

Food allergen detection methods and the challenge to protect food-allergic consumers

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Abstract The detection of allergenic ingredients in food products has received increased attention from the food industry and legislative and regulatory agencies over recent years. This has resulted in the improvement of measures aimed at the protection of food-allergic consumers. The controlled production of food products and control activities executed by food inspection agencies rely on the availability of methods capable of detecting traces of allergenic ingredients. The development of such methods faces a multitude of analytical challenges. Those challenges will be identified and discussed in this review. Furthermore, future developments and trends in analytical methodology as applied to the detection of food allergens are reported.

Keywords Bioanalytical methods · Foods · Beverages · Immunoassays · ELISA · PCR

Introduction

Food allergy is recognised as a major health concern with an estimated 4% of the total population affected by food-allergic disorders [1]. Food allergy occurs even more frequently in infants and children with prevalence levels reaching 8% [2]. Unfortunately there are clear indications that the prevalence of food allergy has risen in recent years with one study finding a doubling of the prevalence of peanut allergy over a period of only 5 years [3].

The prevention of acute and potentially life-threatening reactions triggered by food allergens relies heavily on a

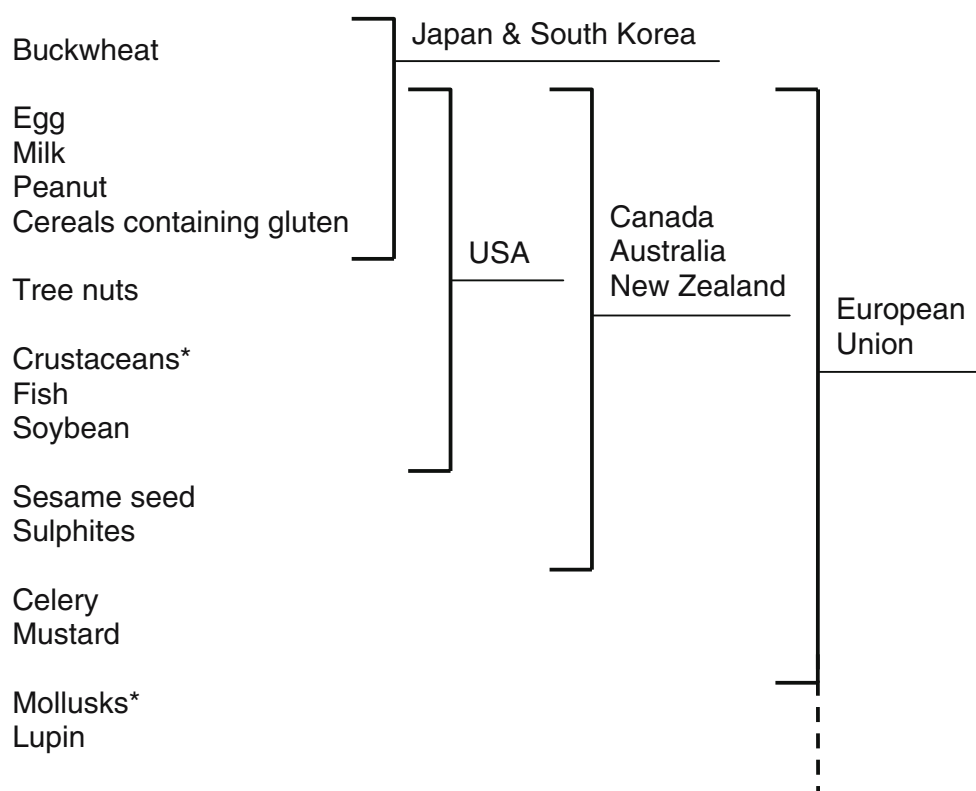
successful avoidance of the offending food. This presents a major challenge for food-allergic consumers that need to obtain information on food ingredients in order to assess whether a food contains allergenic ingredients and therefore can endanger their health. Food labelling plays a crucial role in this by providing the consumer access to the information that he or she requires to implement a successful avoidance strategy. Consequently, clear and accurate labelling practises are needed to allow the allergic consumer to assess the health risks of food products.

Legislation has been put into place in the USA and European Union (EU) aimed at achieving a high level of health protection for allergic consumers. A new labelling law, the Food Allergen Labelling & Consumer Protection Act (FALCPA), came into effect in the USA at the start of 2006 [4]. The FALCPA addresses the most commonly allergenic foods in the USA and requires the identification on the food label of ingredients derived from commonly allergenic sources. For this purpose the FALCPA recognises milk, eggs, fish, crustacean shell fish, peanuts, soybeans, wheat and tree nuts (hazelnut, walnut, almond etc.) [5]. Within the EU, Directives 2000/13/EC and 2003/89/EC [6, 7] require a mandatory declaration of allergenic foods, which includes the above-mentioned major allergenic ingredients and in addition to these celery, mustard, sesame seeds and sulfites. Recently, Commission Directive 2006/142/EC [8] announced the inclusion of lupin and molluscs to the list of the 12 allergenic foods. Figure 1 gives an overview of allergens whose presence in food products needs to be declared in different parts of the world.

The above-mentioned legislation on food labelling only concerns allergenic ingredients that are knowingly and deliberately introduced into food products, in contrast to traces of such allergens whose presence in a food product is unintentional, and that have entered the food product as a result of e.g. contamination during the production process.

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Fig. 1 Major food allergens whose presence in food products currently needs to be declared on the label of packaged foods, or whose presence in food products will have to be declared in this manner in the future (*dashed lines*). *In the USA and Canada crustaceans are grouped with shellfish and therefore include several types of molluscs. In Australia, New Zealand and the EU crustaceans do not include molluscs



The presence of such “hidden allergens” can affect the safety of the food product, since it can pose a threat to the health of (allergic) consumers. Such a presence of non-declared allergens in a food product could therefore be interpreted as a non-compliance with legislation concerning product/food safety which might result in liability of the manufacturer of a food product [9].

Precautionary labelling has been introduced as a means for manufacturers and retailers to voluntarily provide information to consumers by indicating the possible presence of such “hidden allergens”. It is based on the fact that it can be hard for the food industry to guarantee that food products are not contaminated with food allergens, since many different types of food products are stored and produced in the same facilities and often different products are manufactured on the same machinery. Although equipment is cleaned between runs it is difficult (and expensive) to ensure that all contaminating allergens are completely removed by the cleaning process.

In order to safeguard the health of allergic consumers the concerted action of food producers and legislative and regulatory agencies is absolutely required. Tools like allergen management plans that are implemented by the food industry, guidelines on the handling of allergens, and the appropriate use of precautionary labelling combined with legislation on food labelling define the framework that is essential to convey the correct information regarding the allergen content of food products to the consumer. Another

cornerstone is the availability of methods that are designed to identify traces of food allergens on food production equipment and in food products. This review focuses on the analytical challenges presented by the development of such methods.

Sensitivity

Food allergy is characterised by adverse reactions of the immune system when triggered by the consumption of particular foods. Food allergy reactions occur as a result of the recognition of allergenic proteins by allergen-specific immunoglobulin E (IgE) antibodies. The immune system of food-allergic patients has developed hypersensitivity during sensitisation to an allergenic food and thereby primed the immune system to recognise the allergen in the future. Subsequent consumption of the allergen triggers immunological reactions and can result in a variety of clinical symptoms that may include hives, pruritus, atopic dermatitis, swelling of the throat or facial tissues, vomiting, diarrhoea, asthmatic wheeze, difficulty in breathing and hypotension [5]. For some allergic individuals the ingestion of certain food allergens can even provoke life-threatening reactions (severe anaphylaxis). It is estimated that in the USA alone about 30,000 anaphylactic reactions to food are treated in emergency departments and that food allergy results in 150–200 deaths each year [10].

In order to prevent food allergy reactions from occurring it is necessary to know how much, or how little, of an allergenic food is capable of triggering an allergic reaction. A major complication here is the fact that not only the clinical reactions, but also the eliciting doses (EDs) vary widely between individuals. Moreover, symptoms and EDs can change over time for individual allergic patients. An individual's sensitivity can only reliably be determined using food challenge studies, with the double-blind, placebo-controlled food challenge (DBPCFC) being the "gold standard" for the diagnosis of food allergy and the determination of the clinical reactivity to low concentrations of an allergen. Such oral challenge studies are usually solely designed to establish a diagnosis for food allergy rather than to determine the no observed adverse effect level (NOAEL). This problem is addressed by a consensus protocol for DBPCFC aimed at determining EDs [11]. This consensus protocol has been used in a recent study that was designed to determine the NOAEL and ED in a group of children with peanut allergy. In this study population the NOAEL was found to be 2 mg peanut [12]. Although scientific data on NOAELs are scarce, this study seems to confirm the notion that analytical methods should be capable of detecting food allergens in the low milligram per kilo range [13]. The analytical detection of trace amounts of allergenic ingredients can be complicated by difficulties with their extraction, or the presence of other, often very abundant components of the food matrix that can mask the allergen, while the type of food matrix is known to impact the recovery of food allergens [14]. Further complications exist in the detection of allergens in ingredients derived from allergenic sources. In some cases, only a subset of the total proteins from the allergenic source is present; soy lecithin with its over-representation of lipid-soluble proteins would serve as an example. In other cases only minute amounts of allergenic protein are likely to be present in products derived of an allergenic source. This is for instance the case for fats and (refined) oils. Obviously, the available methods can differ in their ability to detect residual allergens in such ingredients.

Target selection and selectivity

The design of methods for the detection of allergenic ingredients in food products requires the identification and selection of target analytes. Allergenic proteins are obvious targets in this case, but many allergenic foods contain several proteins with allergenic potential. In peanut at least eight proteins (Ara h 1, Ara h 2, etc.) have the potential to cause an immunological response [15–22], while in milk all proteins (e.g. caseins, α -lactalbumin, β -lactoglobulin, etc.) possess an allergenic capacity [23]. The selection of a

single allergen for detection of the allergenic food has a clinical relevance. But, the fact that allergic individuals often react to different protein constituents of an allergenic food implies that this clinical relevance is restricted to a subset of allergic patients.

Another important factor reflects on the sensitivity as described above. In order to achieve good sensitivity, the abundance of the target analyte within the allergenic food should be taken into account. Theoretically, high sensitivity can be achieved by the selection of multiple target analytes. Alternatively, targets that do not possess any allergenic potential can be used as markers for the detection of allergenic ingredient in food products, justified by the fact that they are highly abundant in the allergenic commodity.

A major requirement for the selection of target analytes or markers for allergenic commodities concerns their specificity. Is the marker representative for the allergenic food only, or can the same marker be detected in other foods? False positive results can also be obtained when methods designed to target markers for allergenic ingredients also respond positively to other components of food matrices. For this reason target selection requires extensive database searching as well as practical experiments designed to detect possible cross-reactivity.

Despite the fact that selectivity is a major requirement, cross-reactivity can be beneficial in certain circumstances. Some allergenic commodities include a large number of plant or animal species. Clear examples of this are fish and crustaceans, where a large variety of species are capable of triggering allergic reactions. Specificity is an important issue here, since methods for the detection of crustacean and fish residues in food products should be specific so that only the allergenic ingredients are detected. However, a high specificity would imply that every crustacean or fish species requires its own specific method of analysis. Practically, however, it would be preferable to have methods at hand that are able to detect several different crustacean or fish species in a single analysis. In principle, such methods can be developed, since immunological detection methods have clearly shown cross-reactivities of allergens from several different fish species [24], but these methods might not be specific enough and possess potential for cross-reactivities with non-crustacean or non-fish ingredients in the food products. Currently the availability of methods for the detection of crustaceans and fish in food products is limited partly due to the issue of selectivity [25].

Types of methods

Currently multiple technical approaches have been designed to detect the presence of allergenic foods used as ingredients in food products. Several methods do not target

a specific allergenic protein, but rather a marker indicative of the presence of the offending food [13]. In principle any component that is specific for the offending food can serve as a marker to detect its presence in food products. But, in practice, proteins or DNA are targeted for this purpose. Protein-based methods usually involve immunological (antibody-based) techniques. DNA-based methods are based on the amplification of specific DNA fragments by means of the polymerase chain reaction (PCR) in which specificity is achieved by the use of primers that only facilitate amplification of DNA originating from the offending food. A further confirmation that the correct marker has been detected can be achieved by analysis of the amplification product e.g. by means of restriction site analysis, DNA sequencing or hybridization to DNA probes or their peptide homologue PNA probes [26].

The variety of methods currently employed for the detection of food allergens in food products has recently been reviewed [13]. Table 1 presents the most common immunological and molecular biological methods. Immunological methods employ antibodies that are raised in animals against purified allergens or other proteins specific for the allergenic commodity or alternatively, human IgE. However, human IgE is characterized by a variability of sera obtained from individual patients which prevents standardization and its limited availability prevents commercialization of methods based on human IgE.

Table 1 shows that the majority of methods are based on an immunological detection of allergenic ingredients. Some immunological methods are considered to be more labourious or more time-consuming than others. ELISA and dipstick methods for the detection of food allergenic ingredients are commercially available and both methods utilize antibodies raised against allergenic commodities [41, 42]. ELISA is

probably the method that is used most commonly by the food industry and official food control agencies. A multitude of ELISA methods have been developed for the detection of several different food allergens [31, 32, 42–51] and numerous commercial test kits have become available during the last decade. Dipstick-based methods are less labourious and clearly faster than ELISA, but they are not well suited for a quantitative assessment of the allergenic ingredient that they are designed to detect.

The immunological methods listed in Table 1 all lack a separation of proteins and therefore cross-reactivity of antibodies with food matrix components goes unobserved, which can lead to false positive results. An exception to this is SDS PAGE followed by immunoblotting, which can provide information on the molecular weight of proteins that are bound by the antibodies employed for the detection.

Biosensors provide a rather novel approach for the detection of allergenic ingredients in food products [52]. In principle they can be used to detect DNA or protein as analytes; however, they are currently mainly employed to detect protein analytes. This type of method benefits from short analysis times and has a potential for automation [53]. Surface plasmon resonance (SPR)-based biosensors have been reported to be capable of detecting food allergens down to 1–12.5 µg/g in food samples and therefore achieve sensitivities comparable to immunoassays like ELISA [54].

Compared to protein-based methods, DNA-based methods offer both advantages and disadvantages. Whereas the concentration of (allergenic) proteins within an allergenic commodity depends upon variables like the species/variety and the growth conditions, the level of DNA is very stable. On the other hand the main limitation of the use of DNA-based methods lies in the fact that they do not detect the compounds responsible for triggering the allergic reactions.

Table 1 Commonly used methods for the detection of food allergens

Methods	Type of method	Specificity achieved by	Target analyte	LOD/LOQ ^a	References
RAST/EAST	Immunological	Human IgE binding	Allergenic proteins	1 mg/kg	[27, 28]
Immunoblotting	Immunological	Binding to human IgE or antibodies raised in animals	Proteins	2.5–5 mg/kg	[29, 30]
Rocket immuno-electrophoresis	Immunological	Binding to human IgE or antibodies raised in animals	Proteins	2.5–30 mg/kg	[31, 32]
ELISA	Immunological	Binding to human IgE or antibodies raised in animals	Proteins	0.1–2 mg/kg	[33, 34]
Dipstick	Immunological	Binding to antibodies raised in animals	Proteins	1 mg/kg	[35]
Biosensor	Immunological	Binding to antibodies raised in animals	Proteins	0.5–2 mg/kg	[36, 37]
PCR	Molecular biological	Oligonucleotide primers	DNA	1 ppm	[38]
PCR ELISA	Molecular biological and immunological	Oligonucleotide primers	DNA	10 ppm	[39]
Real-time PCR	Molecular biological	Oligonucleotide primers (and probe)	DNA	2–10 ppm	[40, 41]

^a Either the LOD or the LOQ as stated in the references is given. The LOD/LOQ stated here is directly derived from the references and is therefore expressed either in mg/kg or pmm

Furthermore it needs to be stressed that some food allergenic commodities are characterized by high protein content and a low DNA content (e.g. eggs) and therefore DNA-based methods are not well suited for the detection of such allergenic commodities within food products. In contrast, DNA-based methods may be a good choice for commodities where protein content is low (e.g. celery).

Quantification

Both in the USA and the EU the legislation regulating the declaration of allergenic ingredients on the label of food products does not contain threshold levels, below which allergenic ingredients are not required to be revealed on the food label. The limited availability of clinical data on EDs and NOAELs is likely to be the main reason for the fact that safe threshold levels are currently absent in the legislation [55]. Notwithstanding this, it is apparent that the allergen content of a food product is of paramount importance, since both the occurrence and severity of allergic reactions clearly depend on the allergen content of the offending food as well as the amount consumed [5].

Amongst the methods developed for the detection of allergenic ingredients there are methods that provide a qualitative assessment. Examples of such methods include dipsticks and conventional PCR (alternatively called endpoint PCR). Both methods are capable of determining the presence of allergenic ingredients, but do not provide reliable information that would be required to determine the allergen content of a food product.

Examples of methods that have the potential to make a quantitative assessment of the concentration of allergenic ingredients in food products are ELISA and real-time PCR. Such methods are often used for semi-quantitative or quantitative determination of allergenic ingredients within food products. For this purpose the analytical results of food samples are compared to a standard curve obtained by the analysis of a series of standards with known concentrations of the allergen in question. Although this mode of operation allows a quantitative assessment it needs to be noted that a variety of factors influence the accuracy of the analytical results.

The efficiency of the extraction of proteins or DNA specific for the allergenic ingredients clearly affects the quantitative result [56]. This can also be influenced by matrix components that can affect either the extraction or the detection of the analytes. The use of extraction additives like e.g. fish gelatine has been reported to improve allergen extraction by a factor of 30 [57].

Another factor of concern is the reactivity of the method towards the standard as compared to the analytical sample. This issue is apparent for methods employing a broad

specificity, like for example ELISA methods that are able to detect a variety of fish species. Such a method is unlikely to exhibit equal reactivities to the different species of fish [24], which severely impacts the accuracy of the quantitative result. Even when a single species needs to be detected genetic variability between varieties of the same species can impact the reactivity and therefore influence the quantitative result of both protein-based and DNA-based methods.

The allergen detection methods that are routinely used like ELISA and PCR usually cannot, or do not, differentiate between different species or varieties of allergenic commodities. Moreover, different standards are used as a basis for semi-quantitative and quantitative assessments. This highlights the need for reference materials that should be widely available in order to develop common standards. This can be achieved in several ways. For the standardisation of pollen and house dust mite allergens recombinant versions of allergens have been produced within the CREATE project and their potential to be developed into certified reference materials has been investigated [58]. This strategy has not been followed for food allergens. Instead, recently a peanut test material (IRMM-481) was made commercially available by a reference materials producer. IRMM-481 consists of five different types of peanut that vary in variety and their geographical origin. With the introduction of this material a standard peanut material has been made available to the research community which could be employed to standardise semi-quantitative and quantitative methods for the detection of peanut traces in food products.

Reliability

The recent introduction of a multitude of methods for the detection of food allergens, many of which are commercially available, raises questions about their robustness and comparability. International collaborative trial studies can be performed to address this issue in order to provide confidence in the analytical results obtained.

An international harmonised validation protocol for collaborative trial studies exists [59], but adherence to the current protocol may not be the ideal approach for the validation of semi-quantitative and quantitative methods designed for the detection of allergenic ingredients in food products [60]. Nevertheless, the analytical community and especially standardisation bodies are looking for properly validated methods that are able to detect trace amounts of food allergens.

Recently several studies reporting validation by means of interlaboratory studies have been performed. Such studies have mainly focussed on immunological methods

for the determination of the presence of peanut proteins in food products, since peanut allergy is frequently associated with severe anaphylactic reactions.

A study by van Hengel et al. [61] reported an interlaboratory (18 laboratories) validation study of two different types of dipsticks. For this study the guidelines for the validation of qualitative methods were followed [62]. The results showed that both dipstick test kits are capable of correctly identifying test samples containing peanut at levels of 21 mg/kg or more.

Recently the performance of commercially available ELISA test kits for the detection of peanut protein in food matrices has been analysed by several laboratories. Whitaker et al. [63] reported the results of a single laboratory study in which four ELISA kits were evaluated for the detection of peanut proteins at four levels of peanut contamination (including a negative matrix control), in four food matrices (breakfast cereal, milk chocolate, ice cream and cookies). A multiple laboratory validation study in which three ELISA test kits were validated with three laboratories that analysed four different matrices (cereal, milk chocolate, ice cream and cookies) has been reported by Park et al. [64]. In the latter study the focus was placed on the qualitative analysis of samples containing 0 and 5 mg peanut per kg food matrix. Poms et al. [60] have reported an interlaboratory validation study of five ELISA test kits, in which 34 laboratories analysed two food matrices (biscuit and dark chocolate) for the detection of peanut proteins at four levels of peanut contamination (including a negative matrix control). In the three studies the peanut-containing food matrices were prepared in different ways: one study used defatted peanut flour to spike food matrices [63], another used peanut butter to spike [64] and in the third one peanut flour was added to biscuit dough prior to baking [60]. The precise measurement of peanut concentrations in food is known to be affected by variables like the type of test kit, the food matrix, the spiking method, food processing after spiking, and the peanut material used to spike or contaminate the food matrix. Despite the differences in experimental design, two international validation studies [60, 64] have shown that all ELISA test kits used in these studies correctly identified test samples containing 5 mg peanut per kg food matrix. Notwithstanding this, the differences in the experimental design of the studies clearly hamper a direct comparison of the reported results. This highlights the necessity of a common protocol for the design of studies aimed to validate methods for the detection and quantification of food allergens. The development of such a common protocol is currently initiated by a working group consisting of representatives of the Food and Drug Administration (FDA), Health Canada, Food Allergy Research and Resource Program University of Nebraska (FARRP), Food Products Association (FPA), and the European Commis-

sion's Institute for Reference Materials and Measurements (EC-JRC-IRMM).

Food processing

Whereas some allergenic foods are often eaten raw, like apples, others are rarely eaten without having undergone some type of food processing. An example of the latter type is peanut, which is usually consumed after roasting. Other allergenic foods are undergoing food processing to increase their shelf life: the pasteurization or sterilization of milk and dairy products are clear examples of this. A large variety of food processing methodologies are currently being employed by the food industry for example heating, chilling, high pressure treatment, ultra filtration, irradiation, hydrolysis and fermentation.

Food processing is likely to affect the capacity of allergenic foods to trigger allergic reactions by modulating the integrity of allergenic proteins [65]. In sensitized individuals IgE will bind to epitopes on the allergens. Such epitopes can either be linear stretches of amino acids, or conformational epitopes that consist of amino acids that are not sequential but are in each others vicinity as a result of the protein's three-dimensional structure. Food processing that affects the integrity of the allergenic protein by changing its three-dimensional conformation (e.g. heat-induced denaturation) will affect IgE binding to conformational epitopes, whereas food processing methods that cleave the protein (e.g. fermentation) will also affect IgE binding to linear epitopes. One might therefore expect that food processing reduces the allergenic capacity. However, an increase of the allergenic capacity as a result of food processing is also possible, since such processes can reveal hidden epitopes that were previously buried within the three-dimensional structure of the allergen. In addition to this, protein or peptide modifications resulting from food processing might lead to the formation of novel structures with allergenic capacities, resulting in an increased IgE binding [66].

The changes in protein structure as discussed above also affect the detection of allergenic ingredients in food products. The first factor that can be affected is the extraction efficiency. It has been reported that roasting of peanut decreases the protein extraction efficiency and thereby negatively impacts the detection [56]. Secondly, food processing affects the target analytes of methods employed to detect allergenic ingredients in food products, since it can alter the integrity of both protein and DNA targeted by such detection methods. However, whereas food processing can either decrease or increase the allergenic capacity, it generally decreases the sensitivity of detection methods. As a result of this it is obvious that

quantification of the allergen content of food products is also severely hampered by food processing. During the development of allergen detection methods the effects of food processing need to be taken into account. Therefore, it is important to assess the reactivity of detection methods towards processed allergenic foods in order to investigate the performance of such methods in relation to allergen-containing processed food products that the consumer is exposed to. To assist this type of method assessment, a peanut test material (IRMM-481) has been developed that contains peanuts that have undergone different blanching and roasting treatments as applied by the food industry.

A further challenge for the detection of food allergenic ingredients is formed by the increased use of hydrolysates derived from allergenic foods like for instance soy, wheat and milk. By means of hydrolysis, allergen-derived products are generated that are used as ingredients in a large variety of nutraceutical, pharmaceutical and food products [23]. It remains to be investigated to what extent the currently available methods for the detection of food allergens are able to detect such hydrolysates.

Future trends

The correct and unambiguous identification of proteins from allergenic commodities within food matrices is a major challenge. The available methods listed in Table 1 all assist in the protection of the allergic patient. Nevertheless immunological methods can suffer from unexpected cross-reactivities giving rise to problems with regard to method specificity. DNA-based molecular biological methods, on the other hand, are not designed to detect the presence of proteins which are the factors capable of triggering allergic reactions. Therefore employing the use of confirmatory methods can increase fidelity in the analytical results obtained with a single detection method.

In addition to this, a correct identification of allergenic proteins can be achieved by using methods with a very high specificity. This is possible by utilizing mass spectrometry (MS). A combination of liquid chromatography with MS detection can provide an unambiguous identification of proteins or peptides originating from an allergenic food commodity. This type of method has recently been employed successfully for the detection of peanut [67] and milk allergens [68] in food matrices. Despite the need for costly and specialised equipment, MS-based methods provide, in addition to a high specificity, the advantage that MS detection theoretically allows the detection of multiple, unrelated food allergens in a single analysis. Furthermore, the pattern of peanut allergen derived peptides that are detected changes after food processing [69] and therefore MS detection might provide insight into the processing history

of the allergenic ingredients. This is relevant since food processing affects quantification of allergenic ingredients.

The food industry has also reacted to ensure an improved flow of information towards the allergic consumer. This is achieved by adherence to good manufacturing practises and the proper declaration of allergenic ingredients on the label. In addition to this, precautionary labelling warns consumers of the possible presence of allergens that have entered the food product via cross-contamination. However, this type of labelling has to be used appropriately, since blanket use does not contribute to safeguarding the health of allergic consumers.

Recently there has been an increase in the marketing of food products specifically destined for people suffering from food allergies. However, current legal requirements for the use of statements referring to allergen-free claims are scarce. Nevertheless, producers of such products rely on specific production and control methods that should ensure the absence of undesirable allergenic substances in their food products. In Canada the Association Québécoise des Allergies Alimentaires has launched a program whereby the use of a certified allergen control (CAC) logo can be granted to identify food products that comply with rigorous requirements with regard to optimal allergen control. The use of regular allergen detection tests is seen as a cornerstone for this program.

Conclusion

The analytical community is challenged to develop methods for the detection of a multitude of food allergens in food products. Several methods that have now been developed are capable of detecting such allergens at levels that are relevant to improve the protection of the health of allergic consumers. However, this is not yet the case for all major allergens. Furthermore it needs to be recognised that issues regarding method characteristics like specificity, selectivity and reliability require a continued effort.

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