit	Cupin	36

(a): Molecular weight (SDS-PAGE).

MnSOD, manganese superoxide dismutase.

Pis v 1, which belongs to the 2S albumin family, shows structural similarity to cashew allergens with 64 % sequence identity to Ana o 3 and 48 % to Ana o 2 (Wang et al., 2003; Robotham et al., 2005). Pis v 2, a 11S globulin, has similar sequence homology with Jug r 4 of English walnut (50 %), Cor a 9 of hazelnut (47 %) and Ber e 2 of Brazil nut (46 %) (Beyer K. et al., 2002; Wallowitz M et al., 2006). Nineteen out of 28 patients with pistachio allergy showed IgE binding to Pis v 1 and 14 out of 28 (50 %) to Pis v 2 (Ahn et al., 2009).

Pis v 3 is a 7S vicilin-like protein (Willison et al., 2008). Pis v 4 is a manganese superoxide dismutase (MnSOD)-like protein (Ayuso et al., 2007), and Pis v 5 is an 11S globulin acidic subunit (Ahn, 2007). They are all recognised as minor pistachio allergens, showing IgE-binding in 7 out of 19 patients (Willison et al., 2008) and 10 out of 25 patients (Noorbakhsh et al., 2010a), respectively.

17.3.8. Chestnut

European chestnut (*Castanea sativa*) allergens are shown in Table 17.

Table 17: Chestnut (*Castanea sativa*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Cas s 1 [(b)]	PR-10	Bet v 1 (profilin)	22
Cas s 5	Chitinase	(Hevein-like domain)	–
Cas s 8	ns-LTP 1	LTP	12 – 13
Cas s 9	Cytosolic heat shock protein	(Heat shock protein)	17 [(c)]

(a): Molecular weight (SDS-PAGE).
(b): Not included in the IUIS database.
(c): kDa.

Cas s 5 is a chitinase, which contains an N-terminal domain with homology to the hevein-like domain of rubber latex hevein. By analysing recombinant Cas s 5with and without the N-terminal hevein-like domain, it was shown that the majority of the Cas s 5-reactive IgE from patients with the latex-fruit allergy syndrome was directed to this domain, though some evidence for reactivity with the C-terminal catalytic domain was also found (Diaz-Perales et al., 1998). These findings explain why many chestnut-allergic individuals are also allergic to latex (Raulf-Heimsoth et al., 2007).

Studies regarding other chestnut allergens are less common. Cas s 1 is a pollen protein, which shows significant amino acid sequence similarity at the N-terminus with the major birch pollen allergen Bet v 1 and is antigenically closely related to it (Kos et al., 1993). Cas s 8, a member of the lipid transfer protein (LTP) family, has 53 % identity to apple Mal d 3 and 50 % identity to peach Pru p 3 fruit allergens (Lee et al., 2005; Sánchez-Monge et al., 2006). Six out of nine patients (66 %) sensitised to chestnut but not to latex had a positive SPT response to Cas s 8 (Blanco et al., 2006).

Cas a 9 is useful to identify patients with systemic reactions, which are more common in children (De Knop et al., 2011; Verweij et al., 2011).

17.3.9. Macadamia and Queensland nut

There are no designated allergens for macadamia (*Macadamia integrifolia*) and Queensland nut (*Macadamia ternifolia*) to date, although strong serum IgE binding to a protein of 17.4 kDa from both raw and roasted extracts of macadamia has been reported (Sutherland et al., 1999).

17.4. Cross-reactivities

17.4.1. Cross-reactivity among nuts and between nuts and peanuts

Allergies to nuts are generally induced by non-pollen-mediated food sensitisation. However, allergy to hazelnut, almond and, less frequently, other nuts can be induced by sensitisation to birch pollen, plane tree pollen or mugwort pollen (Vieths et al., 2002; Flinterman et al., 2006c).

Allergy to nuts is characterised by a high frequency of life-threatening anaphylactic reactions, so when allergy to a single nut is demonstrated, the patient is often advised to avoid the entire nut group. It is estimated that 20 to 50 % of peanut-allergic patients are also allergic to tree nuts (Ewan, 1996; Sicherer et al., 2003).

It is still disputed if cross-reactivity between peanuts and tree nuts is related to taxonomic proximity or rather results from structural homology of IgE-binding epitopes present in several tree nuts and peanuts (Wallowitz et al., 2004; Maleki et al., 2011). Studies on cross-reactivity among nuts and between nuts and peanuts include mainly *in vitro* studies, but there are some studies on IgE-binding and clinical studies.

Peanut-specific IgE antibodies that cross-react with tree nut allergens and may contribute to the manifestation of tree nut allergy in peanut-allergic subjects have been identified (de Leon et al., 2005). The structurally related cross-reactivity between Ara h 3 and tree nut allergens such as Jug r 4 of walnut, Cor a 9 of hazelnut, or Ana o 2 of cashew nut appears to be unrelated to the botanical origin of the allergens and suggests that individuals allergic to peanut should avoid tree nuts, unless the allergy status in relation to all other nuts is clarified (Barre et al., 2007; Ball et al., 2011).

Cross-reactivities among tree nuts are mostly related to botanical family associations (Hasegawa et al., 2009; Noorbakhsh et al., 2011), although some studies reported IgE cross-reactivity among nuts not showing taxonomic relationship (de Leon et al., 2003). Grouping of tree-nuts into cross-reacting groups (Goetz et al., 2005) has been proposed. Walnut, pecan nut and hazelnut, which are members of the same botanical subclass, form a strongly cross-reactive group, whereas walnut and pecan nut, which are members of the same *Juglandaceae* family, showed the strongest cross-reactivity. Also cashew and pistachio, which showed strong cross-reactivity, are both members of the botanical family *Anacardiaceae*.

17.4.2. Cross-reactivity between nuts and other foods

The major hazelnut allergen Cor a 1 was demonstrated to be cross-reactive with the birch pollen major allergen Bet v 1 (Hirschwehr et al., 1992). The 18 kDa allergens from hazelnut kernel and hazel pollen were cloned and tested using sera from 43 patients with positive DBPCFC to hazelnut (Lüttkopf D. et al., 2002). Four recombinant variants of the major hazelnut allergen Cor a 1.04 were synthesised. These variants showed only 63 % identity and partial IgE cross-reactivity with the major hazel pollen allergen Cor a 1.01, but 85 % identity with the major birch pollen allergen Bet v 1. This suggests that the epitopes of hazelnut Cor a 1.04 are less related to hazel pollen than to birch pollen. The presence of specific IgE to Cor a 1 identifies patients with birch pollen allergy, which is more common among hazelnut-sensitised adults.

In Northern Europe, where birch pollen allergy is common, most cases of hazelnut allergy develop secondarily to birch pollen allergy because of an immunological cross-reactivity between birch and hazelnut. In contrast, in Southern Europe, where birch pollen allergy is rare, hazelnut allergy is more often a primary allergy.

The hazelnut profilin Cor a 2 also displays cross-reactivity with a birch pollen homologous allergen, but the clinical relevance of this immunological cross-reactivity seems, however, low (Hirschwehr et al., 1992; Wensing M. et al., 2002).

Latex-fruit syndrome represents a well known phenomenon of cross-reactivity caused by the presence of chitinase I both in latex (known as hevein Hev b 6.02, Hev b 11) and several food products (especially fruits), although other allergens may be involved. Chitinase I is present also in chestnut (Cas s 5) and shares common epitopes with natural latex antigens, which can explain sensitisation to chestnut in subjects allergic to latex (Blanco et al., 1999; Diaz-Perales et al., 1999; Sánchez-Monge et al., 2000; Blanco, 2003).

Four important nut allergens belong to a family of LTPs: Pru d 3 of almond, Cas s 8 of chestnut, Cor a 8 of hazelnut, and Jug r 3 of walnut. Owing to structural homology, LTPs from different allergen sources are generally IgE cross-reactive. A high degree of IgE cross-reactivity has been observed among allergenic LTPs within the *Rosaceae* family, and particularly with the peach allergen Pru p 3, which possesses more epitopes and/or epitopes with higher IgE-binding affinity compared with other LTPs. Peach represents a primary sensitiser to LTPs (Egger et al., 2010) and the level of specific IgE to peach LTP seems to be a main factor associated with cross-reactivity to other plants, including nuts (Asero, 2011).

17.5. Possible effects of food processing on allergenicity

Nuts are often subjected to a variety of processing conditions, which may affect their allergenic potential.

17.5.1. Thermal processing

Hazelnut, which is often used in pastries, partially loses its allergenicity after roasting. One study investigated the IgE-binding pattern of raw and roasted hazelnut (Pastorello et al., 2002a) and found that the major allergen Cor a 1 loses its IgE-binding capacity in roasted hazelnut, while the minor allergen LTP, recognised by a distinct subset of patients without birch pollinosis, was heat resistant. However, 5 out of the 17 patients sensitised to Cor a 1.04 who underwent a DBPCFC with roasted hazelnut reacted with mild OAS to the challenge (Hansen et al., 2003), whereas in another clinical study, a decreased allergenicity of roasted hazelnut compared with raw hazelnut was observed in patients with birch pollen allergy during an oral challenge (Worm et al., 2009). This suggests that roasting can decrease, but not abolish, clinical reactions to hazelnut in birch pollen-allergic patients sensitised to Cor a 1.04. This is supported by the observation that thermal processing results in partial or complete depletion of the stimulatory activity of basophils only in some subjects with systemic allergic reactions to hazelnut (Cucu et al., 2012a). Similarly, in a DBPCFC heat-processed (roasted) hazelnut and native hazelnut were given orally in increasing amounts. The dosage by which allergic reactions were elicited varied from 0.01 to 2.0 g for native hazelnut, with a median of 0.1 g, and from 0.01 to 10.0 g for roasted hazelnut, with a median of 0.23 g (Worm et al., 2009). *Ex vivo* basophil activation measured by flow cytometry showed that significantly higher allergen extract concentrations were needed to induce 50 % basophil activation in roasted vs. native hazelnut.

Contrary to hazelnut, allergen Ber e 1, belonging to the prolamin superfamily present in Brazil nut, is inherently stable to thermal treatment (Venkatachalam et al., 2008).

The IgE-binding capacity assessed by ELISA and Western blot assays was significantly lower for the protein extract prepared from steam-roasted than from raw and dry-roasted pistachio nuts (Noorbakhsh et al., 2010b).

A meta-analysis based on the results of 32 individual studies found that thermal processing may reduce the IgE-binding capacity of proteins of the PR-10 family present in hazelnut (Cor a 1) and almond (Pru du 1), but has a little influence on the allergens belonging to LTPs and seed storage proteins in hazelnut, almond, cashew, Brazil nut, walnut, pecan and pistachio (Masthoff et al., 2013).

In some cases thermal treatment may enhance the allergenicity of nuts. This was observed in pecan nut, which develops new allergens upon heating as a result of the Maillard reaction (Berrens, 1996). These modifications have a clinical significance: an anaphylactic reaction to cooked pecan nuts was reported by a patient who showed specific IgE antibodies exclusively against allergenic determinants present in aged or heated pecan, but not in fresh pecans (Malanin et al., 1995). On the contrary, the Maillard reaction decreases the IgE- and IgG-binding properties of the hazelnut allergen Cor a 11 (Iwan et al., 2011).

17.5.2. Other treatments

Antigenic stability of proteins in several nuts subjected to various processing methods, including γ-irradiation alone or in combination with blanching, pressure cooking, oven roasting, frying and microwave heating, was demonstrated using Western blotting and ELISA (Su et al., 2004). While heat processing may inactivate certain structural epitopes of hazelnuts, such treatments are unlikely to affect the allergenicity of almonds, cashew nuts and walnuts. In aqueous solutions with pH values between 5.0 and 7.0 and high pressure, a temperature exceeding 110 °C is needed to denature Ber e 1, the major allergen of Brazil nut (van Boxtel et al., 2008).

Enzymatic treatments can also influence the allergenicity. It has been shown that treatment with trypsin or elastase decreases the IgE-binding capacity of hazelnuts (Wigotzki et al., 2000). High stability to pepsin digestion has been shown in relation to Ber e 1, an allergen present in Brazil nut (Moreno et al., 2005). In contrast, mice sensitised to cashew and then undergoing provocation challenges with pepsin-digested cashew proteins showed less severe allergic reactions compared with native cashew proteins (Kulis et al., 2012). Pepsin and trypsin destroy IgE-binding of Bet v 1-related food allergens (including hazelnut allergen Cor a 1.04) but not their T-cell activating properties (Schimek et al., 2005).

There is only limited information available about the influence of different methods used for preparation of nut oils on their allergenicity. Teuber et al. (Teuber et al., 1997) examined a range of nut oils (walnut, almond, hazelnut, pistachio, and macadamia) finding that oils that had undergone less processing at lower temperatures tended to demonstrate higher protein concentration. Those oils with most protein and least processing tended to demonstrate the strongest IgE binding within each group of nut oil extracts.

Despite several effects of processing on the antigenicity of nut allergens, nuts are generally considered to be relatively resistant to processing.

17.6. Detection of allergens and allergenic ingredients in food

17.6.1. Immunological methods

Different ELISAs (sandwich, competitive) have been described for the detection of nut allergens with high sensitivity and LOD as low as 0.1 mg/kg. Numerous kits are commercially available (Poms et al., 2004a; Schubert-Ullrich et al., 2009; Fielder et al., 2010). All tests provide quantitative results, based on in-house reference materials, and present variable cross-reactivity among nuts. Matrix effects and food processing may also affect the detection of nut allergens by ELISA (Garber and Perry, 2010). Lateral flow devices and dipsticks are also commercially available and mostly used for screening purposes (Schubert-Ullrich et al., 2009; Fielder et al., 2010).

17.6.1.1. Hazelnut

Many ELISA methods have been developed for hazelnut by using monoclonal or polyclonal antibodies against raw or processed foods (Holzhauser and Vieths, 1999; Koppelman et al., 1999; Blais and Phillippe, 2001; Scheibe et al., 2001; Stephan et al., 2002). Commercial products containing hazelnuts have been tested, including chocolate (Ben Rejeb et al., 2003). The performance of four commercial ELISA kits was compared in the presence or absence of wheat proteins in processed foods

(Cucu et al., 2011). A significant loss of accuracy was observed for three of the four kits as a result of the Maillard reaction.

A sandwich ELISA with a LOD of 0.7 ng/mL in the range of 1 to 2.5 µg of hazelnut protein/g of food (Akkerdaas et al., 2004), and a hazelnut-specific indirect competitive ELISA based on polyclonal chicken antibodies raised against processed hazelnut proteins (Cucu et al., 2012a), with a LOD of 1.36 µg/mL, have been proposed.

Another sandwich ELISA operating in optical and electrochemical modes and targeting the allergen Cor a 9 was devised (Trashin et al., 2011), with a LOD of 4 ng/mL or 0.1 µg of hazelnut protein/g of food. A time-resolved fluoroimmunoassay for the detection of hazelnut protein traces in food matrices has been developed and validated (Faeste et al., 2006), with a LOD of 0.1 mg/kg and a LOQ of 0.33 mg/kg. A sensitive biosensor based on a highly specific monoclonal antibody is able to detect hazelnut proteins in olive oil (Bremer et al., 2009).

17.6.1.2. Walnut

ELISA kits are available for the detection of walnut-soluble proteins in processed foods with a LOD of 0.39 ng/mL, corresponding to 0.156 µg of walnut-soluble protein/g of food (Doi et al., 2008). Mild cross-reactivity with pecan and hazelnut was observed. An ELISA was built to detect raw and roasted walnut allergens with a LOD of 1 µg/g in several matrices (Niemann et al., 2009). Substantial cross-reactivity was observed with pecan. The performance of a walnut ELISA kit in processed foods was evaluated in an inter-laboratory study by analysing incurred samples with 10 µg of walnut-soluble protein/g of food. The results obtained were reliable (Sakai et al., 2010).

17.6.1.3. Cashew nut

A sandwich ELISA for the detection of anacardein (i.e. the predominant cashew nut protein fraction) with previous immune adsorption showed good specificity when tested against several nuts and seed proteins potentially cross-reactive (Wei et al., 2003). The LOD was 1 µg/g of food in processed food products. A more recent ELISA test with the same LOD but substantial cross-reactivity with pistachio and, to a lesser extent, with hazelnut, has also been published (Gaskin and Taylor, 2011).

17.6.1.4. Brazil nut

An indirect competitive ELISA for the detection of Brazil nut in food based on polyclonal antibodies rose against the 2S albumin, with a LOD of 1 µg/g, showed negligible cross-reactivity with other nuts and legumes (Clemente et al., 2004). A polyclonal competitive inhibition ELISA has been proposed for detecting the other major Brazil nut allergen 11S globulin (Sharma et al., 2009). The LOD was 10–90 ng/mL. A remarkable food matrix effect, which affected protein recovery from spiked samples, was observed.

In order to avoid raising antibodies from animals, probes were produced *in vitro* by isolation of recombinant antibodies specific for the Brazil nut protein and used in an indirect phage-ELISA (de la Cruz et al., 2013). For Brazil nut protein extracts the LOD was 0.9 µg/mL and the LOQ 1.2 µg/mL. The sensitivity of the assay slightly increased for the detection of roasted Brazil nuts (LOD 0.4 µg/mL, LOQ 0.6 µg/mL). In a binary mixture model with wheat flour, the LOD and LOQ were established at 5 and 20 mg/g on account of strong matrix interferences.

17.6.1.5. Pecan nut

Only an ELISA method for detecting pecan proteins in the range of 32 to 800 ng/mL (Venkatachalam et al., 2006) and a more sensitive ELISA with a LOD of 1 ng/mL in complex matrices (Polenta et al., 2010), which showed extensive cross-reactivity with walnut, have been described.

17.6.1.6. Almond

ELISA, LFDs, dipsticks and biosensors are commercially available immunological methods for detection of almonds (Costa et al., 2012b). These tests are generally rapid and sensitive, with a LOD of 0.1 mg/kg of almond protein in food samples for ELISAs and 1 mg/kg for LFDs (Schubert-Ullrich et al., 2009).

17.6.1.7. Multiplex immunoassays

A multiresidue enzyme immunoassay (under a competitive indirect format) was developed for the simultaneous detection of four tree nuts (hazelnut, almond, cashew and Brazil nuts) and peanuts in a single run (Ben Rejeb et al., 2005). The LOD was < 1 µg/g of protein for all allergenic ingredients. The method was applied to chocolate samples.

The performance of three commercial sandwich ELISA kits for the detection of almonds was compared (Garber and Perry, 2010). The LOD for almonds spiked into several cooked foods varied from 3 to 39 mg/kg depending on the food matrix and the ELISA kit.

17.6.2. Mass spectrometry

17.6.2.1. Hazelnut

Determination of hazelnut has been performed by LC-MS/MS through selection and measurement of specific marker peptides. After extraction and trypsin digestion of hazelnut proteins, six peptides were identified by MS/MS as specific for hazelnut and synthesised to be used as standards for developing a LC-MS/MS method in the SRM mode. Depending on the peptide, the lowest concentrations determined were 3.1 or 4.2 ng/mL (Ansari et al., 2012).

17.6.2.2. Pecan

The effect of processing on detectability of pecan proteins by proteomic tools was evaluated (Polenta et al., 2012). Despite the high homology between the majority of pecan and walnut proteins, three proteins were unambiguously identified from pecan origin: 7S vicilin, 11S legumin and a putative allergen 11. Peptides from the tryptic digestion of putative allergen 11 were highly specific for pecan, allowing the detection of the presence of fentomoles (or ng) of proteins with a LOQ of 2.2 ng/mL, comparable with that observed with the ELISA test.

17.6.2.3. Multiplex MS methods

An LC-LIT-MS/MS method for the simultaneous detection and quantification of the five allergens Ana o 2 (cashew nut), Cor a 9 (hazelnut), Pru du 1 (almond), Jug r 4 (walnut) and Ara h 3/4 (peanut) in a single short run has been developed (Bignardi et al., 2010). The method is based on the detection of selected specific marker peptides for every target protein. The peptide mixtures obtained from the tryptic digestion of the protein extract were separated on a particle-packed column, identified and quantified by linear ion trap (LIT) MS detection, under the SRM mode, with LODs from 10 to 55 mg/kg and LOQs of 37 to 180 mg/kg. When performing a preliminary clean-up step by size-exclusion chromatography, before enzymatic digestion of the proteins, the sensitivity was highly improved for every allergen. LODs ranging from 0.1 to 1.3 mg of nut/kg for biscuits and from 5 to 15 mg of nut/kg for chocolate and LOQ values in the 0.3–4.5 mg nut/kg range for biscuits and in the 18–50 mg of nut/kg range for chocolate were obtained (Bignardi et al., 2013).

A multi-method for the detection of seven allergens, including hazelnut, walnut and almond, based on LC-QpQ-MS/MS in MRM mode is available (Heick et al., 2011a). On the basis of selected marker peptides obtained from the tryptic digested extracted proteins, it was possible to detect the seven allergens also in incurred food samples with LOD values of 5 and 3 mg/kg for hazelnut and almond, respectively, and of 70 mg/kg for walnut.

17.6.3. DNA-based methods

PCR-based methods for nut allergen detection have been extensively reviewed (van Hengel, 2007; Monaci and Visconti, 2010).

17.6.3.1. Hazelnut

A real-time PCR method based on the Cor a 11 gene with an absolute LOD of 13 pg hazelnut DNA, corresponding to approximately 27 genome equivalents, has been proposed. When applied to model pastry samples with a defined hazelnut content, a practical detection limit of 0.01 % (w/w) hazelnut was obtained (Piknová et al., 2008). A species-specific real-time PCR protocol was devised with a LOD of 9.6 pg of hazelnut DNA, corresponding to 20 genome copies (D'Andrea et al., 2009). In flour samples spiked with known amounts of hazelnut, the LOD was 0.001 % hazelnut, corresponding to 10 mg/kg. A single-tube nested real-time PCR system allowed a decrease in the LOD to 0.5 pg of hazelnut DNA, corresponding to one DNA copy (Costa et al., 2012a). A high-resolution TaqMan real-time PCR for detecting hazelnut DNA with a LOD of 0.1 mg/kg of the target in food samples has also been published (López-Calleja et al., 2013). A comparative evaluation of the performance of ELISA and real-time PCR in detecting and quantifying hazelnut in a food model system showed that, although ELISA appeared to be more sensitive, both techniques had matrix effects and lack of robustness when detecting hazelnut in processed foods (Platteau et al., 2011a). A duplex real-time PCR for the simultaneous detection of sesame and hazelnut had a LOD of 5 mg/kg for hazelnut (Schoringhumer et al., 2009).

17.6.3.2. Almond

Almond has been detected in foods by applying the single-tube nested real-time PCR system (Costa et al., 2013). The system allowed lowering the LOD of the conventional real-time PCR from 100 mg/kg to 50 mg/kg of spiked almond in food. The absolute LOD was 1.28 pg of almond DNA, corresponding to 3.9 DNA copies. The system showed cross-reactivity with peach and apricot, which belong to the same *Rosaceae* family and have a high homology regarding DNA encoding for the allergen Pru du 6.

17.6.3.3. Brazil nut, walnut, pistachio, pecan, macadamia and cashew nut

A specific real-time PCR method for the detection of Brazil nut in processed food was compared with a commercially available qualitative lateral flow device (Röder et al., 2010), showing a LOD ≤ 5 mg/kg in spiked foods.

A series of real-time PCR methods for the detection of walnut, pistachio, pecan, macadamia and Brazil nut in foods were developed by the same research group (Brežná et al., 2006; Brežná et al., 2008; Brežná and Kuchta, 2008; Brežná et al., 2009; Brežná et al., 2010). The absolute LODs were 0.24 ng DNA for walnut, 0.012 pg DNA for pistachio, 1 pg DNA for pecan nut, 1.45 pg DNA for macadamia nut, and 10 pg DNA for Brazil nut. Using a series of model pastry samples with defined nut contents, practical LODs of 0.01 %, 0.0004 %, 0.01 %, 0.02 %, and 0.1 % for walnut, pistachio, pecan nut, macadamia nut, and Brazil nut, respectively, were estimated. A real-time PCR method for the detection of cashew nuts in confectionery was described by the same group (Piknová and Kuchta, 2007). The absolute LOD was 1.25 pg DNA, corresponding to approximately 2.5 genome equivalents. Using a model pastry sample with defined cashew nut content, a practical LOD of 0.01 % was obtained.

Another more sensitive real-time PCR system was devised with an absolute LOD of 0.5 pg genomic cashew DNA, corresponding to 10 copies DNA. The practical LOD for a pesto Genovese sauce was 2 mg/kg (Ehlert et al., 2008).

17.6.3.4. Multiplex PCR

A LOD of 0.01 % was obtained in two tetraplex real-time PCR assays to simultaneously detect eight allergens, among which were hazelnut and almond (Köppel et al., 2010). In two hexaplex real-time PCR systems proposed by the same group (Köppel et al., 2012), DNA of 12 allergenic foods including

cashew, hazelnut, almond, pistachio and walnut, were detected with a LOD of 0.1 % for all analytes. Detection of cashew nut, pecan nut, pistachio, hazelnut, macadamia nut, almond, walnut and Brazil nut by employing a MLPA method was obtained with a LOD of 5 mg/kg for each allergenic ingredient (Ehlert et al., 2009). By using the same method, hazelnut DNA was detected with a LOD of 1.4 ng, corresponding to 105 DNA copies (Mustorp et al., 2011). In a multiplex real-time PCR for detecting DNA of allergens in foods, the LOD was of 5 pg for almond, hazelnut and peanut and 0.5 pg for cashew, walnut and sesame (Pafundo et al., 2010).

17.6.3.5. PCR coupled to other techniques

An electrochemical low-density DNA array coupled to PCR has been devised, with a LOD of 0.3 and 0.1 nM for Cor a 1.03 and Cor a 1.04, respectively. A PNA-array was used in combination with a duplex PCR for the simultaneous detection of hazelnut and peanut with a LOD of 50 pg DNA (Rossi et al., 2006).

17.7. Minimum (observed) eliciting doses

Nut-induced fatalities have been described after the ingestion of foods apparently free from nuts (Ortolani et al., 2000; Wensing M. et al., 2002), suggesting that even little amounts may elicit severe allergic reactions. Studies that provide information MEDs for nuts are limited to hazelnut, cashew nut and walnut.

In a European multicentre study (Italy, Switzerland and Denmark), DBPCFCs were performed in 86 subjects with clinical history of hazelnut allergy, a positive SPT and specific IgE (Ortolani et al., 2000). Challenges were considered positive when a symptom was reported by the patient or a reactive sign was observed by the investigators. Of the 86 subjects, 67 (77.9 %) reacted to the food challenge, which started with 1.4 to 1.5 g of hazelnut (about one hazelnut) in Italy and Denmark and with 2.7 g in Switzerland. Doses were doubled every 10 to 15 minutes. MEDs ranged from 1.4 g of hazelnut in Denmark and 2.7 g in Switzerland (first dose tested) to 15.3 g in Italy. MEDs in Italy were not reported. In this study, eliciting doses were apparently unrelated to the severity of the reaction.

In a DBPCFC study on 29 hazelnut-allergic patients 3 to 17 years of age, doses eliciting subjective reactions varied from 1 mg to 100 mg of hazelnut protein (equivalent to 6.4 to 640 mg of hazelnut meal), while objective reactions were observed in two patients after 1 mg and 1000 mg of protein, respectively (Wensing M. et al., 2002).

In another DBPCFC study, 28 children sensitised to hazelnut were challenged with increasing doses of defatted hazelnut flour in series: 10 µg, 100 µg, 500 µg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g, and 3 g (protein content, 15.5%). Only 12 children were diagnosed with hazelnut allergy by DBPCFC. Of these, four reported OAS at doses starting at 1.6 mg of hazelnut protein (MED) and eight developed an objective reaction. The MOED was 46.5 mg of hazelnut protein.

DBPCFCs were conducted in 31 cashew nut-allergic, 28 hazelnut-allergic, and 13 walnut-allergic children (Blom et al., 2013). Challenges started with 0.2 mg of protein applied to the oral mucosa and continued with six oral, increasing doses (in mg of protein) until a subjective or an objective reaction occurred (first dose was 1.7 mg for hazelnut, 1.8 mg for walnut and 2.3 mg for cashew nut). Among cashew nut-allergic children, 10 % reacted to the mucosal challenge and 3 % to the first oral dose. Among hazelnut-allergic children, 0.4 % reacted to the mucosal challenge and 15 % to the first oral dose. The MOED for walnut was 0.9 mg of protein.

Data from DBPCFCs shows that minimum doses of nuts eliciting allergic reactions in susceptible individuals may be below 1 mg of protein.

17.8. Conclusion

Nuts are common triggers of systemic allergic reactions, which can be life-threatening. Clinical cross-reactivities among nuts and between nuts and peanuts are frequent, as well as between hazelnut and

birch pollen. Prevalence of nut allergy among the general population varies depending on the nut. Prevalence rates of 2.2 % based on clinical history and food challenges have been reported for hazelnut. Nut allergens are generally resistant to processing, although thermal treatments may reduce the IgE-binding capacity of PR-10 in hazelnut and almond. Many sensitive ELISA, MS, and PCR methods are available for the detection of nut allergens. ELISA kits may present serious cross-reactivities among nuts. Data from DBPCFCs shows that minimum doses of nuts eliciting allergic reactions in susceptible individuals may be below 1 mg of protein.

18. Allergy to peanuts

18.1. Background

Peanut (*Arachis hypogea*) is a member of the legume family, which also includes pea, bean, soybean, lupin, lentil and fenugreek. Peanut consumption has increased during the last decades because of its content of easily digested proteins and its versatility. It can be consumed raw as a vegetable, crushed or ground as "butter", roasted or salted as snack, incorporated into candies, and used to produce oil, extracted by solvents or pressure. The wide uses of peanuts and derived products in processed foods make inadvertent exposure frequent. For example, peanut butter is often used in restaurants to harden soft foods or to "glue down" and close egg rolls; peanuts that have been pressed, deflavoured and reflavoured are sold as, for example, walnuts or almonds (Loza and Brostoff, 1995).

18.2. Epidemiology

18.2.1. Prevalence

Peanut allergy is one of the most common forms of IgE-mediated reactions to food.

18.2.1.1. Europe

Studies on the prevalence of peanut allergy and sensitisation to peanut in unselected populations have been conducted in 10 European countries, including Denmark, France, Germany, Hungary, Iceland, Norway, Sweden, The Netherlands, Turkey and the UK. There is much variation in the type of data available regarding the age ranges considered and the methods used for diagnosis, which makes difficult comparisons among studies (University of Portsmouth, 2013).

The highest prevalence rate of self-reported peanut allergy (15 %) was observed in a group of 15- to 17-year-old French children(Touraine et al., 2002), whereas the lowest, which was close to zero, was observed among 18-month-old young children in Iceland (Kristjansson et al., 1999).

Studies on the prevalence of sensitisation to peanut were based on positive SPT and/or serum-specific IgE levels. In young children (0–3 years old), rates of positive SPT ranged from 0.4 % (Venter et al., 2008) to 2.8 % (Ro et al., 2012). In older children (> 3 years) prevalence of positive SPT ranged from 0.7 % (Mustafayev et al., 2012) to 5.1 % (Nicolaou et al., 2010). Rates of positive SPT in adults were between 6.4 % (Bakos et al., 2006) and 6.8 % (Schafer et al., 2001). For specific IgE levels, the only study from Norway in young children reported a prevalence of sensitisation of 3.4 % (Ro et al., 2012), whereas in older children the prevalence of sensitisation ranged between 2.6 % (Krause et al., 2002) and 12.2 % (Nicolaou et al., 2010). The latter study used a low cut-off point for determining sensitisation (0.2 kU/L), which may explain the higher sensitisation rates observed. The rate of sensitisation determined by SPT in the same study was much lower (5.1 %). In adults, sensitisation rates to peanut were between zero (Bakos et al., 2006) and 3.1 % (Bjornsson et al., 1996) when serum-specific IgE levels were used for diagnosis.

The prevalence of peanut allergy based on OFC was zero (95 % CI: 0.0–4.2%) in the young children in Denmark (Osterballe et al., 2005) and ranged from 0.1 % in Turkey (Mustafayev et al., 2012) to 1.4 % in the older children in the UK (Grundy et al., 2002). No data based on OFC are available in adults.

Data on the prevalence of peanut allergy in young children using DBPCFC are not available. In older children, it ranged from zero in Turkey (Orhan et al., 2009) to 1.8 % in the UK (Hourihane et al., 2007).

18.2.1.2. Outside Europe

More than 50 studies on the prevalence of sensitisation to peanut and peanut allergy have been conducted in different countries outside Europe, mainly in the USA. Again, the use of very diverse methods of diagnosis of peanut allergy makes the comparison among studies difficult. The rates of peanut allergy based on self-reports ranged from zero (Oh et al., 2004) to 8.4 % (Greenhawt et al., 2009). Sensitisation rates based on positive SPT ranged from 0.3 % (Hu et al., 2010) to 8.6 % (Arbes et al., 2005), and on specific IgE levels between 7.6 % (Liu AH et al., 2010) and 13.5 % (Kumar et al., 2011). Lower rates were reported when clinical history and positive SPT were used for diagnosis, ranging from zero (Dalal et al., 2002) to 0.4 % (Woods et al., 2002). Only one study outside Europe used OFC and reported a prevalence of peanut allergy of 2.9 % in a group of Australian children 12 to 15 months old (Osborne et al., 2011).

18.2.2. Natural history

It was considered for a long time that no oral tolerance to peanut developed in peanut-allergic patients. However, some individuals outgrow their peanut allergy (Hourihane et al., 1998). In one study, peanut allergy had resolved in 18 % of individuals participating in oral peanut challenges. The chances of negative results on a challenge despite clear reactions in the past are increased in subjects who do not have allergies to other foods at the time of the challenge. In another study, 21.5 % of individuals aged 4 to 20 years with a serum peanut-specific IgE level < 21 kIU/L who underwent oral food challenges did not develop a reaction, likely indicating resolution of their allergy (Skolnick et al., 2001).

Based on data from several studies, it is estimated that 20 % of peanut-allergic children will outgrow their peanut allergy later in life. Peanut-specific IgE levels can be used to decide which patients with peanut allergy should be considered for a formal OFC (Fleischer et al., 2003). However, peanut allergy may also recur after resolution. A recurrence rate of approximately 8 % was determined in patients who outgrew their peanut allergy (Fleischer et al., 2004).

18.2.3. Time trends

Three sequential cohorts of children (age three to four years) born in the same geographical area (Isle of Wight, UK) were assessed for peanut sensitisation and peanut allergy. Cohort A included children born in 1989 (Tariq et al., 1996) and was assessed at four years of age (n = 981). Cohort B included children born between 1994 and 1996 (Grundy et al., 2002) and was assessed between three and four years of age (n = 1246). Cohort C included children born in 2001–2002 (Venter et al., 2008) and was assessed at three years of age (n = 642). Peanut sensitisation was defined by a positive SPT to peanut, whereas peanut allergy was defined by a positive OFC in patients with a positive SPT or with history of immediate systemic reaction. Peanut sensitisation increased significantly from 1.1 % (95 % CI: 0.7–2.3 %) in cohort A to 3.3 % (95 % CI: 2.4–4.5 %) in cohort B (p = 0.001) before falling back to 2.0 % (95 % CI: 1.1–3.5 %) in cohort C (p = 0.145 as compared with cohort B). Similarly, peanut allergy increased from 0.5 % (95 % CI: 0.2–1.1 %) in cohort A to 1.4 % in cohort B (p = 0.023), with a subsequent fall to 1.2 % (95 % CI: 0.6–2.3 %) in cohort C (p = 0.850 as compared with cohort B).

A prospective study conducted in Olmsted County, Minnesota, indicated an increase of more than 3-fold in the annual incidence of peanut allergy, namely from 2.05 cases per 10,000 children in 1999 to 6.88 cases per 10,000 children in 2007 (Rinaldi et al., 2012). The prevalence of peanut allergy in children in 2007 was 0.65 %. Incident peanut allergy was defined as a positive history of IgE-mediated type 1 reaction to peanuts and at least one of the following criteria: a positive blood test (specific IgE > 0.35 kU/L), a positive SPT (≥ 3.0 mm), a positive food challenge.

Even if the study conducted in the USA indicates an increase in the prevalence of peanut allergy in the past years, the Panel considers that the available data do not allow concluding on whether the prevalence of peanut allergy has changed in the UK between 1993 and 2005 and no data are available from other European countries.

18.2.4. Severe reactions/anaphylaxis

Peanut is the most common cause of severe or fatal food-induced anaphylaxis. The most severe reactions have been observed in subjects with asthma. In an American registry of fatal food-induced anaphylaxis, 37 of the 63 fatalities recorded in a 12-year period were caused by peanut (Bock et al., 2001, 2007). In the UK, 10 out of 37 fatalities to food recorded from 1992 to 1998 were caused by peanut (Pumphrey, 2000). A two-year prospective study in a paediatric population in the UK described three deaths (none caused by peanut) and 55 severe or near-fatal food-allergic reactions, 10 of which caused by peanut (Macdougall et al., 2002).

18.2.5. Factors affecting prevalence of peanut allergy

Allergy to peanuts manifests very early in life. According to a voluntary registry (Sicherer et al., 2001), 89 % of peanut-allergic subjects are younger than 18 years of age (median age 5 years). Most children experience their first allergic reaction to peanuts at a median age of 14 months, which occurs during the first known exposure in 74 % of them. The high incidence of peanut allergy in very young children who do not frequently consume this food suggests the potential role of foetal and infant (through breast milk) exposure to allergens ingested by the mother (Vadas et al., 2001) or even skin application of peanut oil-containing ointment in children with eczema (Lack et al., 2003). The relationship between maternal peanut intake during pregnancy or lactation and allergic disease development in children is controversial.

Two studies in the UK suggest that early oral exposure (< 12 months) to peanuts may decrease the frequency of peanut allergy (Du Toit et al., 2008), while early non-oral exposure may have the opposite effect (Fox et al., 2009). Prospective studies in infants at high risk for food allergy are lacking.

18.3. Identified allergens

Peanut kernels contain over 50 different types of proteins (about 23–27 % protein by weight), 19 of which bind IgE from sera of peanut-allergic subjects (Clarke et al., 1998). Peanut allergens are shown in Table 18.

Ara h 1 and Ara h 2 were identified and characterised in the early 1990s (Burks et al., 1991b; Burks et al., 1992a) and are the most extensively studied. Ara h 1, Ara h 2, and Ara h 3 are considered the major peanut allergens (Burks et al., 1998), as well as Ara h 6 in some studies (Flinterman et al., 2007).

Ara h 1 is a 7S globulin (vicilin) which belongs to the cupin superfamily, whereas Ara h 2, 6 and 7 are 2S albumins (conglutin) which belong to the prolamin superfamily. Ara h 2 has high sequence homology with Ara h 6. As a result of their stability to heat and gastrointestinal digestion, many allergens of the prolamin superfamily may account for severe allergic reactions. Ara h 3 and Ara h 4 are nearly identical isoforms and are 11S globulins (legumins) of the cupin superfamily. Ara h 4 is considered an isoform (Ara h 3.02) of Ara h 3. Ara h 6 and Ara h 7 show a low amino acid sequence identity to each other and to the other peanut conglutin Ara h 2, even though the three proteins belong to the same 2S albumin family. Ara h 6 seems to be responsible for severe allergic reactions (Becker et al., 2001). Ara h 5 belongs to the profilin family. Profilins show high sequence homologies even if from distantly related plants and are known panallergens involved in cross-reactions between pollen and plant foods (Radauer and Breiteneder, 2007). Ara h 8 is a pathogenesis-related protein (PR)–10 and it is of relevance to peanut-allergic patients with birch pollen allergy because of the cross-reactivity to the homologous Bet v 1 allergen (Mittag et al., 2004a). Ara h 9 is a ns-LTPs. Ara h 10

and 11 (oleosins) have more recently been recognised. Allergenic oleosins are found in legumes, nuts, and seeds.

Different immunological patterns of peanut allergy have been observed. For instance, peanut-allergic patients are commonly sensitised to Ara h 1, Ara h 2, and/or Ara h 3 in the USA, to Ara h 9 in Spain and to Ara h 8 in Sweden. This demonstrates heterogeneity in the immunological phenotype of peanut allergy in different geographical areas (Vereda et al., 2011a).

Table 18: Peanut (*Arachis hypogea*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Ara h 1	7S Globulin (vicilin-type)	Cupin	64
Ara h 2	Conglutin (2S albumin)	Prolamin	17
Ara h 3	11S Globulin (legumin)	Cupin	60.34 (fragment)
Ara h 5	Profilin	Profilin	15
Ara h 6	Conglutin (2S albumin)	Prolamin	15
Ara h 7	Conglutin (2S albumin)	Prolamin	15
Ara h 8	PR-10	Bet-v1	17
Ara h 9	ns-LTP	Prolamin	9.8
Ara h 10	Oleosin	Oleosin	16 [(b)]
Ara h 11	Oleosin	Oleosin	14 [(b)]
Ara h 12	Defensin	–	8 kDa (reducing), 12 kDa (non-reducing), 5.184 kDa (mass)
Ara h 13	Defensin	–	8 kDa (reducing), 11 kDa (non-reducing), 5.472 kDa (mass)

(a): Molecular weight (SDS-PAGE).
(b): kDa.

18.4. Cross-reactivities

18.4.1. Cross-reactivity between peanuts and other legumes

Peanut has structurally homologous proteins and share common epitopes with other members of the legume family such as peas, beans, clover, lupin and lentils (Vereda et al., 2011a). Peanut-allergic patients show extensive serological cross-reactivity with members of the legume family (Jensen et al., 2008). Studies demonstrated that 38 % to 79 % of subjects with clinical reactions to a single legume showed IgE-binding (positive SPT/RAST) to a variety of legumes (Sicherer, 2002). This will not necessarily lead to clinical reactions. In a study using oral challenges, only 5 % of patients with peanut allergy had a positive clinical challenge to more than one legume (Sicherer, 2002).

18.4.1.1. Lupin

β-Conglutin (Lup an 1) was the major lupin allergen cross-reacting with peanut proteins, as observed in IgE binding and SPT studies in peanut-allergic individuals (Ballabio et al., 2013).

Significant sequence and molecular homology between Ara h 8 and the pathogenesis-related protein PR-10 of white lupin suggests that these proteins could in part be responsible for some of the reported cross-reactivities in peanut-allergic individuals (Guarneri et al., 2005).

A large study performed in France and Belgium showed that 14.5 % of adults and 17 % of children with peanut allergy had cross-sensitisation with lupin (Gayraud et al., 2009). A study performed in the UK showed that sensitisation to lupin was observed significantly more often in peanut-allergic children and teenagers (34 %) than in non-peanut-allergic patients (4 %) (Shaw et al., 2008).

Cross-reactivity to lupin in peanut-allergic patients is of clinical relevance. In a study of 24 peanut-allergic subjects, 11 (44 %) had positive SPTs to lupin flour and seven out of the eight subjects who underwent a DBPCFC with lupin flour reacted, indicating clinical cross-reactivity between peanut and lupin (Moneret-Vautrin et al., 1999). In a study of 23 peanut-allergic patients who underwent a DBPCFC with lupin flour, 15 (68 %) showed clinical reactions (Leduc et al., 2002). In another study, sensitisation to lupin was found in 82 % of 39 patients allergic to peanut, of whom 35 % showed clinically relevant symptoms after challenge (Peeters et al., 2009).

18.4.1.2. Soybean

Ara h 1 and Ara h 2 cross-react *in vitro* with soybean allergens. In five children with a history of anaphylactic reactions to peanuts, IgE binding to Ara h 1 and Ara h 2 decreased by 79 % and 76 %, respectively, after pre-incubation of the sera with soy extract (Eigenmann et al., 1996; Burks et al., 1998). The soybean glycinin G1 acidic chain shares IgE epitopes similarity with peanut Ara h 3 (Beardslee et al., 2000). However, the prevalence of clinical reactions to soy in peanut-allergic patients is low, < 10 % (Sicherer, 2002). The report from Sweden in 1999 raised concern over soy allergy in peanut-allergic individuals. Four fatalities were reported due to ingestion of foods containing a low concentration of soy in asthmatic patients severely allergic to peanut with no previously known allergy to soy (Foucard and Malmheden Yman, 1999).

18.4.1.3. Peas

A study described three patients with a history of severe allergic reactions after ingestion of pea who had peanut-related symptoms, in one case confirmed by DBP

CFC (Wensing et al., 2003). These patients reacted to the peanut major allergen Ara h 1 and to the pea allergen vicilin. The immunoblotting inhibition experiments demonstrated that pea was probably the first sensitiser, as IgE binding to peanut was inhibited by pea but IgE binding to pea was not, or only partially, inhibited by peanut.

18.4.2. Cross-reactivity between peanuts and tree nuts

It is estimated that 20 to 50 % of peanut-allergic patients are also allergic to tree nuts (Ewan, 1996; Sicherer et al., 2003; Glaspole et al., 2011).

Correlation between serum levels of peanut-specific IgE and hazelnut, Brazil nut as well as almond-specific IgE in peanut-allergic children suggests that cross-reactive immune responses underlie co-allergy to peanut and tree nuts (Glaspole et al., 2011). Peanut-specific IgE antibodies that cross-react with tree nut allergens and may contribute to the manifestation of tree nut allergy in peanut-allergic subjects have been identified (de Leon et al., 2005). The structurally related cross-reactivity between Ara h 3 and tree nut allergens such as Jug r 4 of walnut, Cor a 9 of hazelnut, or Ana o 2 of cashew nut appears to be unrelated to the botanical origin of the allergens and suggests that individuals allergic to peanut should avoid tree nuts, unless the allergy status in relation to all other nuts is clarified (Barre et al., 2007; Ball et al., 2011). The major peanut allergen, Ara h 2, shares common IgE binding epitopes with almond and Brazil nut allergens, which may also contribute to the high incidence of tree nut sensitisation in peanut-allergic individuals (de Leon et al., 2007).

SPT/RAST cross-reactivity between peanut and tree nuts does not imply the occurrence of clinical cross-reactivity. In a study performed in the UK over a 5-year period from 2006, 145 children diagnosed as peanut or tree nut allergic were challenged (Ball et al., 2011). In those with peanut allergy challenged with tree nuts, none of the 72 with negative SPTs to tree nuts reacted on challenge, whilst only 7 of 22 (31.2 %) with positive SPTs did.

18.5. Effects of food processing on allergenicity

18.5.1. Thermal processing

Heat treatments may enhance peanut allergenicity by 90-fold. It has been documented that roasting increases the allergenicity of peanuts more than frying or boiling because of dry heating at high temperature (Maleki et al., 2000; Beyer et al., 2001). Ara h 1 and Ara h 2 increase their IgE binding capacity after roasting owing to structural modifications or functional alterations, although the exact mechanisms are unknown (Maleki et al., 2000). After roasting, Ara h 1 forms highly stable trimers by intermolecular cross-linking, while Ara h 2 forms intramolecular cross-links without forming higher order structures. Ara h 2 functions as a weak trypsin inhibitor, the activity of which increases approximately 3.5-fold after roasting (Maleki et al., 2003). In addition to being more resistant to trypsin digestion itself, Ara h 2 was found to protect Ara h 1 from degradation by trypsin.

The IgE binding capacity of whole peanut protein extracts prepared from boiled peanuts was 2-fold lower than that of extracts prepared from raw or roasted peanuts, as shown using sera of 37 peanut-allergic patients (Mondoulet et al., 2005). The IgE binding capacity of purified Ara h 1 and Ara h 2 prepared from roasted peanuts was also higher than that of their counterparts prepared from raw or boiled peanuts, whereas the IgE binding capacity of purified Ara h 1 and Ara h 2 was particularly increased by roasting. The decrease in allergenicity of boiled peanuts seems to result mainly from a transfer of low molecular weight allergens into the water during cooking. Boiling (100 °C for 15 minutes) resulted in partial loss of Ara h 1 secondary structure and formation of rod-like branched aggregates with reduced IgE-binding capacity and impaired ability to induce mediator release (Blanc et al., 2011), whereas roasted Ara h 1 retained the IgE-binding capacity of the native protein.

The fact that peanuts are most commonly eaten after roasting in the USA could explain the higher prevalence of peanut allergy in this population as compared with China, where peanuts are eaten after frying or boiling (Beyer et al., 2001).

18.5.2. Enzymatic hydrolysis

Hydrolysis of roasted peanut protein extract with proteases decreased the IgE-binding capacity of the soluble protein fraction, with a higher effect of the endoprotease alcalase as compared with that of the exoprotease flavourzyme (Cabanillas et al., 2012). Hydrolysis of peanut flour extracts with alcalase, pepsin, or flavourzyme reduced its IgE-binding capacity. However, the IgE-binding capacity during hydrolysis was retained, suggesting that such hydrolysates are not necessarily less allergenic (Shi et al., 2013).

18.5.3. High-pressure processing

The effect of pressure treatment was investigated on a mixture of Ara h 2 and Ara h 6 (Johnson et al., 2010). The structure of these allergens remained practically unchanged after 700 MPa treatments at 20 °C and 80 °C. Another study showed that dynamic high-pressure microfluidisation treatment changed the secondary structure of Ara h 2 and decreased its IgE-binding capacity (Hu et al., 2011).

18.5.4. Preservation treatments

18.5.4.1. Effect of pH

One study aimed at determining the effects of various pH conditions on the IgE-binding capacity of major peanut allergens (Kim et al., 2012). The IgE-binding capacity of Ara h 1, Ara h 2 and Ara h 3 was significantly reduced after treatment with acetic acid (pH 1.0) or commercial vinegar (pH 2.3), whereas there was no substantial change at pH 3.0 and 5.0 when compared with raw peanuts.

18.5.4.2. Other preservation treatments

Pulsed UV radiation appeared to be effective in reducing the IgE-binding capacity of peanut extracts and liquid peanut butter (Chung SY et al., 2008). The γ-irradiation (1–10 kGy) induced significant

changes in the secondary and tertiary structures of purified Ara h 6, and the IgE-binding capacity of purified Ara h 6 and of whole peanut protein extract were reduced upon increasing the irradiation doses (Luo et al., 2013). However, in another study, γ-irradiation alone was not associated with any change in peanut allergenicity, whereas boiling followed by γ-irradiation significantly reduced the IgE-binding capacity of peanut (Kasera et al., 2012).

18.5.5. Multiple treatments

The effects of ultrasounds, enzyme concentration and enzyme treatment time on the concentrations and IgE-binding capacity of two major allergenic proteins (Ara h 1 and Ara h 2) of roasted peanut kernels have been studied (Li et al., 2013). The ultrasound treatment followed by protease digestion of peanuts significantly decreased the concentrations of Ara h 1 and Ara h 2. The sequential treatment of peanuts by ultrasonication-trypsin-α-chymotrypsin resulted in maximum reductions of Ara h 1/Ara h 2 concentrations, and lowest IgE-binding. Instant controlled pressure drop combining heat and steam pressure (temperature up to 170 °C with a pressure of 6 bar for 3 minutes) was associated with a decreased IgE-binding capacity of peanut extracts, with a higher effect on roasted peanut proteins than on raw peanuts (Cuadrado et al., 2011).

18.5.6. Peanut oil

Peanut oil is a common ingredient of some foods and cosmetics. While it is possible to minimise overt contact with peanuts, peanut oil is more difficult to detect and avoid.

Case reports of allergic reactions in infants fed infant formulas containing peanut oil (Fries, 1982; Moneret-Vautrin et al., 1991), as well as a flare up of dermatitis induced by oral challenge with peanut oil in infants with atopic dermatitis (Moneret-Vautrin et al., 1994), aroused the suspicion that residual allergenic proteins could be contained in peanut oil.

The protein content of crude peanut oil is in the range of 100 to 300 μg/mL, about 100 times higher than in fully refined peanut oils, which are subjected to physical and chemical methods of purification, including degumming, refining, bleaching, and deodorisation (Crevel et al., 2000). However, refined peanut oil may contain sufficient peanut allergenic proteins to elicit a reaction in highly sensitive individuals (Olszewski et al., 1998). One of these proteins was recognised by IgE antibodies from a population of 11 peanut-allergic patients, four of whom reacted to commercially available refined peanut oils from the European market during a DBPCFC. The allergen had a molecular weight of 18 kDa and an isoelectric point of 4.5 similar to those of the major peanut allergen Ara h 2. The allergenicity of crude and refined peanut oils was tested by double-blind, crossover food challenge in 60 subjects with proved allergy to peanuts (Hourihane et al., 1997). Crude peanut oil caused allergic reactions in 10 % of the allergic subjects studied, while refined peanut oil did not trigger allergic reactions in any. Indeed, allergenicity seems to be confined mostly to unrefined, cold-pressed peanut oils (Moneret-Vautrin and Kanny, 2004).

In addition, the two oleosins Ara h 10 and Ara h 11 identified in 2010 may play a role in the allergenicity of peanut oil, although no data are available regarding their contribution to the occurrence of allergic reactions to crude or fully refined peanut oil.

Peanut-allergic individuals are usually cautioned to avoid crude peanut oil because of traces of peanut protein in the oil. However, the risk of severe adverse reactions to highly refined peanut oils seems to be low, although it cannot be ruled out in every highly sensitive peanut-allergic individual.

18.6. Detection of allergens and allergenic ingredients in food

18.6.1. Immunological methods

18.6.1.1. ELISA

Many ELISA methods for the detection and quantification of peanut in foods are commercially available (Fielder et al., 2010; Zeleny and Schimmel, 2010). Some detect total soluble proteins or a mixture of proteins, whereas others are designed to target a specific peanut allergen (Ara h 1 or Ara h 2) (Schmitt et al., 2004). Available ELISA methods differ in the extraction buffer (usually from neutral to slightly alkaline), the sample treatment, the format (sandwich or competitive ELISA), the antibodies used (monoclonal or polyclonal, different host animals), the detection system (enzyme, substrate, secondary antibody) and the calibrant (whole peanut extract or a specific peanut allergen). Such differences lead to high between-kit variations in the absence of certified reference materials (Westphal et al., 2004; Scaravelli et al., 2009; Khuda et al., 2012a). For example, five ELISA kits were used for the detection of peanuts in incurred sugar cookies, which detected concentrations of peanut proteins ranging from 11 to 101.8 % of the incurred levels, depending on the kit (Khuda et al., 2012b). A commercial rapid sandwich ELISA based on polyclonal antibodies with a LOD of 1.5 mg/kg, covering a range of 2.5 to 20 mg/kg, received an AOAC certificate (Immer et al., 2004). The reported LODs for ELISA kits available on the market range from 0.1 mg/kg for Ara h 1, to 0.5 mg/kg for Ara h 2 and 2.5 mg/kg for peanut proteins. The LOQs vary in the range of 1 to 20 and 1 to 15 mg/kg for Ara h 1 and Ara h 2, respectively, and of 3.3 to 90 mg/kg for peanut proteins (Fielder et al., 2010).

The ELISA methods suffer from matrix effects, especially for the detection of peanut allergens in chocolate, from which recovery is problematic (Koch et al., 2003; Poms et al., 2003), as confirmed in a comparison study of four commercial ELISA systems (Hurst et al., 2002). Thermal processing also negatively affects the detection of peanut allergens by commercial ELISA kits, owing to heat-induced changes in the solubility and immunoreactivity of the target proteins (Koch et al., 2003; Park et al., 2005; Poms et al., 2005; Whitaker et al., 2005). Raw peanuts exhibited 3 to 4 times higher responses than oil-roasted peanuts (Koch et al., 2003). Five ELISA kits were evaluated for detecting and quantifying peanut allergens in biscuits and dark chocolate in an inter-laboratory study (Poms et al., 2005). Although all kits performed well in the 5 to 10 mg/kg range, they were dependent on the type of processing and working conditions. Similar results were obtained in another inter-laboratory study evaluating the performance of three ELISA kits on different spiked foods, with a LOD of 5 mg/kg and good sensitivity and specificity (Park et al., 2005). Two commercial ELISA kits for the quantification of proteins in peanut flours, which were subjected to either moist or dry-heat treatments, underestimated the amount of proteins in samples heated at high temperature, in particular the ELISA kit targeting the thermolabile Ara h 1 (Fu and Maks, 2013). Boiling of the incurred peanut flour sample or autoclaving resulted in a decrease of 50 % in the amount of protein extracted, whereas dry-heat treatments induced a decrease in protein solubility as well as binding affinity, but at much higher temperature (> 176 °C).

A new ELISA format based on antibody–dendrimer conjugated magnetic microparticles for the detection of Ara h 3/4 has been described (Speroni et al., 2010). Allergens captured by the magnetic particles are harvested on a magnet, washed, and quantified with a LOD of 0.2 mg peanuts/kg food.

Multi-allergen immunoassays have been developed for the detection of several allergenic proteins including peanut. One assay was applied to chocolate samples with a LOD < 1 mg/kg (Ben Rejeb et al., 2005).

The peanut test material IRMM-481, which contains five peanut varieties from different geographic origins exposed to five different heat treatments, is used as certified reference material for peanuts (Trucksess et al., 2004; Westphal et al., 2004). All kits should report the calibration method and specify which allergenic protein is the target. In case of protein detection, whether the method targets

the total protein or the soluble protein, the relative method of detection should be specified. Conversion factors to calculate the amount of peanut from protein concentrations are only approximately known.

18.6.1.2. Dipsticks and lateral flow devices

A number of fast qualitative methods (dipsticks and LFDs) to detect peanuts for screening purposes are commercially available. Two dipstick-type sandwich ELISAs allow detection of about 10 ng/mL of peanut and hazelnut, corresponding to 1 mg of protein/kg of food (Stephan et al., 2002).

LFDs are based on immune-chromatographic principles and can be in the sandwich or the competitive format. A competitive liposome-based lateral flow assay for detecting Ara h 1 had a calculated LOD of 0.45 µg/mL and a visually determined detection range from 1 to 10 µg/mL (Wen et al., 2005). Two commercial peanut LFDs were tested on cookies in an inter-laboratory trial (van Hengel et al., 2006), with a performance comparable to the ELISA kit (LOD 5 mg/kg). Two commercial LFDs for peanuts also showed comparable and satisfactory specificity and a sensitivity at a level of 3.5 mg/kg in chocolate and cookies (Röder et al., 2009). The buffer used for extraction appears to play a major role on peanut protein detectability by LFDs, particularly when analysing highly processed foods (e.g. dark roasted peanuts). Some buffers are incompatible with LFD performance because of extraction inefficiency or signal inhibition (Rudolf et al., 2012).

18.6.1.3. Biosensors

An electrochemical impedance biosensor for the detection of peanut allergen Ara h 1 has been developed using a gold substrate on which an antibody film has been immobilised (Huang et al., 2008). The LOD was estimated to be < 0.3 nM. A nanobead enhanced optical fibre SPR biosensor was also prepared for the detection of Ara h 1 and compared with a label-free prism based SPR assay and to a commercial ELISA (Pollet et al., 2011). Antibody-linked nanobeads greatly amplify the fibre optic SPR signals from 9 µg/mL to 0.09 µg/mL. The nanobeads enhanced assay had a LOD of 0.1 µg/mL, comparable to the ELISA kit.

18.6.2. Mass spectrometry

MS for the detection of peanut allergens is most often used with the "bottom up" strategy, according to which the extracted protein is digested with enzymes (trypsin in most cases) and several peptides are selected as specific markers for the allergen, separated by HPLC and identified by MS/MS. MS was also used as a confirmatory method for the presence of a specific allergen, such as Ara h 1, in a model food matrix using HPLC/MS/MS (Shefcheck and Musser, 2004). The method was applied for the detection of Ara h 1 in ice cream samples, allowing detection levels as low as 10 mg/kg of the protein. The method was improved and applied to dark chocolate (Shefcheck et al., 2006). Two peptides were chosen as biomarkers of Ara h 1. The pre-extraction digestion led to better results than the post-extraction digestion. The LOD could be reduced to 2 mg/kg by using a QpQ and multiple reaction monitoring (MRM).

Another confirmatory method is the nano-electrospray Q-TOF MS/MS combined with capillary LC, which allowed the detection of a high number of peptides derived from the three peanut allergens Ara h 1, Ara h 2 and Ara h 3 in raw and processed peanuts (Chassaigne et al., 2007). Five peptides, which were stable to roasting, were selected as markers for the three proteins. Roasting affected the LOD of the method for the peptide ions measured in the multiple ion monitoring (MIM) mode. The absolute LOD was set at 7 ng of the protein used for tryptic digestion for raw peanuts, and at 10 ng for mild-roasted peanut extract, whereas the absolute LOD was 40 ng of protein for strong-roasted peanut.

A similar but quantitative method based on LC-ESI-QqQ-MS/MS for the detection of Ara h 2 and Ara h 3/4 was also developed (Careri M. et al., 2007). Four peptides were chosen as specific biomarkers for the two proteins. When applied to spiked rice-crispy and chocolate snacks, the method showed a LOD for Ara h 2 of 5 mg/kg and for Ara h 3/4 of 1 mg/kg.

One paper (Pedreschi et al., 2012) discusses the difficulties encountered when detecting Ara h 1, Ara h 2 and Ara h 3 by MS in different food matrices and the ambiguities observed in some publications. The authors analysed a well characterised processed food matrix (incurred cookies with IRMM-481f) by MS. After enrichment using a commercial kit and digestion, two biomarker peptides from Ara h 3/4 were selected by shotgun proteomics and analysed by nano-LC-ESI-Q-TOF-MS/MS via SRM. The LOD was about 10 mg peanut/kg matrix.

18.6.2.1. Multiplex mass spectrometry methods

A LC-LIT-MS/MS method for the simultaneous detection and quantification of five allergens including Ara h 3/4 in a single short run is available (Bignardi et al., 2010). Sensitivity was significantly improved for all allergens by performing a preliminary clean up step using size-exclusion chromatography before the enzymatic digestion of the proteins. The method is based on the detection of selected specific marker peptides for every target protein. The LOD was 0.1 mg/kg for biscuits and 7 mg/kg for chocolate, with corresponding LOQs of 0.3 mg/kg and 25 mg/kg, respectively (Bignardi et al., 2013).

A multi-method for the detection of seven allergens, including peanut, based on LC and QpQ-MS/MS in MRM mode is available (Heick et al., 2011a). On the basis of selected marker peptides for every allergen it was possible to detect the seven allergens also in incurred food samples with a LOD of 10 mg/kg for peanut.

18.6.3. Immunological methods coupled to mass spectrometry

A non-competitive sandwich ELISA combined with inductively coupled plasma-MS (ICP-MS) was constructed (Careri M. et al., 2007) using polyclonal antibodies as capture reagents and monoclonal antibodies anti-Ara h 1 and anti-Ara h 3/4 for identification (Ab I). Rabbit anti-mouse polyclonal antibodies labelled with europium (Ab II) were used for detection. The LOD for peanut in food was 2 mg/kg.

An antibody magnetic support was developed for enriched extraction of the Ara h 3/4 allergen from food. After a microwave-assisted tryptic digestion of the protein, LC-ESI-IT-MS/MS was used to identify the specific Ara h 3/4 peptide biomarkers. The LOD and LOQ obtained on breakfast cereals were 3 and 10 mg peanuts/kg matrix, respectively (Careri et al., 2008).

18.6.4. DNA-based methods

Real-time PCR methods based on the DNA sequence of the peanut allergen gene Ara h 2 were developed using the TaqMan technology (Hird et al., 2003; Stephan and Vieths, 2004). Seven methods for DNA extraction were examined by Hird with an internal positive control (IPC) kit to have an indication of the amount of PCR inhibitors co-extracted with the DNA. The Hird assay was used to detect peanut in spiked commercial foods and in biscuits baked with 2 mg/kg of roasted peanut powder prepared for the Central Science Laboratory Food Analysis Proficiency Assessment Scheme (FAPAS) food allergen program. The Stephan and Vieths assay is specific and suitable to detect peanut in processed foods with a LOD of < 10 mg/kg.

Commercial PCR kits are also available in the format of real-time PCR, PCR-ELISA and an end-point PCR followed by gel electrophoresis, with a LOD of 10 mg/kg.

Three real-time PCR assays were developed targeting the Ara h 3 gene of peanut (Scaravelli et al., 2008), which are capable of detecting 2.5 pg peanut DNA, corresponding to less than one copy of genomic DNA. The method is quantitative and, when applied to model food samples with a precise peanut content, was able to detect 10 mg/kg peanut. A new version of this method based on single-tube nested PCR was proposed (Bergerová et al., 2011), which is more sensitive when applied to DNA extracted from peanut leaves (LOD of 0.375 pg and LOQ of 0.76 pg DNA), but less sensitive when applied to raw or roasted peanuts (LOD of 31.25 pg DNA).

18.6.4.1. DNA biosensors

An electrochemical DNA biosensor was developed for detecting Ara h 1 (Sun et al., 2012). A stem–loop probe was linked to a gold electrode. Hybridisation to the complementary DNA gave rise to electron-transfer efficiency changes between probe and electrode, as proved by electrochemical impedance spectroscopy (EIS). The detection limit was 0.35 fM. When applied to a peanut milk beverage, the LOD was 3.2×10^{-13} M.

18.6.4.2. Comparison between ELISA and PCR technologies for detecting peanuts in food products

The performance of ELISA and PCR technologies for detecting peanut in food products have been compared in a number of studies (Stephan and Vieths, 2004; Watanabe et al., 2006). Results were generally qualitative owing to the lack of a common reference material. One study (Scaravelli et al., 2009) reported the comparison between two ELISA kits and three real-time PCR methods and all data were normalised to the IRMM-481 peanut test material. Results were similar with both methods, despite a high variability observed between the two ELISA kits and the lower variability among the PCR methods. The ELISA and PCR methods were tested in the analysis of a model food matrix (cookies) to which known amounts of peanut were added before processing. The roasting processes greatly reduced the detectability of both methods according to the baking time.

18.7. Minimum (observed) eliciting doses

Case reports of allergic reactions in peanut-allergic patients after accidental ingestion of foods containing peanuts show that even traces of peanut proteins can trigger severe allergic reactions in these subjects. Data from the US Peanut and Tree Nut Allergy Registry show that most reactions to peanut occurring in restaurants were triggered by foods which contained peanut as an ingredient that could not be identified by patients (e.g. in sauces, dressings, egg rolls). The most common source of exposure was desserts (43 %), followed by entrees (35 %) and appetisers (13 %) (Furlong et al., 2001).

Different types of studies (i.e. diagnostic series, threshold-finding studies and immunotherapy trials) have reported on MED following challenge studies in adults and children combined (Oppenheimer et al., 1992; Leung et al., 2003; Lewis et al., 2005; Anagnostou et al., 2009), mostly in adults (Atkins et al., 1985; Hourihane et al., 1997; Nelson et al., 1997; Patriarca et al., 2006) (Wensing Marjolein et al., 2002), and mostly in children (Flinterman et al., 2006a; Clark et al., 2008; Clark et al., 2009; Blumchen et al., 2010; Nicolaou et al., 2010; Taylor et al., 2010; Wainstein et al., 2010; Blom et al., 2013). Studies are variable in size, challenge protocol used and type of food tested (Taylor et al., 2014). The total number of patients showing objective reactions during the oral challenge in a given study ranged from one to 283. The lowest MOEDs also varied widely among studies, ranging from 0.1 to 1,637 mg of total peanut protein (Remington, 2013).

Four studies (Hourihane et al., 1997; Wensing Marjolein et al., 2002; Lewis et al., 2005; Flinterman et al., 2006a) were specifically designed to assess LOAEL doses and provide accurate information on the doses tested (Taylor et al., 2009b).

Fourteen adult subjects proven by challenge to be allergic to peanut were randomised to receive varying doses of peanut protein administered as peanut flour in a DBPCFC (Hourihane et al., 1997). The challenge started with a dose of 10 µg of peanut protein (21.63 µg of flour), and increased stepwise thereafter to 20 µg, 50 µg, 100 µg, 250 µg, 500 µg, 1 mg, 2 mg, 5 mg, 10 mg, 20 mg, up to a maximum dose of 50 mg (108.15 mg of flour). One subject had a systemic reaction to 5 mg of peanut protein, and two subjects had mild objective reactions to 2 mg and 50 mg of peanut protein, respectively. Five subjects had mild subjective reactions (one to 5 mg and four to 50 mg). All subjects with convincing objective reactions had short-lived subjective reactions to preceding doses, as low as 100 µg in two cases. Five subjects did not react to any dose up to 50 mg.

In another DBPCFC, 26 adult patients with a convincing history of peanut-related allergic reactions, a specific IgE level ≥ 0.7 kU/L, or a positive SPT of ≥ 2 mm to peanut were challenged with varying

doses of peanut protein provided as roasted peanut meal (Wensing Marjolein et al., 2002). Ten doses of peanut protein (30 µg, 100 µg, 300 µg, 1 mg, 3 mg, 10 mg, 30 mg, 100 mg, 300 mg and 1 g) were tested in two separate challenges. The first challenge consisted of the seven lowest doses (30 µg-30 mg). Patients who did not react during this challenge were asked to participate in a second challenge with two overlapping doses (10 and 30 mg) and three higher doses (100 mg, 300 mg, and 1 g). All patients reported oral symptoms (n = 26), of which 14 reported prior subjective gastrointestinal symptoms (n = 14) and other symptoms were observed in five subjects. Reactions started within 30 minutes after ingestion of peanut, but in two patients additional symptoms were delayed by one to two hours. Doses eliciting allergic reactions ranged from a dose of 100 µg up to 1 g of peanut protein. Fifty per cent of the study population had an allergic reaction after ingestion of 3 mg of peanut protein. Patients with severe reactions had lower threshold doses compared with those patients with mild reactions.

Forty peanut-allergic patients older than six years of age were recruited who had a convincing clinical history of an allergic reaction to peanut in the last 3 years before challenge and a SPT for peanut of ≥ 6 mm, in the presence of a negative control (saline), and at least a 3 mm wheal to histamine 1:10 w/v (Berg et al., 2008). In a DBPCFC, subjects received doses of peanut protein of 1, 2, 5, 10, 20, 50, 100, 250, 500 mg, and 1, 2, 4 g as roasted and partially defatted peanut flour. Doses were given between 15 and 30 minutes apart. Eliciting doses varied between 1 mg and 2 g of peanut protein.

A total of 27 children older than 3.5 years sensitised to peanut and on a peanut elimination diet were evaluated by specific IgE measurements, SPT, and DBPCFC (Flinterman et al., 2006a). Nine doses of defatted light roasted peanut flour (10 µg, 100 µg, 500 µg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g and 3 g), which contain about 50 % of peanut protein, were tested at intervals between 15 and 30 minutes. All children tolerated a dose of 1 mg peanut flour, which corresponds to 2 mg peanut. The lowest eliciting dose was 10 mg (n = 2), causing OAS. The eliciting dose for subjective reactions ranged from 10 mg to 3 g of peanut flour, and was significantly lower than that for objective reactions (from 100 mg to 3 g).

Minimum doses reported to elicit objective reactions in peanut-allergic individuals are variable depending on the study population, challenge protocol and food matrix tested. The lowest reported MOED in peanut-allergic patients undergoing DBPCFC was 100 µg of peanut protein, with a NOAEL of 30 µg. Doses of peanut protein inducing OAS in other studies were above that level. Few data are available on the doses that may trigger allergic reactions in patients with a history of severe allergic reactions, since they have often been excluded from oral challenge tests.

18.8. Conclusion

Peanut is a common cause of allergic reactions, which can be severe or even fatal. Prevalence of well documented peanut allergy in Europe varies between 0.1 to 1.8 % depending on the age and country of origin. The available data do not allow concluding on whether the prevalence of peanut allergy has changed in the last years in Europe. The major peanut allergens are well characterised. Roasting may increase the IgE-binding capacity of peanut allergens, whereas boiling may decrease it or leave it unchanged. ELISAs are sometimes unsuitable for the detection of peanut allergens in processed foods. MS and PCR technologies can be used as alternative or complementary methods. The lowest reported MOED in peanut-allergic patients undergoing DBPCFC was 100 µg of peanut protein, with a NOAEL of 30 µg. Few data are available on the doses that may trigger allergic reactions in patients with a history of severe allergic reactions, since they have often been excluded from oral challenge tests.

19. Allergy to soy

19.1. Background

Soy (soybean) (*Glycine max*) is an edible legume belonging to the *Fabaceae* family. The seed contains around 20 % oil and 38 to 40 % protein. Consumption of soy, widespread in Asia and the USA, has increased in Europe during the past years particularly. In vegetarian cuisine soy is consumed as soy

oil, soy flour, soymilk, soy drinks, soy flakes or as fermented soybean products such as miso, okara, soy sauce (Tamari, Shoyu), tempeh or tofu. Soy products are also used in the food industry for technological reasons as texturisers, emulsifiers and protein fillers. As soy is a good and cheap protein source, it may be part of a wide variety of processed foods such as meat products, sausages, bakery goods, chocolate or breakfast cereals (Ballmer-Weber and Vieths, 2008).

Soy is widely consumed also by children. Soy-based formulas were introduced in infant nutrition more than 100 years ago (Katz et al., 2014) and are currently used for the treatment of cow's milk allergy (CMA), lactose and galactose intolerance, among other conditions. However, soy and soy protein-based formulas (SPFs) can induce IgE and non-IgE-mediated food allergy.

The clinical manifestations of soy allergy are similar to those of CMA, ranging from enterocolitis and food protein-induced enterocolitis syndrome (FPIES) (Sicherer, 2005), which are generally not associated with detectable specific IgE antibodies, to atopic dermatitis and IgE-mediated systemic reactions (anaphylaxis).

19.2. Epidemiology

19.2.1. Prevalence

19.2.1.1. Europe

There are 15 studies conducted in Denmark, Germany, Hungary, Iceland, Sweden, The Netherlands and the United Kingdom between 1994 to 2008, which report on the prevalence of soy allergy in unselected European populations (University of Portsmouth, 2013). Ten studies aimed to assess both IgE and non-IgE mediated allergy whereas five focused on IgE-mediated allergy only. All ages were included.

The highest prevalence (0.8–1.2 %) of self-reported soy allergy has been recorded among four- and eight-year-old children in Sweden (Ostblom et al., 2008b; Ostblom et al., 2008a). Self-reported prevalence in one- and two-year-old children in Sweden (Ostblom et al., 2008b) and in other European countries at all ages were ≤ 0.6 % (Young et al., 1994; Brugman et al., 1998; Emmett et al., 1999; Kristjansson et al., 1999; Schafer et al., 2001).

Sensitisation rates assessed by positive SPTs among adults were 7.3 to 8.3 % in Hungary (Bakos et al., 2006) and 1.7 % in Germany (Schafer et al., 2001), but only 0.3 and 0.2 % among UK children of four (Arshad et al., 2001) and eight (Roberts et al., 2005) years of age. Rates of sensitisation based on IgE levels were between 2.1 % and 3.7 % in adults and children in the three geographical areas (Sweden, Hungary and Greenland) for which studies were available (Bjornsson et al., 1996; Krause et al., 2002; Bakos et al., 2006; Ostblom et al., 2008b). When a convincing history was combined with sensitisation, prevalence of soy allergy was zero in 18-month-olds (Kristjansson et al., 1999) and 1.6 % in four-year-olds (Ostblom et al., 2008a) in Sweden.

The only study which assessed soy allergy using either OFC (in subjects younger than three years) or DBPCFCs found a zero prevalence in a large sample (1 272) of Danish children and adults (Osterballe et al., 2005; Osterballe et al., 2009).

19.2.1.2. Outside Europe

In the USA, prevalence of self-reported soy allergy was 2.7 % in children up to three years of age (Bock, 1987) and ranged between 0.1 % and 1.8 % in adults (Vierk et al., 2007; Greenhawt et al., 2009). Soy allergy was reported by < 0.3 % of the children and adults in other parts of the World, including Canada. No studies assessing prevalence of soy allergy using food challenges are available.

19.2.2. Natural history

Most soy-allergic subjects outgrow their allergy. In a retrospective analysis of data in 133 children with soy allergy (88 % of which with concomitant peanut allergy) recruited at a median age of one year and followed up for a median of five years predicted a resolution of soy allergy in 25 % of children at four years, in 45 % at six years, and in about 70 % at 10 years. Absolute soy-specific IgE levels were useful predictors of outgrowing soy allergy (Savage et al., 2010). By age six years, subjects with a peak soy-specific IgE level < 10 kUA/L had > 50 % chance of outgrowing their allergy, whereas peak levels > 50 kUA/L suggested < 20 % chance of tolerance development. Although soy allergy usually manifests early in life, the study identified a subset of patients in which allergy symptoms started after tolerating soy in their diet. It has been hypothesised that such late onset of soy allergy may be related to either birch pollen cross-reactivity or persistent peanut allergy, as indicated by high peanut-specific IgE levels at their last follow-up (Savage et al., 2010).

The prevalence of soy sensitisation progressively increased from 2 % at 2 years to 7 % at 10 years of age in the German Multi-Centre Allergy Study, in which 1 314 children were followed up from birth to 13 years (Matricardi et al., 2008). In patients with soy-induced FPIES, tolerance usually develops within three years of life (Nowak-Wegrzyn and Muraro, 2009), although the rate of tolerance development varies between studies and populations. Occasionally, FPIES may persist into the teenage years. Earlier reports suggested that, by two years of age, 20 % of soy-induced FPIES resolves (Sicherer, 2005). However, a study in 23 Korean infants with FPIES found that 92 % of them tolerated soy at age 10 months (Hwang et al., 2009).

19.2.3. Time trends

There are no studies available, which allow investigating time trends in soy allergy.

19.2.4. Severe reactions/anaphylaxis

Symptoms of soy allergy are generally mild. No severe allergic reactions to soy were reported by a research group in 13 years of experience with DBPCFCs (Sicherer et al., 2000b). However, severe gastrointestinal symptoms upon consumption of SPFs in infants and children and anaphylaxis following oral exposure to soy have also been reported. During a period of four years (1993–1996), a Swedish group (Foucard and Malmheden Yman, 1999) reported 61 cases of severe anaphylactic reactions from a national register. Peanut, tree nuts and soy were deemed to have caused 45/61 reactions. All four children who died from soy anaphylaxis were suffering from asthma and severe peanut allergy. Severe reactions occurred after initially mild symptoms and an almost symptom free interval of about one hour. The foods responsible for allergic reactions in soy-allergic patients were ice cream and hamburger (Host and Halken, 1990), and kebab and soy sauce (Schrander et al., 1993). Peanut-allergic subjects who reacted to kebab and hamburger were highly sensitised to peanut and had soy-specific IgE. However the nature of the study does not allow firm conclusions regarding the true trigger for these fatal reactions and hidden peanut exposure as trigger cannot be ruled out. Anaphylaxis and exercise-induced anaphylaxis to soy have also been reported by others (Pumphrey and Stanworth, 1996; Sicherer et al., 2000a; Adachi et al., 2009).

In a study on allergic reactions during in-patient OFCs, three (7 %) soy challenges required administration of adrenaline (Jarvinen and Chatchatee, 2009). Higher frequency of severe reactions (25 %), including throat or chest tightness, has been reported in patients with soy and birch pollen allergy during DBPCFCs with soy (Mittag et al., 2004b).

19.2.5. Factors affecting prevalence of soy allergy

Prevalence of soy allergy appears to be higher among subjects with atopic dermatitis than in the general population. In a study conducted in the USA, 21 out of 165 children with atopic dermatitis had a positive SPT to soy (13 %) and three (1.8 %) reacted to soy in a DBPCFC (Kattan et al., 2011). Two Italian studies (Giampietro et al., 1992; Magnolfi et al., 1996) report a positive RAST in 22 % and a positive SPT in 23 % of the 1075 food-allergic and atopic children investigated, of which only 3 %

and 6 % reacted in a DBPCFC or OFCs, respectively, representing 1.1 % of children referred for atopic disease. A higher prevalence of soy allergy has also been reported in delayed onset enterocolitis and enteropathy syndromes (Kattan et al., 2011), and in birch pollen and peanut-allergic subjects owing to cross-reactivities with soy allergens.

19.3. Identified allergens

Soybean contains approximately 38 % protein. At least 16 IgE-binding protein fractions of soy have been identified in the Allergome database. However, only eight soybean allergens appear in the IUIS database (Table 19).

The main storage proteins in soybean are glycinin (11S) and β-conglycinin (7S), which account for about 70 % of the total seed protein. β-Conglycinin is a trimeric glycoprotein of molecular weight 180 kDa, which consists of three sub-units, α, α′ and β, all N-glycosylated (Vu Huu and Shibasaki, 1978). Only the α-subunit is allergenic, although the α′- and β-subunits have 90.14 % and 76.2 % homology with it, respectively. Glycinin is a hexamer of molecular weight 360 kDa. Each subunit is composed of an acidic and a basic polypeptide linked by a disulphide bond (Staswick et al., 1981). The five subunits form three groups according to the combination of acid and basic peptides (Maruyama et al., 2004). Subunits Gy1 and Gy5 are considered main epitopes for this protein (Schiller et al., 2014).

Table 19: Soy (*Glycine max*) allergens

Allergen	Protein	Molecular weight [(a)]	Superfamily/family
Gly m 1	Hydrophobic protein	7	Hydrophobic seed protein
Gly m 2	Defensin	8	Defensin
Gly m 3	Profilin	14	Profilin
Gly m 4	PR-10 protein	17	Bet v 1 related protein
Gly m 5	β-Conglycinin (7S globulin, vicilin) Subunit α Subunit α′ Subunit β	 67 71 50	Cupin
Gly m 6	Glycinin (11S globulin, legumin) Subunit Gy1 Subunit Gy2 Subunit Gy3 Subunit Gy4 Subunit Gy5	 53.6 52.4 52.2 61.2 55.4	Cupin
Gly m 7	Seed biotinylated protein	76.2 [(b)]	
Gly m 8	2S Albumin	28	Prolamin

(a): Molecular weight (SDS-PAGE).
(b): kDa.

Other soy proteins have been characterised and proposed as allergens, including the thiol-protease Gly m Bd 30k (Ogawa et al., 1991; Helm et al., 1998; Helm et al., 2000), and the Kunitz trypsin inhibitor (Moroz and Yang, 1980; Gu et al., 2001).

Several authors report on *in vitro* IgE-binding studies in patients suffering from peanut or soy allergy. IgE-binding to Gly m 1 has been reported in > 90 % of patients (Djurtoft et al., 1991), to Gly m 4 in 86 % (Baur et al., 1996), and to Gly m 3 in 69 % (Rihs et al., 1999). Later studies reported IgE to Gly m 4 in 70 % to 100 % of soy-allergic patients (Mittag et al., 2004b; Ballmer-Weber et al., 2007; Fukutomi et al., 2012). IgE to Gly m 5 and 6 was detected in 5 to 67 % and 5 to 58 % of patients, respectively (Holzhauser et al., 2009; Ito et al., 2011; Fukutomi et al., 2012). The frequency of IgE to Gly m 5 and Gly m 6 was lower in adults than children (5 % vs. 67 % for Gly m 5 and 5 % vs. 58 % for Gly m 6, respectively) (Ito et al., 2011; Fukutomi et al., 2012). Children with primary, more severe

soybean allergy are usually sensitised to Gly m 5 and Gly m 6 (Fukutomi et al., 2012). However, raised levels of IgE against Gly m 5 and 6 were related to mild symptoms in adults, and higher levels of Gly m 4 were related to soy milk allergy (Klemans et al., 2013).

Soy components were also studied in relation to the severity of soy allergy. IgE to Gly m 5 and Gly m 6, which contains linear epitopes, was identified as a potential diagnostic marker for severe soy allergy (Holzhauser et al., 2009), whereas raised levels of IgE to Gly m 4, which contains a conformational epitope, were detected in patients with anaphylactic reactions to soy drinks (van Zuuren et al., 2010; Kosma et al., 2011). The use of soy-specific components for the diagnosis of soy allergy was investigated in case–control studies where controls were not suspected of being soy allergic (Ito et al., 2011; Vissers et al., 2011; De Swert et al., 2012; Fukutomi et al., 2012) and in a soy-allergic patients only (Mittag et al., 2004b; Ballmer-Weber et al., 2007) (Holzhauser et al., 2009; van Zuuren et al., 2010; Kosma et al., 2011). IgE to Gly m 8 had the best accuracy in diagnosing adult soy allergy, IgE to Gly m 5 and 6 was related to mild symptoms, and Gly m 4 to soy milk allergy (Klemans et al., 2013).

19.4. Cross-reactivities

Serological cross-reactivities against other legumes in soy-allergic individuals have been described in relation to peanut (70–90 %), green pea (~ 80 %), lima bean (~ 50 %), string bean (~ 40 %) (Bernhisel-Broadbent and Sampson, 1989; Bernhisel-Broadbent et al., 1989) and wheat flour in soybean-sensitised bakers (Baur et al., 1996), although these do not correlate with clinical cross-reactivities.

Immediate-type allergic reactions in patients with birch pollen allergy after consuming soy protein-containing food can result from cross-reactivity between Bet v 1 specific IgE and the homologous PR-10 protein SAM 22 Gly m 4 (Kleine-Tebbe et al., 2002; Holzhauser et al., 2009). In a study of 50 Bet v 1 allergic individuals (Treudler et al., 2008), eight reactions to soy protein were reported in subjects with high IgE levels against Gly m 4.

Clinical symptoms in peanut-allergic patients after soy intake are likely to result from cross-reactivity between Ara h 3 and Gly m 6 (11S globulin) (Beardslee et al., 2000). Peanut-allergic children (with or without anaphylaxis) had significantly higher IgE binding to Ara h 1–3 (peanut allergens) and Gly m 5–6 (soy allergens) than asymptomatic children sensitised to peanut (Hong et al., 2012).

Clinical cross-reactivity between peanut and soy is rare despite the high degree of cross-sensitisation based on IgE-binding and SPTs (Sicherer et al., 2000a; Kattan et al., 2011). Clinical co-reactivity to soy was reported in 1 % to 6.5 % of peanut-allergic individuals in placebo controlled studies (Burks et al., 1998). Of 140 peanut-allergic patients, 7 % were allergic to soy as determined from a combination of clinical history, serum IgE levels, SPT, and OFCs in another study (Green et al., 2007). Soy intake does not appear to be a risk factor for peanut allergy (Koplin et al., 2008).

Co-sensitisation to soy is common in patients with CMA, but clinical co-allergy is rare (Zeiger et al., 1999). Co-sensitisation without clinical reactivity to soy milk was noted in 17 % of patients with CMA (Osterballe et al., 2009). Several studies suggest that the majority of subjects with IgE-mediated CMA tolerate soy or soy formula, and that clinical reactions in subjects who do not tolerate soy are mainly non-IgE mediated (EFSA, 2004). The soybean Gly m Gy4 and Gly m 5 subunit α cross-react with casein (Rozenfeld et al., 2002; Curciarello et al., 2014). Out of 10 children with a positive milk challenge, six also had a positive soy challenge. There was a challenge order effect, which needs to be considered when designing and reporting food challenge studies (Niggemann and Beyer, 2007).

19.5. Possible effects of food processing on allergenicity and derived products

The effects of different processes on the allergenic potential of soy products, including lecithins and soybean oil, have been reviewed (Besler et al., 2001). Storage, heat treatments, fermentation and high

pressure processing (HPP) affect the IgE-binding activity of sera obtained from peanut and soy-allergic patients.

19.5.1. Heat treatments

Most exposures to heat between 80 to 120 °C for 60 minutes lead to a reduction in IgE-binding (Burks et al., 1991a; Burks et al., 1992b; L'Hocine and Boye, 2007). Combinations of heat and steam pressure, such as instant controlled pressure drop (DIC), were shown to decrease the IgE-binding to legumes, including soy proteins, proportionally to the increase in steam pressure and duration of treatment (Cuadrado et al., 2011). Conversely, heat treatment and storage was reported to increase allergenicity of soybean hull through the formation of two neoallergens (Codina et al., 1998), and thus the conditions and duration of thermal treatments may affect the allergenicity of soy products in different ways.

19.5.2. Fermentation

Natural or induced fermentation in soybean meals significantly reduced IgE-binding up to 89 %, in particular if the resulting proteins were < 20 kDa (Song et al., 2008). Yoghurts showed the lowest antigenic activity, followed by miso and tempeh. The lowest IgE-binding was observed with liquid fermentation of soybean flour (Frias et al., 2008). The extent of hydrolysis of soybean formulas (powder vs. liquid) may affect the outcome of challenge studies in children with enterocolitis. Out of the 43 children challenged with soy formula in one study (Burks et al., 1994), 14 (33 %) reacted to a powdered soy formula and 13 reacted to a liquid formula. Allergenicity was retained in a soy sauce, a fermented product containing both wheat and soy (Hefle et al., 2005).

19.5.3. High hydrostatic pressure

The application of HHP treatments (100–30 MPa for 15 minutes) to soy "whey", a by-product from the preparation of tofu, reduced the immunoreactivity of soy proteins towards antibodies against Gly m 1 (Peñas Elena et al., 2006). HHP treatment of soy seeds led to lower immunoreactivity of the resulting soy sprouts, probably owing to the increased susceptibility to enzymatic hydrolysis during germination (Peñas et al., 2011). The influence of HHP treatments on IgE-binding capacity of soy allergens has also been studied in soybean protein isolates (SPI) for infant formula (Li et al., 2012). The processing conditions (300 MPa for 15 minutes) significantly reduced immunoreactivity by 48.6 % compared with the untreated SPI, which was linked to structural modifications of the proteins.

19.5.4. Soy-derived products

19.5.4.1. Soy lecithin

Soy lecithins are used as stabilisers and emulsifiers in a wide range of foods, drugs and other industrial products. Soy lecithins are mostly obtained by hexane extraction during the manufacturing of soy oil. Crude lecithins are separated from the oils by degumming and their composition is variable. Lecithins are complex mixtures composed mainly by phospholipids, glycolipids and fatty acids (phospholipid complex), but they also contain residual proteins in variable amounts depending on the manufacturing process. Proteins present in lecithins may trigger allergic reactions in sensitive individuals (Palm et al., 1999).

Residual proteins have been determined in commercial lecithins in the range of 100 to 1 400 mg/kg (Gu et al., 2001; Martin-Hernandez et al., 2005). Soy lecithin was shown to contain a number of IgE-binding proteins (Gu et al., 2001), among which a methionine-rich protein (12 kDa) belonging to the 2S albumin family, the Kunitz trypsin inhibitor (20 kDa) and a protein of 39 kDa, attributed to the acidic subunits of glycinin (Müller et al., 1998). The SDS-PAGE protein pattern of the standard soy lecithin was very similar to that of soy flour (Martin-Hernandez et al., 2005). The MWs of the main proteins in soy lecithins and soy flour determined by MALDI-MS ranged from 10.5 to 52.2 kDa and were identified by ESI-MS/MS to belong to the 11S globulin fraction, corresponding to glycinin A acid subunits (35 kDa), glycinin B basic subunits (18–20 kDa) and glycinin A5 subunit (10 kDa). The

seed maturation protein P34 (32 kDa) from the 7S globulin fraction of soy proteins was also identified. An IgE-binding protein (16kDa) and weak bands (< 14 kDa) were also observed (Paschke et al., 2001).

19.5.4.2. Soybean oil

Soybean oil is used in cooking and food formulations. The presence of protein in soy oil depends on the degree of refining, as for other seed oils. Both cold-pressed and fully refined oils have been shown to contain proteins (0.35–0.78 mg/kg) (Hidalgo and Zamora, 2006). Although most publications suggest that refined oils do not induce allergic reactions in sensitive individuals (Bush et al., 1985; Crevel et al., 2000), an adverse reaction to soy oil in an infant has been reported (Moneret-Vautrin DA et al., 2002). IgE-binding proteins with MWs of 53 and 58 kDa were identified in three unrefined soybean oils (Paschke et al., 2001). A 56 kDa allergenic protein was also found in cold-pressed and deodorised soybean oils (Errahali et al., 2002), which was later (Errahali et al., 2004) identified as soybean β-amilase (7S globulin), together with the 20 kDa allergen Kunitz trypsin inhibitor (KTI). The protein profile of the cold-pressed soy oil was similar to that of soy flour, with seven bands in a wide molecular range (94–14 kDa) (Martin-Hernandez et al., 2008). The soy lecithin seed maturation protein P34 from the 7S globulin fraction (35 kDa) and β-amylase (56 kDa) were identified. There is a certain consensus that fully refined soybean oils are not allergenic (Taylor et al., 2004).

19.6. Detection of allergens in food

Many methods are available to detect allergens in soy products. A critical aspect is the extraction of proteins from soy lecithins and soy oils. Extraction with hexane-isopropanol-water was found most suitable to extract protein from lecithin (Martin-Hernandez et al., 2005) and acetone-hexane from oil (Martin-Hernandez et al., 2008).

19.6.1. Immunological methods

19.6.1.1. ELISA

Several ELISA methods for detection of soybean allergens are commercially available (Gatti and Ferrett, 2010). These are based on antibodies raised against native soybean proteins, against a single protein such as Gly m Bd 30 K/P34, β-conglycinin, glycinin, the KTI or the Bowman–Birk inhibitor (BBI), or against denatured/renatured soybean proteins.

ELISA methods for the detection of soy proteins in processed foods were thoroughly reviewed (Koppelman et al., 2004). In order to improve sensitivity, a competitive ELISA based on polyclonal antibodies which used preliminary extraction with a buffer at pH 12 was applied to soy ingredients and soy-containing foods processed in different ways. The LOD was 0.4 mg/kg and the LOQ was 1 mg/kg.

Polyclonal and monoclonal antibodies have been raised against β-conglycinin. Two competitive ELISA quantification of β-conglycinin in processed foods and seeds are available. One is based on a specific rabbit anti-β-conglycinin polyclonal antibody (Moriyama et al., 2005) and the second on a monoclonal antibody obtained by using a conjugated chicken ovalbumin with a synthetic peptide that corresponded to one epitope sequence of β-conglycinin as the immunogen (You et al., 2008). The LOD of the latter was 2.0 ng/mL. A sandwich ELISA for the determination of β-conglycinin in food has also been developed, with a LOD of 1.63 ng/mL (Hei et al., 2012).

A competitive ELISA based on the monoclonal antibody 4B2 against glycinin exhibited high-sensitivity, with a LOD of 0.3 ng/mL of glycinin (Ma et al., 2010).

A sandwich ELISA for the detection and quantification of the soluble soybean protein in processed foods was developed using polyclonal antibodies raised against the protein P34 (Gly m Bd 30K) as a soybean marker protein (Morishita et al., 2008). The method was highly specific, with a LOD of 0.47 ng/mL (equivalent to 0.19 mg/kg in foods) and a LOQ of 0.94 ng/mL (equivalent to 0.38 mg/kg in foods). Polyclonal antibodies against the recombinant P34 fusion protein were used for an indirect

ELISA able to determine the P34 content of soybean products (Liu B et al., 2012), which is deemed to be very specific and accurate, but no LOD has been reported (the lowest concentration tested was around 2.5 ng/mL, as deduced from the calibration curve).

The detection of soy proteins by commercial ELISA kits is variable and strongly influenced by processing. A competitive ELISA targeting renatured soy proteins and a sandwich ELISA determining the trypsin inhibitor in the food sample showed high sensitivity (LOD 2µg/mL and < 1µg/mL, respectively) when applied to soy proteins undergoing hydrolysis and glycation during food processing (L'Hocine et al., 2007). However, both methods showed drawbacks related either to interferences with the food matrix and specificity (cross-reactivity with chickpeas) or to accuracy, which hampered the detection of soy proteins in processed foods. Indeed, ELISA kits do not perform well in heat-treated foods, where glycated proteins are formed by the Maillard reaction (Platteau et al., 2011b). Antibodies against modified protein extracts are more suitable to detect soy allergens in processed foods than antibodies raised against the native protein (Cucu et al., 2012b). If antibodies are raised against a single native allergen, this should be stable during processing conditions.

The performance of seven different assays for the detection of soy was compared on several commercial food products (Pedersen et al., 2008). The difficulties of detecting soy proteins in processed foods was evidenced for all immunological methods, whereas the detection of DNA with a soy-specific real time-PCR offered the advantages of a good sensitivity (LOD 10 mg/kg) and a high specificity. In particular, a sandwich ELISA showed a very good sensitivity (LOD 0.05 mg/kg), but only towards native proteins; a competitive ELISA recognised denatured/renatured proteins, although with a lower sensitivity (LOD 20 mg/kg); enzyme-allergosorbent test (EAST) inhibition and histamine release (HR) tests were also utilised with a good sensitivity (LOD = 0.8–12 mg/kg and 0.2 mg/kg, respectively), although they were very variable depending on patient sera and donor basophils, as well as potentially cross-reactive with other legumes.

19.6.1.2. Immunosensors

An optical biosensor (BIACORE) was used to develop a biosensor immunoassay (BIA) based on polyclonal antibodies for the simultaneous detection of soy, pea, and soluble wheat proteins in milk powder (Haasnoot et al., 2001). The LODs were < 0.1 % of plant protein relative to the total milk protein content. An automated fluorescent microsphere-based flow cytometric triplex inhibition immunoassay was developed for the same purpose (Haasnoot and du Pre, 2007). It is faster than the BIA and allows the simultaneous analysis of several samples with the same LOD.

A direct homogeneous aggregation immunoassay involving the use of gold nanoparticles (AuNPs) adsorbed to polyclonal anti-soy protein antibodies and light scattering detection has been described for soy protein determination in food samples (Sánchez-Martinez et al., 2009). When the method was applied to fruit juice and "non-milk yoghourt" samples, the results were similar to those obtained with a commercial ELISA kit, but the time for analysis was shorter and the LOD was about 10 times lower (65 ng/mL). A heterogeneous competitive fluoroimmunoassay with antibody capture for the determination of soy protein involving Nile blue-doped silica nanoparticles (NPs) bound to anti-soy protein antibodies was found to be very sensitive, with a LOD of 0.05 mg/L (Godoy-Navajas et al., 2011).

19.6.2. Mass spectrometry

Although soy proteins could be analysed by RP-HPLC (Mujoo et al., 2003), the advent of LC coupled to MS allowed to determine the presence of allergens with high specificity and good sensitivity, and to measure multiple proteins simultaneously.

Two quantitative proteomic methods, spectral counting and LC-MS/MS were used to calculate the relative and absolute quantities, respectively, of eight soybean allergens in 20 soy varieties (Houston et al., 2011). The total proteins extracted were digested with trypsin and the peptides analysed by LC-MS/MS in the MRM mode. Absolute quantitation was carried out by spiking the peptide mixture with

isotope labelled synthetic peptide standards, previously designed as markers for each allergen, according to the protein absolute quantification (AQUA) strategy. The concentration of the eight allergens in soy seeds ranged approximately from 0.5 to 5.7 µg/mg of soy protein. The impact of food processing on these specific peptides was investigated in another study (Cucu et al., 2012b), which aimed at identifying soybean-derived tryptic markers stable to processing (e.g. denaturation, Maillard reaction, oxidation) using MALDI-TOF/MS and MS/MS. Although several peptides were modified by the treatments, the most stable to processing were one from Gy1 glycinin (Gly m 6) and one from the α'-chain of β-conglycinin (Gly m 5). The study was not designed to provide LODs, but to provide the basis for a future quantitative method.

Two methods for the detection of soybean proteins in skimmed milk powder (SMP) have been developed for control purposes. One, based on nano-LC-MS/MS (Luykx et al., 2007), could detect 1 to 5 % of plant proteins in SMP (mainly glycinin and β-conglycinin), but was restricted only to the insoluble plant proteins in the borate buffer used for enrichment. The second was untargeted and analysed peptide mixtures resulting from the trypsin digestion of the entire SMP samples by a comparative RP nano-LC/Q-TOF MS, in combination with data dependent LC-MS/MS (Cordewener et al., 2009). No detection limits were provided.

A screening method for the simultaneous detection of seven allergens, including soy, based on LC-QpQ-MS, showed a LOD of 24 mg/kg for soy (Heick et al., 2011a).

19.6.3. DNA-based methods

End-point and real-time PCR for the detection of soybean in food products are available as alternative/complementary to ELISA. Most PCR tests are based on the amplification of the gene of soy lectin, and few on the gene of the soybean allergen Gly m Bd 30K DNA (Torp et al., 2006).

One study (Gryson et al., 2008) was aimed at detecting soy in bread following the addition of various soy ingredients (i.e. full-fat soybean flour, defatted soybean flour, toasted soy, soy protein isolate and soy fibre). Although DNA was partially degraded during the baking process of bread, the detection of soy by end-point PCR was still possible in the full-fat and defatted soybean flour (practical LOD 0.2 %) and in the soy protein isolate (practical LOD 1 %), whereas no amplification was possible for the soybean fibre and toasted flour.

An optimised end-point PCR protocol targeting the soybean lectin gene was able to detect soy DNA in 0.1 % and 0.5 % of hydrated textured protein, corresponding to 0.01 % and 0.06 % (w/w) of soybean protein in unprocessed and heat-processed pork meats, respectively (Soares et al., 2010). The absolute LOD reached a level of 10 pg of soybean DNA.

Two methods based on end-point and real time-PCR techniques were compared for detecting soy protein in commercial processed products by amplification of the lectin gene (Espineira et al., 2010). Both assays were specific, but the real time-PCR was more sensitive. The absolute LOD of end-point and real time-PCR was 100 pg and 10 pg of DNA for raw soy, 0.06 % and 0.05 % for soybean powder added to canned fish, and 100 and 10 mg/kg for soy flour contained in maize flour, respectively. A commercially available real time-PCR method for detection and quantification of soy in boiled sausages was validated in a ring trial (Siegel et al., 2012). The method was reproducible, allowing detecting a spike level of 10 mg/kg.

For a quantitative real time-PCR assay targeting the genomic regions of the soy allergens Gly mBd 28K and GlymBd 30K, the LOD was determined as 3.2 pg of genomic soy DNA, corresponding to 2.8 copies; the LOQ was 6.4 pg of DNA, corresponding to 5.7 copies for both copies (Platteau et al., 2011c)

A duplex real time-PCR method allowing the simultaneous detection of lupin and soy in processed (bakery and vegetarian) food products targeted DNA sequences coding for a mitochondrial gene which, being present in multiple copies per cell, increases the probability of obtaining positive results

(Gomez Galan et al., 2011). The PCR platform is specific and sensitive, allowing the detection of lupin and soy at a level of 2.5 mg/kg food matrix.

A multiplex PCR by using Ligation-dependent probes targeting soy among other allergens was able to detect 13.6 ng of soy DNA (Mustorp et al., 2011). Two quantitative multiplex real time-PCR systems simultaneously determining DNA of eight allergenic foods, including soy, exhibited good specificity and sensitivity in the range of 0.01 % (LOD 10 mg/kg) (Köppel et al., 2010).

19.7. Minimum (observed) eliciting doses

Six clinical studies have assessed MEDs in soy-allergic patients using food challenges with increasing doses of soy protein. In five studies, challenges were conducted for diagnostic purposes (Magnolfi et al., 1996; Zeiger et al., 1999; Fiocchi et al., 2003b; Rolinck-Werninghaus et al., 2012; Blom et al., 2013) and the fifth was a threshold-finding study (Ballmer-Weber et al., 2007).

In an Italian study (Magnolfi et al., 1996), 131 children aged one month to 18 years with clinical history of soy allergy and a positive SPT to soy were challenged with powdered soy formula in fruit juice using a DBPCFC design and rice or maize flour as placebo. Up to 200 mL of soy formula (13 %, 1.8 soy protein/100 mL) were administered at increasing doses (one drop, 1 mL, 5 mL, 10 mL, 50 mL and 134 mL) every 20 minutes. Alternatively, up to 40 capsules containing soy flour (88 mg soy protein/capsule) were administered every 20 minutes at increasing doses (1, 2, 3, 5, 10 and 19 capsules). Children with no symptoms were given 200 mL of soy formula for the following days. Clinical reactions to the challenge were observed in eight (6 %) of the children. Immediate reactions (3–15 minutes) were observed in five children, of whom two reacted to the first dose of 88 mg soy protein. Delayed (gastrointestinal) reactions were noted in three children (at four hours and three and seven days after ingestion).

In another Italian study (Fiocchi et al., 2003b), 18 children with CMA who developed clinical reactions to a soy-based formula after a 2 to 18 months' treatment were recruited and challenged with a soy formula in a diagnostic DBPCFC. None of the children had history of analphylaxis to soy. Doubling doses of 12, 24, 48 and 96 mL of soy-based formula were used, unless symptoms occurred. There were 7 cases of immediate reactions and 11 delayed reactions occurring between 2 and 48 hours following the DBPCFC with soy-based formula. All delayed reactions were observed with the maximum cumulative dose used (180 mL), whereas immediate reactions were already observed at the lowest dose tested in one subject, and at cumulative doses of 36 mL and 84 mL in three subjects each.

In a study conducted in the USA (Zeiger et al., 1999), eight children (age ≤ 3.5 years) with CMA and history of soy allergy were challenged for diagnostic purposes (confirmation of soy allergy) by DBPCFC (n = 8) or OFC (n = 2) with six to seven doubling doses of soy-based formula (starting from one drop to 5 mL depending on the child sensitivity) given at 10 to 15 minute intervals up to 100 mL. Children with convincing history of anaphylaxis and a high level of soy IgE (> 10 U/mL) (n = 2) were excluded from the challenge. The minimum cumulative dose eliciting an allergic reaction was 29 mL of soy-based formula.

In a multicentre trial conducted in Germany, Italy and Denmark (Ballmer-Weber et al., 2007), 30 patients with a history of soy allergy (age range 1–69 years) underwent a titrated DBPCFC with 9 increasing doses containing 0.002, 0.008, 0.148, 0.296, 1.183, 2.367, 4.734, 9.47, and 31.8 g of soy flour in a chocolate bar, respectively, administered at intervals of 15 minutes until objective allergic reactions or ingestion of the whole meal occurred. Five patients were included on the basis of a convincing history of anaphylaxis to soy. Twelve patients experienced subjective symptoms (e.g. OAS, nausea, gastrointestinal pain or thoracic tightness), whereas objective reactions (e.g. blistering of the oral mucosa, rhinoconjunctivitis, urticaria, flush, diarrhoea, decrease in blood pressure) occurred in 11. Since none of the patients reacted to the first dose, the NOAEL was 2 mg of soy flour (1.1 mg of soy protein). The LOAEL for subjective reactions was 10 mg of soy flour (5.3 mg of soy protein) and the LOAEL for objective reactions 454 mg of soy flour (240.6 mg of soy protein).

In the study by Blom et al. (2013), 10 soy-allergic children underwent DBPCFCs for diagnostic purposes. Children were challenged with 0.2 mg of soy protein (mucosal) and oral doses of 1.8, 3.5, 14, 70, 350, and 1 750 mg of soy protein (cumulative dose 2 190 mg). The LOAELs (expressed as discrete doses) for subjective reactions were 0.2 for one, 1.8 mg for three, 14 mg for one, 350 mg for one and 1 750 mg for four children. Only three children had objective reactions (at 0.2 mg, 350 mg and 1 750 mg, respectively).

The aim of the last study (Rolinck-Werninghaus et al., 2012) was to evaluate the relationship between eliciting allergen doses, IgE levels and predictive factors, and the outcome of food challenges in children. Oral food challenges were performed in 317 children (median age 1.1 years; age range 0.3–16.1 years) with a suspected history of soy allergy, i.e. objective clinical reactions in conjunction with soy-specific IgE. Seven increasing doses were administered at 30-minute intervals using a semi-log scale. The first and last doses of soy protein were 4 mg and 3.6 g (0.1 and 100 mL of soy milk), respectively. The severity of objective clinical reactions was graded following a five-level grading system, from grade I (skin symptoms and/or gastrointestinal tract symptoms with no respiratory, cardiovascular, neurological symptoms) to grade V (skin symptoms and/or gastrointestinal tract symptoms plus respiratory symptoms plus cardiovascular symptoms). Objective reactions occurred at all doses of soy tested (grade I for doses 4 mg; 11 mg; 36 mg; 110 mg and 360 mg of soy protein), but the majority of patients reacted only after the higher doses were given (grades II and III at 1.1 g of soy protein; grades I to IV at 3.6 g of soy protein). The MED in this study was 4 mg of soy protein, the lowest dose tested.

Minimum doses reported to elicit reactions in soy-allergic individuals are variable depending on the study population, challenge protocol and food matrix tested. The lowest MED reported in soy-allergic patients undergoing DBPCFC was 0.2 mg of soy protein, although the majority of patients only reacted to higher doses. Few data are available on the doses that may trigger allergic reactions in patients with anaphylactic reactions to soy, which were often excluded from challenge tests, or on the doses which may trigger non-IgE mediated, late and mostly gastrointestinal reactions, which are difficult to assess and has not been done prospectively. It is also unclear whether these patients may react to small amounts of allergen over a prolonged period of exposure.

19.8. Conclusion

Severe and/or fatal anaphylaxis reactions to soy and soy containing foods seem to be rare. Higher rates of anaphylactic reactions to soy protein have been reported among peanut-allergic patients. The prevalence of clinically confirmed soy allergy in unselected populations in Europe appears to be low, although available studies are scarce. Serological and clinical cross-reactions have been described between soy and other legumes, with the pollen allergen Bet 1 v, and with bovine casein. Thermal processing, HHP treatments and fermentation have been shown to reduce the IgE-binding capacity of soy proteins, depending on the conditions and duration of the processes. The detection of soy proteins by ELISA is variable and strongly influenced by processing, while methods based on DNA are more robust and present good sensitivity and specificity. Quantitative determination of soy allergenic proteins by MS is possible, but not yet suitable for the analysis of large numbers of samples. The lowest MED reported in soy-allergic patients undergoing DBPCFC was 0.2 mg of soy protein, although the majority of patients only reacted to higher doses. Few data are available on the doses which may trigger allergic reactions in patients with anaphylactic reactions to soy, which were often excluded from challenge tests, or on the doses which may trigger non-IgE mediated, late and mostly gastrointestinal reactions, which are difficult to assess and has not been done prospectively.

20. Allergy to fish

20.1. Background

Fish are water-living non-mammalian vertebrates, breathing with permanent gills, with finger-less fins. In the regulatory literature, the terms finned fish or finfish are often used to distinguish fish from shellfish (crustaceans and molluscs).

Fish is a common food in all European countries. However, fish intake varies considerably between different regions, depending on local traditions and supplies. Traditionally, consumption has been highest in coastal areas, but this pattern may have become less pronounced. Fish consumption also appears to vary greatly between families and individuals (Welch et al., 2002; Wennberg et al., 2012).

Fish allergy was demonstrated in a classic study early in the history of allergology (Prausnitz and Küstner, 1921) and fish is considered one of the eight most common allergenic foods, which are collectively considered to be responsible for about 90 % of food-allergic reactions (Hebling et al., 2012). The route of exposure appears to determine whether food allergy or respiratory allergy to fish develops. Food allergy to fish is thought to be induced and triggered mainly via the peroral–gastrointestinal route (Untersmayr et al., 2007). In some fish-processing workplaces, respiratory allergy to fish has been a considerable problem because of inhaled allergen (Douglas et al., 1995; Rodríguez et al., 1997; Jeebhay et al., 2000).

This section addresses IgE-mediated food allergy to fish only. However, two important differential diagnoses should be mentioned, namely scombroid poisoning (histamine poisoning) and allergic reactions to the fish parasite *Anisakis simplex* (Sharp and Lopata, 2013). Scombroid poisoning can be caused by some fish species if stored under suboptimal conditions, owing to the conversion of histidine to histamine by bacterial enzymes (Prester, 2011; Demoncheaux et al., 2012). Allergic reactions caused by *Anisakis simplex* in infested fish (EFSA Panel on Biological Hazards (BIOHAZ), 2010; Pravettoni et al., 2012) can be very severe and clinically similar to fish allergy. Up to 35 % of allergic reactions experienced after ingestion of fish in Spain have been reported to be caused by *Anisakis* (Añíbarro et al., 2007). Allergy owing to *Anisakis* and scombroid poisoning will not be further discussed in this opinion.

20.2. Epidemiology

20.2.1. Prevalence

Data on the prevalence of well-documented fish allergy in the general population are scarce. The majority of prevalence estimates have been gathered using questionnaire-based methods (self-reported, clinical history, diagnosis by a clinician), although sensitisation rates (SPT, specific IgE) have also been assessed in some studies. Comparisons between studies are difficult owing to differences in the methodologies used and age ranges assessed.

20.2.1.1. Europe

Prevalence of self-reported fish allergy ranged from 3.5 to 7 % in Finnish children up to four years of age (Kajosaari, 1982; Pyrhonen et al., 2009), although only 1 % reported a clinician-diagnosed fish allergy (Pyrhonen et al., 2009). Prevalence of self-reported allergy to fish in children was lower in other Northern European countries with high fish consumption, like Iceland (1.5–2.2 %) (Kristjansson et al., 1999), Norway (1.5 %) (Eggesbo et al., 1999) or Sweden (1.2–3.2 %) (Eggesbo et al., 1999; Kristjansson et al., 1999). Self-reported allergy to cod in children ranged from 0.3 % in the UK (Venter et al., 2006a) to 1.6 % in Sweden (Ostblom et al., 2008a). In children > 4 years old and adolescents, prevalence of self-reported allergy ranged from 0.3 % and 2.3 % in Turkey (Orhan et al., 2009; Mustafayev et al., 2012), Greece (Zannikos et al., 2008), the UK (Pereira et al., 2005), Sweden (Ostblom et al., 2008b) and the Netherlands (Brugman et al., 1998) and was highest in France (4 %) (Touraine et al., 2002) and Spain (6.9 %) (Martínez-Gimeno et al., 2000). The scarce data available for adults come from southern countries, where a prevalence between 0.4 % (Turkey) (Gelincik et al., 2008) and 1.9 % (Greece) (Sakellariou et al., 2008) has been reported.

In the European Community Respiratory Health Survey (Burney et al., 2010), prevalence and country distribution of sensitisation to 24 allergenic foods were determined in sera collected around the year 2000 in 4 522 young adult individuals of the general population in 13 countries. The average prevalence of sensitisation to any food was 16.2 %. The prevalence of sensitisation to fish (0.2 %) was the lowest among the 24 foods tested, and was considerably lower than the prevalence of sensitisation

to crustaceans (shrimp, 5.4 %). The highest prevalence was reported in Germany (0.9 %), followed by Spain (0.5 %), Norway and Sweden (both with 0.3 %). Sensitisation rates (positive SPT) to cod in children 1 to 6 years of age in the UK were up to 1 % (Arshad et al., 2001; Roberts et al., 2005; Venter et al., 2006a), and up to 1.4 % to any fish in adolescents (Pereira et al., 2005).

The few studies available using DBPCFC to confirm a clinical history of fish allergy consistently show a zero prevalence of fish allergy (Denmark (Osterballe et al., 2005); Turkey (Gelincik et al., 2008) except in six-year-old Finnish children, among whom it was up to 1 % (Kajosaari, 1982).

20.2.1.2. Outside Europe

Prevalence of fish allergy using questionnaire-based methods in countries with Western lifestyle outside Europe is generally below 1 % and no studies are available using DBPCFC to confirm a clinical history of fish allergy (University of Portsmouth, 2013).

20.2.2. Natural history

In contrast to clinical allergies to milk, egg, wheat and soy, which are typically outgrown despite frequently persistent positive SPT, clinical allergy to fish is often "life-long" (Bock, 1982; Eigenmann and Sampson, 1998; Priftis et al., 2008). However, fish allergy may sometimes resolve (Solensky, 2003; Pite et al., 2012), as reported for 3.5 % of fish-allergic patients in one American study (Sicherer et al., 2004). Thus, fish-allergic patients should be re-evaluated from time to time.

20.2.3. Time trends

Few studies are available to examine time-trends for fish allergy. The prevalence of self-reported allergy to fish in Finnish children one to three years of age was assessed in 1980 (Kajosaari, 1982) and in 2001 (Pyrhonen et al., 2009). Prevalence of self-reported fish allergy slightly decreased from 7.5 % to 3.5 % in one-year-olds, from 6 % to 5 % in two-year-olds and from 5 % to 3.6 % in three-year-olds within that time frame. In 1980, 4.5 % of children four years old reported allergy to fish, but only 1 % of children six years old did so in 2001. In the UK, 0.7 % of a four-year-old cohort and 0.5 % of a three-year-old cohort had a positive SPT in 1993 (Arshad et al., 2001) and 2001 (Venter et al., 2008), respectively.

There are no more recent data available to identify any time trends in fish allergy.

20.2.4. Severe reactions/anaphylaxis

Food-allergic reactions to fish can be severe and sometimes fatal. Fish meat is one of the foods most commonly provoking severe anaphylaxis (Sampson, 2000; Lopata and Lehrer, 2009). There are also a number of reports on anaphylactic reactions to caviar and roe from various fishes (Untersmayr et al., 2002; Makinen-Kiljunen et al., 2003; Flais et al., 2004; Kondo et al., 2005; Escudero et al., 2007; Perez-Gordo et al., 2008; Chen et al., 2009; Gonzalez-De-Olano et al., 2011).

20.2.5. Factors affecting the prevalence of fish allergy

Fish allergy often manifests in young children, but it can also manifest at any age. In one study of 79 patients with clinical fish allergy (Pascual et al., 1992), the age t onset was 0 to 6 months in 24 %, 7 to 12 months in 51 %, 13 to 18 months in 8 %, 19 to 24 months in 6 %, and > 24 months in 11 % of subjects. Age distribution in a patient study can be biased for several reasons, but these data are in conformity with the perception that fish allergy tends to develop in the first year of life, although somewhat later than allergy to cow's milk and hen's egg.

20.3. Identified allergens

Fish allergens included in the IUIS database are shown in Table 20.

Table 20: Fish (*Animalia: Chordata*) allergens

Biochemical name	Allergen	Common name	Scientific name	Source	Molecular weight [(a)]
β-Parvalbumin	Clu h 1	Atlantic herring	*Clupea harengus*	Fish meat	12 [(b)]
	Cyp c 1	Carp	*Cyprinus carpio*		
	Gad c 1	Codfish	*Gadus callarias*		
	Gad m 1	Atlantic cod	*Gadus morhua*		
	Lat c 1	Barramundi	*Lates calcarifer*		
	Lep w 1	Whiff	*Lepidorhombus whiffiagonis*		
	Onc m 1	Rainbow trout	*Oncorhynchus mykiss*		
	Sal s 1	Atlantic salmon	*Salmo salar*		
	Sar s 1	Pacific pilchard	*Sardinops sagax*		
	Seb m 1	Ocean perch	*Sebastes marinus*		
	Thu a 1	Yellowfin tuna	*Thunnus albacares*		
	Xip g 1	Swordfish	*Xiphias gladius*		
Tropomyosin	Ore m 4	Mozambique tilapia	*Oreochromis mossambicus*	Fish meat	33 [(c)]
β-Enolase	Gad m 2	Atlantic cod	*Gadus morhua*	Fish meat	47.3 [(c)]
	Sal s 2	Atlantic salmon	*Salmo salar*		47.3 [(c)]
	Thu a 2	Yellowfin tuna	*Thunnus albacares*		50
Aldolase A	Gad m 3	Atlantic cod	*Gadus morhua*	Fish meat	40
	Sal s 3	Atlantic salmon	*Salmo salar*		40
	Thu a 3	Yellowfin tuna	*Thunnus albacares*		40
Vitellogenin (β′ component)	Onc k 5	Chum salmon	*Oncorhynchus keta*	Fish roe	18[b]

(a): Molecular weight (SDS-PAGE).
(b): Approximate—slight variation exists between species.
(b): kDa

Parvalbumin is a major fish allergen, which is found in all fish species (panallergen). However, fish also contains a number of other allergens, some of which have been recently characterised and identified. For example, Baltic codfish contains other allergens than the parvalbumin Gad c 1 (Aukrust et al., 1978a; Aukrust et al., 1978b; Dory et al., 1998). A total of 18 IgE-binding bands in freshly prepared codfish extract were identified by immunoblotting (Hansen et al., 1996). In one study, only one of eight tuna fish-allergic patients had IgE-binding to parvalbumin (Yamada et al., 1999).

Cod parvalbumin is widely used as a general test allergen for sensitisation to fish, and IgE to cod is widely used as a marker of sensitisation to fish (de Martino et al., 1990). However, for patients with allergy to a single or a limited number of fish species, test allergens should be chosen according to the patient's history to avoid 'false negative' results (Kuehn et al., 2013).

While fish muscle and fish skin appear to share allergens, fish roe contains allergens not found in muscle or skin. Fish roe-allergic individuals will often tolerate fish (Makinen-Kiljunen et al., 2003; Escudero et al., 2007; Chen et al., 2009).

20.3.1. Fish muscle and skin

20.3.1.1. Parvalbumins

Parvalbumin was first described in codfish (Aas and Jebsen, 1967; Elsayed and Apold, 1983) as one of the first allergens characterised at the molecular level. Parvalbumin is a vertebrate-specific, mainly cytosolic, globular acidic (pI 3.9–5.5), calcium-binding small protein (106–113 residues, molecular weight ~ 12 kDa) of the EF-hand superfamily, and is expressed in fast-twitch muscles and, to some extent, in certain other cells and organs (Arif, 2009). Two phylogenetic lineages of parvalbumin, α and β, differ in isoelectric point (pI > 5 and pI < 5 for α- and β-parvalbumin, respectively) and features of

amino acid sequence, but share a similar tertiary structure. Both have 30-residue long sub-domains, each containing a central loop flanked by short amphipathic α-helices (Nakayama et al., 1992). Allergenicity of fish is related to the presence of β-parvalbumins. The high structural stability of the calcium-loaded form confers relative resistance to cooking and digestion in the gastrointestinal tract. Allergenicity of parvalbumins is greatly reduced by calcium depletion (Bugajska-Schretter et al., 2000) owing to conformational changes in the calcium-binding region, which is an IgE-binding epitope (Declercq et al., 1991). Some allergenic determinants in parvalbumin appear to be sequential, which may explain the low tendency for remission of fish allergy. Biologically active parvalbumin has been demonstrated in serum samples as early as 10 minutes after ingestion, peaking after one to two hours, suggesting some pre-gastric absorption (Untersmayr et al., 2007).

Parvalbumins have been reported to be the major and only fish allergen for 95 % of patients suffering from IgE-mediated fish allergy (Swoboda et al., 2002). Parvalbumins differ among fish species and belong to the second (after tropomyosin) largest animal food allergen family, with at least 18 parvalbumins described as allergens and a large number of isoallergens. Isoallergens per definition show at least 67 % sequence identity, whereas isoforms with pairwise sequence identity above 90 % are referred to as 'microheterogeneous isoforms' (Chapman et al., 2007). Since even microheterogeneous isoforms of some allergens may induce very different responses by human T and B cells (Wagner et al., 2008) and microheterogeneous isoforms are common in parvalbumin (Lapteva et al., 2013), allergic immune responses may be elicited by a wide repertoire of molecular forms of the same allergen (Lapteva et al., 2013). Certain species of fish (e.g. African catfish) display up to eleven parvalbumin isoforms (Huriaux et al., 2002). Further, parvalbumins have a predisposition for intrinsic disorder (Permyakov et al., 2008) which may contribute to their allergenicity (Xue et al., 2011).

There is evidence that the allergenicity of different fish species may differ to some extent, with, for example, hake and cod reportedly being among the more allergenic, and albacore tuna being among the less allergenic (Bernhisel-Broadbent et al., 1992; Pascual et al., 1992; Pascual et al., 2008). This is in part due to the different levels of expression of parvalbumin in different fish species (Kuehn et al., 2009). Further, parvalbumin content differs in various locations within the whole fish, decreasing in the anterior to posterior direction (Lee et al., 2012), and white fish muscle expresses higher parvalbumin levels than dark fish muscle (Lim et al., 2005; Kobayashi et al., 2006).

20.3.1.2. Collagen

Collagen is ubiquitously found as an extracellular matrix protein in animals. Native collagen is composed of three homo or hetero α-chains twisted together to form a triple helix, and is insoluble in water at low temperature. If collagen is denatured, each α-chain is released from the triple helix, and the denatured form of collagen, i.e. gelatine, is water soluble. Fish skin from several fish species such as cod, pollock, tuna and salmon is used for the preparation of fish gelatine (Taylor et al., 2004). Some muscle tissue is likely to adhere to the skin used for collagen preparation (Koppelman et al., 2012). Isinglass is derived from the swim bladder of certain fish and consists predominantly of collagen.

Although fish collagen has been proposed to be an allergen based on IgE-binding studies and two clinical case reports (Sakaguchi et al., 1999; Sakaguchi et al., 2000; Hamada et al., 2001; Kuehn et al., 2009; Liu R et al., 2012), data from two DBPCFCs studies in fish-allergic patients (Andre et al., 2003; Hansen et al., 2004) suggest that its clinical importance is very limited. Thus, whereas fish collagen is a sensitiser, its ability to trigger allergic reactions is uncertain, in contrast to mammalian collagen, which can cause severe allergic reactions (Fritsche et al., 2010; Land et al., 2013). Mammalian and fish collagens do not cross-react (Hamada et al., 2001).

20.3.1.3. Tropomyosin

Tropomyosin is a major allergen in crustaceans and molluscs, but has only been described as an allergen in one fish, i.e. tilapia (*Oreochromis mossambicus*) (Liu et al., 2013).

20.3.1.4. Enolase and aldolase

β-Enolase and aldolase are enzymes which have been described as allergens of clinical relevance in, for example, codfish, salmon and tuna (Hajeb and Selamat, 2012; Liu R et al., 2012; Kuehn et al., 2013; Sharp and Lopata, 2013; Tomm et al., 2013).

20.3.2. Fish roe allergens

Some clinical reports on anaphylactic reactions and local symptoms to caviar and roe are well documented, but no published DBPCFC studies on fish roe or caviar are available. The reports consistently suggest that roe allergens are different from fish meat allergens because fish roe-allergic patients were often not allergic to fish meat. Several authors have identified vitellogenin (fragments) as allergens in fish roe (Untersmayr et al., 2002; Perez-Gordo et al., 2008; Shimizu et al., 2009). In one case, the presumed allergen (based on immunoblotting) showed homology with α-S1-casein from cow's milk (Chen et al., 2009).

20.4. Cross-reactivities

20.4.1. Cross-reactivity among fish species

20.4.1.1. Fish meat and skin allergens

Parvalbumin is responsible for most of the extensive cross-reactivity among fish species. However, owing to the broad repertoire of molecular forms of parvalbumin and the small role of other allergens, patterns of serological and clinical cross-reactivity are difficult to predict (Hansen et al., 1997; Helbling et al., 1999; Van Do et al., 2005). Extensive serological and clinical cross-reactivity has been observed among closely related as well as among more distant fish species (Bernhisel-Broadbent et al., 1992; Pascual et al., 1992; Hamada et al., 2003). It has been reported that about 50 % of individuals allergic to one type of fish will react to a second fish species, and that up to 40 % of patients sensitised to one or more species of fish do not present symptoms when consuming some other species (Torres Borrego et al., 2003). Fishes in the *Scombroideae* family, which includes tuna, appear to be the best tolerated.

Sera from eight patients clinically allergic to codfish showed cross-reactivity with cod, mackerel, herring and plaice. Clinical cross-reactivity was verified by DBPCFC (Hansen et al., 1997). Extensive cross-reactivity among 17 fish species as determined by SPT, RAST, and clinical history was also observed in fish-allergic patients (de Martino et al., 1990). SPT, serological studies and DBPCFC in nine subjects with clinical symptoms attributed to fish allergy confirmed the broad serological and clinical cross-reactivity among fishes, but also showed that individuals with high specific IgE values to some fish species can have low or undetectable levels of specific IgE to other fishes (Helbling et al., 1999).

Bernhisel-Broadbent Bernhisel-Broadbent et al. (1992) studied clinical cross-reactivity to 10 fish species in 11 patients, each of whom was challenged with four to six fish species. Oral challenges were positive to one fish only in seven patients, to two fishes in one patient, and to three fishes in two patients, suggesting that most fish-allergic patients are able to eat one or more fish species without symptoms. However, cross-reactivity among fishes is so broad and unpredictable that fish-allergic subjects should be advised to avoid all fish species until a fish species has been proven safe by food challenge (Helbling et al., 1999).

The variation in cross-reactivity among individuals can be explained by differences between IgE-binding parvalbumin epitopes in different fish species, combined with different allergen and epitope preferences by different individuals. There are only two case reports describing cross-reactivity between fish species (bluefin tuna and marlin; pangasius and tilapia) without parvalbumin being involved (Kondo et al., 2006; Ebo et al., 2010a). Collagen is assumed to play a role in cross-sensitisation between fishes (Kuehn et al., 2013), but there are no studies specifically addressing this question. Cases with apparent monosensitisation to one fish species or group of fish have been

described, e.g. to swordfish, to tuna, to pollock, to codfish, to tropical sole, and to salmonid fishes (Mata et al., 1994; Kelso et al., 1996; Galland et al., 1998; Asero et al., 1999; Yamada et al., 1999; Kuehn et al., 2011), suggesting that some allergens may be species specific (e.g. specific for yellowfin tuna as compared with albacore tuna) (Yamada et al., 1999).

20.4.1.2. Fish roe

Different patterns of serological cross-reactivity against roe from different fish species (e.g. salmon, cod, trout, hake, herring, pollock) have been observed in various studies, consistent with the fact that different individuals show different allergen and epitope preferences (Makinen-Kiljunen et al., 2003; Escudero et al., 2007; Shimizu et al., 2009). However, broad cross-reactivity between roe from different species appears to be common (Shimizu et al., 2009).

20.4.2. Cross-reactivity between fish and other species

20.4.2.1. Fish meat and skin allergens

Allergenicity in fish is found with β-parvalbumins, while in frog and chicken it is associated with α-parvalbumins. However, both forms of parvalbumin appear to be present in all species mentioned. Some antibodies react with both α- and β-parvalbumins, and rare cases of cross-allergy between fish and chicken (Gonzalez-de-Olano et al., 2012) and between fish and frog (Hamada et al., 2004; Hilger et al., 2004) have been reported, claimed to be caused by cross-reactivity between fish β-parvalbumin and chicken and frog α-parvalbumins, respectively.

There is no IgE cross-reactivity between fish and mammalian collagens (Hamada et al., 2001), and no cross-reactivity between fish and bovine or porcine gelatines has been demonstrated (Sakaguchi (Sakaguchi et al., 1999; Andre et al., 2003).

There is no information on whether tilapia fish tropomyosin cross-reacts with tropomyosins of other species.

20.4.2.2. Fish roe allergens

IgE cross-reactivity between salmon roe and the phylogenetically distant sea urchin roe has been reported (Kondo et al., 2011). Vitellogenin, identified as an allergen in fish roe, is also an allergen in chicken’s egg (Gal d 6), but there is no IgE cross-reactivity between fish roe and chicken’s egg (Koyama et al., 2006; Kondo et al., 2011).

20.5. Possible effects of food processing on allergenicity

20.5.1. Heat and other food treatments

Parvalbumin is resistant to boiling and other high temperature processing. It has “sequential” epitopes which keep their IgE-binding capacity and allergenicity after heating at 100 °C for 10 minutes, and after digestion with proteolytic enzymes or denaturation with chemicals (Elsayed and Aas, 1971; Elsayed and Apold, 1983). The calcium-binding region is an IgE-epitope (Declercq et al., 1991) and is highly stabilised by calcium binding (Bugajska-Schretter et al., 2000).

Using sera from fish-allergic patients, IgE-binding to fresh and processed (smoked, salted/sugar-cured, canned, lye-treated and fermented) cod, haddock, salmon, trout, tuna, mackerel and herring, and to hydrolysates based on salmon and whiting, was investigated using immunoblot and inhibition ELISA. The various treatments often, but not consistently, reduced IgE-binding, whereas some treatments and some sera also showed increased IgE-binding (Sletten et al., 2010).

Bigeye tuna collagen was found to be very thermostable as to its IgE-binding capacity (Hamada et al., 2001). Even when denatured to gelatine by heating in a boiling water bath for 120 minutes, the bigeye tuna collagen retained 90 % of its original binding capacity for specific IgE.

A number of other fish allergens are temperature-sensitive, e.g. some enzymes. Although IgE-binding proteins were observed for cooked or canned tuna, the biologic function was absent when tested in the histamine release assay. This may explain why cooked or canned fish (e.g. salmon, tuna) may be tolerated by individuals who react to undercooked or raw fish (Bernhisel-Broadbent et al., 1992). It should be noted that some patients appear to react to cooked fish but not to raw fish (Prausnitz and Küstner, 1921).

Glycosylation of parvalbumin appears to increase IgE binding without affecting digestibility, and thus food processing under certain conditions may increase the allergenicity of parvalbumin (de Jongh et al., 2013).

In conclusion, heat treatment during food preparation and some other types of traditional food processing may reduce IgE-binding capacity in some cases, but do not represent a reliable method to render fish less allergenic.

20.5.2. Highly processed and novel fish-based products

20.5.2.1. Surimi

Surimi is a product made of minced and thoroughly washed fish meat which is cooked only briefly at low temperature (Musmand et al., 1996). It has numerous applications in food industry. It can be made from one (e.g. cod, Alaskan pollock, mackerel) or more fish species and retains much of its allergenicity after processing (Helbling et al., 1992; Mata et al., 1994; Musmand et al., 1996), 1992). In one study (Mata et al., 1994) a 63 kDa protein in codfish was the single allergenic protein detected in surimi, whereas the 13 kDa parvalbumin was presumably washed out during surimi preparation. Allergy to surimi has been verified by DBPCFC in a patient who reacted to 1 g of surimi (Musmand et al., 1996).

20.5.2.2. Ice-structuring proteins

Ice-structuring proteins (ISPs) are naturally occurring proteins that bind to ice and structure ice crystal formation. Their function is to help protecting organisms in cold habitats from ice crystal damage. ISP can be isolated from fish living in or near Arctic waters, they have been produced using recombinant baker's yeast, and are a novel food ingredient with a number of commercial applications in the food industry. ISP preparations did not bind specific IgE to fish, and other evidence for allergenicity has not been found (Baderschneider et al., 2002; Bindslev-Jensen et al., 2003; Crevel et al., 2007a).

20.5.2.3. Fish oils

Fish oils might contain fish allergens. However, no analytical studies are available and only one clinical study has been published. Six subjects (23 to 64 years, three female) who reported fish allergy (throat constriction, urticaria and angioedema) and had a positive SPT to at least three of six common fish species showed negative SPT to undiluted, unfiltered liquid extracted from softgel fish oil supplement capsules of two different brands (Mark et al., 2008). An oral challenge with one 1 000 mg capsule of each supplement (one hour apart) did not induce subjective or objective reactions.

20.5.2.4. Fish gelatine and isinglass

Fish gelatine is derived from the skin of several fish species. After thorough washings to remove remaining muscle tissue, bones, salts and off-flavours, the skin is subjected to heating, acidic extraction and acidic and/or enzymatic hydrolysis. Fish gelatine is mostly used by dairy, confectionery, and pharmaceutical industries. Both fish gelatine and isinglass are widely used as fining agents in the production of beverages, such as wine and beer. Isinglass is derived from the swim bladders of fish by washing and cleaning, conditioning in hot water, removing muscle layers and blood vessels, and treating with hydrogen peroxide.

Fish gelatine and isinglass generally contain 80 to 95 % of collagen and related peptides. Depending on the degree of hydrolysis, they may contain molecules ranging from native collagens (MWs > 117 kDa) to short peptides (MWs < 40 kDa). Isinglass particles can be removed from the wine or beer by sedimentation and/or filtration. Low levels of parvalbumin have been reported in isinglass but not in gelatine (Weber P et al., 2009).

Fish gelatine-specific IgE antibodies have been identified in fish-allergic patients (Sakaguchi et al., 2000). No adverse reactions were observed in two independent DBPCFC studies (Andre et al., 2003; Hansen et al., 2004) with gelatine derived from tuna and codfish. Neither research group observed allergic reactions of clinical relevance in a total of 33 fish-allergic patients tested with a cumulative dose of 5 g fish gelatine (three patients) or with 14.6 g of fish gelatine (30 patients), respectively, although very few of the patients tested had specific IgE to collagen.

20.6. Detection of allergens and allergenic ingredients in food

Fish parvalbumins are abundant and stable proteins in fish meat. However, the measurement of fish parvalbumin concentrations in foods is complicated by the fact that parvalbumins show differences in different fish species and consequently IgG antibodies raised against them will show different binding patterns (Lee et al., 2011).

Immunological methods (mainly ELISA), mass spectrometry and PCR techniques have been used to detect parvalbumin in foods.

20.6.1. ELISA

A specific sandwich ELISA for the quantitative determination of fish using a polyclonal rabbit anti-cod parvalbumin antibody for capture and detection (Faeste and Plassen, 2008) was reported, with a LOD of 0.01 mg parvalbumin/kg food (or 5 mg fish/kg food). The recovery was very variable, ranging from > 50 % for nine fish species down to < 1 % for some others.

Purified parvalbumins from several fish species were used to develop a competitive indirect ELISA based on commercial PARV-19 anti-parvalbumin antibodies raised against frog muscle parvalbumin. The method allowed to detect all the parvalbumins investigated within a range of 0.1 to 0.5 mg/L (Weber P et al., 2009). ELISA methods to detect the presence of parvalbumin in fish gelatine and isinglass have been described (Lifrani et al., 2009; Weber et al., 2010; Koppelman et al., 2012) and used to detect fining agent residues in wine.

A sandwich ELISA for the determination of fish protein in processed foods used a polyclonal antibody raised against Pacific mackerel parvalbumin (Shibahara et al., 2013). The LOD was estimated to be 0.23 mg fish protein/kg food and the LOQ was 0.70 mg fish protein/kg food. The method was validated in inter-laboratory tests with a good recovery (69.4–84.8 %) and sufficient sensitivity and specificity. It could measure fish protein in 18 of the 21 processed foods tested, but not in fermented foods, where parvalbumin can be highly degraded to peptides.

Different types of antibodies have been used to develop ELISAs which show varying specificities for different fish species. Among three anti-parvalbumin IgG antibodies, a polyclonal anti-cod parvalbumin antibody, and the commercially available monoclonal anti-frog and monoclonal anti-carp antibodies, the polyclonal anti-cod parvalbumin antibody showed reactivity to the widest range of fish species (Lee et al., 2011). In general, polyclonal antibodies are more suitable than monoclonal antibodies to detect different fish species in processed foods.

ELISA methods seem to better recognise conformational epitopes than linear epitopes. Parvalbumin being a calcium-binding protein, calcium depletion in the assay buffer may considerably change the conformation of the protein, thus decreasing the sensitivity of ELISAs.

Polyclonal antibodies have also been raised against fish collagen and a sensitive indirect ELISA for the detection of fish gelatine and isinglass has been described (Weber et al., 2010). The LOD was ≤ 0.11 mg/mL.

20.6.2. Mass spectrometry

A method based on mass spectrometry for the rapid and direct detection of fish parvalbumin in food products has been described (Carrera et al., 2012). Parvalbumin is extracted, purified and digested by trypsin in High Intensity Focused Ultrasound (HIFU) equipment. Nineteen selected peptide biomarkers are separated by LC and monitored by Selected MS/MS Ion Monitoring (SMIM) in a linear ion trap mass spectrometer. The method was applied to several processed foods and allowed the identification of parvalbumin unequivocally, although no quantification has been provided.

20.6.3. PCR

PCR-based assays have been developed to detect fish DNA from different species. DNA is present in all tissues, stable at high temperature, and allows differentiation of closely related fish species.

A comparative study of methods for the extraction of DNA from fish muscle which utilise different principles of separation reported high variability in the extraction efficiency (Cawthorn et al., 2011). One method consistently extracted DNA with the highest yield from all fish species tested, but DNA purity was satisfactory for only 50 % of the extracts. Conversely, the method with the lowest yield provided the highest DNA purity.

Parvalbumin DNA of Pacific mackerel (*Scomber japonicus*) has been detected in food by conventional PCR (Choi and Hong, 2007). Parvalbumin DNA has been detected in different tissues of sturgeon species (*Acipenser*) by reverse-transcription (RT)-PCR (Rehbein and Lopata, 2011). Conventional PCR methods have also been set for the specific detection of salmonoid fish (Ishizaki et al., 2012) and Atlantic herring (Rencova et al., 2013) in processed foods, with LODs of 0.02 fg DNA/µL (corresponding to 10 DNA copies) and of 10 pg DNA/µL, respectively.

A real-time PCR assay (Sun et al., 2009) detects parvalbumin DNA from several fish species with a LOD of 5 pg DNA, but it is not species specific. An assay which identifies eight fish species in food was developed by using PCR for amplification of fish parvalbumin introns and the multianalyte profiling (xMAP^{TM}) technology with probes targeting species-specific sequences (Hildebrandt, 2010). The LODs for the eight fish species ranged from 0.01 % to 0.04 %. The assay showed no cross-reactivity with other species.

20.7. Minimum (observed) eliciting doses

Different fish species may differ with regard to the minimal eliciting dose, because of allergen heterogeneity and differences in allergen concentration in the flesh (see section 20.3.1.1). In a DBPCFC study (Hansen and Bindslev-Jensen, 1992), the minimum dose needed to elicit a reaction (oropharyngeal itching and swelling) was 6 mg of codfish (starting dose 5 mg). Urticaria was observed after 56 mg of codfish. In a DBPCFC (Untersmayr et al., 2007), adverse reactions to codfish protein extract digested in simulated gastric fluid were studied. After digestion at pH 2.0, one subject experienced subjective symptoms after 2.11 mg protein, while after digestion at pH 3.0, one subject reported subjective symptoms after 1.11 mg and one subject had objective signs after 2.11 mg of codfish protein.

The Panel notes that MEDs reported in DBPCFCs are 6 mg of codfish and range from 1.11 mg of codfish protein for objective symptoms to 2.11 mg for objective signs. Few data are available on the doses that may trigger allergic reactions in patients with history of severe allergic reactions, since they have often been excluded from challenge tests.

20.8. Conclusion

Fish is widely consumed in all European countries and is among the foods most commonly triggering IgE-mediated allergic reactions, which can be severe and occasionally fatal. Fish meat is one of the foods most commonly triggering severe anaphylaxis. Prevalence of well documented fish allergy in the general population is well below 1 %. The major fish allergens are well characterised and heat-resistant. Parvalbumin is responsible for most of the extensive and unpredictable cross-reactivity among fish species. ELISA and MS, as well as PCR techniques, have been used successfully to detect parvalbumins and DNA belonging to different fish species in foods. The minimum doses observed to elicit a clinical reaction were 6 mg of codfish and 1.1 mg of codfish protein. Few data are available on the doses that may trigger allergic reactions in patients with history of severe allergic reactions to fish.

21. Allergy to crustaceans

21.1. Background

Crustaceans are common food in all European countries. Few accurate consumption data are available, but seafood intake in general is considered to vary considerably between different regions, depending on local traditions and supplies. Similar to fish, the route of exposure for crustaceans appears to determine whether food allergy, skin allergy or respiratory allergy develops. In some food processing workplaces, respiratory allergy to crustaceans has been a considerable problem owing to inhaled allergen (Lopata and Jeebhay, 2013).

Decapod crustaceans (e.g. shrimp, prawn, crab and lobster) are of main interest as allergenic foods. However, some non-decapod crustaceans like krill, mantis shrimp and barnacles have recently been reported to be allergenic and may become more important because of food trends. Krill is mainly consumed as unrefined oil in Europe, whereas in, for example, Russia, Japan and Korea, krill is used also in various processed foodstuffs.

21.2. Epidemiology

21.2.1. Prevalence

21.2.1.1. Europe

Studies reporting on the prevalence of allergy to crustaceans in the general (unselected) European population are scarce.

In the few studies available, the prevalence of self-reported crustacean-related adverse reactions to food in children ranged from 0.1 % and 0.3 % in Greece (Zannikos et al., 2008) and the UK (Pereira et al., 2005) to 5.5 % in France (Touraine et al., 2002). Figures reported from the Netherlands (Brugman et al., 1998), Sweden and Iceland (Kristjansson et al., 1999) were within that range (0.7–1.5 %). Prevalence of self-reported allergy to shrimps was 0.5 % in 2- to 14-year-old Finch children (Rancé et al., 2005).

In adults, estimated sensitisation rates to crab in Germany (Schafer et al., 2001) based on positive SPTs were similar to those reported in Hungary (Bakos et al., 2006) based on specific IgE testing (1.9 % and 1.8 %, respectively).

Prevalence rates of allergy to crustaceans based on clinical history and positive SPT in the German general population were much lower (0.2 %) (Zuberbier et al., 2004). Only one study conducted in Denmark reported challenge proven prevalence data for shrimp allergy, which ranged from zero in subjects < 22 years to 0.3 % in subjects > 22 years (Osterballe et al., 2005).

21.2.1.2. Outside Europe

Most prevalence data available from Western countries outside Europe have been collected using questionnaire-based methods (self-reported, physician's diagnosis) and do not relate to crustaceans specifically but rather to "seafood" or "shellfish". In Canada, rates of total self-reported allergic reactions to shellfish among children were about 0.5 %, and 0.1 % when diagnosis was made by a clinician (Ben-Shoshan et al., 2010). Self-reported allergic reactions to shellfish in adults varied in the USA, ranging from 1.7 % (Vierk et al., 2007) to 9 % (Greenhawt et al., 2009). In Australia, self-reported allergic reactions to shrimp were 3.3 % (Woods et al., 2002).

Higher rates of self-reported allergic reactions were observed in some Asian countries, both for adults (24.5 %; China) (Sai et al., 2011) and children (8.7 % and 11.6 %, respectively; Philippines) (Connett et al., 2012). However, the only study which used OFC to confirm diagnosis reported a prevalence of clinical allergy to crab and shrimp of 0.2 % and 0.9 %, respectively, among three- to seven-year-olds in Thailand (Lao-araya and Trakultivakorn, 2011).

21.2.2. Natural history

There are no data available regarding the natural history of crustacean allergy, apart from a report suggesting that shrimp sensitisation decreases with age (Ayuso et al., 2010).

21.2.3. Time trends

The only information available on time trends comes from China and relates to the prevalence of sensitisation to shrimp in infants and young children, which was zero and 0.3 % in 1999 and 2009, respectively (Hu et al., 2010).

The data available are insufficient to conclude on time trends regarding the prevalence of allergy to crustaceans in Europe.

21.2.4. Severe reactions/anaphylaxis

Crustacean allergy was early recognised to cause severe allergic reactions and occasionally fatal anaphylaxis (Yunginger et al., 1988; Bock et al., 2001). In a multicentre study of anaphylactic reactions to food (Moneret-Vautrin and Kanny, 1995), crustaceans accounted for 11 % (9/81) of cases. Crustaceans were incriminated on the basis of clinical history, SPT and serum-specific IgE in 17 % of 60 cases of severe, near-fatal reactions in another study (Andre et al., 1994).

21.2.5. Factors affecting prevalence of crustacean allergy

Allergy to crustaceans mostly affects the adult population (Crespo and Rodriguez, 2003; Skypala, 2011). Nevertheless, shellfish (crustaceans and molluscs) appears to be responsible for a majority of emergency department visits for food allergy also in children ≥ 6 years of age in the USA, and represents a significant cause of allergic reactions in children one to six years of age (Ayuso et al., 2009).

Crustacean-allergic children show higher levels of specific IgE to shrimp allergens and a broader epitope repertoire than adults, indicating a higher IgE reactivity (Ayuso et al., 2010). It has been suggested that children tend to react more to raw crustaceans and adults to boiled crustaceans (Ayuso et al., 2010), and that children more frequently react to allergens like sarcoplasmic calcium-binding protein (SCBP) and myosin light chain (MLC) than adults (Ayuso et al., 2008; Ayuso et al., 2009).

Sensitisation to crustaceans appears to be influenced by sensitisation to molluscs and non-molluscan invertebrates, like mites, cockroaches and the fish parasite *Anisakis*, and allergy to crustaceans may sometimes be secondary to allergy to cross-reactive organisms (Ayuso et al., 2008).

21.3. Identified allergens

21.3.1. Decapod crustaceans

Decapod crustaceans are of main interest as allergenic foods. The Allergome database lists 375 crustacean allergens (isoallergens included), whereas only 30 allergens (plus 33 isoallergens) are listed in the IUIS database (Table 21).

Crustaceans contain many IgE-binding proteins. Raw black tiger prawn and king prawn showed 14 and 11 IgE-binding proteins, respectively (Sahabudin et al., 2011), while giant freshwater prawn also had 11 (Yadzir et al., 2012). Some of these proteins, e.g. sarcoplasmic calcium-binding protein (SCBP), were found able to activate basophils even more than tropomyosin (Ayuso et al., 2009). Although the clinical relevance of most IgE-binding proteins is uncertain, some crustacean-allergic individuals only show IgE-binding to non-tropomyosin allergens (Abramovitch et al., 2013).

Several major allergens have been described in crustaceans, including tropomyosin, arginine kinase, sarcoplasmic calcium-binding protein and myosin light chain 1 and light chain 2 (Ayuso et al., 2008; Ayuso et al., 2009; Bauermeister et al., 2011; Sahabudin et al., 2011).

Table 21: Crustacean (Animalia Arthropoda) allergens

Biochemical name	Allergen	Common name	Scientific name	Molecular weight [(a)]
Tropomyosin	Cha f 1	Crab	*Charybdis feriatus*	34
	Cra c 1	Common shrimp	*Crangon crangon*	38 [(b)]
	Hom a 1	American lobster	*Homarus americanus*	–
	Lit v 1	European white shrimp	*Litopenaeus vannamei*	36
	Mac r 1	Giant freshwater prawn	*Macrobrachium Rosenbergii*	37 [(b)]
	Met e 1	Greasyback shrimp	*Metapenaeus ensis*	–
	Pan b 1	Northern red shrimp	*Pandalus borealis*	37 [(b)]
	Pan s 1	Spiny lobster	*Panulirus stimpsoni*	34
	Pen a 1	Brown shrimp	*Panulirus stimpsoni*	36
	Pen i 1	Indian shrimp	*Penaeus indicus*	34
	Pen m 1	Black tiger prawn	*Penaeus monodon*	38
	Por p 1	Blue swimmer crab	*Portunus pelagicus*	39 [(b)]
Arginine kinase	Cra c 2	Common shrimp	*Crangon crangon*	45 [(b)]
	Lit v 2	European white shrimp	*Litopenaeus vannamei*	
	Pen m 2	Black tiger prawn	*Penaeus monodon*	40
Myosin light chain 2	Hom a 3	American lobster	*Homarus americanus*	23
	Lit v 3	European white shrimp	*Litopenaeus vannamei*	20
	Pen m 3	Black tiger prawn	*Penaeus monodon*	
Sarcoplasmic calcium-binding protein, SCBP	Cra c 4	Common shrimp	*Crangon crangon*	25
	Lit v 4	European white shrimp	*Litopenaeus vannamei*	20
	Pen m 4	Black tiger prawn	*Penaeus monodon*	–
	Pon l 4	Narrow clawed crayfish	*Pontastacus leptodactylus*	24 [(b)]
Myosin light chain 1	Art fr 5	Brine shrimp	*Artemia franciscana*	17.5
	Cra c 5	Common shrimp	*Crangon crangon*	17.5
Troponin C	Cra c 6	Common shrimp	*Crangon crangon*	21
	Hom a 6	American lobster	*Homarus americanus*	20
	Pen m 6	Black tiger prawn	*Penaeus monodon*	–
Troponin l	Pon l 7	Narrow clawed crayfish	*Pontastacus leptodactylus*	30 [(b)]
Triosephosphate isomerase	Arc s 8	Shrimp	*Archaeopotamobius siberiensis*	28 [(b)]
	Cra c 8	Common shrimp	*Crangon crangon*	28

(a): Molecular weight (SDS-PAGE). Approximate—some variation exists between species.
(b): kDa

21.3.1.1. Tropomyosin

Tropomyosin is a well-characterised, major crustacean allergen, which shows high sequence homology (up to 98 %) among crustaceans (Leung and Chu, 1998). Tropomyosin was the first allergen (Pen a 1) identified in shrimp (Hoffman et al., 1981), its IgE-binding epitopes have been identified, and at least 80 % of shrimp-allergic subjects react to tropomyosin (EFSA, 2004; Zheng et al., 2011). Monosensitisation to tropomyosin appears to be frequent. In one study, 12 of 31 subjects exhibited detectable specific IgE exclusively to tropomyosin (Bauermeister et al., 2011).

21.3.1.2. Arginine kinase

Arginine kinase (France et al., 1997) is an enzyme that plays a key role in energy metabolism in invertebrates (García-Orozco et al., 2007; Yu et al., 2011). It is a panallergen (Binder et al., 2001), a major allergen in at least some crustaceans, and has also been identified in other invertebrates, such as moth and cockroach (Ayuso et al., 2008).

21.3.1.3. Sarcoplasmic calcium-binding protein

The water-soluble SCBP has been identified as a shrimp allergen (Shiomi et al., 2008; Ayuso et al., 2009; Chen et al., 2013). SCBP functions as an EF-hand calcium-binding protein in invertebrates, analogous to parvalbumin in fish. Like parvalbumin, SCBP shows high polymorphism. All isotypes and subunits of SCBP show IgE-binding capacity (Chen et al., 2013). In some studies SCBP is a major allergen (Ayuso et al., 2009), and some shrimp-allergic individuals are exclusively sensitised to SCBP (Bauermeister et al., 2011).

21.3.1.4. Myosin light chains

Myosin light chains (MLC) are components of the macromolecular complexes, which constitute myosins. The 177-amino acid muscle protein myosin light chain (MLC) is a major allergen in shrimp (Ayuso et al., 2008) and has also been identified in other invertebrates. There seems to be independent groups of allergenic MLC (Bauermeister et al., 2011), as the sequence identity between two MLC proteins studied (light chain 1 and light chain 2) is only 13 % (Ayuso et al., 2008; Bauermeister et al., 2011). MLC is an EF-hand calcium-binding protein. In one study, the recombinant protein was recognised by serum IgE from 17 out of the 19 shrimp-allergic individuals tested, and was almost the only allergen recognised by some shrimp-allergic patients (Ayuso et al., 2008).

21.3.1.5. Other crustacean allergens

Troponin C is a novel IgE-binding protein in crustaceans (Bauermeister et al., 2011), which was also identified in the cockroach (Bla g 6). Troponin 1 is an allergen also in crayfish, a major allergen of the fish parasite *Anisakis* (Ani s 1), and an allergen in the cockroach. Triosephosphate isomerase is a novel crustacean allergen (Bauermeister et al., 2011), but members of this protein family had already been reported as IgE-binding proteins in fish, midges and various plants.

21.3.2. Non-decapod crustaceans

The small shrimp-like krill species Pacific krill (*Euphausia pacifica*) and Antarctic Krill (*Euphasia superba*) have the allergens Eup p 1 and Eup s 1 listed in the Allergome database, both with one variant. These tropomyosins show extensive IgE-binding cross-reactivity with shrimp, crab and lobster tropomyosins (Nakano et al., 2008). Krill tropomyosins and mantis shrimp tropomyosin have 82.3 to 89.8 % and > 90 % sequence identity with decapod tropomyosins, respectively (Motoyama et al., 2008). Tropomyosin is also the main allergen in acorn barnacle (*Balanus rostratus*) and goose barnacle (*Capitulum* (*Pollicipes*) *mitella*). These non-decapod crustaceans belong to the class Thecostraca, which is taxonomically remote from the decapods, but cross-react with decapod tropomyosin (Suma et al., 2007).

21.4. Cross-reactivities

At least four crustacean allergens are, like the main fish allergen parvalbumin, EF-hand calcium-binding proteins, namely SCBP, myosin light chain (MLC), troponin C, and troponin 1. Some homologues of these crustacean proteins are allergens in, for example, cockroaches and the fish parasite *Anisakis* (Bla g 6, Bla g 8, Ani s 1), and in plants. Cross-reactivity between EF-hand proteins is limited to phylogenetically closely related species, suggesting that different families of calcium-binding allergens possess specific epitopes (Ayuso et al., 2009).

21.4.1. Cross-reactivity among crustaceans

In a crustacean-allergic individual, the probability of reacting to another crustacean species has been estimated to be 75 % (Torres Borrego et al., 2003). Although tropomyosin is the allergen most frequently involved in cross-reactions among crustaceans (Kamath et al., 2013), SCBP shows high sequence identity among crustaceans and also contributes to serological cross-reactivity (Ayuso et al., 2009; Mita et al., 2013). Cross-reactivities among crustaceans are, however, not always observed, possibly because of selective IgE-binding to species-specific epitopes of major allergens, or to minor allergens (e.g. haemocyanin) which may be species specific (Piboonpocanun et al., 2011; Abramovitch et al., 2013).

21.4.2. Cross-reactivity between crustaceans and molluscs

Tropomyosin is important for cross-reactions between crustaceans and molluscs, but tropomyosin sequence identity between the two is markedly lower than within crustaceans or within classes of molluscs, which may explain why cross-reactivity between crustaceans and molluscs is more limited than within either crustaceans or classes of molluscs (EFSA, 2006b; Lu et al., 2007; Tsabouri et al., 2012; Kamath et al., 2013). The IgE-binding regions of Pen a 1 partly or completely overlap with those of Pen i 1 from the shrimp *P. indicus*, Tur c 1 from the snail *T. cornutus* and Cra g 1 from the oyster *C. gigas*, supporting the notion that tropomyosin is a major cause of serological and clinical cross-reactivity between crustaceans and molluscs (Lehrer and McCants, 1987; Reese et al., 1999; Ayuso et al., 2002a). SBCP shows only 14 % identity between shrimp and scallops and does not appear to be involved in cross-reactivity between crustaceans and molluscs (Ayuso et al., 2009), whereas some other non-tropomyosin allergens may play a role (Boquete et al., 2011).

Cross-reactivity between crustaceans and molluscs is often restricted to a few species. For example, there are several reports of individuals clinically reacting to squid and shrimp (Carrillo et al., 1992) while tolerating other crustaceans and molluscs. A similar situation is observed with regard to serological cross-reactivity. Serological and clinical cross-reactivities between crustaceans (e.g. shrimp, lobster and crab) and the mollusc squid (e.g. *Todarodes pacificus*, *Loligo vulgaris*), and between crustaceans and oyster, e.g. *Crassostrea gigas* appear to be frequent (EFSA, 2006b).

The extent to which mollusc allergy may be secondary to crustacean allergy, or vice-versa, is uncertain. Out of 38 patients with shellfish allergy, 25 were sensitised to crustaceans and molluscs, 12 to crustaceans only, and one to molluscs only (Laffond Yges, 1996). Further, out of 24 shellfish-allergic children, 23 were allergic to crustaceans, while 10 were allergic to molluscs (Crespo et al., 1995a). Using sera from nine crustacean-allergic individuals, IgE-binding to tropomyosin in the muscle extract of all 10 molluscs tested was observed (Leung and Chu, 1998). These findings suggest that some patients reacting to molluscs could be primarily sensitised to crustaceans, and vice-versa, although some cross-inhibition studies suggest dual sensitisation (van Ree et al., 1996a; Goetz and Whisman, 2000).

All the mentioned clinical cross-reactivities between crustaceans and molluscs, however, are based on case histories, sometimes including elimination diets, and not on DBPCFC.

21.4.3. Cross-reactivity between crustaceans and non-molluscan invertebrates

Sensitisation to shrimp tropomyosin may induce allergy to mite and cockroach (Reese et al., 1999), whereas IgE reactivity to shrimp has been observed in unexposed populations allergic to mite and/or cockroaches (Fernandes et al., 2003). Serological cross-reactivity between crustaceans, mollusc, mite and cockroach tropomyosins may cause difficulties in the diagnosis of, for example, shrimp allergy (Shafique et al., 2012).

Clinically relevant cross-reactivity between crustacean and house dust mite allergens has been described as the "mite–crustacean–mollusc–syndrome" (Witteman et al., 1994; Kütting and Brehler, 2001). Primary sensitisation is most often against mite. However, allergy to mites or cockroaches subsequent to sensitisation to crustaceans has also been described (van Ree et al., 1996a; Ayuso et al., 2002a). Shrimp tropomyosin Pen a 1 IgE-binding regions show high sequence homology with corresponding regions of Per a 7 (cockroach) and Der p 10 (mite) (60–100 %) (Ayuso et al., 2002a). Immunotherapy with mite allergen has been reported to increase the risk for anaphylactic reactions to crustaceans (van Ree et al., 1996a; Pajno et al., 2002).

Cases of clinical cross-reactivity between crustaceans (shrimp), molluscs (oysters in the case cited) and the fish parasite *Anisakis* (Pascual et al., 1997; Gonzalez Galan et al., 2002) are supported by IgE-binding studies (Martínez et al., 1997; Pascual et al., 1997; Asturias et al., 2000) and by tropomyosin sequence homology data (Ayuso et al., 2002a; Ivanciuc et al., 2002). *Anisakis* also shares other allergenic molecules with crustaceans, e.g. troponin 1, which may explain cases of apparent cross-reactivity between crustaceans and molluscs, and between them and *Anisakis*-infested fish (Torres Borrego et al., 2003). Shrimp tropomyosin also cross-reacts with the phylogenetically distant sea urchin roe used in, for example, sushi (Pascal et al., 2012).

The involvement of non-tropomyosin allergens in cross-allergy between shellfish and mites is suggested by a number of case reports. Serum from a man with primary sensitisation to house dust mites and selective allergy to lobster showed no IgE-binding to tropomyosin, but recognised four proteins in lobster and two and three allergens in two different mite species (Iparraguirre et al., 2009). The panallergen arginine kinase (Der p 20 and Pen m 2 in mite and shrimp, respectively) show 78 % amino acid sequence homology and appear to be involved in cross-reactivity between crustaceans, molluscs and mites (Boquete et al., 2011). Myosin light chain 1 (MLC 1) may also be involved in cross-reactivity between crustaceans and cockroach and possibly mites (Ayuso et al., 2008).

21.4.4. Cross-reactivity between crustaceans and vertebrates

In vertebrates, tropomyosin is considered to be non-allergenic (Leung et al., 1996; Restani et al., 1997), with the exception of the recent description of tropomyosin from the fish tilapia (*Oreochromis mossambicus*) as an allergen (Liu et al., 2013). Extracts of salmon, tuna, trout, pollock and mackerel failed to significantly inhibit a shrimp RAST (O'Neil et al., 1993), indicating that unique IgE epitopes are present among crustacean tropomyosins, which may explain the general absence of cross-reactivity between fish and crustaceans. Similar considerations can be made for the other crustacean allergens, e.g. EF-hand calcium-binding muscle proteins (Ayuso et al., 2008).

21.5. Effect of food processing on allergenicity

21.5.1. Heat treatment

The antigenicity of crustacean allergens may be unchanged, increased or decreased after heat treatment depending on the allergen. One study in giant freshwater prawn showed that, out of the 11 IgE-binding proteins observed in the raw shrimp extract, only 5 could be identified after cooking (Yadzir et al., 2012).

Crustacean tropomyosin re-folds after heating (Usui et al., 2013) and is heat resistant (Shanti et al., 1993; Leung et al., 1994; Crespo et al., 1995b; Samson et al., 2004). Whereas tropomyosin increases its IgE-binding capacity after boiling (Liu AH et al., 2010; Shriver et al., 2011; Kamath et al., 2013),

extracts from boiled shrimp showed reduced IgE-binding, which points to a role of other heat-sensitive allergens in IgE-binding to the extract. Heating markedly increased IgE-binding of extracts from blue swimmer crab and black tiger prawn (Abramovitch et al., 2013). The Maillard reaction has been reported to increase the antigenicity of scallop tropomyosin (Nakamura et al., 2005). How these findings reflect on clinical allergenicity is unknown.

SCBP has been found stable during thermal and acid/alkali treatment (Chen et al., 2013). In addition, MLC binds IgE in both raw and cooked shrimp extracts (Ayuso et al., 2008). One potentially important heat-sensitive allergen is arginine kinase (Yu et al., 2003; García-Orozco et al., 2007).

21.5.2. Other treatments

Proteinase digestion reduced the IgE-binding capacity of prawn, shrimp and crab tropomyosins. The effect of boiling, of combined ultrasounds and boiling, and of high pressure steaming on the digestibility of crab tropomyosin under simulated gastric and intestinal conditions has also been investigated (Liu GM et al., 2010a; Liu GM et al., 2010b; Yu et al., 2011). High-pressure steaming was most efficient in accelerating digestion and correspondingly reducing IgG/IgE antibody binding.

Pulsed ultraviolet light (PUV), a novel technology most commonly used for microbial inactivation, has been reported to reduce the antigenicity of some foods. PUV-treated shrimp extracts showed reduced IgE-binding to tropomyosin (Shriver et al., 2011), and this effect was maintained in extracts undergoing gastric and intestinal digestion in models intended to mimic real-life conditions (Yang et al., 2012).

Krill is mainly consumed as unrefined oil, which may contain allergenic proteins. Tropomyosin was found in samples of krill oil, but not in oil from the small crustacean-like zooplankton *Calanus finmarchius* (found in North Atlantic Ocean, which is similar to krill found in the South Atlantic) (Vang et al., 2013).

21.6. Detection of allergens and allergenic ingredients in food

21.6.1. Immunological methods

All ELISA methods described for the detection of crustacean allergens targeted tropomyosin, the most important allergen present in crustaceans and molluscs. While some immunological methods have been developed to detect crustaceans and molluscs and are not able to differentiate between these two types of shellfish, others are specific for crustaceans or even for single crustacean species (mostly biosensors). Monoclonal antibodies are preferred for targeting a single species, whereas polyclonal antibodies are more useful for detecting the entire group of crustaceans and/or molluscs.

21.6.1.1. ELISA

Methods of detection specific for crustaceans

The first method developed was a mAb sandwich ELISA for the quantification of the major shrimp allergen tropomyosin Pen a 1 (brown shrimp) (*Penaeus aztecus*) (Jeoung et al., 1997). The method is sensitive (LOD 1ng/mL), reproducible and non-cross-reactive with oyster, cockroach or house dust mites. It is suitable to detect Pen a 1-like molecules in extracts from other crustacean species, such as crab and lobster, but it was not tested for the detection of other shellfish in cooked foods.

A number of sandwich ELISAs using polyclonal antibodies have been developed. One against Western King prawn (*Penaeus latisulcatus*) tropomyosin is able to detect both cooked and raw crustaceans in foods (Fuller et al., 2006). The method did not show cross-reactivity with molluscs, fish or mammalian meat. The LOD was about 1 mg/kg (prawn, lobster).

Another sandwich ELISA based on a polyclonal anti-tropomyosin capture antibody raised against shrimp (*Pandalus borealis*) and the biotinylated conjugate of the same antibody for detection has been

validated using spiked samples and commercially available food products (Werner et al., 2007). It is specific for crustaceans, though cross-reacting to some extent with cockroach. The LOD is 1mg/kg.

One sandwich ELISA which used monoclonal and polyclonal antibodies against black tiger prawn tropomyosin (Seiki et al., 2007) was specific for the Decapoda group, apart from minor cross-reactivities with other crustacean and molluscs. This method has a good accuracy and precision, with a LOD of 0.71 ng/mL, corresponding to 0.29 mg of crustacean protein/kg food sample. A similar sandwich ELISA using a monoclonal antibody against the same tropomyosin showed no cross-reactivity with molluscs. The LOD was 0.16 mg/kg (Shibahara et al., 2007).

Few commercial test kits are available for the detection of soluble crustacean tropomyosins in foods. A rapid and sensitive ELISA test kit for the detection of crustacean residues in food samples has been commercialised as screening method, with a LOQ for tropomyosin of 0.05 mg/kg by using a microwell reader (Schubert-Ullrich et al., 2009). LFDs for the qualitative detection of crustaceans are also commercially available.

Methods of detection for crustaceans and molluscs

While the above mentioned ELISA methods are specific for crustaceans, others detect tropomyosin in crustaceans and molluscs. A commercial ELISA based on a tropomyosin-specific monoclonal antibody was tested to detect crustaceans and molluscs, as well as to investigate the impact of heating on the detection (Abdel Rahman et al., 2013). Tropomyosin was detected in all crustacean species, but only partially in molluscs. Heating of shellfish increased recognition of multiple tropomyosin variants in both crustaceans and molluscs. The effect was attributed to the modification of the protein by the Maillard reaction. A potentially specific antibody targeting the N-terminal region of tropomyosin was identified to enable the differentiation between crustaceans and molluscs.

A sandwich ELISA based on the monoclonal antibody CE7B2 obtained against an IgE epitope, which is shared by several shellfish tropomyosins, was developed in order to detect tropomyosin in both crustaceans and molluscs (Zhang et al., 2014). The mAb CE7B2 reacts to intact tropomyosin, but also recognises fragmented peptides with a specific IgE epitope sequence, which is shared by a number of shellfish tropomyosins. The LOD is 0.09 ng/mL for kuruma prawn and 0.64 ng/mL for molluscs and house dust mite samples.

A lateral flow test for crustaceans and molluscs (including crab, lobster, brown shrimp, tiger prawn, langoustine, crayfish, scallop, oyster, mussel, cockle, and squid) has been marketed with a LOD around 5 mg/kg (Schubert-Ullrich et al., 2009).

21.6.1.2. Protein chip

A sandwich protein chip assay, which used rabbit antisera as the capture reagent and a biotin-labelled mAb as detector reagent, was developed to quantify shrimp allergens in food matrices (Zhenxing et al., 2010). The resulting antigen–antibody complexes were visualised in the presence of streptavidin labelled with Cy3, which produced a fluorescence signal suitable for quantification. The LOD was 0.054 mg tropomyosin/kg and the LOQ 0.096 mg tropomyosin/kg. The protein chip was cross-reactive with allergens from other crustaceans.

21.6.1.3. Biosensors

A rapid method based on a quartz crystal microbalance immunosensor, which uses a polyclonal antibody specific to shrimp allergens, was able to detect shrimp allergens with a LOD of 0.333 µg/mL (Xiulan et al., 2010). The electrode was modified based on self-assembly with 1,6-hexanedithiol (HDT) and nanogold.

In a cell-based electrochemical biosensor for the quantification of shrimp tropomyosin (Pen a 1) (Jiang et al., 2013), rat basophilic leukaemia (RBL-2H3) mast cells encapsulated in type I collagen were immobilised on a self-assembled L-cysteine/gold nanoparticle(AuNPsCys)-modified gold electrode

and pre-sensitised by specific anti-shrimp tropomyosin IgE. In the presence of the antigen, mast cells exhibit morphological changes which indicate degranulation, thus inducing dose-dependent impedance signals which can be detected by EIS. The impedance value increased with the concentration of purified tropomyosin, with a LOD of 0.15 µg/mL.

21.6.2. Mass spectrometry

Two methods for the characterisation and quantification of tropomyosin and arginine kinase from snow crab using isotope-labelled mass spectrometry (LC-MS/MS) are available (Abdel Rahman et al., 2010; Abdel Rahman et al., 2011). A comprehensive proteomic strategy (allergenomics) for characterising and quantifying allergenic proteins in crustaceans (northern shrimp) has also been described by the same authors (Abdel Rahman et al., 2013). Tropomyosin, arginine kinase and sarcoplasmic calcium-binding protein were targeted for quantification. The marker peptides for each protein were selected and synthesised in the light and heavy form and used in the development of the AQUA LC-MS/MS (MRM mode) approach. The peptide mixture could be analysed with a LOD < 0.25 nM. These methods are very selective, reproducible and accurate, and were applied to measure the level of these allergens in air samples, but not in foods.

21.6.3. DNA-based methods

DNA-based methods can discriminate among crustaceans by targeting specific nucleotidic sequences, while commercially available ELISA kits targeting tropomyosin are generally not able to differentiate between shrimp and crab species owing to the high homology of the tropomyosins.

Detection and species identification of crustacean DNA was achieved with a PCR restriction fragment length polymorphism (PCR-RFLP) method (Brzezinski, 2005). A fragment of the 16S rRNA gene in crustacean species was amplified by PCR and digested with differential restriction endonucleases to determine the species to which the DNA belongs. The specificity of the method was demonstrated by analysing shrimp, crab, lobster and crawfish. The LOD was < 0.1 % for shrimp in a raw meat mixture.

A shrimp end point-PCR method with post-amplification digestion and a crab end point-PCR method that specifically amplifies a fragment of the 16S rRNA gene allowed differentiating between shrimp and crab (Taguchi et al., 2011). The sensitivity and specificity of these PCR methods were verified by using incurred foods and commercial food products. Both methods could detect 5 pg of DNA extracted from the target species and 50 ng of genomic DNA extracted from incurred foods containing 10 mg/kg total protein of shrimp or crab.

A fast (40 minutes) real-time PCR for crustacean detection in differently processed foods has been developed using a locked nucleic acid probe (LNA) which, on account of its high affinity, allows specific recognition of even short DNA sequences (Herrero et al., 2012). No cross-reactivity was observed with molluscs or fishes, but it did not distinguish among crustacean species. The method was able to recognise 1.25 pg of crustaceans in highly processed foods.

Two real-time PCR assays aimed to detect penaeid shrimp and blue crab targeting mitochondrial genes, which could provide more sensitive assays owing to the high copy number (Eischeid et al., 2013). The assays were tested using shrimp and crab meat spiked into several types of foods at levels ranging from 0.1 to 106 mg/kg and analysed either raw or cooked. Cooking methods used to simulate thermal processing of foods had little effect on the assay performance. LODs were between 0.1 and 1 mg/kg.

21.7. Minimum (observed) eliciting doses

There is little information on the lowest doses of any crustacean triggering a clinical allergic reaction upon ingestion. In two DBPCFCs, one patient developed objective signs after consuming 14 g of shrimp (Bernstein et al., 1982) and four individuals reacted with objective signs to 32 mg of shrimp extract, corresponding to 16 g of shrimp (four shrimps of medium size) (Daul et al., 1988). Atkins et

al. (1985), in a study using open food challenges and objective signs as the outcome, reported reactions in four individuals to 25, 30 and 100 g of shrimp and 100 g of crab.

21.8. Conclusion

Crustaceans are a common trigger of food-allergic reactions, which are sometimes severe. The most important major allergen, tropomyosin, is well characterised. Tropomyosin and other crustacean allergens are heat-resistant. Prevalence of crustacean allergy in unselected populations in Europe has been estimated to be 0.2 to 0.3 %, although available studies of clinically confirmed crustacean allergy are scarce. Immunological methods such as ELISA, protein chips and biosensors, as well as mass spectrometry and DNA-based methods have been developed for the detection of crustacean allergens in processed foods. The lowest doses of crustaceans reported to elicit an allergic reaction in sensitive individuals are 14 g of shrimp and 32 mg of shrimp extract.

22. Allergy to molluscs

22.1. Background

The phylum *Mollusca* is second only to the phylum *Arthropoda* (which includes the class *Crustacea*) in the number and diversity of species (> 100 000) (Levinton, 2001; Hickman et al., 2004). Molluscs live in salt water, fresh water and on land, range in size from less than one millimetre to nearly 20 metres (giant octopus), and may weigh up to 900 kg (giant squid, *Architeuthis*). Molluscs are commonly classified into eight classes, of which three are of particular importance as food, namely gastropods, bivalves and cephalopods. The largest class, *Gastropoda*, counts more than 70 000 species (Hickman et al., 2004). A non-exhaustive list of mollusc species relevant in relation to food allergy is given in Table 22.

Table 22: Taxonomic classification of relevant molluscs (*phyllum Mollusca*) in the context of food allergy

Class	Major organisms	Common name	Scientific name
Gastropoda	Abalones	Small abalone	*Haliotis diversicolor*
		Red abalone	*Haliotis rufescens*
			Haliotis rubra
		Abalone	*Haliotis midae*
		Northern disc abalone	*Haliotis discus hannai*
	Limpets	Limpet	*Patella piperata*
			Patella vulgata
		Grand keyhole limpet	*Fissurella maxima Sowerby*
	Land (terrestrial) snails	Land snail	*Eupariphra pisana*
			Limax agrestis
			Eobania vermiculata
			Cernualla virgata
			Helix aperta
			Helix terrestre
			Helix pomatia
			Helix aspersa aspersa
			Helix aspersa maxima
			Helix lucorum
	Marine snails	Turban shell	*Turbo cornutus*
	Whelks	Whelk	*Hemifusus ternatana*
			Buccinum undatum
			Neptunea arthritica
Bivalvia	Clams	Clam	*Lutraria philipinarum*
			Tapes decussates
			Tapes japonica
		Razor clam	*Tagelus plebius*
	Oysters	Pacific oyster	*Crassostrea gigas*

Class	Major organisms	Common name	Scientific name
		Eastern oyster	*Crassostrea virginica*
	Mussels	Green mussel	*Perna viridis*
		Blue mussel	*Mytilus edulis*
		Mussel	*Mytilus galloprovincialis*
	Scallops	Scallop	*Chlamys opercularis*
			Chlamys nipponensis
			Patinopecten yessoensis
	Cockles	Cockle	*Cardium edule*
Cephalopoda	Squids	Squid	*Toradodes pacificus*
			Loligo edulis
			Loligo vulgaris
			Loligo japonica
			Loligo forbesi
			Loligo opalescens
			Loligo pealei
	Octopuses		*Octopus luteus*
			Octopus vulgaris
	Cuttlefishes		*Sepia madokai*
			Sepia latimanus
			Sepia officinalis

Source: adapted from Hickman et al. (2004) and Taylor (2008).

Molluscs are often grouped together with crustaceans under the term “shellfish” in the literature dealing with food allergy and food consumption data. However, data on allergy to “shellfish” do not provide information about the allergenicity of molluscs and thus have not been considered in this section.

Molluscs are a common food in most European countries, although consumption data are sparse. Whereas their use as added ingredients appears to be limited, they can be found in some processed foods, like soups and sauces, and in products like surimi.

22.2. Epidemiology

22.2.1. Prevalence

Data on the prevalence of mollusc allergy should be interpreted with particular caution for a number of reasons. First, adverse reactions to molluscs caused by infectious agents or toxins (e.g. algal toxins in blue mussels), in particular gastroenteritis-like symptoms (Myrmel et al., 2004; Hungerford, 2005), may lead to overestimation of the prevalence of mollusc allergy in studies using questionnaire-based methods. Second, the serological cross-reactivity between molluscs and crustaceans, mites, insects and helminths, may lead to overestimation of sensitisation to molluscs. Third, mollusc species often do not cross-react with each other, and thus the absence of a reaction to the one or few molluscs species used for testing in clinical studies does not exclude sensitisation or clinical allergy to other mollusc species, leading to an underestimation of the prevalence of mollusc allergy in these studies.

No prevalence studies using food challenges to confirm allergy to molluscs are available.

22.2.1.1. Europe

Data on the prevalence of mollusc allergy in unselected European populations are scarce.

In a questionnaire-based survey among children (2716 responders) in France (Rancé et al., 2005), two children (0.8 % of cases) reported allergy to mussels, one to snails and one to oysters (0.4 % of cases each), giving a prevalence of self-reported mollusc allergy of about 0.15 % (4/2716). In another French study (Touraine et al., 2002), 1.5 % of 5- to 17-year-olds reported an allergy to oysters. In Denmark, 0.4 % of 22-year-olds (n = 843) reported allergy to octopus, with 0.1 % confirmed by food

challenge (Osterballe et al., 2009). One study (Zuberbier et al., 2004) was based on positive SPT and a convincing clinical history and reported zero prevalence of mussel allergy in adults (n = 4093) in Germany.

Prevalence data of mollusc allergy in food-allergic patients are more abundant, but are only indicative of the relative importance of molluscs as a trigger of allergic reactions to food. In Gran Canaria, Spain, 33 (27.5 %) out of 120 food-allergic subjects reported adverse reactions to squid (second most common allergenic food after shrimp), 12 (10 %) to oyster, 10 (8 %) to clam, and 10 (8 %) to mussel (Castillo et al., 1996). Also in Spain, molluscs caused 1.6 % of 608 allergic reactions in 355 children based on clinical history, SPT and specific IgE (Crespo et al., 1995a).

Among 163 severe food-allergic reactions reported by allergologists in France in 2001, six (3.8 %) were caused by molluscs (Moneret-Vautrin D et al., 2002). In a questionnaire-based study among 1139 subjects with food hypersensitivity in Baltic countries, 6.2 % indicated allergic reactions to clam, 3.2 % to oyster and 1.4 % to snail, but < 50 % of the subjects had ever eaten clam, oyster or snail (Eriksson et al., 2004).

22.2.1.2. Outside Europe

In a random cross-sectional telephone survey on the prevalence of seafood allergy in the USA (Sicherer et al., 2004), 14 948 individuals completed the survey. Sixty-seven persons reported reactions to scallops, clams, oysters, or mussels, representing about 0.4 % of the study population (67/14 948) and 20 % of all doctor-diagnosed or “convincing” seafood allergy.

In Thailand, self-reported mollusc allergy was 0.2 % in three- to seven-year-olds (Lao-araya and Trakultivakorn, 2011). In Taiwan, mollusc allergy defined by a clinician’s diagnosis varied from 0.1 % in under three-year-olds to 1.5 % in adults (Wu et al., 2012).

22.2.2. Natural history

There are no data available regarding the natural history of mollusc allergy.

22.2.3. Time trends

There are no studies available, which allow investigating time trends in mollusc allergy.

22.2.4. Severe reactions/anaphylaxis

Anaphylactic reactions and death have been reported in mollusc-allergic patients, including cases of food-dependent exercise-induced anaphylaxis (EFSA, 2006b). Of the 107 fatal or near-fatal reactions to food reported by the Allergy Vigilance Network for 2002 mainly in France, five were to snails (4.7 % of all reactions) (Moneret-Vautrin et al., 2004). In a report on 67 consecutive cases of anaphylaxis from an immunology/allergy centre in Singapore (Thong et al., 2005), 30 cases were triggered by food and 11 of these (36.7 %) by molluscs (limpet and abalone).

22.2.5. Factors affecting the prevalence of mollusc allergy

A number of case reports and patient series suggest that allergic reactions to molluscs most commonly occur in school age children and young adults (EFSA, 2006b), and thus may tend to develop later than the common childhood allergies This may be explained in part by the later introduction of molluscs into the diet, and by the fact that cross-reactive respiratory allergies, e.g. to mites, tend to develop relatively late.

22.3. Identified mollusc allergens

Whereas the Allergome database records 155 entries under molluscs allergens, only three allergens are listed in the IUIS database (Table 23), which illustrates the relative lack of systematic studies relative to mollusc allergens.

Table 23: Mollusc (*Animalia: Mollusca*) allergens [(a)]

Biochemical name	Allergen	Common name	Scientific name	Molecular weight (kDa) [(b)]
Tropomyosin	Buc u 1	Common whelk	*Buccinum undatum*	36–38
	Chl n 1	Japanese scallop	*Chlamys nipponensis*	
	Cra g 1	Oyster	*Crassostrea gigas*	
	Ens m 1	Razor clam	*Ensis macha*	
	Hal d 1	Abalone	*Haliotis discus*	
	Hal m 2	Abalone	*Haliotis midae*	
	Hel as 1 [(c)]	Garden snail	*Helix aspersa*	
	Hal r 1	Abalone	*Haliotis rufescens*	
	Mim n 1	Scallop	*Mimachlamys (Chlamys) nobilis*	
	Oct v 1	Octopus	*Octopus vulgaris*	
	Per v 1	Mussel	*Perna viridis*	
	Pin a 1	Fan shell	*Pinna atropurpurea*	
	Tod p 1 [(c)]	Japanese flying squid	*Todarodes pacificus*	
	Tur c 1	Turban shell	*Turbo cornutus*	
NA	Hal m 1 [(c)]	Abalone	*Haliotis midae*	49

(a): The list is not comprehensive;
(b): Molecular weight (SDS-PAGE). Slight variation between species.
(c): Allergens listed in the IUIS database.
NA, not assigned.

Tropomyosin is considered to be the most important mollusc allergen (Chu et al., 2000; Motoyama et al., 2006; Emoto et al., 2009). The muscle protein tropomyosin is a highly conserved panallergen among invertebrate animal species including molluscs, crustaceans, arachnids (e.g. mites), and insects (e.g. cockroaches) (Reese et al., 1999). Tropomyosin has also been described as an allergen in one fish, i.e. tilapia (*Oreochromis mossambicus*) (Liu et al., 2013). The carboxyl-terminal region of tropomyosins is highly conserved across molluscan species, whereas other parts of the molecule are more variable. This is also reflected at the epitope level, with some epitopes being shared and cross-reactive among mollusc species whereas others are more or less species specific (Chu et al., 2000).

Tropomyosins are allergens in many mollusc species (Table 23). However, in some species, tropomyosin appears to be a minor allergen, e.g. in land snail (Asturias et al., 2002) and non-tropomyosin allergens are present in most, if not all, food-relevant molluscs (Taylor, 2008). Non-tropomyosin mollusc allergens include hemocyanin (Koshte et al., 1989; Mistrello et al., 1992), myosin heavy chain (Martins et al., 2005), arginine kinase (Shen et al., 2012) and amylase (Azofra and Lombardero, 2003). Myosins have been reported to be a major allergen in snail (Martins et al., 2005). However, little information is yet available on the clinical importance of mollusc non-tropomyosin allergens.

22.4. Cross-reactivities

Vertebrate tropomyosins, that generally are not allergenic, show between 50 and 60 % amino acid sequence identity with all invertebrate tropomyosins. All molluscan tropomyosins show identity ranging from 68 to 88 %, and even higher within the individual mollusc classes: 91 to 100 % among cephalopods, 70 to 100 % among bivalves, and 85 to 97 % among gastropods. The amino acid sequence identities between crustacean and mollusc tropomyosins range from 56 to 68 %. Mite and cockroach show 56 to 66 % sequence identity with mollusc tropomyosins, similar to crustaceans (Motoyama et al., 2006; Taylor, 2008; Emoto et al., 2009). This, and the yet ill-defined role of non-tropomyosin allergens in cross-reactivity, makes the serological and clinical picture of cross-reactivity in relation to molluscs' complex.

22.4.1. Cross-reactivity among molluscs

Some individuals appear to react to all common molluscs, whereas others react to few or one species only. Although clinical cross-reactivity among molluscs is limited and tends to fall into a number of loosely defined clusters that, to a limited extent, reflect the taxonomic classification, it is often unpredictable (EFSA, 2006b). Among 67 individuals reacting to scallops, clams, oysters, or mussels, 34 (51 %) reacted to one, 13 (19 %) to two, 5 (8 %) to three, and 15 (22 %) to all four species (Sicherer et al., 2004). Case reports have been published on isolated allergy to octopus (Caiado et al., 2009), snail (San Miguel-Moncin M et al., 2007), razor shell clam (Martín-García et al., 2007) and clam (Rodríguez-Del Rio et al., 2009). In the three first cases, there was evidence that the triggering allergen was not tropomyosin.

22.4.2. Cross-reactivity between molluscs and crustaceans

See section 21.4.2 on crustaceans.

22.4.3. Cross-reactivity between molluscs and non-crustacean invertebrates

A number of examples of simultaneous clinical and serological reactivity to molluscs, insects, arachnids and nematodes with IgE-binding to tropomyosin suggest that tropomyosin is an important allergen triggering clinical cross-reactivity among invertebrates. The house dust mite allergens Der p 10, Lep d 10 and Der f 10, and the cockroach (*Periplaneta americana* and *Blatella germanica*) allergens Per a 7 and Bla g 1, are tropomyosins (EFSA, 2004, 2006b).

Clinically relevant cross-reactivity between snail, limpet, mussel and house dust mite has been described as the "mite–crustacean–mollusc–syndrome" (Kütting and Brehler, 2001; EFSA, 2006b). Primary sensitisation is most often against mite. However, allergies to mites or cockroaches subsequent to sensitisation to molluscs (e.g. snail) have been reported (van Ree et al., 1996a; Ayuso et al., 2002a; Martins et al., 2005). Tropomyosin (Der p 10) may play a role in snail–mite cross-reactivity (Ayuso et al., 2002b; Ayuso et al., 2002a), but non-tropomyosin allergens are also involved, e.g. Der p 4 (amylase), Der p 5, Der p 7, and haemocyanin (Mistrello et al., 1992; Bessot et al., 2010), and these allergens appear to be of importance in cross-reactivity between molluscs, cockroach and other insects (van Ree et al., 1996a; Guilloux et al., 1998). Immunotherapy with mite allergen has been reported to increase the risk of anaphylactic reactions to snails (van Ree et al., 1996a; Pajno et al., 2002).

Similar to crustaceans, clinical cross-reactivity between molluscs and the fish parasite *Anisakis* has been observed (Gonzalez Galan et al., 2002). Individuals reacting clinically to fish because of *Anisakis* infestation may also react to molluscs, which would appear as fish–mollusc cross-reactivity.

22.4.4. Cross-reactivity between mollusc and vertebrates

Vertebrate tropomyosins appear to be non-allergenic (Leung et al., 1996; EFSA, 2004, 2006b), with the exception of tropomyosin from the fish tilapia (*Oreochromis mossambicus*) (Liu et al., 2013). Cross-reactivities for tilapia tropomyosin have not yet been reported. Invertebrate myosins, a major snail allergen, did not cross-react with chicken, pig, rabbit, cow or horse myosins (Martins et al., 2005).

22.5. Effects of food processing on allergenicity

Tropomyosin is heat resistant, whereas some non-tropomyosin mollusc allergens may be destroyed by heating (Yadzir et al., 2010; Shen et al., 2012). Mollusc overall allergenicity is not reliably reduced by heat treatment. In some cases, heating may increase the allergenicity of molluscs (EFSA, 2004, 2006b). One study on scallop tropomyosin (Nakamura et al., 2005) identified the Maillard reaction as one mechanism by which the IgE-binding capacity may be increased after heating.

22.6. Detection of allergens and allergenic ingredients in food

Only few (mainly immunological) methods for the detection of mollusc allergens, primarily tropomyosin, have been reported. However, tropomyosin is a major cross-reactive allergen among crustaceans and molluscs, and thus immunological methods of detection may not be able to discriminate between the two groups of shellfish. Only methods specific for the detection of molluscs allergens are described here. No MS methods for the detection of mollusc allergens have been reported. PCR methods have been used mostly for the identification and authentication of different mollusc species. No limits of detection have been reported.

22.6.1. Immunological methods

22.6.1.1. ELISA

A sandwich ELISA based on a monoclonal antibody obtained using the Japanese flying squid (*Todarodes pacificus*) tropomyosin as immunogen has been described (Shibahara et al., 2010). The method is specific for cephalopods and does not recognise other molluscs and crustaceans. The LOD was 0.24 mg/kg.

22.6.1.2. Rapid methods

A specific immunostick assay based on monoclonal antibodies against abalone (*Haliotis midae*) enabled differentiation between several abalone species (Lopata et al., 2002), although it showed less reactivity to heat treated abalone. No binding was observed for other mollusc or crustacean species analysed. No limit of detection has been reported.

22.6.2. PCR methods

Various PCR methods have been used for the identification of molluscs in food products, mostly for traceability and authentication. In particular, forensically informative nucleotide sequencing (FINS), multiplex PCR and single-strand conformation polymorphism (PCR-SSCP) have been used to identify abalone (Aranceta-Garza et al., 2011; Chan et al., 2012). FINS and restriction fragment length polymorphism (PCR-RFLP) have been used for the identification of cephalopod species (Chapela et al., 2003). However, no data are reported on limits of detection and quantification.

22.7. Minimum (observed) eliciting doses

Specific data on doses of mollusc triggering allergic reactions are rare. Exercise-induced anaphylaxis has been reported after ingestion of 100 g of canned oysters (Maulitz et al., 1979) and fatal anaphylaxis after consumption of three snails (Wu and Williams, 2004). In a DBPCFC, the cumulative dose of dried snail causing 20 % decrease in forced expiratory volume (FEV1), a measure of systemic allergic reaction, was 120 mg on one child and 400 mg in another(Pajno et al., 2002).

22.8. Conclusions

Molluscs can cause severe and occasionally life-threatening food-allergic reactions. Prevalence data in unselected populations are scarce and mostly limited to self-reported prevalence. The most important allergen of molluscs is tropomyosin, which has been well characterised in several mollusc species. Molluscs also contain a number of other allergens. Tropomyosin is heat resistant and mollusc allergenicity is not reliably reduced by food processing. Only few immunological methods specific for mollusc allergens have been described. ELISA methods to detect both crustaceans and molluscs are available. There is limited information on the lowest dose of mollusc that can elicit a clinical reaction. In the only DBPCFC available, reactions were observed to cumulative doses of dried snail in the low hundred milligram range.

23. Allergy to celery

23.1. Background

Celery (*Apium graveolens*) belongs to the *Apiaceae* family (or *Umbelliferae*). The celery plant is composed of a root or tuber, also called celeriac, and of an aerial part, the sticks or stalks. Celery (tubers and sticks) is consumed raw (e.g. in salads), cooked (e.g. on its own, in sauces and soups), and dried as a spice, and it is a common ingredient in processed foods.

Celery can induce allergic reactions of immediate type, from oral contact urticaria to anaphylactic shock. About 30 % of patients with OAS are allergic to celery. The first evidence for IgE celery-specific antibodies in sensitised subjects was reported using SPTs (Dechamp et al., 1984; Pauli et al., 1985). Diagnostic tests like SPT with raw celery and allergen extracts have high positive predictive values, while the negative predictive values are low.

23.2. Epidemiology

23.2.1. Prevalence

23.2.1.1. Europe

Four studies published between 2001 and 2006 reported prevalence data on celery allergy in Europe in unselected populations (Schafer et al., 2001; Touraine et al., 2002; Zuberbier et al., 2004; Bakos et al., 2006).

Reported rates of positive SPTs to celery were 9.1 % in adults in Germany (Schafer et al., 2001), and 11.1 % and 3.7 % in adults and elderly people in Hungary, respectively (Bakos et al., 2006). Sensitisation rates measured by serum-specific IgE levels to celery in Hungary were 2.8 % for adults and 9.2 % for the elderly (Bakos et al., 2006). The prevalence of self-reported allergy to celery was 5.5 % among 5- to 17-year-olds in France (Touraine et al., 2002). The prevalence of celery allergy in Germany (all ages) based on positive SPT and clinical history of allergy to celery was 2.7 % (Zuberbier et al., 2004).

Allergy to celery was not confirmed with food challenges in any of the studies available reporting on prevalence in unselected populations.

23.2.1.2. Outside Europe

One study conducted in Taiwan reported that 1.8 % of six to eight-year-olds suffer from celery allergy based on positive serum-specific IgE levels and clinical history (Wan and Chiu, 2012).

23.2.2. Natural history

There are no data available regarding the natural history of celery allergy.

23.2.3. Time trends

There are no studies available, which allow investigating time trends in celery allergy.

23.2.4. Severe reactions/anaphylaxis

Celery root may trigger severe anaphylactic reactions in some of patients with celery allergy (Wuthrich et al., 1990). In one study of French patients attending a specialist allergy clinic, about 30 % of 580 food-allergic patients showed specific IgE to celery and 30 % of the 60 severe, near-fatal allergic reactions to food occurred between 1984–1992 appeared to be due to celery (Andre et al., 1994). Systemic and severe reactions to celery in subjects allergic to pollen (pollen–food allergy syndrome) have been reported by several authors (Schöll and Jensen-Jarolim, 2004).

23.2.5. Factors affecting prevalence of celery allergy

Patients with birch pollen allergy may develop allergic reactions to celery. In Germany, 70 % of patients with a pollen-related food allergy were reported to have a positive SPT or RAST to celery (Jankiewicz et al., 1996). In Italy, about 10 % of 262 patients with OAS to fresh fruit and vegetables had a clinical history of allergic reactions to celery, and about 3 % experienced severe reactions to celery, such as laryngeal oedema (Ortolani et al., 1988).

23.3. Identified allergens

The major celery antigen is Api g 1. Other identified allergens in celery are Api g 2, Api g 4, and Api g 6 (Table 24). The cross-reactive carbohydrate determinants also seem to be allergenic.

Table 24: Celery (*Apium graveolens*) allergens

Source	Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Tuber/root	Api g 1	PR-10	Bet v 1	15
Sticks/stalk	Api g 2	ns-LTP 1	Prolamin	9 [(b)]
Sticks/stalk	Api g 3	Chlorophyll a-b binding protein		–
Tuber/root	Api g 4	Profilin	Profilin	14
Tuber/root	Api g 5	FAD-containing oxidase		58
Tuber/root	Api g 6	ns-LTP 2	Prolamin	7[b]

(a): Molecular weight (SDS-PAGE).
(b): kDa.

The panallergen profilin Api g 4, a cross-reacting protein present in several plant foods, has been shown to be involved in celery allergy (Vallier et al., 1992). The cross-reactive carbohydrate determinants, structures containing α-1,3-fucose and β-1,2-xylose bound to proteins via N-glycoside linkages, are highly immunogenic in mammals; some celery-allergic patients exclusively display IgE-binding to these determinants of molecular weight > 45 kDa. However, any clinical significance of cross-reactive carbohydrate determinants-specific IgE is still a matter of debate (Aalberse, 1998; Fotisch et al., 1999).

Ganglberger et al. (Ganglberger et al., 2000) described two high molecular weight allergens of celery recognised by the sera of five patients with positive case histories, SPTs and RASTs to celery and birch. These 55 and 58 kDa proteins represent members of a protein family not described so far, as no homologous sequences were found in the databases. The 58 kDa allergen, included in the IUIS nomenclature as Api g 5, may correspond to a 60 kDa allergen identified by Heiss et al. (Heiss et al., 1996), the function of which was not determined. Bublin et al. (Bublin et al., 2003) found the complete abolition of binding of serum IgE from all 14 patients tested by chemical deglycosylation of the Api g 5 glycoprotein allergen and observed that native Api g 5 other than the deglycosylated protein completely inhibited the IgE-binding to high molecular weight allergens in protein extracts from birch pollen, mugwort pollen and celery. These results suggest that IgE directed to cross-reactive carbohydrates may be capable of eliciting allergic reactions.

23.4. Cross-reactivities

Three different structures are responsible for cross-reactions between pollen and plant foods, namely Bet v 1 and related plant proteins, profilin, and carbohydrate determinants. Cross-reactivities of celery allergens with pollen and other plant foods, which have been described in the literature, are depicted in Table 25.

Api g 1 and Bet v 1 belong to a class of intracellular PR proteins and their structure shows 40 % identity and 60 % similarity (Breiteneder et al., 1995; Schirmer et al., 2005). Api g 1 is the major allergen for patients with birch pollen/celery sensitisation (Vieths et al., 1995). Whereas two isoforms

of Api g 1, Api g 1.0201 and Api g 1.0101 cross-react with Bet v1 from birch pollen, Api g 1.0201 shows a weaker IgE-binding capacity than Api g 1.0101 (Hoffmann-Sommergruber and Mills, 2009). Api g 1 also shows high identity (61.4 %) and homology (79.1 %) with the two parsley PR proteins PcPR1–1 and PcPR1–3 (Somssich et al., 1988). Both celery and parsley belong to the Apiaceae family.

Celery profilin, Api g 4, has high sequence identity (71–82 %) to known allergenic plant profilins, which may be responsible for allergenic cross-reactivity between celery and other plant foods or pollens (Table 25). Its IgE cross-reactivity with the minor birch pollen allergen Bet v 2, identified as a profilin (Valenta et al., 1992), explains the birch–mugwort–celery syndrome (Wuthrich et al., 1990; Bauer et al., 1996; Scheurer et al., 2000).

Table 25: Cross-reactivities of celery allergens with pollen and other plant foods

Celery allergen	Cross-reactivity	Allergen	Detected by	Reference
Api g 1	Birch	Bet v 1	Sequence homology IgE	(Hoffmann-Sommergruber et al., 2000; Lüttkopf et al., 2000; Schirmer et al., 2005; Wangorsch et al., 2007)
Api g 1	Parsley	PcPR1–1 PcPR1–3	Sequence homology	(Somssich et al., 1988)
Api g 2	Peach Mugwort	Unknown	IgE	(Gadermaier G. et al., 2011)
Api g 4	Birch Olive	Bet v 2 Ole e 2	Sequence homology IgE	(Valenta et al., 1992; Bauer et al., 1996; Asturias et al., 1997; Asturias et al., 1998; Scheurer et al., 2000)
Api g 4	Timothy grass Bermuda grass Sunflower Soy Peanut Pear Cherry	Phl p 11 Cyn d 12 Hel a 2 Gly m 3 Ara h 5 Pyr c 4 Pru av 4	IgE	(Scheurer et al., 2000; Scheurer et al., 2001)

Sensitisation to celery is frequently associated with birch and/or mugwort pollinosis, hence the term “birch–mugwort–celery syndrome”. Wüthrich et al. (Wuthrich et al., 1990) hypothesised that the association between celery and birch is due to a common thermolabile allergen while the common allergen between celery and mugwort is thermostable. A confirmation of this assumption is the fact that RAST with cooked celery extract were negative in patients with birch allergic rhinitis, while it remains positive in those with mugwort allergic rhinitis.

There is evidence that birch pollen and celery allergy are highly related in Central Europe (Hoffmann-Sommergruber et al., 1999), while celery allergy is most frequently related to mugwort pollen allergy in Southern Europe. Ballmer-Weber et al. (Ballmer-Weber et al., 2000) reported that all patients with positive DBPCFC for celery were sensitised to either birch (91 %) or mugwort (64 %) pollen, and that only two out of 22 patients did not show any sensitisation to birch pollen. Also in a previous study performed in Swiss patients, 8 % of celery-allergic subjects were not sensitised to rBet v 1 or rBet v 2 (Wuthrich and Straumann, 1997). Api g 2 was shown to cross-react with peach and mugwort pollen and was recognised by IgE in 25 % of 786 LTP sensitive Italian subjects (Gadermaier Gabriele et al., 2011).

In one study (Lüttkopf et al., 2000), the allergens recognised by IgE from 22 patients with positive DBPCFCs to celery were identified. Cross-reactivities with pollen allergens were also assessed. Api g 1, cross-reactive carbohydrate determinants, and celery profilin were recognised by IgE of 59 %,

55 %, and 23 % of patients, respectively. IgE-binding to all three structures in a celeriac extract was inhibited by birch pollen extract, whereas mugwort pollen extract could only inhibit IgE reactivity to Api g 4 and cross-reactive carbohydrate determinants. Cross-inhibitions with extracts of birch pollen, mugwort pollen, timothy grass pollen, and lychee, demonstrated the ubiquitous presence of cross-reactive carbohydrate determinants and profilin, while Api g 1 was only cross-reactive with birch pollen. Homologues of Api g 4 and cross-reactive carbohydrate determinants were also present in tree pollen and pollens from weeds, Graminaceae and other plant families.

Allergies to carrot and spices, predominantly of the Umbelliferae family, are strongly associated to celery allergy, known as "celery–carrot–mugwort–spice syndrome". This syndrome is frequently described in the German literature (Wuthrich and Dietschi, 1985). The only studies carried out in patients with celery allergy confirmed by positive DBPCFC pointed out a sensitisation to carrot in 77 % of patients with CAP > 0.7 kU/L (Ballmer-Weber et al., 2000).

23.5. Possible effects of food processing on allergenicity

Jankiewicz et al. (Jankiewicz et al., 1997) investigated the immunochemical stability of allergens in celery roots after various treatments such as microwaving, drying, γ-irradiation, ultra-high pressure and high-voltage impulse, and demonstrated that the cross-reactive carbohydrate determinants are the most heat-stable allergens, followed by profilin and Api g 1. The risk of clinical reactivity to cooked celery could not be deduced from the sensitisation pattern to individual celery allergens. The majority of patients reacting to cooked celery recognise the heat-stable allergens Api g 4 and cross-reactive carbohydrate determinants, but a minority of them are exclusively sensitised to Api g 1, which is more heat-labile. Patients exclusively sensitised to Api g 1 react to higher doses of cooked celery than the other patients. In addition, Api g 2, a member of the lipid-transfer protein family primarily expressed in celery stalks, is thermally stable and resistant to gastrointestinal digestion (Gadermaier G. et al., 2011).

EAST inhibition data also shows that Api g 1 is the most heat-labile allergen in celery, that celery profilin Api g 4 is more stable under thermal processing, and that the IgE reactivity to cross-reactive carbohydrate determinants is not affected by heating (Ballmer-Weber et al., 2002). In this study, a total of 12 patients with history of allergic reactions to raw or to raw and cooked celery were recruited. Of these, 11 (3 with reported allergic reactions to raw, but not cooked, celery) underwent a DBPCFC with cooked celery root and six reacted. An increasing intensity of the heat treatment did not lead to unequivocal reduction of allergenicity. Only 10 patients agreed to undergo a DBPCFC with raw celery root and all reacted. There were no patients reacting to cooked celery root without symptoms to the raw root. This may indicate that no neoallergens are created by the heating process, and that residual activity of the native celery allergens is responsible for the allergenic activity of the cooked vegetable. Also, a DBPCFC with celery spice, dried and pulverised celery was performed in five of the patients recruited. All patients reacted with symptoms comparable to those observed during the DBPCFC with raw extract. However, symptoms occurred at a lower provocation dose compared with raw celery, probably because of higher protein content in celery spice (about 4.5 times higher). This study suggests that, in some subjects, allergic reactions to cooked celery will take place even when high temperatures are used, and that celery spice is allergenic for individuals allergic to raw celery.

23.6. Detection of allergens and allergenic ingredients in food

Several methods for the detection of celery have been described (Stephan et al., 2004; Hupfer et al., 2007), including ELISA and PCR.

23.6.1. ELISA

ELISA methods are generally non-specific as cross-reactivity has been shown with other vegetal species. However, a sandwich ELISA for the detection and quantification of celery proteins with monoclonal antibodies obtained against the recombinant fusion allergenic protein rApi g 1.01 has been published (Wang H et al., 2011). The method has a high specificity, except a low cross-reactivity with

carrot, and a good sensitivity (LOD = 1.9 µg of celery soluble protein/g food sample; LOQ = 5.6 µg whole celery protein/g food sample). The method gave reliable results on both raw and heat processed foods.

23.6.2. PCR

PCR methods are generally more specific than ELISA. Both conventional PCR and real time PCR (Hupfer et al., 2007; Mustorp et al., 2008) methods target the gene encoding for mannitol dehydrogenase. A novel real time PCR method (Fuchs et al., 2013) allows the detection of traces of celery in complex food matrices targeting the gene coding for the *Apium graveolens* NADPH (nicotinamide adenine dinucleotide phosphate)-dependent mannose-6-phosphate reductase. It allows the detection of three varieties of celery commonly used. It is specific and sensitive (LOD = 10 pg celery DNA). The best performance concerning DNA extraction was obtained with the Wizard method, which produced no false negative results. The method is applicable to commercially available foodstuffs (e.g. noodles, sauces, sausages, soups and spice mixes). In another report (Pafundo et al., 2011), the amount of DNA recovered from celery with the GK-resin method was less variable than with other methods, although the yield was lower. By using Fast SYBR PCR, DNA analysis was performed on raw celery and food products containing celery (LOD = 10 mg/kg). The results obtained are repeatable, reproducible and suitable for routine analysis. A duplex PCR was also carried out by the same authors by combining the primer pair 18SR as an internal control, and the primer pairs specific for celery. This method, although only qualitative, can be used to detect celery in highly processed foods.

A multiplexed real-time PCR method for the detection of DNA from 12 allergenic ingredients, including celery, is also available (Köppel et al., 2012). Two hexaplex real-time PCR systems were developed to amplify DNA simultaneously from six allergenic ingredients each. The method is specific and allows detecting 0.1 % of each analyte, but the corresponding amounts of protein on a weight basis have not been reported.

An innovative method based on optical thin film biochips for multiplex detection of eight allergenic ingredients, including celery, has also been developed (Wang W et al., 2011). Eight target fragments were amplified by two tetraplex PCR and spotted on the microarray. This method has a high throughput and allows visualising the results with the naked eye (no need of expensive instruments), but needs further development for routine use.

23.7. Minimum (observed) eliciting doses

Data from DBPCFCs conducted in 32 patients with history of an allergic reaction to celery have been published (Ballmer-Weber et al., 2000). Patients underwent SPT and specific IgE was measured in serum. In addition, DBPCFC with raw celery root was performed. Two different drinks were prepared for the test meal, i.e. an active drink with celery and a placebo. The foods were identical in colour, consistency and taste, and all ingredients except for celery were known to be tolerated by each patient. Patients were first challenged in a single-blind way with a placebo drink (5 mL), which they had to retain in their mouth for 5 minutes. Individuals reacting were excluded. Patients who did not react underwent a two-step DBPCFC, during which (i) they had to retain increasing amounts of the celery extract from raw celery root and placebo drinks in the mouth for one minute at 15-minute intervals and (ii) if the patients did not complain about symptoms during this "spit" phase, they were asked to swallow the drinks in increasing amounts. Twenty-two patients showed reactions to the active preparation: 11 complained about symptoms strictly localised to the oral cavity (OAS) and 11 showed systemic reactions. The minimum dose to elicit a subjective reaction was 0.7 g of celery root, corresponding to the first dose tested, whereas the minimum doses to elicit a systemic (objective) reaction were 1.9 g (nausea and dyspnoea) and 5.6 g (urticaria). The eight non-responders underwent an open challenge with celery, and four of them had OAS with 5 g.

The same authors confirmed allergy to cooked celery in six out of eight patients with a positive case history (Ballmer-Weber et al., 2002) using DBPCFC with raw celery, cooked celery, and celery spice.

Seven of these complained about OAS during the local mucosal challenge ("spit" phase) of raw celery at a minimum dose of 0.7 g (first dose tested), and five at a minimum dose of 0.9 g of cooked celery. Reactions were systemic in three patients at provocation doses of 28.5 g of raw celery, and in one patient at the provocation dose of 34.5 g of cooked celery. DBPCFC with celery spice were performed in five of the patients recruited: all of them were responders. Two had OAS at the provocation dose of 0.16 g of celery spice and three had systemic reactions during the local mucosal challenge at 0.16 g, 0.32 g and 5.85 g of celery spice.

23.8. Conclusion

Celery tuber/root is an important source of food allergens in Central Europe. Celery stalks are often consumed in Mediterranean countries. Celery tuber/root could be found as food ingredients in several pre-packed food and spice mixes, as it is widely used in the food industry owing to its aromatic flavour. The prevalence of celery allergy based on positive SPT and clinical history has been estimated to be 2.7 % in Germany. Celery root may trigger severe anaphylactic reactions in some of patients with celery allergy. Patients with birch pollen allergy may develop allergic reactions to celery and react with severe symptoms, such as laryngeal oedema. Allergenicity to cooked celery seems to be reduced, though not abolished. There are currently no specific immunochemical methods commercially available for celery detection, on account of cross-reactivity with other species. At the moment, the method of choice, suitable for quantification of both raw and cooked celery, is the detection of DNA by conventional (semi-quantitative) and real-time PCR. Only two studies reported minimum doses eliciting local symptoms (at 0.7 g) or systemic reactions (at 1.9 g), which were similar for raw and cooked celery but corresponded to the first dose tested in the study. It cannot be excluded that some subjects would react to lower doses. Celery spice can trigger systemic reactions also at low doses (0.16 g).

24. Allergy to lupin

24.1. Background

Lupin (genus *Lupinus*, family *Leguminosae*) is a legume, which includes over 450 species. It is widely grown as a flowering plant for animal feed and farmland management. The usual garden species are poisonous. Some species, like *Lupinus luteus* (yellow lupin, Central Europe), *Lupinus albus* (white lupin, Mediterranean countries), and *Lupinus angustifolius* (blue lupin, Australia) are used as whole seed flour, or as lupin derived drinks (lupin milks), for human and animal consumption. Lupin seeds are a common snack in several European countries. The yellow lupin variety, because of its colour, is used as egg substitute. The above varieties are known as sweet lupins.

The nutritional value of lupin and its potential as a human food has been under consideration for about 30 years owing to its low cost, high protein quality, and the associated increased protein efficiency ratio compared with other members of the legume family. Lupin protein contains the essential amino acids (lysine, leucine and threonine) but it is low in methionine, the addition of which improves the protein efficiency ratio. Lupin flour is an excellent source of protein (39 %–45 %, depending on the lupin species). Lupin does not contain gluten and can be used in gluten-free foods (Marss, 1996; Kanny et al., 2000). Since its introduction as an ingredient in wheat flour in the late 90s, lupin flour became a more widely consumed food ingredient. Lupin flour is used in biscuits, pasta, sauces, dietetic products sold as milk and soy substitutes. Owing to its emulsifying properties, lupin concentrates are also used in meat and cold-cut industry (EFSA, 2006a).

24.2. Epidemiology

24.2.1. Prevalence

No population-based studies investigating the prevalence of primary allergy to lupin are available, and thus the prevalence of primary allergy to lupin in the general population is unknown.

Allergic reactions to lupin in Europe have emerged following its introduction in processed foods in the late 1990s. The possibility of under-reporting of allergic reactions to lupin cannot be excluded because, until recently[10], it was a hidden (undeclared) ingredient in bakery and other food products.

In 1040 consecutive patients attending an allergy clinic in the Netherlands with a suspected food allergy, 372 were skin prick tested with peanut, soy and lupin flour. A total of 135, 58 and 22 patients were SPT positive for peanut, soy and lupin flour, respectively. Nine patients sensitised to lupin flour underwent a DBPCFC, which was negative in eight cases. The estimated prevalence of lupin allergy among patients with a suspected food allergy was 0.27–0.81 %. In most cases, sensitisation was not clinically relevant and was most likely caused by cross-sensitisation to peanut (de Jong et al., 2010).

Since allergic reactions to lupin have mostly been documented in peanut-allergic individuals (Moneret-Vautrin et al., 1999; Leduc et al., 2002), the clinical relevance of primary allergy to lupin has been questioned (Lindvik et al., 2008; de Jong et al., 2010). However, cases of allergic reactions following lupin consumption as a bread ingredient or as snacks were reported in subjects with no prior allergy to peanut and negative SPT for this food (Smith et al., 2004; Peeters et al., 2007; Quaresma et al., 2007). This is corroborated by specific binding of IgE from sensitised individuals to isolated lupin globulins (Klos et al., 2010). Sensitisation to lupin via inhalation has also been reported in individuals with no immunologic reactivity to other legumes (Novembre et al., 1999; Prieto et al., 2010), and for occupationally exposed adults with no allergy to peanut (Crespo et al., 2001; Parisot et al., 2001; Campbell et al., 2007). Individuals sensitised via inhalation to lupin flour may react to lupin flour after ingestion (Crespo et al., 2001; Crespo et al., 2002).

24.2.2. Natural history

There are no data available regarding the natural history of lupin allergy.

24.2.3. Time trends

The prevalence of allergy to lupin is likely to depend on local eating habits and the level of exposure through other routes. Lupin consumption appears to be increasing in several European countries, but no data are available on time trends for lupin allergy.

24.2.4. Severe reactions/anaphylaxis

Severe anaphylactic reactions have been described upon oral exposure to lupin both in subjects primarily sensitised to lupin (Brennecke et al., 2007) and in legume-allergic individuals, including peanut-allergic subjects (Matheu et al., 1999). It is currently estimated that, with respect to the cases of anaphylaxis reported after oral exposure to lupin, the ratio between pre-existing legume allergy (mostly peanut) and *de novo* sensitisation to lupin seeds is around 1:1 (Jappe and Vieths, 2010).

24.2.5. Factors affecting prevalence of lupin allergy

The main population at risk of developing allergic reactions to lupin is peanut-allergic individuals. Most reports of allergic reactions to lupin refer to patients with known allergy to peanuts attending specialist medical services (Hefle et al., 1994; Moneret-Vautrin et al., 1999; Faeste et al., 2004). A study conducted in 47 peanut-allergic children and adolescents and 46 atopic controls, 4 % of peanut-allergic patients showed clinical reactions to lupin when challenged orally, whereas none of the atopic controls reacted (Shaw et al., 2008).

24.3. Identified allergens

L. angustifolius contains seed storage proteins which include two major protein types, β-conglutin (vicilin-like protein, or acid 7S globulin) and α-conglutin (legumin-like protein or 11S globulin), and two minor components, δ-conglutin (2S albumin) and γ-conglutin (basic 7S globulin). The four

[10] Labelling Directive 2000/13/EC, as amended by Directive 2003/89/EC that entered into force on 25 November 2005, which applies only to pre-packaged foodstuffs.

fractions (named on the base of their electrophoretic mobility) are glycosylated (Foss and Frökiær, 2005) (Table 26).

Table 26: Lupin (*Lupin angustifolius*) allergens

Superfamily/ family	Biochemical name	% of total protein	Molecular weight (kDa)	Allergen	Molecular weight [(a)] (kDa)
α-Conglutin [(b)] (legumin-like)	11S Globulin	33	69–89	Lup-2[2]	20
β-Conglutin (vicilin-like)	7S Globulin	45	19–60	Lup an 1	55–61
				Lup-1 [(c)]	34.5
γ-Conglutin [(b)]	7S Globulin	6	46		46
δ-Conglutin [(b)]	2S Albumin	12	14		14

(a): Molecular weight (SDS-PAGE).
(b): www.allergome.org
(c): Present also in *Lupinus albus*.

β-Conglutin is a non-covalently associated heterogeneous trimer consisting of a number of polypeptides ranging from 16 to 70 kDa (Duranti et al., 2005). β-Conglutin from *L. angustifolius* seed was recognised by the IgE of most (8/12) lupin-allergic subjects (Goggin et al., 2008) and is considered the major lupin allergen and is the only lupin allergen (Lup an 1) reported in the IUIS allergen nomenclature. A polypeptide of the β-conglutin fraction from *L. albus* has been also identified and designated as Lup-1 (Alvarez-Alvarez et al., 2005; Guillamon et al., 2010). Lup-1 has been shown to be a major allergen in patients with allergy following lupin ingestion (Sanz et al., 2010) and is highly homologous to soy β-conglycinin and peanut Ara h 1.

In *L. angustifolius*, α-conglutin consists of four subunits, which are non-covalently linked. The subunits are in a 55 to 80 kDa range and contain a 20 kDa disulphide-bound moiety. A polypeptide of 20 kDa, called Lup-2, has been isolated from the α-conglutin fraction, characterised and identified as a major allergen in *in vitro* IgE-studies (Guillamon et al., 2010). In *L. albus*, α-conglutin is a heterogeneous mixture composed of a hexamer of basic and acid trimers linked by two disulphide bridges with molecular weight in the range 47 to 54 kDa linked to a basic polypeptide of ca. 20 kDa (Magni et al., 2005). α-Conglutin is highly homologous to the peanut allergen Ara h 3 (Foley et al., 2011).

γ-Conglutin consists of two disulphide linked polypeptides of 30 and 17 kDa, respectively. Specific IgE-binding from lupin-allergic individuals to γ-conglutin has been described (Klos et al., 2010). α-, β- and γ-Conglutin include two cupin domains. δ-Conglutin is a monomeric protein with two disulphide linked polypeptides of 9 and 4 kDa, respectively, which is highly homologous to the peanut allergen Ara h 2.

IgE-binding proteins of lupin with molecular weight of 43 to 45 kDa were shown by immunoblotting in studies involving lupin-allergic patients (Moneret-Vautrin et al., 1999; Novembre et al., 1999; Parisot et al., 2001; Holden et al., 2008).

Other potential allergens with MWs of 13, 29, 34, 38, and 66 kDa were isolated from lupin seeds by chromatographic methods (Dooper et al., 2007) and were studied for IgE-binding affinities (Magni et al., 2005; Holden et al., 2008).

24.4. Cross-reactivities

Several legumes (e.g. peanut, soybean, lentils, beans, chickpeas and peas) may cross-react with lupin *in vitro* (IgE-binding) (Bernhisel-Broadbent and Sampson, 1989; Bernhisel-Broadbent et al., 1989; Ballabio et al., 2013).

In vitro cross-reactivity between lupin and other legumes has been tested mostly using serum from peanut-allergic individuals. β-Conglutin (Lup an 1) was a major allergen in patients with allergy following lupin ingestion (Sanz et al., 2010) and the major lupin allergen cross-reacted with peanut proteins, as observed in IgE-binding and SPT studies in peanut-allergic individuals (Ballabio et al., 2013). The basic subunits of the 11S globulin α-conglutin may also be responsible for cross-reactivity between lupin and other leguminous plants (Ballabio et al., 2010; Sirtori et al., 2011). The high level of amino acid sequence homology between Lup-1 and Lup-2 and major allergens of some legumes explains the cross-reactivity between lupin and other legumes.

Although the amino acid sequence of the lupin γ-conglutin does not align with any known peanut protein, γ-conglutin also bound specific IgE from peanut-allergic individuals (Sirtori et al., 2011), suggesting that the IgE-binding epitopes for this protein and for peanut allergens are similar. Conversely, the lupin proteins γ-conglutin (2S albumin) and 11S globulin were shown to cross-react with homologous polypeptides of other legumes (Magni et al., 2005).

Cross-reactivity between Ara h 2 and δ-conglutin and between Ara h 1 and β-conglutin were also described using sera from lupin-allergic patients (Dooper et al., 2009). Significant sequence and molecular homology between Ara h 8 of peanut and the pathogenesis related protein PR-10 of white lupin suggest that these proteins could in part be responsible for some of the reported cross-reactivities in peanut-allergic individuals (Guarneri et al., 2005).

In vitro cross-reactivities between members of the legume family are of clinical relevance in about 5 % of legume-allergic individuals, notably cross-reactivity to peanut (EFSA, 2006a). The risk of clinically relevant cross-reactions with peanuts in lupin-allergic patients is higher than with other legumes, whereas cross-reactivity to lupin in peanut-allergic patients is also of clinical relevance (Hefle et al., 1994; Moneret-Vautrin et al., 1999; Kanny et al., 2000; Faeste et al., 2004).

In a study of 44 peanut-allergic subjects, 11 had positive SPTs to lupin flour and seven out of the eight subjects who underwent a DBPCFC with lupin flour reacted, indicating clinical cross-reactivity between peanut and lupin (Moneret-Vautrin et al., 1999). In a DBPCFC, 68 % (15/23) of patients allergic to peanuts showed clinical reactions to lupin flour (Leduc et al., 2002).

Although a major lupin allergen belonging to the PR-10 is homologous to the birch pollen allergen Bet v 1 superfamily (17–22 kDa), with common secondary structures, there is no information as to the likelihood of clinical reactions to lupin in these individuals. The scarcity of clinical reports from countries with a high birch pollen sensitisation rate (e.g. Sweden) may suggest that this structural similarity is not of clinical relevance in these populations. The Panel is not aware of systematic studies, which address the relationship between lupin pollen allergies and reactions to lupin flour after ingestion.

24.5. Effects of food processing on allergenicity

A common feature of most legume allergens is their relative resistance to thermal, chemical, and proteolytic degradation (Lalles and Peltre, 1996; Mills et al., 2009). The IgE-binding capacity of lupin seeds after boiling (up to 60 minutes), autoclaving (121 °C, 1.18 atmospheres, up to 20 minutes and 138 °C, 2.56 atmospheres, up to 30 minutes), microwave heating (30 minutes), and extrusion cooking has been assessed (Alvarez-Alvarez et al., 2005). An important reduction in IgE-binding capacity was reported after autoclaving at 138 °C for 20 minutes and absence of IgE-binding after autoclaving for 30 minutes.

After harsh industrial processing involving mechanical, high-pressure homogenisation and lyophilisation and thermal (200 °C for 30 minutes) treatments, α-, β-, and δ-conglutin were still able to release stable IgE-binding peptides (Sirtori et al., 2010). However, the *in vitro* IgE-binding of lupin was reduced when an instantaneous controlled pressure drop was applied to lupin cotyledons (Guillamon et al., 2008). Removal of oligosaccharides from intact lupin seeds by ethanol extraction reduced the content of γ-conglutin in the lupin protein fraction (Martínez-Villaluenga et al., 2006). *In vitro* hydrolysis of the major globulins found in sweet lupin by the enzymes pepsin and trypsin appears to reduce their IgE-binding capacity (Sormus de Castro Pinto et al., 2009).

Lupin allergens are generally resistant to thermal, chemical, and proteolytic degradation. The effects of different processing methods on the allergenicity of lupin have not been systematically investigated.

24.6. Detection of allergens and allergenic ingredients in food

24.6.1. ELISA

A quantitative sandwich ELISA that uses polyclonal rabbit anti-lupin antibodies was used as a routine method to detect lupin in food products with a LOD of 1 mg lupin protein per 1 kg of food (Holden and Egaas, 2005). A kit for this ELISA test is commercially available. Another test based on monoclonal/polyclonal sandwich ELISA has also been described with a LOD of 1 mg/kg, suitable for both raw and processed proteins (Holden et al., 2007). Kaw's assay (Kaw et al., 2008) is based on the rabbit antisera as the capture reagent and the sheep antisera as detector reagent, with a LOQ of 1mg/kg. It has been applied to model and reference food standard with reliable results. Minor cross-reactivities have been observed with soy (*Glycine max*) and black bean (*Castanospermum australe*). The extensive immunological cross-reactivities between legumes represent a problem in the development of lupin-specific immunological assays (Bernhisel-Broadbent and Sampson, 1989; Bernhisel-Broadbent et al., 1989).

24.6.2. Mass spectrometry

Protein detection by mass spectrometry has emerged as an alternative method to ELISA. A semi-quantitative study was performed on lupin trypsin digested proteins using HPLC/ESI-MS/MS (Locati et al., 2006). β-Conglutin was detected in a linear 0.025 to 1.5 mg/mL concentration range. The same group (Brambilla et al., 2009) developed a nanoHPLC-chip-MS/MS stable isotope label-free (SIF) method for the simultaneous characterisation and quantitation of *L. albus* seed storage proteins in four lupin cultivars and a HPLC-chip-ion trap MS/MS for the quantitation of γ-conglutin in a lupin seed protein extract (Resta et al., 2012).

A rapid shotgun proteomic LC-ESI-MS/MS method was proposed for the simultaneous and unequivocal confirmation and quantitation of α-, β-, γ- and δ-conglutins in pasta and biscuits (Mattarozzi et al., 2012). The allergenic proteins were identified by monitoring target tryptic peptides specific and unique for the four proteins. The LOD was in the same range as shown for ELISA and PCR (around 1 mg/kg). The LOQ varied for the different proteins (4–42 mg/kg) and depending on the matrix.

24.6.3. PCR

Real-time PCR assays for the detection of lupin genomic DNA in food products have been developed with different LODs (0.1–10 mg/kg) (Demmel et al., 2008; Scarafoni et al., 2009; Gomez Galan et al., 2010; Demmel et al., 2011) on the base of sequence tags publicly available. Also, a qualitative duplex real-time PCR method for the simultaneous detection of lupin and soy mitochondrial DNA is available, with a LOD of 2.5 mg/kg in processed foods (Gomez Galan et al., 2011).

The performance of ELISA and real-time PCR methods for the detection of lupin in different food matrices (biscuits, bread, noodles and rice patties) was assessed by developing in-house one sandwich ELISA, two competitive ELISA and one real-time PCR method (Ecker and Cichna-Markl, 2012). The most sensitive method was the sandwich ELISA, with a LOD of 10 mg/kg for all matrices, both before

and after heat treatment. Both ELISA and PCR methods gave different results according to the lupin cultivars (Röder et al., 2013).

24.7. Minimum (observed) eliciting doses

Available data on the lowest doses of lupin triggering clinical allergic reactions have been obtained almost exclusively in peanut-allergic individuals.

In a DBPCFC, six children allergic to peanut were challenged orally with lupin flour (Moneret-Vautrin et al., 1999). Five children showed allergic reactions at doses of lupin flour ranging from 265 to 1000 mg (two children with clinical response to the labial challenge did not undergo the oral challenge). The youngest child (1.5 years) did not have a positive SPT but responded with deterioration of her atopic dermatitis. In this study, the minimum doses of allergenic food eliciting a subjective clinical response were similar for peanut and lupin (5–965 mg and 265–1000 mg, respectively).

Twelve children with history of clinical allergic reactions to peanut were evaluated by SPT, the ImmunoCAP test, immunoblotting, and DBPCFC. In the DBPCFC, patients received lupin-enriched pasta every 30 minutes at increasing doses of 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 g of macaroni (the last dose at 3.5 h), totalling 12.7 g of lupin protein for children under 13, with a ninth dose of 51.2 g of pasta at 4 h for children over 13 years of age for a total of 25.6 g of lupin protein. Carrot-containing pasta was used as control. Positive clinical subjective reactions were recorded in two children at doses of 0.2 and 6.4 g of pasta, corresponding to 50 mg and 1.6 g of lupin proteins, respectively. β-Conglutin was the protein most involved in SPT positivity (Fiocchi et al., 2009).

Peeters et al. (Peeters et al., 2009) studied 39 peanut-sensitised patients by SPTs and ImmunoCAP to lupin, pea, and soy. Eighty-two per cent of the study population was sensitised to lupin, 55 % to pea, and 87 % to soy. Clinical reactivity was measured by DBPCFC for lupin, and by history for pea and soy. Clinical reactions to lupin, pea, or soy occurred in 35 %, 29 %, and 33 % of the study population, respectively. The MED for lupin inducing mild subjective symptoms was 0.5 mg of lupin flour, and the NOAEL was 0.1 mg of flour. The amount of protein corresponding to this amount of flour was not reported.

A case of anaphylaxis and deteriorating lung function in a peanut-allergic, 13-year-old girl after oral challenge with a cumulative dose of 965 mg of a crude lupin flour extract has been reported (Kanny et al., 2000). This quantity could be present in 100 g of bread if wheat flour contains 10 % lupin, as it was allowed by some national legislation.

24.8. Conclusion

The frequency of allergic reactions to lupin in the general population is unknown. Allergic reactions to lupin have been documented in peanut-allergic as well as in individuals with primary sensitisation to lupin. The possibility of under-reporting of allergy cases cannot be excluded, as until recently lupin was an unlabelled ingredient in various bakery and meat products. Lupin allergens have been identified. Lupin allergens are generally resistant to thermal, chemical, and proteolytic degradation. The effects of different processing methods on the allergenicity of lupin have not been systematically investigated. ELISA, MS and PCR methods are available to detect and quantify lupin in foods. Clinical reactions to lupin range from mild symptoms to anaphylaxis. Doses of lupin protein triggering clinical reactions in peanut-allergic individuals vary widely (from 50 mg to 1.6 g). Subjective symptoms have been reported to 0.5 mg of lupin flour.

25. Allergy to sesame

25.1. Background

Sesame (*Sesamum indicum* L.) is a plant originally from tropical Africa, which is now universally cultivated for its seeds. It is the most important species in the *Sesamum* genus of the Pedialaceae

family. The seeds are used in several food products, especially in bakery products, fast-foods, processed meat, vegetarian and ethnic dishes. The oil obtained from the seeds is used for cooking and salad dressing in Oriental, Chinese and South American cuisines, and is also employed by the pharmaceutical industry as a vehicle of medications for intramuscular injection.

In some countries, sesame is one of the major causes of food allergy. In Israel, where sesame seed-based foods (halva and tehina) are included in the diet of infants and young children as a source of proteins and iron, sesame is the third common cause of IgE-mediated food allergy and the second most common cause of anaphylaxis (Dalal et al., 2002; Dalal et al., 2012).

25.2. Epidemiology

25.2.1. Prevalence

25.2.1.1. Europe

Eight studies from Europe (in France, Germany, Hungary and the UK) are available between 1999 and 2008 and all ages were studied.

Self-reported diagnosis of sesame allergy was investigated in three studies. The highest prevalence was observed in France, where 1.5 % of 5- to 17-year-olds self-reported an adverse reaction (Touraine et al., 2002), whereas a zero prevalence was reported in the UK across all ages (Emmett et al., 1999).

Sensitisation to sesame measured by SPT was reported in four studies. In the UK the lowest sensitisation rates were reported in 7-year-old children (0.1 %) (Roberts et al., 2005), and the highest in three-year-olds (1.4 %) (Venter et al., 2008). Only one study determined specific IgE levels to sesame and found zero prevalence of sensitisation among 60- to 97-year-olds in Hungary (Bakos et al., 2006). In Germany, a population based study reported prevalence rates based on a positive SPT plus a convincing clinical history of 1.7 % (Zuberbier et al., 2004).

In the UK, two studies challenged subjects suspected of sesame allergy and showed prevalence between 0.1 % in six-year-olds (Venter et al., 2006a) and 0.6 % in three--year-olds (Venter et al., 2008).

25.2.1.2. Outside Europe

In the rest of the world, self-reported sesame allergy ranged between 0.07 and 0.23 % in Canada (Ben-Shoshan et al., 2010) and was 0.1 % in the US general population (Sicherer et al., 2010).

In Israel, where exposure to sesame occurs earlier in life than in European countries, 0.18 % of young children up to two years of age had positive SPT with clinical history of sesame allergy (Dalal et al., 2002).

One Australian study based on OFC reported a prevalence of food allergy to sesame of 0.7 % in children 12 to 15 months old (Osborne et al., 2011).

25.2.2. Natural history

Sesame allergy appears to be persistent similar to allergy to fish or peanuts (Agne et al., 2004). However, Cohen et al. (Cohen et al., 2007) reported that 20 % of 74 sesame-allergic paediatric patients in Israel developed tolerance during the follow-up period of 1.8 to 14 years (median 6.4 years). Clinical scoring and severity of symptoms were not found to be predictive in the development of the tolerance. Similarly, in a cohort of 234 children, sesame was the third most common allergenic food after milk and egg, but unlike milk and egg allergy, sesame allergy resolved in only 30 % of patients (Aaronov et al., 2008).

25.2.3. Time trends

There were limited studies on sesame allergy, with only two studies worldwide using food challenges (in the UK and Australia), so no time trends can be reported.

25.2.4. Severe reactions/anaphylaxis

Information about documented severe reactions to sesame is scarce. In the UK, a questionnaire-based survey suggested that sesame was responsible for severe reactions. Among 280 replies received, 54 % reported reactions to sesame; 89 % of reactive subjects reported other atopic diseases and notably 84 % were also nut/peanut-allergic. A total of 17 % had suffered potentially life-threatening reactions, with 65 % of severe reactions happening on first known exposure (Derby et al., 2005). Many publications on sesame allergy are case reports describing anaphylaxis or case series on anaphylaxis presenting to emergency departments (Dalal et al., 2012). Although systemic reactions occur, a single case of death owing to an allergic reaction to sesame has been reported (Pumphrey and Gowland, 2007). Anaphylactic shocks with 1 and 2 mL of sesame oil have been reported in two out of five patients with a positive DBPCFC (Morisset et al., 2003b).

25.2.5. Factors affecting prevalence of sesame allergy

Sesame allergy appears to be present more frequently during childhood, although onset maybe at any age (Dalal et al., 2012).

In one study using SPT and specific IgE testing for diagnosis in children with sesame-seed allergy, a significant association was found with allergy to other seeds, especially poppy seed, in 17 % of subjects (Foong et al., 2013). Sesame-seed allergy was also significantly associated with tree-nut allergies but not with peanut allergy. Patients with sesame allergy have a high likelihood of having multiple allergies and it is recommended to test sesame-allergic patients at least for tree nut and peanut allergens (Dalal et al., 2012).

25.3. Identified allergens

Sesame seeds contain 50 to 60 % oil and 19 to 25 % proteins. Most protein present in sesame seeds are storage proteins composed of globulins (67.3 %), albumins (8.9 %), prolamins (1.4 %) and glutelins (6.9 %) (Rivas R et al., 1981). The water-insoluble 11S globulins and the soluble 2S albumins are the two major storage proteins, constituting 80 to 90 % of the total seed proteins in sesame.

Seven proteins have been identified as allergens in sesame seeds and are reported in the IUIS database (Table 27).

Table 27: Sesame (*Sesamum indicum*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Ses i 1	2S Albumin (sulphur-poor)	Prolamin	9
Ses i 2	2S Albumin (sulphur-rich)	Prolamin	7
Ses i 3	7S Vicilin-like globulin	Cupin	45
Ses i 4	Oleosin	Oleosin	17
Ses i 5	Oleosin	Oleosin	15
Ses i 6	11S Globulin	Cupin	52
Ses i 7	11S Globulin	Cupin	57

(a): Molecular weight (SDS-PAGE).

The first allergen identified and sequenced in sesame seeds was a 2S albumin named Ses i 1, a seed storage protein, which was recognised by the 10 patients studied (Pastorello et al., 2001b). All patients showed high levels of sesame-specific IgE and highly positive SPT with fresh seeds and commercial extracts. In contrast, only a minority of the 20 patients with systemic reactions tested in a second study recognised proteins with a molecular weight in the range of Ses i 1 (Beyer Kirsten et al., 2002). In a third study conducted in Israel, which evaluated 24 subjects with symptoms and specific IgE to sesame, 22 recognised the 14 kDa 2S albumin precursor, confirming Ses i 1 as a major sesame allergen (Wolff et al., 2003). 2S albumins are typically heterodimeric proteins with small and large subunits linked by disulphide bonds. Several reacting epitopes were found on the peptide corresponding to the residues 24–94 (Wolff et al., 2004). Ses i 1 has 47 % homology with the Brazil nut Ber e 1, 41 % with ricin nut Ric c 1 and 40 % with sunflower seeds (Pastorello et al., 2001b). Other authors (Fremont et al., 2002) reported homology of Ses i 1 with Sin a 1 of yellow mustard, and with Bra j 1 of oriental mustard.

The two sesame allergens Ses i 2 and Ses i 3 (Beyer Kirsten et al., 2002) belong to the family of seed storage proteins. Ses i 2 is a 2S albumin and a sulphur-rich protein (Tai et al., 1999), 47 % homologous and 35 % identical to Ses i 1, which is a sulphur-poor protein (Tai et al., 2001). Ses i 2 was recognised only by 30 % of the patients. It has a sequence homology of 38 % with the walnut allergen Jug r 1, 40 % with the Brazil nut Ber e 1 and 34 % with the peanut allergen Ara h 1 (Beyer Kirsten et al., 2002).

Ses i 3, which constitutes approximately 5 % of the total sesame protein, is a 7S vicilin-like globulin, formed by polypeptides non-covalently linked (Orruño and Morgan, 2007). It was recognised by 75 % of patients and is a major allergen of sesame (Beyer Kirsten et al., 2002). Ses i 3 showed a 41 % sequence homology to the walnut allergen Jug r 2 and 36 % homology to the peanut allergen Ara h 1.

Two oil body-associated proteins (oleosins), which were recognised by IgE from most sesame-allergic patients (29 out of 32 patient sera), were sequenced and named Ses i 4 and Ses i 5 (Leduc et al., 2006). They represent 80 to 90 % of total oil body proteins and correspond to only 1 to 2 % of total seed proteins. Ses i 4 and Ses i 5 are highly hydrophobic and may remain residually present in oil and bind specific IgE. Anaphylactic shocks have been reported after ingestion of a few millilitres (1 and 5 mL) of sesame oil (Morisset et al., 2003b). Homology between oleosin of different species has been found for a Chinese spice shiso (*Perilla frutescens*, 75 % identity) and for carrot oleosin (64 % identity). Lower levels of identity have been observed with peanut and soybean oleosins (56 and 51 %, respectively).

Two additional sesame seed allergens Ses i 6 and Ses i 7, which are 11S globulins of high-molecular weight sharing only 36 % identity, have been obtained by cloning (Beyer et al., 2007). The recombinant proteins were screened with sera of 24 patients with sesame allergy. Thirteen patients showed a strong IgE binding to Ses i 6 and ten patients to Ses i 7. Clinical reactions were observed in these patients involving the skin (n = 19), the gastrointestinal tract (n = 11), the respiratory system (n = 6) and several organ systems (n = 12).

The stability of the allergenic proteins to gastrointestinal digestion varied widely and consistently with the reported IgE-binding data (Orruño and Morgan, 2011). The 2S albumins were highly stable to digestion by all the enzymes tested. The 7S and 11S globulins were relatively labile to pepsin, but generated stable polypeptides after digestion with trypsin and chymotrypsin.

25.4. Cross-reactivities

Few data are available on the clinical and immunological cross-reactivity of sesame seeds.

Children sensitised to sesame had a high prevalence of sensitisation to peanuts (84.8 %), hazelnut (82.9 %), egg (81.5 %), walnut (80.6 %) and almond (76.3 %) (Stutius et al., 2010). Both cross-sensitivity and clinical cross-reactivity were observed between sesame, peanut and tree nuts. Children

sensitised or allergic to both peanuts and tree nuts may be more likely to be sensitised or allergic to sesame (Stutius et al., 2010).

By using a modified basophil activation test (mBAT), the sesame 11S globulin Ses i 6 showed partial immunological cross-reactivity with walnut (Wallowitz ML et al., 2006; Wallowitz et al., 2007).

25.5. Effects of food processing on allergenicity

The major sesame allergen Ses i 1 was thermo-stable up to 90 °C at neutral and acid pH, showing minimum conformational alterations which were reversible on cooling, as shown by circular dichroism (CD) and Fourier transform-infrared spectroscopy (FTIS) (Moreno et al., 2005). It was also highly resistant to digestion in an *in vitro* gastrointestinal model system. However, no tests on the antigenicity or the allergenicity of heated samples have been reported.

Protein extracts of sesame seeds prepared using different conditions (NaCl concentration, pH) showed different immunological responses (Achouri and Boye, 2013). The immunoreactivity was higher for isolates extracted with water and lower salt concentration (0.2 M NaCl) as compared with those extracted at higher salt concentration (0.6 M and 1 M), on account of the different solubility of the proteins extracted (Achouri et al., 2012). At higher salt concentration, salting out and aggregation could have also prevented the interaction of the epitopes with the IgG antibodies.

High pressure treatments (from 100 MPa to 500 MPa) markedly decreased the antigenicity of sesame allergens at all pH values (in particular at pH 7 and 10), probably owing to the unfolding of the proteins with loss of conformational epitopes, as monitored by FTIR (Achouri and Boye, 2013). Thermal processes, such as boiling and dry roasting, increased the antigenic response, whereas microwaving decreased it. IgE-binding capacity of sesame storage proteins was not significantly altered by the application of γ-irradiation with doses up to 10 kGy (Zoumpoulakis et al., 2012).

Subjects reacting to 100 mg up to 7 g of sesame seeds also reacted to a few milligrams of proteins in sesame oil (Morisset et al., 2003b). Sesame oil is generally unrefined and contains proteins, in particular lipophilic proteins (oleosins) in the lipid matrix, which may explain the higher allergenicity.

25.6. Detection of allergens and allergenic ingredients in food

25.6.1. ELISA

Immunochemical assays for the detection of sesame allergens with a LOD < 1 mg/kg of food have been reported (Brett et al., 1998) and detection kits are commercially available (Poms et al., 2004a). An indirect competitive ELISA, based on polyclonal antibodies, has been developed (Husain et al., 2010). In crisp bread, crackers, cereals and snacks, the LOD was found to be 5 mg of sesame protein/kg of food, corresponding to 28 mg sesame/kg of food, with a LOQ of 30 mg sesame protein/kg of food, corresponding to 165 mg sesame/kg of food. In fresh bread and rolls, the LOD was 11 mg of sesame protein/kg food, corresponding to 61 mg sesame/kg of food, with a LOQ of 49 mg/kg sesame protein/kg of food, corresponding to 270 mg sesame/kg of food. No cross-reactivity with other allergens was observed. It was not possible to detect sesame in sesame oil or in sesame roasted at 250 °C with this method.

A sandwich ELISA was also developed which did not show any cross-reactivity with 19 food ingredients commonly found in sesame containing foodstuffs (Redl et al., 2010). In wholegrain bread, crisp toasts and snacks the LOD was 0.5, 0.5 and 0.3 mg sesame protein/kg and the LOQ was 0.6, 0.8 and 1.4 mg sesame protein/kg, respectively. The sandwich ELISA showed significantly lower LOD and LOQ than the competitive ELISA previously described. Sesame roasted at 250°C for 10 minutes could not be detected. This method was not tested for the detection of allergens in sesame oil.

25.6.2. PCR

PCR test kits for detecting sesame are commercially available. Selective real-time PCR methods for the detection of sesame in food are able to detect 5 pg of purified sesame seed DNA (Brzezinski, 2007). Another method targeting the gene coding for Ses i 1 (Schoringhumer and Cichna-Markl, 2007) was able to recognise white, brown and roasted sesame, but not sesame oil, probably owing to the low concentration of DNA in oil. A good linearity was obtained down to 10 pg/µL, corresponding to an absolute amount of 50 pg or, assuming a haploid sesame genome size of 0.97 pg, 52 genomic copies. The assay did not show cross-reactivity with 17 common food ingredients.

Another quantitative and sensitive real-time PCR method for the detection of sesame in food was developed (Mustorp et al., 2008) targeting the genes encoding for the two 2S albumins, Ses i 1 and Ses i 2, which show only a limited sequence homology. The assay gave a good performance with solid foods (wheat flour, barbecue spice) at a spiking level of 0.005 % (w/w), detecting template DNA below approximately 1 molecule. It was not tested for the detection of sesame in sesame oil.

25.6.3. Multiplex DNA-based methods

Multiplex DNA-based methods based on different approaches have been developed for the simultaneous determination of several allergens, including sesame.

A duplex real-time PCR method to simultaneously detect sesame and hazelnut in food did not show cross-reactivity with 25 common food ingredients and allowed detection in spiked foods down to 0.005 % of both sesame and hazelnut (Schoringhumer et al., 2009). A method for the simultaneous detection of celery and sesame in foods by means of an end-point PCR protocol in connection with a microchip CE platform (Coïsson et al., 2010) had a LOD of 1 mg/kg DNA for sesame.

Two tetraplex qPCR for the detection of eight allergenic ingredients had a specificity and sensitivity for sesame in the range of 0.01 % (Köppel et al., 2010), whereas two quantitative hexaplex real-time PCR systems for the detection and quantification of 12 allergens in food (Köppel et al., 2012) had a LOD of 0.1 % for all analytes. Another six-plex qPCR able to detect six allergenic ingredients (Pafundo et al., 2010) had a LOD of the template sesame DNA of 0.5 pg.

A multiplex ligation-dependent probe amplification (MLPA) method for the detection of 10 different nuts and sesame (Ehlert et al., 2009)had a reported LOD in the lower mg/kg range for all allergenic ingredients. A quantitative 10-plex competitive MLPA method for the detection of 10 allergenic ingredients, including sesame, with an internal positive control (IPC) had a high sensitivity (Mustorp et al., 2011) for sesame, with a of LOD 3.8 ng DNA, corresponding to 173 gene copy number (± 93) and similar or higher sensitivity for spiked foods.

Optical thin-film biochips for multiplex visible detection of eight allergenic ingredients in food have been developed based on two tetraplex PCR systems (Wang W et al., 2011). The absolute LOD was 0.5 pg sesame DNA, and the practical LOD for sesame concentration in a blended mixture was 0.001 %, the lowest value observed up to now.

25.7. Minimum (observed) eliciting doses

The four studies available in the literature which have reported on oral challenges in sesame-allergic patients have been conducted for diagnostic purposes by the same research group in France (Kanny et al., 1996; Kolopp-Sarda et al., 1997; Morisset et al., 2003b; Leduc et al., 2006).

In one study (Morisset et al., 2003b), haemodynamic modifications and respiratory symptoms were observed in 8 % and in 42 %, respectively, of the 12 positive oral challenges (SBPCFC or DBPCFC) to sesame seeds analysed. A cumulative reactive dose ≤ 65 mg of solid food (equivalent to 12.4 mg of sesame proteins) was found in 8 % of sesame-allergic patients. The lowest eliciting dose was observed

at ≤ 30 mg of sesame seeds (equivalent to 5.1 mg of sesame proteins). Five out of six DBPCFCs with sesame oil were positive, and two patients had an anaphylactic shock with 1 and 5 mL, respectively.

In a subsequent study (Leduc et al., 2006), 32 patients (15 children and 17 adults) with positive history, labial test or DBPCFC to sesame seeds were challenged (DBPCFC or labial test) with either sesame seeds or sesame oil. Five were not challenged because of history of severe allergic reactions to sesame. Of the 27 patients undergoing the oral challenge, four did not react to the highest doses (965 mg and 7 g of sesame seeds). Two patients reacted with objective signs to 0.7 and 1 mL of sesame oil, respectively. One patient also reacted with objective signs to 6 mg of sesame seeds, corresponding to 1 mg of sesame protein. Systemic reactions were noted in some subjects at higher doses (965 mg, 7 g and 10 g of sesame seeds). Objective reactions to higher doses of sesame proteins were reported in the two previous studies by the same group (Kanny et al., 1996; Kolopp-Sarda et al., 1997).

25.8. Conclusion

Allergy to sesame seeds is well documented, especially in countries such as Israel where exposure occurs early in life. Sesame seeds contain major allergens, which can cause severe anaphylactic reactions. Both sensitivity and clinical cross-reactivity were observed between sesame and peanut and tree nut. Thermal processes, such as boiling and dry roasting, increased the IgE-binding capacity, whereas microwaving decreased it. High pressure treatments markedly decreased the IgE-binding capacity of sesame allergens at all pH values. ELISA and real-time PCR methods are available to detect sesame in foods. DBPCFC studies show that both sesame seeds and sesame oil can elicit allergic reactions in sensitised patients, with MEDs ranging from 6 mg to 10 g of sesame seed, and few millilitres (1–5 mL) of sesame oil. A few milligrams of sesame protein is enough to trigger severe allergic reactions.

26. Allergy to mustard

26.1. Background

The mustard plant belongs to the *Brassicaceae (*formerly *Cruciferae)* family. White/yellow (*Sinapis alba* L.), black (*Brassica nigra* L.) and brown/oriental mustard (*Brassica juncea* L.) are the main types of mustard seeds used in cuisine and food processing. Mustard powder commercially available is usually a mixture of ground white and black mustard seeds. White and brown seeds are blended to make the English style mustard. White mustard seeds are the main ingredient in Nord-American mustard, while the brown seeds are mainly used in Europe and China. Black mustard is mostly used in Indian cuisine. Mustard oil is also widely used as an edible oil and as a flavouring agent in India.

Mustard is used on some meat dishes, such as hot-dogs and hamburgers, and is very often an added ingredient in spices, sauces, salads and other foods. For example, mayonnaise as well as ketchup and curry mixtures may contain mustard. Mustard is also used in traditional remedies (Rancé et al., 2000). Mustard consumption in different countries varies according to local food habits.

26.2. Epidemiology

26.2.1. Prevalence

Prevalence data from DBPCFC for allergy to mustard are scarce owing to the difficulty of masking the strong taste of mustard. In addition, oral challenges to confirm mustard allergy have been considered an unethical health risk owing to the severity of systemic reactions reported following ingestion of mustard in allergic individuals.

Only one population-based study based on self-reported diagnosis of mustard allergy could be found in the literature (Touraine et al., 2002), where 3 % of teenagers (5- to 17-year-olds) in France reported adverse reactions to mustard.

Data on the prevalence of mustard sensitisation, clinical mustard allergy and the incidence of severe allergic reactions to mustard in the general population are lacking.

Data obtained in patient populations are particularly abundant in France, where sensitisation rates form 1 % to 28 % have been reported in food-allergic adults and children between 1983 and 2001 (Moneret-Vautrin and André, 1983; Andre et al., 1994; Rancé et al., 1999).

The prevalence of mustard allergy in clinical studies using DBPCFC or OFC varies between 23.3 % among children with a previous history of a clinical reaction to mustard (Morisset et al., 2003a), 42 % among previously sensitised children (Rancé et al., 2000; Rancé et al., 2001), and 58 % among atopic adults (Figueroa et al., 2005).

Studies testing sensitisation to mustard in patient populations based on SPT, mustard-specific IgE and RAST are also available. In Spain, 18.2 % of a patient population (n = 269), who visited their allergy clinic for other reasons, had a positive SPT to mustard (Leanizbarrutia et al., 1988). Among a subpopulation of pollen SPT positive patients from the same study, 54.2 % (n = 49) were SPT positive to mustard, but only four (8 %) complained of symptoms associated with mustard ingestion. In another Spanish study, mustard allergy (unequivocal history with positive SPT or serum-specific IgE (CAP-System) and absence of symptoms after mustard elimination from the diet) accounted for 7 % of food allergy consultations (8 of 120 subjects) in the Canary Islands (Castillo et al., 1996). In Finland, a series of 1 120 atopic and 380 non-atopic patients were SPT tested for allergy to spices including curry (Niinimäki and Hannuksela, 1981). When 71 of the curry-positive subjects were SPT tested with curry components, 23 (32.4 %; 2.2 % of all atopic subjects tested) had positive reactions to mustard. However, as in Spain, only five patients reported clinical reactions when eating seasoned food. In another Finnish study, 58 % of the 50 subjects with a history of reaction to spices and pollen tested with SPT and RAST showed positive reaction to mustard in at least one test (Niinimäki et al., 1989).

It is to be noted that prevalence to mustard in atopic children varies: 22 of 49 subjects (44 %) in Finland (Niinimäki et al., 1995), 23 out of 83 (28 %) in southwest France (Rancé et al., 2002) and lower in other parts of France (1 %) (Moneret-Vautrin, 2001).

26.2.2. Natural history

There are no data available regarding the natural history of mustard allergy.

26.2.3. Time trends

Since only one population-based study on the prevalence of self-reported allergy to mustard is available, no time trends for mustard allergy can be derived.

26.2.4. Severe reactions/anaphylaxis

The potential severity of mustard allergy has been described by several authors. The risk of severe reactions and anaphylaxis appears to be higher in adults than in children based on results of DBPCFC (Morisset et al., 2003a; Figueroa et al., 2005). Anaphylactic reactions have been reported in 2 % of children (Rancé et al., 1998; Rancé et al., 1999) and in up to 48 % of adults with confirmed mustard allergy (Caballero et al., 2002).

Based on specific IgE, mustard was incriminated in 11 % of food allergy reactions and 3 % of the anaphylactic reactions in 580 patients (480 adults, 100 children) with a "pathological reaction" to food, 60 of which had severe, near-fatal reactions (Andre et al., 1994). In a multicentre survey of food-induced anaphylactic shocks in France, 2 of the 81 reported cases were identified as being caused by mustard (Moneret-Vautrin and Kanny, 1995). In a report published by Health Canada in 2009[11], 22 individual cases of allergic reactions to mustard were described in 13 international case reports, 15 of which reported anaphylactic-type reactions that required emergency medical intervention. Other

[11] http://www.hc-sc.gc.ca/fn-an/alt_formats/pdf/pubs/label-etiquet/mustard-moutarde/index-eng.pdf

severe reactions described in case reports included laryngeal oedema, generalised urticaria and bronchial asthma.

26.2.5. Factors affecting prevalence of mustard allergy

The occurrence of mustard allergy symptoms was observed in children under the age of three years (Amlot et al., 1987; Rancé et al., 2000; Rancé et al., 2001) in France, which is one of the largest consumers in Europe and where mustard was previously present in certain commercial foods for toddlers. However, despite a low consumption of mustard in Finland and no information related to the introduction of mustard in baby foods or a significant consumption by young children in this country, 100 % positive SPT and specific IgE to mustard was reported in 14 Finnish children with atopic dermatitis and positive challenge to turnip rape, which belongs to the *Brassicaceae* family (Poikonen et al., 2009). Five of these children (36 %) had a positive oral challenge to mustard. The 2S albumin allergens present in the seeds of certain plants from the *Brassicaceae* family (mustard, oilseed rape, turnip rape) were considered to be highly cross-reactive and to play a role as sensitisers (via oral or respiratory route), particularly in children with atopic dermatitis.

Positive SPTs were reported in three children aged 12 to 18 months, breastfed until the age of 11 months, who had never consumed mustard (Niinimäki et al., 1989). It has been suggested that sensitisation *in utero*, during lactation and early consumption in baby foods may occur, as with peanut and sesame seed. It has been reported that the prevalence of mustard allergy increases with age in children (Guillet and Guillet, 2000).

The presence of mustard allergy in small children may be taken as an indication of primary sensitisation to mustard in at least some food allergies. The reported cross-reactivities with pollens and with other members of the *Brassicaceae* family may influence the prevalence of specific IgE and SPT positivity, lead to an overestimation of the prevalence of sensitisation to mustard, and may possibly also influence the occurrence of oral allergy syndrome-like symptoms elicited by mustard. Another factor contributing to a possible overestimation of mustard sensitisation and allergy, as determined by SPT or labial provocation challenge, is the presence of irritating substances in mustard that may cause false positive allergy-like reactions.

26.3. Identified allergens

Several mustard allergens have been identified and characterised (Table 28).

Table 28: Mustard allergens

Scientific name (common name)	Allergen	Biochemical name	Superfamily/family	Molecular weight [(a)]
Sinapis alba (yellow mustard)	Sin a 1	2S Albumin	Prolamin	14
	Sin a 2	11S Globulin	Cupin	51 [(b)]
	Sin a 3	nsLTP	Prolamin	12.3 [(b)]
	Sin a 4	Profilin	Profilin	13–14 [(b)]
Brassica juncea (oriental mustard)	Bra j 1	2S Albumin	Prolamin	14 [(b)]

(a): Molecular weight (SDS-PAGE).
(b): kDa.

Sin a 1 and Sin a 2 are the main protein components of yellow mustard seeds. Sin a 1 is a 2S seed storage albumin constituted by two disulphide-bonded subunits of 10 and 4 kDa (Menéndez-Arias et al., 1988). Immunologic mapping of Sin a 1 with 10 monoclonal antibodies showed two immunodominant regions, one located in the large chain (a continuous epitope) and the other in the

hypervariable region of the molecule (Menéndez-Arias et al., 1990). Sin a 1 is thermostable and resistant to digestion by trypsin and other proteolytic enzymes (EFSA, 2004; Palomares et al., 2005). Sin a 1 interacts with cell membranes, facilitating its uptake in the intestine. Sin a 1 was the first food allergen to be cloned and expressed by molecular biology techniques (Gonzalez de la Peña et al., 1993; Gonzalez De La Peña et al., 1996).

Sin a 2, an 11S globulin of the cupin superfamily, is a multimeric protein (Palomares et al., 2005; Palomares et al., 2007). The single-chain nsLTP Sin a 3 and the profilin Sin a 4 are contained in yellow mustard seeds in very low amounts (Sirvent et al., 2009).

Bra j 1 is a 2S seed storage albumin similar to Sin a 1 in structure and amino acid composition (Gonzalez de la Peña et al., 1991).

A detailed clinical characterisation of 34 patients with mustard allergy combined with component-resolved diagnosis was performed by using yellow mustard extract and the four purified mustard allergens Sin a 1, Sin a 2, rSin a 3, and rSin a 4 (Vereda et al., 2011b). A SPT was performed and the specific IgE level in serum was measured by ELISA. All patients reported a clear immediate allergic reaction with mustard within the first 30 minutes of consumption and had positive SPT to mustard extracts. Twenty-seven patients developed immediate systemic reactions after the ingestion of mustard, 28 had symptoms with other plant foods and 24 were allergic to pollen. Twenty-five patients had a positive SPT to Sin a 1, and 19 to Sin a 2. Twenty-five of the 34 tested sera had positive IgE to Sin a 1, 16 to Sin a 2, 14 to rSin a 3 and eight to rSin a 4. A significant positive correlation was found between SPT and ELISA for Sin a 1 and Sin a 2, but not for rSin a 3 and rSin a 4.

Specific IgE against Sin a 1 was the most suitable diagnostic marker to determine genuine sensitisation to yellow mustard and specific IgE against Sin a 2 was an useful marker to predict the severity of reactions, whereas specific IgE against Sin a 3 and Sin a 4 were associated with sensitisation to other plants of the *Rosaceae* family and to *Artemisia vulgaris* pollen (Vereda et al., 2011b).

Mustard also contains a number of irritating substances, such as isothiocyanates in *B. nigra*, sinalbin in *S. alba*, and capsaicin, which may trigger non-immune mediated reactions mimicking allergic reactions. These may lead to false positive SPT and difficult interpretation of labial provocation tests. For example, capsaicin induces release of substance P, which may trigger non-IgE-mediated mast cell degranulation (Rancé et al., 2000).

26.4. Cross-reactivities

Specific IgE for both Sin a 1 and the 2S fraction of *Brassica juncea* were detected in 10 sera from mustard sensitive individuals. Also six monoclonal antibodies and a rabbit polyclonal serum specific for Sin a 1 recognised the 2S fraction of *Brassica juncea*, suggesting that Bra j 1 and Sin a 1 may share a homologous epitope and that subjects allergic to one type of mustard may also react to other types (Gonzalez de la Peña et al., 1991; Monsalve et al., 1993; Palomares et al., 2007).

The Brassicaceae family includes a number of common vegetables, such as cabbage, cauliflower, Chinese cabbage, Brussels sprouts, broccoli, turnip, rutabaga and radishes, and rape (Monreal et al., 1992). High *in vitro* cross-reactivity between Sin a 1 and the major allergen of rapeseed (*Brassica napus*) Bra n 1 has been reported (Bartolome et al., 1997; Asero et al., 2002). Bra n 1 is a close homologue of Sin a 1, with 94 % of sequence homology. The antigenic properties of Bra n 1 and Sin a 1 were studied using sera from mustard and rapeseed-sensitive patients. The recombinant rapeseed 2S pronapin precursor protein bound to IgE in sera from mustard-allergic patients. Also, a Sin a 1-specific polyclonal rabbit antiserum was able to bind IgE in serum from a rapeseed-allergic patient (Palomares et al., 2002), which indicates serological cross-reactivity between mustard and rapeseed.

Sensitisation and allergy to turnip rape (*Brassica rapa* subsp. *oleifera*), oilseed rape (*B. napus* subsp. *oleifera*) and mustard seeds (*S. alba*) was observed in Finnish and French children with atopic dermatitis (AD) (Poikonen et al., 2009). Turnip rape challenge was positive in 5 (36 %) French and all the 14 Finnish children, although the frequency of positive mustard challenge was the same (36 %). Specific IgE antibodies from the challenged children, measured by ImmunoCAP and ELISA, were cross-reactive with the purified 2S albumin allergens present in the seeds of these plants (Bra r 1, Bra n 1 and Sin a 1, respectively), which are highly homologous.

Few reports of clinical cross-reactivity between mustard and foods other than Brassicaceae species are available (Asero et al., 2002; Caballero et al., 2002). A significant association between allergies to nuts and spices has been found (Castillo et al., 1996). At the molecular level, the 11S globulin Sin a 2, which is associated to severe adverse reactions in mustard-allergic patients, shares IgG epitopes with 11S globulins of almond, walnut, pistachio and hazelnuts, but not from peanuts, and is involved in IgE cross-reactivity with tree nuts and peanuts (Sirvent et al., 2012).

Sin a 3 and Sin a 4 showed IgE cross-reactivity with fruits of the *Rosaceae* family, such as peach and melon (Sirvent et al., 2009). An association between specific IgE to rSin a 3 and allergy to *Rosaceae* fruits (mainly peach) or *Artemisia vulgaris* pollen was observed in patients with mustard allergy (Vereda et al., 2011b).

An association between mustard allergy and pollen allergy has been observed as part of the "celery–mugwort–birch–spice syndrome" (Bauer et al., 1996). Food homologues of the major mugwort allergen Art v 1 may be responsible. An analogous "mugwort–mustard allergy syndrome" has been proposed (Figueroa et al., 2005) owing to the high association found between sensitisation to *Artemisia vulgaris* pollen and mustard allergy (37 out of 38 patients). Partial cross-reactivity between mustard and mugwort pollen was confirmed by CAP-inhibition assays. The same patients (92.1 %) were sensitised to other pollens belonging to weeds (*Chenopodium* and *Chrysanthemum*), grasses (*Poa*, *Lolium* and *Anthoxanthum*) and trees (*Ulmus* and *Platanus*), although at lower rates.

26.5. Effects of food processing on allergenicity

No clinical studies addressing the allergenicity of mustard after food processing are available. However, the stability of mustard allergens to thermal treatments is well documented. Sin a 1 showed the characteristic resistance of the 2S albumin family to denaturation upon heat treatments, keeping the integrity of IgE and IgG epitopes because the global folding is maintained. Sin a 3, a nsLTP, despite its conformational change after strong heating (95 °C for 30 minutes), also maintained the IgE and IgG reactivity. Both Sin a 1 and Sin a 3 belong to the prolamin superfamily and share a stable structural folding, with four α-helices stabilised by disulphide bridges.

Sin a 1 extracted from yellow mustard seeds and recombinant Sin a 3 were resistant to gastric digestion and partially resistant to intestinal digestion in an *in vitro* model, retaining significant IgE-binding (Gonzalez De La Peña et al., 1996; Sirvent et al., 2012). The resistance of LTP proteins to *in vivo* proteolytic degradation within the gastrointestinal tract may explain the severe allergic reactions of LTP-sensitised patients (Fu et al., 2002). Sin a 1 and Sin a 3 could reach unaltered the gut immune system and trigger systemic reactions (Moreno, 2007). In contrast, the profilin Sin a 4 was completely digested by gastric enzymes and its secondary structure was irreversibly unfolded by heat treatment.

Edible oils may be produced from mustard seeds by different processes. Depending on the type of process and the degree of refinement, oils may contain various amounts of proteins. In one study (Koppelman et al., 2007), no allergenic proteins were detected in a mustard seed oil using an ELISA method. However, the solvent used for the extraction of the proteins (aqueous buffer at pH 8) was not appropriate for the purpose of extracting lipophilic proteins present in the oil.

26.6. Detection of allergens and allergenic ingredients in food

A number of methods of detection, based on either ELISA or PCR analysis, have been developed for mustard.

26.6.1. ELISA

Koppelman et al. (Koppelman et al., 2007) described an inhibition ELISA for detecting the presence of allergens from *B. juncea* in a mustard oil with a LOD of 1.5 mg/kg. Proteins were extracted at pH 8 with a TRIS buffer. Weak cross-reactivity with soy (0.016 %) and milk (0.28 %) was reported.

Two quantitative sandwich ELISAs have been developed for detecting the three varieties of mustard seeds with a LOQ of 1 to 3 µg/mL (Lee et al., 2008). Their performance was tested in retail foods (Lee et al., 2009). The detectability of mustard allergens was much lower in acidic salad dressings, probably owing to the decreased solubility of the proteins, highlighting the importance of evaluating the applicability of the ELISAs to the different food matrices.

A less sensitive sandwich ELISA targeting the Sin a 1 allergen from *S. alba*, with a LOD of 0.3 µg/mL for the purified protein, has been described (Shim and Wanasundara, 2008). The Sin a 1 content of six mustard seed genetic lines was in the range of 0.82 to 2.94 mg/g when extracted with phosphate buffer saline (PBS) at pH 7.4. The sample extraction conditions for full recovery of Sin a 1 need to be considered when assessing its allergenicity.

Limitations of these ELISA methods are the partial recovery of the proteins and cross-reactivity (up to 50 %) with rapeseed (*Brassica napus*) (Monsalve et al., 1997; Lee et al., 2008).

ELISA kits for mustard detection are commercially available. One has been recently validated in an inter-laboratory study (Cuhra et al., 2011) with a LOQ of 1.8 mg/kg and a LOD of 0.5 mg/kg. The method did not show cross-reactions with other allergenic foods.

26.6.2. PCR

A real-time PCR method (Fuchs et al., 2010) specifically targeted the gene coding for white mustard (Sin a 1). The method is specific and does not show cross-reactivity with other biological species, including members of the *Brassicaceae* family. The LOD was 1 pg of white mustard DNA/µL, corresponding to 5 pg of white mustard DNA. In model sausages, the LOD was 10 mg/kg. The method was successfully applied to a series of commercial products. Another real-time PCR method targeted the simultaneous detection of black mustard (*B. nigra*) and brown mustard (*B. juncea*) in food (Palle-Reisch et al., 2013). The DNA of both mustard species could be detected down to 0.1 pg. The method applied to brewed sausages allowed the detection of 5mg/kg of black and brown mustard. No cross-reactivity was observed with other Brassicaceae species, with the exception of white mustard. A real-time PCR method (Mustorp et al., 2008), which targeted the mustard SinA gene showed some cross-reactivity with other *Brassica* species.

A quantitative multiplex ligation-dependent probe amplification (MLPA) method (Mustorp et al., 2011) for the simultaneous detection of eight allergenic ingredients including mustard also detected other members of the *Brassicaceae* family such as cabbage, radish and broccoli. The LOD was 5 to 400 gene copies.

Mustard DNA has also been detected by two hexaplex real-time PCR systems targeting twelve allergenic ingredients in foods (Köppel et al., 2012). The two tests exhibited a good specificity and a LOD of at least 0.1 % of total DNA for all analytes, but no quantitative results relative to the allergenic ingredient by weight could be given.

Optical thin-film biochips for multiplex visible detection of eight allergenic ingredients, including mustard, have been developed based on two tetraplex PCR systems (Wang W et al., 2011). The

method is fast, high throughput, and the results are visible at the naked eye. The LOD for mustard was not reported.

26.7. Minimum (observed) eliciting doses

Two DBPCFC studies (Morisset et al., 2003a; Figueroa et al., 2005) and one SBPCFC (Rancé et al., 2002) documenting mustard allergy and anaphylactic reactions to mustard have been published.

A study was performed on 38 mustard hypersensitive patients (mean age 21.9 ± 8.6 years; age range 3–39 years old), 10.5 % of whom had reported systemic anaphylaxis after mustard ingestion (Figueroa et al., 2005). Fourteen patients were not tested with DBPCFC because of severe reactions or denial of consent. The remaining 24 patients underwent DBPCFCs with a commercial mustard sauce mixed in vanilla-lemon flavoured yoghurt to mask its strong taste. The mustard sauce was composed of water, *S. alba* seeds (14 % w/v), vinegar, salt, turmeric, paprika and cloves. Increasing doses of the yoghurt mixture (80, 240, 800, 2400 and 6480 mg) were administered at 15-minute intervals until symptoms appeared or a cumulative dose of 10g of mustard sauce was reached. The test resulted positive in 14 cases (58 %) and the most frequent symptom observed was OAS (10 subjects; 71 %). The MOED was 156.8 mg of mustard sauce in most severe cases (one case of angioedema and bronchial asthma and one case of systemic anaphylaxis), while the MOED for milder symptoms (OAS) was 44.8 mg of mustard sauce. A significant relationship between the SPT and the DBPCFC results was reported.

A non-randomised DBPCFC was conducted in mustard seasoning on 24 patients (age 3–20 years) recruited on the basis of being SPT positive to mustard, and not on clinical reactions (Morisset et al., 2003a). Six additional patients were tested with a SBPCFC. Increasing doses of seasoning (10, 30, 100, 300 and 900 mg) were given every 20 minutes, to a total cumulative dose of 1340 mg. Seven patients tested positive, indicating that 23 % of the SPT positive patients had clinical allergy to mustard. SPTs were performed with four different mustard seasoning preparations, and specific serum IgE to mustard was determined (RAST). The MOED was 440 mg of mustard seasoning, while a dose of 40 mg of mustard seasoning triggered a reaction in a subject experiencing rhinitis and urticaria. The 40 mg dose corresponds to 13.5 mg of mustard seeds (*Brassica juncea*), which is roughly equivalent to 0.8 mg of mustard proteins (*B. juncea* is considered to contain an average of 6 % proteins).

In a SBPCFC with mustard seed powder (including *S. alba* and *B. juncea*), 36 SPT positive children (average age 5.5 years: range from 10 months to 15 years) were tested and compared with 22 control subjects with no history of food allergies (Rancé et al., 2000; Rancé et al., 2001). A mustard seed powder containing both *S. alba* and *B. juncea* was used for the SPT. Progressive doses of mustard (1, 5, 10, 20, 50, 100, 250, and 500 mg) were given. Of the 36 challenged subjects, 15 had positive reactions (42 %) and 21 were not allergic to mustard. The mean cumulative reactive dose was 153 mg of mustard powder. Eight of the subjects with a positive reaction (53 %) had exhibited symptoms of allergy to mustard under the age of 3 years.

26.8. Conclusion

Mustard is commonly used all over the world. Mustard allergy and anaphylactic reactions to mustard have been documented by DBPCFC studies, and the major allergens in mustard have been characterised. The prevalence of mustard allergy in the general unselected population is unknown because frequency estimates are mainly based on patient series. The major mustard allergens are heat-resistant and food processing is unlikely to alter their immunogenic properties. A number of methods of detection, based on either ELISAs or PCR analysis, have been developed for mustard. Protein doses triggering allergic reactions in mustard-allergic patients are around 1 mg.

27. Adverse reactions to sulphites

27.1. Background

Sulphites, or sulphiting agents, are defined as sulphur dioxide and several inorganic sulphite salts that may liberate SO_2 under appropriate conditions. These include sodium and potassium metabisulphites

($Na_2S_2O_5$, $K_2S_2O_5$), sodium and potassium bisulphites ($NaHSO_3$, $KHSO_3$) and sodium and potassium sulphites (Na_2SO_3, Na_2SO_3) (Simon, 1998).

Sulphites can occur naturally in foods as a consequence of fermentation (e.g. of wine) (Taylor et al., 1986), may be added to foods as preservatives or colours (Bush et al., 1986a), and may be found in medications, including those used for the treatment of allergic reactions (Nicklas, 1989). Sulphites have been used for centuries in the preservation of alcoholic drinks (e.g. cider, wine and beer), but their use has expanded to several other products (Bush et al., 1986b). Sulphiting agents used as food additives are given in Table 29.

When added to foods, sulphites inhibit enzymatic browning (e.g. in fresh fruits and vegetables, shrimps and raw potatoes) as well as non-enzymatic browning (e.g. in dried foods and dehydrated vegetables) (Taylor et al., 1997; Simon, 1998). They also have antimicrobial activity (as in wine and beer), dough-conditioning properties (as in frozen pies and pizza crusts) and bleaching effects (as in maraschino cherries) and they are used as processing aids in beet sugar (Bush et al., 1986b; Simon, 1998).

The levels of sulphites contained in foods range from < 10 mg/kg (e.g. frozen dough, maize syrup, jellies) to 60 mg/kg (e.g. fresh shrimp, pickles, fresh mushrooms) and 100 mg/kg (e.g. dried potatoes, wine vinegar). The highest levels of sulphites (up to 1 000 mg/kg) can be found in dried fruit, wine, fruit juices (e.g. lemon, lime, grape) and certain freshly prepared sauces available from retailers (Simon, 1998).

Table 29: Sulphiting agents used as food additives

Type	E number	Sulphiting agent
Preservatives	E 220	Sulphur dioxide
	E 221	Sodium sulphite
	E 222	Sodium hydrogen sulphite
	E 223	Sodium metabisulphite
	E 224	Potassium metabisulphite
	E 226	Calcium sulphite
	E 227	Calcium hydrogen sulphite
	E 228	Potassium hydrogen sulphite
Colours	E 150b	Caustic sulphite caramel
	E 150d	Sulphite ammonia caramel

Directive 95/2/EC[12] on food additives other than colours and sweeteners states maximum levels of sulphites for several foods and beverages expressed as SO_2 equivalents in mg/kg or mg/L, which refer to the total quantity available from all sources. The capacity to release SO_2 varies between the salts. In Directive 95/2/EC, in case of "an SO_2 content of no more than 10 mg/kg or 10 mg/L, SO_2 is considered not to be present". Regulation (EC) No 606/2009 states maximum levels of SO_2 in wines[13]. Sulphur dioxide and sulphites in foods and beverages at concentrations of more than 10 mg/kg or 10 mg/L expressed as SO_2 equivalents are subject to mandatory labelling in Europe[14]. The basis of this threshold was the LOD of sulphites in foods and beverages with the methods of detection available at that time.

[12] European Parliament and Council Directive 95/2/EEC on food additives other than colours and sweeteners. OJ L 061, 18.03.1995, p. 1–40.
[13] Commission Regulation (EC) No 606/2009 of 10 July 2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions. OJ L 193, 24.07.2009, p. 1–59.
[14] Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. OJ L 308, 25.11.2003, p. 15–18.

Indeed early assessments of consumer's exposure to sulphites in foods were difficult owing to shortcomings of the methods of measurement and to the fact that storage and preparation of food affects the final content of sulphites. Average daily intakes in the USA were estimated to be 19 mg of sulphur dioxide equivalents (SDE; 297 µmol of sulphite), with the 99th percentile of the population consuming daily 163 mg of SDE (~ 2.5 mmol of sulphite) (FDA, 1985). In France, average daily intakes were estimated as 20 mg SO_2, reaching 31.5 mg/day among consumers of cider, beer and wine (Mareschi et al., 1992), and as 23 mg SO_2 (0.78 mg/kg/day) in children and 50 mg/day (0.84 mg/kg/day) in adults in Italy, where dried fruits and wine (in adults) were the major contributors (Leclercq et al., 2000). Average intakes estimated in European countries using more sensitive methods for the detection and quantification of sulphites in foods and beverages are 0.16 to 0.17 mg/kg/day in adults (95th percentile 0.59–0.60 mg/kg/day) and 0.031 to 0.04 mg/kg/day in children (95th percentile 0.12–0.14 mg/kg/day) in France (Bemrah et al., 2012); 0.59 to 0.62 mg/kg/day in adults and 0.24 mg/kg/day in children in Austria (Mischek and Krapfenbauer-Cermak, 2012); and 0.34 mg/kg/day (97.5th percentile 1.1 mg/kg/day) in adults in Belgium (Vandevijvere et al., 2010).

Adverse reactions to ingested sulphites were first reported in 1976 (Prenner and Stevens, 1976). Data on adverse reactions to sulphites have accumulated in the 80s and 90s, but very few reports have become available since the year 2000, and particularly after their labelling became mandatory in the USA and the EU.

27.2. Frequency

The prevalence of sensitivity to sulphiting agents in the general population is unknown (Bush et al., 1986a).

Prevalence estimates of sulphite sensitivity among asthmatics reported in the literature (1–4 % among all asthmatics; 5–10 % among steroid-dependent asthmatics) are generally based on series of patients referred to allergy clinics and may overestimate prevalence in the general population (SCF, 1994), as do estimates of the percentage of asthmatics sensitive to oral challenges with sulphites, which range from less than 4 % up to 66 % depending on the selection criteria and challenge protocol used (EFSA, 2004; Vally et al., 2009). The FDA estimates that 1 % people in the general population are sulphite sensitive, and that 5 % of those who have asthma are also at risk of suffering an adverse reaction to sulphites (FDA, 1996).

The average age individuals experiencing asthma after exposure to sulphites is 40 years, and women appear to be more sensitive (Gunnison and Jacobsen, 1987; Simon, 1989). Adverse reactions to sulphites are less commonly reported in pre-school children, possibly owing to their lower consumption of foods with high sulphite content, including alcoholic beverages (Lester, 1995).

27.3. Clinical features

27.3.1. Signs and symptoms

Most reactions to sulphites are characterised by bronchospasm, occasionally severe, which can occur within minutes after ingestion of sulphite-containing foods. In restaurants, the sudden choking sensation may incorrectly be attributed to aspiration of food (Nicklas, 1989). Bradycardia, flushing and prominent gastrointestinal symptoms (Sheppard et al., 1980; Schwartz, 1983), 1983), as well as urticaria, angioedema, hypotension (Prenner and Stevens, 1976; Habenicht et al., 1983; Schwartz, 1983) and shock (Lester, 1995) have also been reported (Vally et al., 2009).

27.3.2. Diagnosis

A careful clinical history, though important in detecting sulphite sensitivity, is not sufficient for diagnosis, whereas skin testing (prick puncture or intradermal technique) allows identifying only a small fraction of patients. Challenge protocols for the diagnosis of sensitivity to ingested sulphites have been developed. The standard practice has been to use oral challenges with < 100 mg/mL of

sulphites for asthmatics and a maximum dose of 200 mg for subjects with a history of sulphite-induced urticaria or anaphylaxis (Simon, 2003). Unlike non-asthmatic individuals, most subjects with asthma are sensitive to inhaled SO_2, so that oral challenges with acid solutions may induce false positive results in terms of oral sensitivity if the dosage is high (Simon, 1998). Substances used for the challenges are usually contained in opaque capsules, which are consumed orally, and thus negative results do not exclude sensitivity to inhaled sulphates.

27.4. Pathogenesis

The pathogenesis of adverse reactions to sulphites has not been clearly documented. Three possible mechanisms have been invoked: an IgE-mediated reaction, a sulphite-induced cholinergic response and low levels of the enzyme sulphite oxidase (Bush et al., 1986b; Nicklas, 1989; Lester, 1995).

Several studies have not been able to demonstrate an IgE-mediated mechanism (Gunnison and Jacobsen, 1987; Nicklas, 1989) or the presence of a specific antibody (Lester, 1995). In addition, an IgE-mediated reaction to sulphites is unlikely, given the nature of the molecule.

The bronchoconstrictive effect of inhaled SO_2, mediated by parasympathetic nerve endings in the bronchi, has been studied with respect to environmental pollutants. Whether gastroesophageal reflux of SO_2 causes bronchospasm in sulphite sensitive patients is not clear. It has been hypothesised that sulphites can induce a cholinergic response and stimulate release of gastrin and other active mediators in sulphite sensitive patients (Nicklas, 1989).

Low levels of the mitochondrial enzyme sulphite oxidase have been demonstrated in some sulphite sensitive patients (Stevenson and Simon, 1984). Absorbed sulphites are added to those produced endogenously and increase the demand placed on the enzyme sulphite oxidase. It is possible that when this demand is not met, sulphite sensitive patients exhibit symptoms. It has also been hypothesised that a number of food additives, including sulphites, induce intolerance because of their aspirin-like properties (Williams et al., 1989), and an association between respiratory reactions to aspirin and those to sulphites has been reported (Sabbah et al., 1987; Hassoun et al., 1994).

Two DBPCFCs performed to address the role of sulphites in wine-induced asthma suggest that the changes in bronchial hyperresponsiveness observed in some of the patients tested could not be attributed only to the presence of sulphites in wine (Vally et al., 1999; Vally et al., 2007).

27.5. Possible effects of food processing on adverse reactions to sulphites

The amounts of sulphites initially used to treat foods do not reflect residue levels after processing. Storage and preparation of food also affects the final amount of sulphites consumed. Mechanisms of loss include volatilisation to SO_2 in acidic conditions, leaching, auto-oxidation, as well as the irreversible reactions with food constituents (Gunnison and Jacobsen, 1987).

Sulphites can react with food constituents, including sugars, proteins and lipids, to form adducts or derivatives. Some of these reactions are reversible, while others are not. The former lead to compounds that may serve as reservoirs for free sulphite, while the latter remove sulphites permanently from the pool of available free SO_2. Since free SO_2 is the most likely cause of adverse reactions to sulphiting agents, these chemical reactions have significant implications regarding foods that may cause difficulty in sensitive patients (Bush et al., 1986b; Simon, 1998). The likelihood of a particular food provoking a reaction depends upon the ratio of free to bound sulphite. For example, lettuce has few components to which sulphites can react, therefore most of the sulphite in lettuce remains in the free inorganic state and this explains why lettuce (salad bars) seems to provoke sulphite sensitive reactions frequently (Martin et al., 1986; Simon, 1998). In contrast, sulphites added to shrimp and potatoes tend to be bound and are not as likely to produce reactions in sulphite sensitive subjects.

27.6. Methods of detection of sulphites in foods

Many methods are available for the determination of sulphites in foods and beverages, the most commonly used being titration, flow injection analysis (FIA) and spectrophotometry, but also CE, HPLC, ion-exclusion chromatography (IC), gas chromatography (GC), fluorometry, chemiluminescence, anodic stripping voltammetry, sensors and biosensors. The available methods for the detection of sulphites in foods have been extensively reviewed (Isaac et al., 2006; Ruiz-Capillas and Jiménez-Colmenero, 2009; Pundir and Rawal, 2013). There are several Official AOAC methods for the detection of sulphites in meats (qualitative), fruits and wines (http://www.eoma.aoac.org/).

Sulphites can be present in foods and beverages as free, reversely bound adducts or irreversibly bound compounds according to the matrix and processing conditions, since they can react with other food constituents (e.g. aldehydes, ketones, sugars). Thus, it is necessary to recover the sulphites either as free or total sulphites (i.e. free plus reversibly bound sulphites). Most methods require preliminary extraction of sulphites from the solid or liquid food through, for example, distillation or acid or alkaline extractants, with the risk of substantial losses of SO_2. In few cases, liquid foods (e.g. water, wines, and fruit juice) can be analysed without preliminary treatments, although the risk of interfering agents must be considered.

27.6.1. Titration

The most commonly used techniques, mostly applied to the analysis of sulphites in wine, are based on titration according to the classic optimised Monier–Williams method (OMW) (AOAC, 1995), and to the Ripper procedure (Vahl and Converse, 1980). The OMW method requires distillation of SO_2, after acidification and oxidation to sulphuric acid with hydrogen peroxide. The sulphuric acid is then titrated with sodium hydroxide. The method is robust, with a LOD $\geq$ 10 mg/kg, but is time consuming and subject to SO_2 losses and/or co-distillation of other oxidisable volatile compounds present in food.

The Ripper procedure is based on iodometric titration. The bound sulphite is degraded by alkali, then the solution is acidified and the resulting total sulphurous acid is oxidised with iodine to sulphuric acid, while iodine is reduced to iodide. The remaining iodine is titrated with a solution of sodium thiosulphate in the presence of starch as an indicator. The LOD is 5 mg/kg. The method lacks accuracy and precision owing to the fact that iodine can react with other oxidisable compounds present in foods and cannot be used for coloured matrices.

27.6.2. Flow injection analysis

Flow injection procedures provide fast analytical responses in real time and are suitable for the routine analysis of a large number of samples (Ruiz-Capillas and Jiménez-Colmenero, 2009). To assess total sulphites, the sample undergoes an alkaline treatment to release most of the bound sulphite, which is then extracted by, for example, a tetracloromercurate solution to form a stable sulphite–mercury complex, with a good recovery. Stabilisers, e.g. EDTA, are added to the solution to avoid oxidation of sulphites by atmospheric oxygen. Once injected into a continuous flow of a strong acidic donor solution, the released SO_2 diffuses through a membrane, commonly via gas diffusion (GD), and dissolves into an acceptor solution, chosen according to the detector to be used, e.g. spectrophotometric, fluorometric, amperometric, potentiometric. For spectrophotometric detectors, which are robust and available in most control laboratories, reagents developing a colour or changing colour are used as acceptors of SO_2 and the difference in absorbance, measured at a defined wavelength, is taken as linearly proportional to the amount of sulphite. Malachite Green is the acceptor reagent most commonly used. A FIA-spectrophotometric method for the analysis of sulphites is accepted by the AOAC for the analysis of sulphites in food, with a LOD $\geq$ 5 mg/kg (AOAC, 2005).

27.6.3. Chromatographic methods

27.6.3.1. Ion chromatography

A method adopted by the AOAC (AOAC, 2000), which uses ion-exclusion chromatography with direct current amperometric detection, has a LOD ≥ 10 mg/kg and can be applied to liquid samples after filtration. However, fouling of the platinum electrode occurs rather quickly, leading to a significant decrease of the response.

A method using an anion-exchange column eluted with sodium carbonate and sodium hydroxide with conductivity detection, when applied to the determination of sulphites in fresh meats and shrimps, provided good results with respect to linearity, LOD (2.7 mg/kg) and LOQ (8.2 mg/kg), expressed as SO_2 (Iammarino et al., 2010). Higher sensitivity was achieved when release of SO_2 from bound sulphites by addition of sodium carbonate, acidification and distillation with vapour and CO_2 stream preceded IC (Zhong et al., 2012). The distilled SO_2 was captured in a sodium hydroxide solution and measured as sulphite by IC, with a LOD of 0.013 mg/L. This method was also used to detect sulphites in preserved foods, dried vegetables and wines.

27.6.3.2. Gas chromatography

GC was applied to the separation of free and combined sulphites in foods. Tartaric acid allows the selective extraction of free sulphites, whereas an alkaline extractant containing potassium sodium tartrate allows detecting total sulphites. The LOD is 0.5 mg/kg (Pundir and Rawal, 2013).

27.6.3.3. High-performance liquid chromatography

Free and total sulphites can be separated by HPLC and quantified by a ultraviolet/visible spectroscopy detector with a LOD of 0.5 mg/L (McFeeters and Barish, 2003), and with a fluorometric detector (via derivatisation with *o*-phthaldialdehyde) with a LOD of 5 mg/kg (Chung SW et al., 2008). Coupling HPLC with an immobilised enzyme reactor (HPLC–IMER), the method became more sensitive (LOD 0.01 mg/L SO_3^{2-}) and specific (Theisen et al., 2010).

27.6.4. Spectrophotometry

Spectrophotometric methods for the analysis of sulphites are simple, sensitive and convenient, although some require preliminary distillation and relatively large amount of samples (Hassan et al., 2006). They measure the absorbance of a coloured compound resulting from reaction of sulphites with a reagent such as *o*-phthaldialdehyde in the presence of ammonia (LOD 0.04 mg/L), *p*-rosaniline-formaldehyde (LOD 0.03 mg/L), or 5,5-dithiobis(2-nitrobenzoic acid) (LOD 0.10 mg/L).

The performance of batch and flow injection spectrophotometric modes for sulphite detection in beverages has been compared (Hassan et al., 2006). The method is based on the reaction of SO_2 with a diaquacobester reagent, resulting in a sulphite aquacobester (SO_3Cbs) complex. Changes of the absorbances at 313, 349, 425 and 525 nm are linearly related to sulphite concentrations, with an average LOD of 10 µg/L. The flow injection system allowed analysis of 50 samples per hour.

A method based on ultraviolet/visible spectroscopy–fibre optic–linear array detection spectrophotometry was developed with a preliminary dispersive liquid–liquid microextraction (DLLME) in order to pre-concentrate sulphite ions from aqueous samples. The procedure is based on the colour reaction of sulphite with *o*-phthaldialdehyde (OPA) in the presence of ammonia to form isoindole, which is extracted by the DLLME technique. The LOD was 0.2 µg/L (Filik and Cetintas, 2012).

27.6.5. Sensors and biosensors

The (bio)sensing methods have experienced a great development in the last few years, since they allow selective, rapid and highly sensitive detection and a wide working range.

Electrochemical sensors are based on non-enzymatic metal complexes, which catalyse production or consumption of ions or electrons, thus changing the electrical properties of the solution, which are used as measuring parameters.

Biosensors are based on immobilised enzymes, such as sulphite oxidase (SO) from chicken liver or plant leaves or bacterial sulphite dehydrogenase (SDH), which catalyse oxidation of sulphite to sulphates with high selectivity. The aerobic oxidation of sulphites by sulphite oxidase produces H_2O_2, which undergoes electrochemical breakdown under high voltage generating electrons, i.e. current, which is directly proportional to the sulphite concentration. Coating the electrode with a polymeric film, in which the enzyme is embedded, has been shown to retain selectivity, since the polymer acts as a selective barrier allowing only H_2O_2 to reach the electrode. Direct electrocatalytic voltammetry of SDH immobilised on a pyrolytic graphite electrode or mediated by cytochrome c as an electroactive relay between the enzyme and a gold electrode have been reported (Kalimuthu et al., 2010). (Bio)sensors may be classified into several "classes" according to the type of transducers used (e.g. dissolved oxygen metre, electrochemical, amperometric, conducting polymer, nanoparticle-based, fill and flow channel, chemiluminescence, optical, screen printed electrode, sol–gel based, gas diffusion-sequential injection, FIA-based). The principles, characteristics, advantages and disadvantages of the different biosensors have been reviewed (Pundir and Rawal, 2013).

27.6.5.1. Chemiluminescence biosensors

Chemiluminescence biosensors combine the specificity of the biological reaction with the sensitivity of light emitting reactions. They are mostly based on sulphite oxidase immobilised on different supports, using chemiluminescent oxidation of luminol for detection. The LODs vary from 0.0003 µM (Sasaki et al., 1997) to 1 µM (Yaqoob et al., 2004) and 4.7 µM of sulphite (Navarro et al., 2010).

27.6.5.2. Electrochemical sensors

Sulphites have been oxidised at simple electrodes (platinum, gold, carbon and metal oxides), or at electrodes modified with enzymes (sulphite oxidase, sulphite dehydrogenase) or metal complexes (iron and copper-cobalt hexacyanoferrate, cobalt porphyrins, ferrocenedicarboxilic acid) in order to overcome the lack of selectivity at the bare electrode. The catalysts are immobilised on the electrode as mono/multilayer films or incorporated within the body of composite electrode materials, such as sol–gel. The LODs are in the range 0.3 to 1 µM. The relative methods have been reviewed (Isaac et al., 2006; Pundir and Rawal, 2013).

Ion-selective electrodes are not suitable for sulphite detection owing to the lack of sufficiently selective ionophores. Redox indicators have been used to avoid interference with other anions, but the selectivity depends also on the food matrix. The problem of non-specific oxidation of interfering species can be avoided by integrating highly specific oxidising enzymes into the transducing surface. A combination of sulphite dehydrogenase, horse heart cytochrome c (cyt c), and a self-assembled monolayer of 11-mercaptoundecanol cast on a gold electrode led to a LOD of 44 pM using an amperometric detector (Kalimuthu et al., 2010).

27.6.5.3. Screen-printed electrode-based biosensors

Screen printed carbon electrodes have been devised in order to construct portable or disposable electrodes. The active substrate (enzyme, metal complex) is deposited onto inert backing supports, such as PVC or ceramic materials. One of these, based on sulphite oxidase and an osmium redox polymer as mediator, is able to work at a desirable low potential, thus avoiding co-oxidation of interferents, with a LOD of 0.08 µM (Spricigo et al., 2010).

27.6.5.4. Biosensors based on conducting polymer matrices

Different types of conducting polymers have been used as support for construction of sulphite oxidase electrodes. Enzyme molecules can be entrapped during electropolymerisation, resulting in a uniformly coverage of the electrode working surface. These biosensors are sensitive, specific and rapid. A

biosensor using sulphite oxidase entrapped in ultrathin polypyrrole film and amperometric detection showed a LOD of 0.9 μM (Ameer and Adeloju, 2008).

27.6.5.5. Nanoparticle-based biosensors

Nanoparticles increase the electroactive surface of electrodes, enhance the electron transport between the electrolyte medium and the electrode, and increase the loading capacity for the enzyme. Electrochemical biosensors modified with sulphite oxidase/magnetic nanoparticles (Fe_3O_4@GNPs)/Prussian blue nanoparticles on gold or/indium tin oxide electrodes, have been produced, with LODs of 0.15 to 0.1 μM (Rawal and Pundir, 2013).

27.6.6. Methods for the detection of sulphites in wine

Free and bound sulphites are present in wine. Free sulphites include SO_2, bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) in a chemical equilibrium according to the pH. At the pH of wine (3.2–4.0) bisulphite is the predominant species of free sulphite, which can bind reversibly to carbonyl compounds, such as aldehydes, and in particular acetaldehyde, ketones, ketoacids and sugars.

Titration, spectrophotometry, FIA, sensors and biosensors can be used to measure sulphites in wine. The most commonly used is OMW (AOAC, 1995). The procedure is time consuming, cannot be used for high-throughput analysis, can overestimate sulphite levels owing to the presence of volatile acidic compounds in wine, or may underestimate them owing to loss of SO_2 during distillation. The Ripper procedure lacks accuracy and precision, particularly for coloured matrices such as red wine.

In order to overcome such limitations, two methods based on FIA with spectrophotometric detection have been adopted by the AOAC: the AOAC-990.29 method for total sulphites and the AOAC-990.30 method for free sulphites. The LOD is ≥ 5 mg/kg.

A GD multicommuted FIA for the determination of free and total sulphites in white and red wine has been reported (Oliveira et al., 2009). The methodology is based on two spectrophotometric reactions with malachite green (MG) and *para*-rosaniline (PRA), with no previous treatment of the sample. The procedure with mg is more sensitive but less accurate than with PRA, because of the negative interference with acetaldehyde. LODs of 0.3 and 0.6 mg/L and LOQs of 1.1 and 1.8 mg/L for free SO_2, LODs of 0.7 and 0.8 mg/L and a LOQ of 2.5 mg/L for total SO_2 were obtained with GM and PRA, respectively. These methods use low cost instrumentation, have high sample throughput and are easy to manipulate.

A sensitive sensor based on electrodes supported on ion-exchange membranes acting as a solid polymer electrolyte (SPE), which allows gaseous electroactive analytes to be detected, was used as an amperometric detector for a flow injection system (Toniolo et al., 2010). The LODs were 0.04 and 0.02 mg/L for free and total SO_2.

Another method based on head-space gas chromatography (HS-GC) with electron-capture detection (ECD) has been developed for the determination of free and total sulphites in wine (Aberl and Coelhan, 2013). Formation of gaseous SO_2 is achieved by acidification and heating in the presence of 2,4-dinitrophenylhydrazine. The LOD is 1 mg/L, the LOQ 5 mg/L. The method is quick, accurate, and requires minimum sample preparation.

27.7. Minimum doses eliciting adverse reactions

Toxicity studies in non-asthmatic individuals have been conducted primarily through oral challenges and inhalation studies (Bush et al., 1986b). Small numbers of individuals have ingested doses of up to 400 mg of SO_2 equivalents per day without adverse effects (Taylor et al., 1986). However, doses of 4 to 6 g per day predictably caused nausea, vomiting, gastric irritation and occasional gastrointestinal bleeding (Schwartz, 1984; Bush et al., 1986b). Sulphite sensitivity was confirmed in a patient after the ingestion of a total dose of 10 mg of $NaHSO_3$ solution (Prenner and Stevens, 1976). Case reports of positive oral challenges with encapsulated sulphites at doses of 10 mg (Schwartz, 1983) and 25 mg

(Habenicht et al., 1983) are available. Challenge studies in larger numbers of non-asthmatic subjects suggest that few react to the ingestion of sulphites in foods or beverages (Meggs et al., 1985; Sonin and Patterson, 1985; Bush et al., 1986b).

Among asthmatics, the amount of sulphite required to produce a response also varies and quantities as low as 1 to 5 mg of ingested potassium metabisulphite (equivalent to 3.7 mg of free SO_2) have been reported to trigger a reaction in sulphite sensitive asthmatics (Stevenson and Simon, 1981). Ingestion of sulphited solutions is more likely to precipitate asthma than ingestion of encapsulated sulphites, perhaps owing to inhalation of volatilised SO_2 (Bush et al., 1986b).

Most sulphite sensitive individuals will react to ingested metabisulphite in quantities ranging from 20 to 50 mg (Simon, 1989; Lester, 1995). However, minimum eliciting doses have not been systematically assessed and the lowest concentration of sulphites able to trigger a reaction in a sensitive person is unknown.

The Panel notes that the studies available do not allow concluding on the lowest concentrations of sulphites that are able to trigger a reaction in a sensitive person.

27.8. Conclusion

The prevalence of sulphite sensitivity in the general population is unknown, but it appears to be rare among non-asthmatics. Most reactions to sulphites are characterised by severe bronchospasm, which can occur within minutes after ingestion of sulphite-containing foods. Average daily sulphite consumption in adults has been estimated to be approximately between 20 mg and 50 mg of sulphur dioxide equivalents, but interindividual variability is high. Most sulphite sensitive individuals will react to ingested quantities of metabisulphite within this range. Labelling of foods containing sulphiting agents in concentrations > 10 mg/kg or 10 mg/L is mandatory in the EU, which was based on the LOD of the detection method available at the time. Many very sensitive and reliable methods are now available for analysis of sulphites in foods, with LODs well below 10 mg/kg. However, MED have not been systematically assessed and the smallest concentration of sulphites able to trigger a reaction in a sensitive person is unknown.

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APPENDIX

Appendix A. Population thresholds calculated for some allergenic foods/ingredients [(a)]

Food	No. of patients	Objective reactions ED01 (95 % CI)	Objective reactions ED05 (95 % CI)	Objective reactions ED10 (95 % CI)	Any reaction ED01 (95 % CI)	Any reaction ED05 (95 % CI)	Any reaction ED10 (95 % CI)	Reference
Hen's egg	53 (children)	0.07 (0.01–0.79)	1.51 (0.3–7.7)	5.82 (1.6–21.4)	0.04 (0.005–0.35)	0.75 (0.2–3.3)	2.75 (0.8–9.2)	(Blom et al., 2013)
	206 (mostly children)	0.0043–0.056 [(b)] (NA)	0.21–0.44 [(b)] (NA)	1.2–1.6 [(b)] (NA)	–	–	–	(Remington, 2013)
	120 (≤ 3.5 years)	–	–	0.6–1.3 (0.1–4.8)	–	–	–	(Defernez et al., 2013)
	21 (> 3.5 years)	–	–	20.4–27 (4.4–134.5)	–	–	–	
	155 (mostly children)	–	2.08 (1.1–4.0)	5.36 (3.0–9.6)	–	–	–	(Eller et al., 2012)
Cow's milk	93 (children)	0.05 (0.01–0.30)	1.07 (0.3–3.8)	4.24 (1.6–11.6)	0.007 (0.001–0.06)	0.27 (0.1–1.1)	1.31 (0.4–4.2)	(Blom et al., 2013)
	351 (mostly children)	0.016–0.14 [(b)] (NA)	0.57–1.9 [(b)] (NA)	2.8–5.1 [(b)] (NA)	–	–	–	(Remington, 2013)
	80 (≤ 3.5 years)	–	–	0.1–0.2 (0.02–1.1)	–	–	–	(Defernez et al., 2013)
	13 (> 3.5 years)	–	–	5.3–7.6 (0.1–269.0)	–	–	–	
	42 (mostly children)	–	59.3 (29.1–109.9)	100.2 (52.7–190.5)	–	–	–	(Eller et al., 2012)
Peanut	135 (children)	0.15 (0.04–0.51)	1.56 (0.7–3.6)	4.42 (2.3–8.5)	0.007 (0.002–0.03)	0.14 (0.1–0.4)	0.52 (0.2–1.2)	(Blom et al., 2013)
	750 (adults/children)	0.015–0.13 [(b)] (NA)	0.5–1.5 [(b)] (NA)	2.3–4.1 [(b)] (NA)	–	–	–	(Remington, 2013)
	51 (adults/children)	–	–	2.8–6.6 (0.2–51.7)	–	–	–	(Defernez et al., 2013)
	149 (mostly children)	–	18.9 (13.0–27.6)	32.9 (23.6–45.9)	–	–	–	(Eller et al., 2012)
	41 (children)	–	–	87 (31.4–NR)	–	–	–	(Wensing Marjolein et al., 2002)

Food	No. of patients	Objective reactions			Any reaction			Reference
		ED_{01} (95 % CI)	ED_{05} (95 % CI)	ED_{10} (95 % CI)	ED_{01} (95 % CI)	ED_{05} (95 % CI)	ED_{10} (95 % CI)	
Hazelnut	28 (children)	0.01 (0.00–0.56)	0.29 (0.0–4.6)	1.38 (0.2–12.0)	0.001 (0–0.05)	0.05 (0.0–0.6)	0.22 (0.0–1.8)	(Blom et al., 2013)
		0.038–0.42 (b) (NA)	1.2–2.6 (b) (NA)	5.2–7.9 (b) (NA)	–	–	–	(Remington, 2013)
	90 (mostly adults)	–	–	8.5–10.1 (2.1–51.0)	–	–	–	(Defernez et al., 2013)
	59 (mostly children)	–	8.7 (4.5–16.8)	15.9 (8.9–28.4)	–	–	–	(Eller et al., 2012)
Cashew nut	31 (children)	1.30 (0.18–9.57)	7.41 (1.9–28.7)	16.0 (5.4–47.4)	0.02 (0.002–0.25)	0.32 (0.1–1.8)	1.07 (0.3–4.5)	(Blom et al., 2013)
		1.4–2.8 (b) (NA)	8.9–11.5 (b) (NA)	16.8–22.7 (b) (NA)	–	–	–	(Remington, 2013)
Soy	80 (adults/children)	0.078–3.1 (b) (NA)	4.7–22.2 (b) (NA)	28.2–63.4 (b) (NA)	–	–	–	(Remington, 2013)
	23 (adults/children)	37.2 (NR)	–	–	0.21 (NR)	–	–	(Ballmer-Weber et al., 2007)
Wheat	40 (adults/children)	0.14–1.1 (b) (NA)	2.0–4.3 (b) (NA)	6.6–10.2 (b) (NA)	–	–	–	(Remington, 2013)
Mustard	33 (adults/children)	0.022–0.097 (b) (NA)	0.32–0.46 (b) (NA)	1.0–1.2 (b) (NA)	–	–	–	(Remington, 2013)
Lupin	24 (adults/children)	0.83–3.7 (b) (NA)	7.8–19.1 (b) (NA)	20.8–33 (b) (NA)	–	–	–	(Remington, 2013)
Sesame	21 (adults/children)	0.10–0.67 (b) (NA)	2.1–3.8 (b) (NA)	7.6–10.6 (b) (NA)	–	–	–	(Remington, 2013)
Shrimp	48 (adults)	3.7–6.1 (b) (NA)	73.6–127[2] (NA)	284–500 (b) (NA)	–	–	–	(Remington, 2013)
	28 (mostly adults)	–	–	~2 500 (1 100–6 300)	–	–	–	(Defernez et al., 2013)
Fish	34 (adults and children)	–	–	25.8–32.6 (4.8–203.8)	–	–	–	(Defernez et al., 2013)
Celery	41 (mostly adults)	–	–	1.6–2.8 (0.2–30.0)	–	–	–	(Defernez et al., 2013)

(a): Expressed as mg of total protein from the allergenic food, unless otherwise specified.
(b): Depending on the distribution model used (e.g. log-logistic, log-normal or Weibull).
NR, not reported; NA, not available numerically, reported only graphically in the original publication.

GLOSSARY AND ABBREVIATIONS

2D	two dimensional
3D	three dimensional
1DE	one-dimensional electrophoresis
2DE	two-dimensional electrophoresis
2D-SDS-PAGE	two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis
Absolute LOD	For DNA-based methods, LOD expressed as absolute amounts of DNA detected (e.g. in pg, ng, or number of DNA copies)
ALA	α-lactalbumin
Allergen	Proteins or peptides responsible for the allergenicity of allergenic foods/ingredients
Allergenic food	Substances listed under Annex IIIa of Directive when considered as such
Allergenic ingredient	Substances listed under Annex IIIa of Directive when considered as part of complex foods
Allergenicity	Ability to trigger immune-mediated clinical reactions
Antigenicity	Ability to combine specifically with the final products of the immune response, e.g. specific IgE antibodies
AOAC	Association of Analytical Communities
AQUA	Absolute quantification of proteins using stable isotope-labelled peptides by MS
Atopy	Familial tendency to produce IgE antibodies in response to low doses of allergens
BIA	biosensor immunoassay
BLAST	basic local alignment search algorithm
BLG	β-lactoglobulin
BMD	Bench Mark Dose
BSA	bovine serum albumin
CAS	casein
CAP (or ImmunoCAP)	A brand name of a test for the diagnosis of atopy in patients with allergy-like symptoms. It measures specific IgE antibodies in human serum

CAP-FEIA	CAP-fluorenzymeimmunoassay
CAP-RAST	CAP-radioallergosorbent test
CCDs	cross-reactive carbohydrate determinants
CD	circular dichroism
CE	capillary electrophoresis
CI	confidence interval
CITP-CZE	capillary isotachophoresis–capillary zone electrophoresis
CMA	cow’s milk allergy
CMP	cow’s milk proteins
Coeliac disease	Autoimmune adverse reaction to food triggered by the ingestion of gluten and related to prolamins found in wheat, barley and rye
CRIE	cross-radioimmunoelectrophoresis
CRM	certified reference material
DAS-ELISA	double antibody sandwich enzyme-linked immunosorbent assay
DBPCFC	double-blind placebo-controlled food challenge
DIGE	difference gel electrophoresis
DLLME	dispersive liquid–liquid microextraction
DNA	deoxyribonucleic acid
Dot-blot	dot-immunoblotting
EAST	enzyme allergosorbent test
ED*p*	population-based eliciting dose
EIS	electrochemical impedance spectroscopy
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
ESI-MS	electrospray ionisation–mass spectrometry
FAPAS	Food Analysis Proficiency Assessment Scheme
FARRP	Food Allergen Research and Resource Program
FDEIA	food-dependent exercise-induced anaphylaxis

FIA	flow injection analysis
Food allergy	Adverse health effect arising from a specific immune-mediated response that occurs reproducibly on oral exposure to a given food, which can be mediated by food-specific IgE antibodies or not
Food intolerance	Non-immune-mediated adverse reactions to food
FPIES	food protein-induced enterocolitis syndrome
FT-ICR	Fourier transform ion cyclotron resonance
FTIR	Fourier transform infrared spectroscopy
GC	gas chromatography
GD	gas diffusion
GLP	germin-like proteins
GNPs	gold nanoparticles
HHP	high hydrostatic pressure
HMW	high molecular weight
HPLC	high-performance liquid chromatography
HPLC/ESI-MS/MS	high-performance liquid chromatography–electrospray ionisation–tandem mass spectrometry
HR-MS	high-resolution mass spectrometry
HS-GC	head-space gas chromatography
IgG	immunoglobulin class G antibodies
IgE	immunoglobulin class E antibodies
IC	ion-exclusion chromatography
ICD	International Classification of Diseases
ICP-MS	inductively coupled plasma–mass spectrometry
ICSA	Interval-censoring survival analysis. Method used to determine individual thresholds when the exact dose that provokes a reaction in an individual is not known, but it is known to fall into a particular interval. Individuals reacting to the first dose in a challenge trial are left-censored: the NOAEL is set to zero with the LOAEL set as that first dose. Individuals not experiencing a reaction after the largest challenge dose are right-censored: the NOAEL is set to that largest challenge dose and the LOAEL is set to infinity. In all other cases, interval censoring occurs bounded by the NOAEL and LOAEL

IEF	isoelectric focusing
Immunogenicity	Ability to induce a humoral and/or cell-mediated immune response
Incurred sample	A sample in which a known amount of the authentic specimen is added before treatment (cooking or any industrial process)
IPC	internal positive control
IRMM	Institute for Reference Materials and Measurements
ISAAC	International Study of Asthma and Allergies in Childhood
IT	ion trap
IUIS	International Union of Immunological Societies
LAB	lactic acid bacteria
LC	liquid chromatography
LC-ESI-MS	liquid chromatography–electrospray ionisation–mass spectrometry
LC-ESI-MS/MS	liquid chromatography–electrospray ionisation-tandem mass spectrometry
LC-ESI-IT-MS/MS	liquid chromatography–electrospray ionisation–ion traps–tandem mass spectrometry
LC-ESI-QqQ-MS/MS	liquid chromatography–electrospray ionisation–triple quadrupoles–tandem mass spectrometry
LC-ESI-Q-TOF-MS/MS	liquid chromatography–electrospray ionisation–quadrupole–time of flight–tandem mass spectrometry
LC/HR-MS	liquid chromatography–high-resolution mass spectrometry
LC-LIT-MS	liquid chromatography–linear ion trap–mass spectrometry
LC-LIT-MS/MS	liquid chromatography–linear ion trap–tandem mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LCQ^{DECA} nLC-MS/MS IT	LCQ^{DECA} nanoflow liquid chromatography–ion trap mass spectrometry
LC-QpQ-MS/MS	liquid chromatography–triple quadrupoles–tandem mass spectrometry
LF	lactoferrin
LFD	lateral flow device
LMW	low molecular weight

LIT	linear ion trap
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
LTP	lipid transfer proteins
mAb	monoclonal antibody
MALDI	matrix-assisted laser desorption ionisation
MALDI-TOF	matrix assisted laser desorption ionisation–time of flight
MED	minimum eliciting dose
MIM	multiple ion monitoring
MLC	myosin light chain
MLPA	multiplex ligation-dependent probe amplification
MnSOD	manganese superoxide dismutase
MoE	Margin of Exposure
MOED	minimum observed eliciting dose
MRM	multiple reaction monitoring
MRM-MS	multiple reaction monitoring–mass spectrometry
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NOE	nuclear overhauser effect
NSAID	non-steroidal anti-inflammatory drug
nsLTP	non-specific lipid transfer proteins
OAS	Oral allergy syndrome is an IgE-mediated immediate type allergic reaction characterised by symptoms within several minutes of contact with food, involving the mouth and the pharynx

OFC	open-label food challenge
OMW	optimised Monier–Williams method
PAD	pulsed amperometric detection
Panallergens	Usually classified as minor allergens, these are homologous molecules that originate from a multitude of organisms and cause IgE cross-reactivity between evolutionarily unrelated species
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction–restriction fragment length polymorphism
PFF	peptide fragment fragmentation
PFU	PCR-forming unit
PMF	peptide mass fingerprinting
PNA	peptide nucleic acid
PR proteins	pathogenesis-related proteins
PUV	pulsed ultraviolet light
PVDF	polyvinylidene difluoride
PWG	Prolamin Working Group
qPCR	quantitative polymerase chain reaction
QpQ	triple quadrupole
Q-TOF	quadrupole–time of flight
RIE	rocket immunoelectrophoresis
RP-HPLC	reversed-phase high-performance liquid chromatography
RP-HPLC/nESI-MS/MS	reversed-phase high-performance liquid chromatography/nano electrospray ionisation-mass spectrometry
RT-PCR	reverse transcriptase polymerase chain reaction
SBPCFC	single-blind placebo-controlled food challenge
SCBP	sarcoplasmic calcium-binding protein
SCORAD	SCORing Atopic Dermatitis
SDAP	structural database of allergenic proteins

SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SELDI	surface-enhanced laser desorption ionisation
SELDI-TOF/MS	surface-enhanced laser desorption ionisation–time of flight–mass spectrometry
Sensitisation	Positive SPTs or specific IgE to the offending food
SGF	simulated gastric fluid
SLIT	sublingual immunotherapy
SOTI	systemic oral tolerance induction
SPI	soybean protein isolate
Spiked sample	A sample in which a known amount of the authentic specimen is added before the analysis
SPF	soy protein-based formula
SPR	surface plasmon resonance
SPRI	surface plasmon resonance imaging
SPT	skin prick test
SRM	selective reaction monitoring
TLP	thaumatin-like proteins
TOF	time of flight
TOF^2	tandem time of flight
WDEIA	wheat-dependent exercise-induced anaphylaxis
WHO	World Health Organization