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# 4

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***Abstract***

Food allergens are proteins that are well tolerated by most, but can cause severe reactions in sensitive individuals. Since there is no cure for food allergy, strict adherence to an allergen-free diet is the only safe choice currently available for allergic consumers. Accurate food labeling can help consumers avoid foods containing an allergenic ingredient. Regulatory agencies have mandated the labeling of major food allergens on packaged foods to help with safe food choices. However, the inadvertent presence of an allergen in food due to cross-contact and labeling error can jeopardize consumer health. Analytical methods are developed for allergen detection and quantitation to ensure food safety and labeling compliance. These methods are mostly based on immunochemistry, mass spectrometry and genomic amplification. This chapter details the general principles and advances in the development of allergen detection methods. The validation of these analytical methods and challenges associated with accurate allergen quantitation is also discussed.

***Keywords***: Food allergens, immunoassay, mass spectrometry, PCR

## 4.1 Introduction

Food allergy has become a major health concern for consumers due to the increase in reported cases of food allergy sensitization in a wide

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variety of foods. Currently, adherence to a strict allergen-free diet is the only reliable mode of treatment for allergic consumers. Recent reports suggest the prevalence of food allergy is approximately 5% in adults and 8% in children [1, 2]. Most food allergies are caused by specific classes of proteins in food that are otherwise harmless to a non-allergic person. The amount of allergen needed to trigger an allergic reaction varies among individuals and different allergens. Recent studies have sought to identify the minimum eliciting dose levels for many food allergens [3, 4]. Though more than 160 foods have been associated with food allergies, major food allergens, including milk, egg, fish, crustacean shellfish, peanut, tree nuts, wheat and soy, account for about 90% of food allergies [5, 6]. Various allergenic proteins have been identified in these foods (Table 4.1). The Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 mandated the declaration of these major food allergens on labels of foods regulated by the U.S. Food and Drug Administration. Inclusion of additional food allergens may depend on factors such as allergy prevalence and severity in a particular geographic region. For example, the European Union includes sesame, shellfish/mollusks, mustard, celery, and lupine as priority food allergens in addition to the "Big 8" [5]. Allergic consumers use food labels to identify

**Table 4.1** Proteins identified as food allergens in major allergenic food sources[a].

| **MILK** | | | |
|---|---|---|---|
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| Bos d 4 | $\alpha$-lactalbumin | Bos d 9 | $\alpha$S1-casein |
| Bos d 5 | $\beta$-lactoglobulin | Bos d 10 | $\alpha$S2-casein |
| Bos d 6 | Serum albumin | Bos d 11 | $\beta$-casein |
| Bos d 7 | Immunoglobulin | Bos d 12 | κ-casein |
| Bos d 8 | Caseins | | |
| **EGG** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| Gal d 1 | Ovomucoid | Gal d 4 | Lysozyme C |
| Gal d 2 | Ovalbumin | Gal d 5 | Serum albumin |
| Gal d 3 | Ovotransferrin | Gal d 6 | YGP42 |
| **FISH[b]** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| *Yellowfin tuna* | | *Atlantic cod* | |
| Thu a 1 | $\beta$-parvalbumin | Gad m 1 | $\beta$-parvalbumin |
| Thu a 2 | $\beta$-enolase | Gad m 2 | $\beta$-enolase |
| Thu a 3 | Aldolase A | Gad m 3 | Aldolase A |

**Table 4.1** Cont.

| *Atlantic salmon* | | *Baltic cod* | |
|---|---|---|---|
| Sal s 1 | $\beta$-parvalbumin 1 | Gad c 1 | $\beta$-parvalbumin |
| Sal s 2 | $\beta$-Enolase | | |
| Sal s 3 | Aldolase A | | |
| **CRUSTACEAN SHELLFISH[b]** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| *Black tiger shrimp* | | *American lobster* | |
| Pen m 1 | Tropomyosin | Hom a 1 | Tropomyosin |
| Pen m 2 | Arginine kinase | Hom a 3 | Myosin light chain 2 |
| Pen m 3 | Myosin light chain 2 | Hom a 6 | Troponin C |
| Pen m 4 | Sarcoplasmic Ca binding protein | *Spiny lobster* | |
| Pen m 6 | Troponin C | Pan s 1 | Tropomyosin |
| *Crab* | | | |
| Cha f 1 | Tropomyosin | | |
| **PEANUT** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| Ara h 1 | 7S globulin | Ara h 10 | 16 kDa oleosin |
| Ara h 2 | 2S albumin | Ara h 11 | 14 kDa oleosin |
| Ara h 3 | 11S globulin | Ara h 12 | Defensin |
| Ara h 4 | renamed Ara h 3.02 | Ara h 13 | Defensin |
| Ara h 5 | Profilin | Ara h 14 | Oleosin |
| Ara h 6 | 2S albumin | Ara h 15 | Oleosin |
| Ara h 7 | 2S albumin | Ara h 16 | nsLTP2 |
| Ara h 8 | PR-10 | Ara h 17 | nsLTP1 |
| Ara h 9 | nsLTP1 | | |
| **TREE NUTS[b]** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| *Almond* | | *Brazil nut* | |
| Pru du 3 | nsLTP1 | Ber e 1 | 2S albumin |
| Pru du 4 | Profilin | Ber e 2 | 11S globulin |
| Pru du 5 | 60s acidic ribosomal protien P2 | *Hazelnut* | |
| Pru du 6 | Amandin, 11S globulin | Cor a 1 | PR-10 |
| *Cashew nut* | | Cor a 2 | Profilin |
| Ana o 1 | 7S globulin | Cor a 8 | nsLTP1 |
| Ana o 2 | 11S globulin | Cor a 9 | 11S globulin |
| Ana o 3 | 2S albumin | Cor a 11 | 7S globulin |
| *Pecan* | | Cor a 12 | 17 kDa oelosin |
| Car i 1 | 2S albumin | Cor a 13 | 14-16 kDa oleosin |

(*Continued*)

**Table 4.1** Cont.

| | | | |
|---|---|---|---|
| Car i 2 | 7S globulin | Cor a 14 | 2S albumin |
| Car i 4 | 11S globulin | *English walnut* | |
| *Pistachio* | | Jug r 1 | 2S albumin |
| Pis v 1 | 2S albumin | Jug r 2 | 7S globulin |
| Pis v 2 | 11S globulin | Jug r 3 | nsLTP1 |
| Pis v 3 | 7S globulin | Jug r 4 | 11S globulin |
| Pis v 4 | manganese superoxide dismutase | Jug r 5 | PR-10 |
| Pis v 5 | 11S globulin | | |
| **WHEAT** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| Tri a 14 | nsLTP1 | Tri a 37 | $\alpha$ purothionin |
| Tri a 18 | Agglutinin isolectin 1 | Tri a 40 | $\alpha$ amylase inhibitor |
| Tri a 19 | $\omega$-5 gliadin | Tri a 41 | Mitochondrial ubiquitin ligase activator of NFKB 1 |
| Tri a 20 | $\gamma$ gliadin | Tri a 42 | Hypothetical protein |
| Tri a 25 | Thioredoxin | Tri a 43 | Hypothetical protein |
| Tri a 26 | High molecular weight glutenin | Tri a 44 | Endosperm transfer cell specific PR60 precursor |
| Tri a 36 | Low molecular weight glutenin GluB3-23 | Tri a 45 | Elongation factor 1 (EIF1) |
| **SOY** | | | |
| **Allergen** | **Biochemical name** | **Allergen** | **Biochemical name** |
| Gly m 3 | Profilin | Gly m 6 | 11S globulin |
| Gly m 4 | PR-10 | Gly m 7 | Seed biotinylated protein |
| Gly m 5 | 7S globulin | Gly m 8 | 2S albumin |

[a]Adapted from http://www.allergen.org/index.php; accessed on September 14, 2016.
[b]Select common sources of fish, crustacean shellfish, and tree nuts are listed.

allergens in packaged foods and make safe food selections. Undeclared allergens, however, can inadvertently appear in a product from cross-contact during manufacturing, ineffective equipment sanitation, and incorrect labeling. To effectively safeguard the food-allergic population, the food industry and regulatory bodies require reliable analytical methods for allergen detection.

The methods commonly used for the detection of allergens in food are based on the detection of markers (i.e., proteins, peptides, DNA) to indicate the presence of allergenic ingredients (Figure 4.1). Despite the abundance of analytical tools, the selection of an appropriate method for allergen detection can be challenging, due in part to the inherent complexity of food. Food composition and the manner in which the food has been processed can mask or alter allergen markers, thereby impairing the solubility, detection, and quantitation of food allergens. Other factors that affect

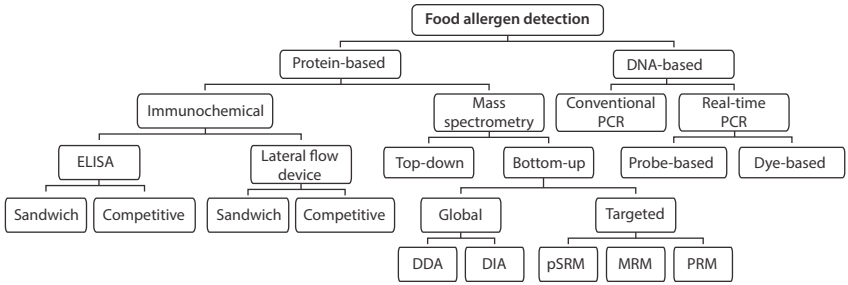


**Figure 4.1** Classification of methods commonly used for food allergen detection in foods.

allergen quantitation in foods include allergen reference materials, target analyte selection, and the reporting units used in quantitation. Quantitative methods must be rigorously evaluated using incurred reference materials (allergen ingredient added prior to processing) and characterized in numerous commercially relevant target matrices. The major analytical methods, including enzyme-linked immunosorbent assay (ELISA), mass spectrometry (MS) and polymerase chain reaction (PCR) are discussed in detail in this chapter. While the majority of commercially available allergen detection methods are single allergen assays, multi-allergen detection methods have recently been developed using a multiplex enzyme immunoassay [7–10], MS [11–16] or DNA amplification [17, 18]. Understanding the limitations of available methods for food allergen quantitation, specifically with respect to sample extraction, thermal processing, and biomarker selection, will improve method selection, establish appropriate allergen control plans, and ultimately protect allergic consumers.

## 4.2 Immunochemical Methods

### 4.2.1 Lateral Flow Device (LFD)/Dipstick

The LFD/dipstick is a qualitative or semi-quantitative method commonly implemented in food analysis due to the relative ease of use, portability, and cost-effectiveness. This method uses a membrane (usually nitrocellulose, nylon, or polyvinylidene difluoride) on which test antigen/analyte and antibody are applied. The role of different components of LFD and their use in food allergen detection have been discussed by Baumert and Tran [19]. The assay can be a sandwich [20–22] or competitive format [23]. In the sandwich assay, immunoreactants [analyte and detector antibody (enzyme labeled or coupled to latex or colloidal metal)] migrate along a test strip. This complex reacts with an immobilized analyte-specific capture antibody

(test zone) and with an immobilized detector antibody-specific antibody (control zone), producing color at each zone. The colorimetric intensity at the test zone is proportional to the amount of analyte present in the sample. For a competitive assay, the immobilized analyte is used as a capture reagent at the test line that competes with the analyte in the migrating analyte-detector antibody complex. Hence, for competitive assays, the intensity of the test line color is inversely related to the amount of analyte present in the sample.

### 4.2.2 ELISA

The most commonly used method platform for both qualitative and quantitative detection of allergens in food is ELISA [24, 25]. Laboratories and food manufacturers prefer ELISA to monitor food products for the presence of allergen residues because of its high level of sensitivity and ease of use [26]. ELISAs use monoclonal or polyclonal antibodies generated in mammals that recognize select food proteins as markers for the presence of an allergenic food. Monoclonal antibodies are specific for a particular protein epitope whereas polyclonal antibodies can detect multiple epitopes on either a single protein or a mixture of proteins. Allergenic foods constitute a number of allergenic and non-allergenic proteins. Moreover, allergenic foods such as egg, milk and peanut have several major allergenic proteins while shrimp and fish have primarily one major allergenic protein. Allergenic proteins are commonly targeted by ELISA as an appropriate analyte for food allergen analysis. The affinity and specificity of the generated antibodies towards the target analyte is vital for the development of a sensitive and robust ELISA.

The ELISA format can be either a sandwich (s-ELISA) or competitive (c-ELISA). The selection of an ELISA format depends on various factors including the food matrix, desired sensitivity, and characteristics of selected antibody and target analyte [27, 28]. In s-ELISA, food allergens in the sample are captured by an immobilized antibody on the microwell plate and detected by a second enzyme-labeled allergen-specific antibody. The intensity of the colored product generated after adding the substrate is proportional to the amount of allergen in the food sample. The c-ELISA is an approach where the target allergen in the sample binds to the specific antibody in solution and competes with the immobilized allergen on the well of the plate. In this format, the intensity of the colored product is inversely proportional to the concentration of allergen in the food sample. These two assay formats can be direct, indirect, or enhanced. The detector antibody is labeled with an enzyme (hydrogen peroxidase or alkaline phosphatase) for direct ELISA and unlabeled for indirect ELISA, where

the detector antibody binds with a second labeled antibody that is immunoglobulin specific. For enhanced ELISA formats, the detector antibody is attached to a molecule, such as biotin, which binds with four molecules of enzyme-conjugated streptavidin, thereby enhancing sensitivity [29, 30].

The sensitivity and specificity of all ELISA formats is highly dependent on the biophysical and chemical properties (e.g., solubility, structure, conformation, and chemical alteration) of target allergens. Food processing may cause allergen conformational changes, denaturation, aggregation, chemical modification of epitopes, or interactions with food matrix components. These changes impact protein extractability and antibody recognition of allergenic proteins. Several reports have shown thermal processing during food manufacture can markedly affect the performance of commercial ELISA kits, resulting in reduced food allergen recovery [15, 31–35]. Nonthermal processing, such as hydrolysis, can also alter the epitope-binding region of target proteins, affecting the antibody interaction necessary for accurate quantitation [36, 37]. Complete extraction of allergenic proteins is a difficult task from complex processed food matrices. Denaturing (e.g., sodium dodecyl sulfate or guanidine hydrochloride) and reducing (e.g., $\beta$-mercaptoethanol) agents have been used to increase the extraction efficiency in thermally processed and complex food matrices for improved food allergen recovery by ELISA [38–40]. The selection of appropriate target analytes and detection antibodies along with suitable extraction methods are the key components to improve the sensitivity and specificity of immunochemical methods. Some examples of protein markers used for major food allergen detection by ELISA are discussed below.

#### *4.2.2.1 Milk*

The major milk-protein fractions are casein (80%) and whey (20%). Casein is a thermostable protein and further subdivided into $\alpha$, $\beta$ and κ isoforms. On the other hand, $\beta$-lactoglobulin from whey is thermolabile and irreversibly denatured or aggregated with casein micelles and $\alpha$-lactalbumin upon heat treatment [41–43]. Hefle and Lambrecht [44] developed an s-ELISA using rabbit (capture) and goat (detector) anti-casein antibodies with a limit of detection (LOD) of 0.5 parts per million (ppm; μg/g) casein, which was successful in quantifying casein in all food products associated with milk-allergic consumer complaints. Comparison of the ELISA formats using anti-$\beta$-lactoglobulin antibodies revealed a lower detection limit by the sandwich format, whereas the $\beta$-lactoglobulin concentration measured by the competitive format was 3 to 5 times higher than that by the sandwich format for skim milk powder in cured sausage, bread and pâté [45]. It was suggested that this difference was due, in part, to the properties of

the ELISA format, thermal processing conditions, and the use of whole anti-serum (competitive) or $\beta$-lactoglobulin adsorbed antibodies (sandwich) in the ELISA. Several commercial ELISA kits are available to quantify milk allergen residues. These assays employ different extraction buffers and use monoclonal or polyclonal antibodies mostly directed against casein or $\beta$-lactoglobulin to quantify milk proteins in foods. Polyclonal antibodies directed against potassium caseinate have been successfully developed to detect casein fining residues in wines by ELISA [46, 47].

#### *4.2.2.2 Egg*

Proteins from egg white are more allergenic than those from egg yolk. Allergenic proteins ovalbumin, ovotransferrin, ovomucoid, and lysozyme account for 54, 12, 11, and 3.4% of egg white protein, respectively [48]. ELISA kits based on polyclonal antibodies with specificity to a single egg protein (ovomucoid or ovalbumin) or multiple egg proteins are commercially available. Although these assays may have less than a 1 ppm limit of quantitation (LOQ), their use in egg quantitation may be governed by antibody specificity. For example, an ELISA targeting egg white proteins may fail to detect egg yolk proteins in foods and thus is not suitable for foods that may have cross-contact with egg yolk proteins alone. Monoclonal antibody-based ELISAs targeting egg allergens such as ovalbumin [49] and lysozyme [50] with an LOD of 0.51 ng/mL and 2.73 ng/mL, respectively, have also been developed. Food processing dramatically reduced the performance of commercial ELISA kits in baked foods and pasta [15, 33, 51]. In general, antibodies generated against processed or denatured egg proteins showed higher affinity for egg proteins extracted from processed food samples [32, 33, 40, 50, 52–54]. The abundance and associated allergenicity of ovalbumin and ovomucoid makes them suitable as effective markers for detection of egg by ELISA.

#### *4.2.2.3 Fish*

In the U.S., fish allergy is most frequently associated with tuna, catfish, and salmon [55]. Parvalbumins ($\beta$-subtype), a major fish allergenic protein, show high structural homology across different marine and freshwater fish [55]. Research on quantitative detection of fish and fish roe by ELISA using an anti-parvalbumin antibody and other fish proteins for antibody generation have been developed in recent years [56–61]. An ELISA employing an anti-cod parvalbumin antibody has been reported to detect a wide range of fish species, which may be a useful screening tool for fish allergens [56, 62]. However, the parvalbumin content in fish varies with the species and muscle type (white or dark) [58, 60]. This may affect the quantitation of fish in

foods depending on the fish source used for antibody generation and calibration standards in ELISA. Variable cross-reactivity with 45 different fish extracts from 17 fish orders has been observed for polyclonal antibodies raised against parvalbumins from different fish species [63]. Fish proteins other than parvalbumin have also been used as a target analyte for detection of fish in foods. Polyclonal antibodies raised against a thermostable 36 kDa muscle protein purified from equal amounts of muscle from 10 different fish species reacted to 63 raw and cooked fish species and the developed s-ELISA had an LOD of 0.1 ppm [59]. Shimizu *et al.* [61] developed an s-ELISA with LOD of 0.78 ppm using polyclonal IgG antibodies against the chum salmon $\beta'$-component to detect chum salmon yolk protein from different processed foods.

#### *4.2.2.4 Crustacean Shellfish*

Shrimp, crab, and lobster are common sources of crustacean shellfish allergens. Tropomyosins were identified as major allergens, exhibiting a high degree of molecular homology between shellfish species. Fewer immunoassays have been developed for the detection of crustacean in food as compared to other allergens. Polyclonal antibodies raised against tropomyosin from prawn (*Penaeus latisulcatus*) [64] and shrimp (*Pandalus borealis*) [65] have been used to develop an s-ELISA with a 1 ppm LOD for the detection of crustacean shellfish protein in foods. Seiki *et al.* [66] developed an s-ELISA with a 0.29 ppm LOD using monoclonal (capture) and polyclonal (detector) antibodies against black tiger prawn tropomyosin with 28.5–114.3% reactivity to Decapoda group (prawn, shrimp, lobster, crab) and negligible reactivity with select mollusk groups (Cephalopoda, Bivalvia, Gastropoda). Thermal treatment has been reported to increase the immunoreactivity of tropomyosin from crustacean and mollusk species with monoclonal anti-insect tropomyosin antibody [67]. The relative abundance of tropomyosin in shellfish makes it a suitable candidate marker for detection of crustacean shellfish in foods by ELISA, but its homology and conserved structure may result in cross-reactivity with mollusk and insects [65, 67, 68]. The epitope from the N-terminal region of crustacean tropomyosin was suggested to react with specific monoclonal antibodies that do not bind molluskan tropomyosin, making these antibodies potential tools for use in labeling compliance of crustacean shellfish allergens in foods [67].

#### *4.2.2.5 Peanut*

Various allergens belonging to different protein families have been identified in the peanut kernel (Table 4.1). Ara h 1 and Ara h 2 allergens can cause 95% of peanut allergy reaction in sensitive individuals [69]. The abundance

and allergenicity of these proteins does not necessarily correlate with the detectability by the immunoassay. Peanut allergens vary in their protein conformation and chemical modification by commercial food processing procedures, posing a significant challenge in the selection of candidate peanut protein markers for immunoassay development. Changes in protein solubility and immunoreactivity resulting from thermal processing has been shown to limit the ability of ELISA kits to accurately quantify the amount of peanut protein in roasted peanut flour [70–72]. Ara h 1 is susceptible to heat and thermal process, such as roasting, induced rapid denaturation or aggregation of this protein [73], whereas Ara h 2 and Ara h 6 are relatively heat stable. The degree and manner of processing limits the extractability of peanut proteins when compared to their extractability from raw peanuts [74]. ELISA methods for the detection of peanut residues in food employ polyclonal or monoclonal antibodies against raw peanut, processed peanut, or purified peanut proteins [75, 76]. Most commercial ELISA kits employ polyclonal antibodies in a sandwich format to detect peanut proteins with LOQs from 0.3 to 2.5 ppm. Investigation of antibody reactivity of six commercial ELISA kits against purified peanut allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6) demonstrated that five commercial kits were most sensitive in detecting Ara h 3 followed by Ara h 1, whereas one kit showed greater sensitivity in the detection of Ara h 2 and Ara h 6 [77].

#### *4.2.2.6 Tree Nuts*

Various ELISA methods have been developed for commonly consumed tree nuts, including almond [78–81], Brazil nut [81–83], cashew nut [81, 84, 85], hazelnut [81, 86–88], macadamia nut [89], pecan [90], pistachio [91], and walnut [92, 93]. As with peanut, the associated allergenicity and abundance of seed storage proteins in tree nuts make them candidate proteins for the detection of tree nuts in foods. Amandin, an 11S globulin, is the major storage protein in almond and has been used as a marker protein for almond detection by ELISA with an LOD of 3 ng almond protein/mL [80]. The presence of amandin in different almond varieties has been reported, though immunoreactivity varied significantly among different almonds by s-ELISA using a rabbit anti-almond polyclonal as the capture antibody and a mouse anti-amandin monoclonal as detector antibody [94]. A sensitive s-ELISA based on chicken yolk antibodies against hazelnut 11S globulin (Cor a 9) with an LOD of 4 ng/mL was successful in detecting hazelnut protein in cookies spiked with as low as 1 ppm hazelnut protein [95]. The formation of advanced glycation end

products (Maillard reaction) following the thermal processing of hazelnut proteins in the presence of glucose reduced the recovery of hazelnut measured by four different commercial ELISA kits [96]. Ben Rejeb *et al.* [81] developed a c-ELISA for the simultaneous detection of almond, Brazil nut, cashew and hazelnut along with peanut in chocolate matrix with an LOD of 1 μg/g protein for each allergen. The antibodies used in their ELISA did not display cross-reactivity with other foods tested, except that the almond antibody exhibited slight cross-reactivity with a cashew protein extract. Antibody cross-reactivity is commonly found among proteins from different tree nuts due to homologous amino acid sequences among tree nuts belonging to the same family, such as walnut and pecan [92] and cashew nut and pistachio [85].

#### *4.2.2.7 Wheat (Gluten)*

Wheat proteins are traditionally grouped as albumin, globulin, gliadin, and glutenin, based on their differences in solubility. The gliadin and glutenin fractions collectively form gluten. Although several wheat allergens belong to the albumin and globulin fractions, most immunochemical methods employ gluten as a protein marker for detection of wheat in foods. This is partly because gluten also causes celiac disease in genetically predisposed individuals. For regulatory compliance, gluten is defined as the storage proteins from wheat, rye, barley, and their crossbreeds that is insoluble in water and dilute salt solutions. Hence the ELISA methods used for gluten detection in foods utilize antibodies that bind to common gluten epitopes found in wheat, rye, and barley. Some of the well-characterized monoclonal antibodies used in commercial ELISA kits include Skerritt or 401/21 [97], R5 [98], and G12 [99]. The variable reactivity of these anti-gluten antibodies towards gluten from different grain sources of wheat, rye, and barley may result in under- or overestimation of gluten in foods [100, 101]. Since gluten is not soluble in common aqueous buffers, the extraction of gluten from foods for quantitation by ELISA is achieved by either aqueous ethanol alone or in combination with denaturing and reducing agents at high temperature. ELISA methods using aqueous alcohol alone may have significantly reduced gluten extraction efficiency in thermally processed foods, resulting in an underestimation of gluten [39, 102]. Moreover, using gliadin as a calibrant may compromise gluten quantitation from rye and barley if the antibody affinity to gluten varies with the grain source. A well-characterized calibrant and an antibody displaying equal affinity towards gluten from wheat, rye, and barley will help improve current ELISA methods for gluten quantitation in foods.

#### *4.2.2.8 Soy*

Soy or its derivative is extensively used as an ingredient in a wide variety of food formulations. Apart from soy allergens listed in Table 4.1, other allergens identified in soybean include Gly m Bd 30K (vacuolar storage protein P34), Gly m Bd 28K (26kDa glycoprotein), and Kunitz trypsin inhibitor (KTI) [103]. ELISA methods and commercial assay kits have been developed for the detection of soy using antibodies against total soy protein [104] or individual soy proteins, such as glycinin [105, 106], $\beta$-conglycinin [107, 108], Gly m 4 [109], Gly m Bd 30K [110–113], Gly m Bd 28K [114, 115], and KTI [116, 117]. Soy proteins are often modified by processing, which may affect their interaction with antibody and quantitation by ELISA. A significant reduction in soy protein immunoreactivity was observed by a commercial ELISA upon hydrolysis with papain and bromelain or glycation of soy proteins [118, 119]. Recently, an anti-trypsin inhibitor-antibody-based s-ELISA was developed to quantify soy proteins in surimi and fish balls with 100–122% recovery [117]. KTI may serve as a marker for the detection of soy traces in processed food as its thermal denaturation is reversible upon cooling [120], which may help maintain the conformation needed for antigen-antibody interactions. However, the characteristics (native or modified) of the antigen used for antibody generation and that of the target analyte in food may dictate the suitability of a particular ELISA application. A c-ELISA developed using antibodies produced in eggs (IgY) from hens immunized with soybean proteins modified by the Maillard reaction and interaction with lipid oxidation products demonstrated improved recovery in spiked cookies as compared to antibodies against KTI [121], emphasizing the importance of protein marker selection in immunochemical method development.

## 4.3 Mass Spectrometry (MS) Methods

Mass spectrometry has served a prominent role in the field of biological proteomics promoting large-scale identifications, characterization, and quantitation of peptides and proteins [122]. Due to advancements in MS technology and improvements to data informatics, food allergenomics has emerged as a complementary technology to immunochemical and genomic-based methodologies for the detection of allergens in complex food samples. MS for allergen detection encompasses both discovery-based proteomics and target-analyte methods providing an analysis platform for highly-multiplexed allergen detection with molecular-level specificity.

In a discovery-based proteomics platform, protein identification is performed using either a top-down or bottom-up approach. Top-down proteomics uses gas-phase ionization and fragmentation of intact proteins for high-resolution mass measurement of analytes. The direct analysis of intact allergen proteins enables the elucidation of higher-order protein structure (isoforms and post-translational modifications) and rapid screening methodologies for allergen detection using a matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) source coupled to a high-resolution mass analyzer [123–128]. The conventional peptide-based bottom-up proteomics platform incorporates a site-specific endoprotease to digest allergen protein extracts into component peptides. Early pioneering bottom-up proteomic studies applied two-dimensional gel electrophoresis with western blotting enrichment for the identification of allergen proteins by MS [129–136]. Electrophoretic-based experiments; however, are hindered by limitations in resolution, protein bias, and dynamic range, making relative quantitation between multiple protein samples and parallel experiments nontrivial.

In recent years, bottom-up discovery-based proteomic methods have been transformed by significant instrumental advances, specifically as it applies to sensitivity, throughput, mass accuracy, and mass resolution [137]. Given the versatility and tunability of available MS platforms, careful consideration should be given to the type of instrument, fragmentation method, and overall strategy with respect to the contingent analytical inquiry. In a traditional bottom-up proteomics method, proteolytic peptides are chromatographically separated and introduced as gas phase ions into a mass spectrometer. Precursor ions are selected based upon user-defined criteria (data-dependent acquisition, DDA) and fragmented via collisions with uncharged gas atoms (collision-induced dissociation or higher-energy collisional dissociation). An alternative to DDA is data independent acquisition (DIA) whereby MS/MS scans are collected systematically and independently of precursor information. Product ions are detected in a mass analyzer and searched against custom protein databases to identify peptide sequences and subsequently infer the presence of a particular protein using statistical scoring algorithms [138–140].

Bottom-up proteomic experiments enable the identification of allergen proteins including sequence-specific variations between protein isoforms and the characterization of post-translational modifications [26, 141–143]. A limitation of many immunochemical methods is the inability to differentiate between homologous, cross-reactive allergens. Global proteomic screening methodologies; however, can be performed to compare allergen-containing food samples to spectral libraries generated from

target reference materials, providing a distinct advantage for molecular identification between closely related species [144]. Compared to model organisms, such as yeast (*Saccharomyces cerevisiae*) and humans, proteomic research in plants has not advanced at the same rate. As a result, the limited availability of non-redundant and accurately annotated genomes for many of the allergen species restricts the comprehensive identification of proteins and corresponding isoforms.

Relative quantitation of proteins using a differential bottom-up proteomics platform can be performed using *in-vivo* metabolic labeling with stable isotope-labeled amino acids (SILAC) [145], chemical labeling (e.g., isotope-code affinity tag (ICAT) [146], isobaric tags for relative and absolute quantitation (iTRAQ) [147], and tandem mass tags (TMT) [148]), or label-free methods. Label-free comparative proteomics uses MS1 ion current or MS2 spectral counting to identify differentially abundant peptides [149–151]. In a differential proteomics experiment, ion-abundance ratios are compared between two or more samples for the relative quantitation of post-translational modifications, processing-induced changes in allergen protein content, and varietal differences between allergen materials [152–154]. Characterizing the fundamental changes in protein chemistry induced by food processing using a global proteomics platform enables the selection of specific allergen peptide targets (biomarkers) for reliable allergen detection and improved analytical performance in complex food systems.

A paradigm shift to targeted MS methods has been driven by the need for orthogonal confirmatory technologies for allergen quantitation. Targeted $MS^n$ experiments harness the capability of MS for multiplex quantitation in a single analytical experiment. In triple quadrupole selected- or multiple-reaction monitoring (SRM or MRM) experiments, the first and third quadrupoles act as filters to select predefined *m/z* values corresponding to the precursor ion (Q1) and product ion (Q3) of a peptide, where the second quadrupole serves as the collision cell. Each peptide undergoes collision-induced dissociations (CID) to produce characteristic b- and y-ions. Combinations of intact peptide ions (precursors) and resulting fragment ions (products) constitute a transition pair that is specific for the monitored peptide sequence. The peak area for MRM experiments are integrated to infer peptide abundance and, in combination with peptide ion ratios and retention time alignments, serve as the basis for quantitative analysis. Variants of MRM assays can also exist for ion trap instruments (pseudo-selected reaction monitoring, pSRM) or quadrupole-Orbitrap hybrid instruments (parallel reaction monitoring, PRM). PRM is a targeted proteomics strategy where all products of a precursor peptide are

simultaneously monitored under conditions that offer high resolution and high mass accuracy [155]. Preliminary reports suggest that PRM analyses exhibit dynamic range and performance characteristics that rival those of MRM analyses performed on triple quadrupole instruments [155, 156].

Targeted allergen methods depend on the pre-selection of proteotypic peptides for monitoring analytes in fortified (spiked) or allergen-incurred food matrices. The selection of representative peptides, typically ≤5 peptides per protein, is assigned as a fingerprint for the protein of interest. While proteotypic peptides exhibit a range of physiochemical properties (size, charge, hydrophobicity, and ionization efficiency) and chemical stabilities, the co-selection of multiple peptides across the full-protein sequence validates high specificity to the targeted protein. Recommended criteria for signature peptide marker selection include: unique amino acid composition, protein specificity, proteolytic cleavage reproducibility, optimization of chromatographic and mass spectrometric performance, and characterization of protein post-translational modifications [157]. Considering the diversity of proteotypic peptide structural and chemical behaviors, the selection of appropriate peptide targets must balance theoretical guidelines with practical limitations [158].

For complex food samples, processing effects (e.g., thermal and nonthermal), relative allergen protein abundance, isoform equivalence, and structural diversity introduce additional considerations for allergen target selection. Characterizing the effects of processing, with respect to the biophysical, chemical, and immunological modifications of allergen proteins, by MS facilitates the development of reliable extraction and allergen detection methods in industry-processed food samples [153, 159–162]. Whereas no single extraction condition may be optimally effective for all food allergens, matrix components, and processing conditions, MS promotes the use of more stringent extraction conditions for protein solubilization in thermally processed foods when used in conjunction with adequate sample cleanup procedures.

The challenge of target-analyte methods is the requirement for internal standards and reference materials for reliable protein quantitation. Stable isotope-labeled internal standards (e.g., AQUA peptides, concatenated peptide constructs, and recombinant proteins) are commonly utilized for robust protein quantitation with consistent linearity spanning 4–5 orders of magnitude, measurement coefficients that vary <10%, and LODs in the sub-ppm range. Nonradioactive stable isotope labels such as $^{13}C$ and $^{15}N$ are commonly incorporated for synthetic enrichment. The absolute quantification (AQUA) of peptides relies on the selection and chemical synthesis of isotope-labeled peptide surrogates. With respect to retention time,

ionization efficiency, and fragmentation mechanism, AQUA peptides are chemically and physically indistinguishable from their endogenous native counterpart [163–165]. Synthetic peptide standards are typically incorporated into the sample prior to proteolysis or directly preceding LC-MS analysis. Since the standard is added at late stages of the analytical process, labeled peptide methods are often less compatible with sample preparation platforms requiring pre-fractionation. Solubilization and stability of synthetic peptide standards are sequence-dependent and often negatively impact measurement precision (e.g., degradation or modification during storage). To optimize quantitative efficiency, individual peptide standards for stable isotope dilution must meet the demands of high chemical purity (>95%) and concentration standardization by amino acid analysis [166] prior to investigation. Concatenated peptides (QConCAT) [167, 168] are chimeric proteins comprising different proteotypic peptides from multiple protein targets. QConCAT constructs are synthesized to empirically balance the order, codon selection, and natural flanking sequences to maximize expression yield and emulate the native protein [169–171]. Concatamers are typically added to the sample immediately prior to proteolysis whereby endoprotease cleavage induces the release of isotope-labeled peptides and allows parallel quantitation of multiple peptides in a single analytical experiment. A third labeling methodology, protein standard absolute quantification (PSAQ), is a strategy which relies on *in-vitro* synthesis of isotope-labeled, full-length proteins as standards [172]. The synthesized standards can be introduced at the onset of the experiment, thus providing flexibility in extraction optimization, endoprotease selection, and target peptide assignment while limiting variability of digestion yields between the isotopic standard and the endogenous protein.

The choice of an MS-based approach towards protein quantitation depends on the application, associated cost, and reliability of the method. While the majority of current MS methods are based upon single analyte detection, as reviewed with representative experiments from each major allergen class below, multi-allergen LC-MS/MS methods have recently emerged as an efficient alternative for method development. The first qualitative LC-MS/MS screening method for the simultaneous detection of seven different allergenic materials (almond, egg, hazelnut, milk, peanut, soy, and walnut) was published by Heick *et al.* [11]. Unique tryptic peptide markers were selected through the survey of reference standards and a triple-quadrupole MRM method was developed to detect allergen concentrations ranging from 10–1000 μg/g in a processed bread material [11]. Using isotopically labeled synthetic peptide standards, Parker *et al.* [15] compared the quantitation of egg, milk, and peanut in industrial processed

allergen-incurred foods at various processing stages using ELISA kits and a multi-allergen MRM method. Protein extraction from allergen-incurred cereal bars and muffins was optimized for egg (ovalbumin and lysozyme C), milk (αS1-casein and β-lactoglobulin), and peanut (Ara h1, Ara h 2, and Ara h 3) allergens, considering influences from thermal processing and matrix interference. The custom LC-MS/MS-based method demonstrated unbiased protein extraction for egg, milk, and peanut, with minor concessions to sample recovery for the final product (baked) cereal bars and muffins [15]. As MS-based methods transition towards use as confirmatory or quantitative applications for allergen detection, the need for harmonization between methods and validation through interlaboratory trials will ultimately help to establish robust analytical methods in support of allergen management in the food industry [173].

### 4.3.1 Milk

Huber *et al.* [174] used capillary electrophoresis (CE)-ESI-MS to perform early experiments on quantitating allergenic whey proteins using external calibration curves derived from commercial whey beverages. Optimizing sample preparation using ion exchange chromatography and a centriprep device, Weber *et al.* [175] developed a DDA method for the detection of αS1-casein in milk-containing cookie matrices on a quadrupole time-of-flight mass spectrometer. Further, SRM experiments were developed for the quantitation of milk peptides from αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, and/or β-lactoglobulin found in milk-spiked wine and food samples [176–181]. Lutter *et al.* [182] designed a method for the quantitation of αS2-casein, β-casein, κ-casein, and β-lactoglobulin using $^{13}C^{15}N$-labeled peptide standards. A simplified extraction containing ammonium bicarbonate and urea was validated in protein-rich infant cereals without additional enrichment or solid-phase purification. Optimizing the detection of αS1-casein, allergen peptides derived from milk-incurred cookie samples were quantitated using $^{13}C^{15}N$-labeled peptide standards and a stable isotope-labeled protein [183]. Isotope-labeled $^{15}N$-αS1-casein improved SRM analysis with regards to extraction recovery; however, it did not eliminate the underestimation of allergen concentration arising from thermal processing during baking. Extraction conditions were optimized for the detection of casein in allergen-incurred cookie samples with an LOQ < 3 ppm of nonfat dry milk and an estimated recovery between 60–80% [183]. Alternatively, Zhang *et al.* [184] designed a peptide construct for α-lactalbumin with flanking amino acid sequences at the C- and N-termini. The internal standard was added prior to sample extraction and

cleaved into the surrogate proteotypic peptide after digestion; however, matrix influences on tryptic digestion prevented accurate quantitation. Comparing methods for milk quantitation, Chen *et al.* [185] developed an MRM assay for the detection of five signature peptides from bovine $\beta$-casein. Three standards were evaluated including a stable isotope labeled peptide, a stable isotope-labeled peptide construct (with proteolytically cleavable flanking sequences), and a human $\beta$-casein homolog. While the synthetic isotope-labeled peptide was successful in many baked foods—for items containing egg, cacao, or a high level of oil—the extended stable isotope-labeled peptide was down-selected as the preferred strategy for quantitating bovine $\beta$-casein [185].

### 4.3.2 Egg

Food processing and matrix interactions have been shown to reduce percent recovery in egg-containing food products [52, 186, 187]. Azarnia *et al.* [51] used LC-MS/MS to identify marker peptides suitable for the determination of ovalbumin before and after thermal treatment in egg-incurred pasta. Hindered by the presence of interfering phenolic compounds, tannins, and polysaccharides, LC-MS/MS assays were developed for the detection of egg proteins in various red [188] and white fined wines [177]. Commercial wine samples were screened and allergen detection confirmed by extracted ion chromatograms of selected tryptic peptides. Complimentary methods have been developed for the simultaneous determination of allergenic milk casein and egg proteins (lysozyme and ovalbumin) in commercial wines [177, 181, 188].

### 4.3.3 Fish and Crustacean Shellfish

Parvalbumins (fish) and tropomyosins (crustaceans) are the major allergens responsible for eliciting an adverse immunological response in seafood allergic patients. Carrera *et al.* [189, 190] developed a rapid detection method for the purification of $\beta$-parvalbumin via heat treatment and accelerated in-solution trypsin digestion under an ultrasonic field. Peptide markers were monitored using selected ion monitoring MS and enabled the unequivocal identification of closely related fish species in processed seafood products.

The molecular weight, sequence information, and peptide markers of tropomyosin were characterized in snow crab and black tiger prawns using MS [191, 192]. Isotope dilution MS was utilized to quantitate concentrations of snow crab tropomyosin in an industrial processing plant

using a $d_3$-L-alanine peptide homolog [192, 193]. Due to the homology of tropomyosin sequences in crustaceans, Ortea *et al.* [194, 195] developed a method to distinguish among seven different Decapoda prawn species using the secondary allergen arginine kinase. Incorporating tropomyosin and arginine kinase marker peptides from snow crab as deuterated chemical surrogates for MRM quantitation, a method for occupational allergen testing in a crab processing plant was developed [192, 196]. Similarly, a targeted LC-MS/MS method was established for tropomyosin and arginine kinase in crustacean shellfish, promoting the differentiation from species such as krill or insects [197].

### 4.3.4 Peanut

Shefcheck *et al.* [198, 199] selected Ara h 1 peptides for the detection of peanut in vanilla ice cream and dark chocolate using selected ion monitoring. Increasing the selectivity of allergen identification, optimal markers for the detection of peanut allergens Ara h 1, Ara h 2, and/or Ara h 3 varied based upon selection criteria, including peptide abundance, epitope recognition, thermal processing, and isoform equivalence [200–202]. Using MS-based methods, the propensity for thermal treatments to induce advanced glycation end product (AGE) modifications was identified for peanut allergens Ara h 1 and Ara h 3 [153, 162]. Hebling *et al.* [153] concluded the incorporation of a protein denaturant (urea) augmented protein solubility in thermally processed peanut flour as compared to more traditional (e.g., phosphate-buffered saline) extraction systems. Recently, Monaci *et al.* [203] developed a high-resolution MS method suitable as a screening tool for the detection of peanut in a mixture of tree nuts down to 4 μg/g of matrix.

### 4.3.5 Tree Nuts

Due to cross-reactivity between homologous botanical families, concurrent allergen sensitization to more than one tree nut is common among food-allergic patients [204]. A multiplex MS assay for the simultaneous analysis of almond (Pru du 1), cashew (Ana o 2), hazelnut (Cor a 9), peanut (Ara h 3), and walnut (Jug r 3) was evaluated in breakfast cereal, biscuit, and dark chocolate samples [13, 205]. Samples were fortified prior to extraction and quantitation was performed by monitoring two selected peptides for each target protein. Improving the selectivity for hazelnut, marker peptides from Cor a 8, Cor a 9, and Cor a 11 were monitored using LC-MS/MS in SRM mode [206]. Analytical method performance was compared by

Costa *et al.* [207] for hazelnut-spiked chocolate samples by LC-MS/MS, ELISA, and PCR, providing appropriate quantitation at 1 mg/kg for all methods. Commercial food samples were evaluated using a comprehensive LC-MS/MS assay developed by Sealey-Voyksner *et al.* [14] for the simultaneous detection of 11 tree nuts (almond, Brazil nut, cashew, chestnut, coconut, hazelnut, macadamia nut, pecan, pine nut, pistachio, walnut) and peanut. To confirm peptide identity and provide relative quantitation of tree nut concentration, isotopically labeled peptide standards were selected and synthesized. Peptide markers were chosen based on conserved peptide sequence and extraction recovery in thermally processed flours [14].

### 4.3.6 Wheat

MS-based methods have been developed for the characterization of chemical changes in gluten proteins upon industrial food preparation [208] and the determination of clinically immunogenic peptides [209–211]. Using a pepsin, trypsin, and chymotrypsin protease cocktail to model gastric and duodenal protein digestion in humans, consumer products were surveyed for gluten using quantitation by six immunogenic peptides [210]. Identifying grain-specific (wheat, barley, and rye) chymotryptic peptide markers, Fiedler *et al.* [212] demonstrated low ppm detection of wheat contamination of oat flour in ethanol protein extracts. In fermented beverages, the absence of reference materials for hydrolyzed gluten complicates the development of analytical methods for quantitation. Confirmatory LC-MS/MS methods for hydrolyzed gluten detection in beer have been developed [213–215] and continue to be explored [37] for the detection of barley and wheat-specific peptide markers in fermented beverages.

### 4.3.7 Soy

Houston *et al.* [216] evaluated the natural variation of ten soy allergens among twenty commercial soybean varieties. Relative quantitation was performed with a spectral counting method referencing bovine serum albumin as an internal standard, and absolute quantitation was performed using an MRM method with isotopically labeled peptide standards. The isotope dilution method reduced technical variance, confirming differential expression for targeted allergens across soybean varieties. To improve the detection of soybean in processed food, Cucu *et al.* [217] used MALDI-TOF/MS and MS/MS to identify tryptic peptide markers: $^{401}$Val-Arg$^{410}$ from G1 glycinin (Gly m 6) and the $^{518}$Gln-Arg$^{528}$ from the $\alpha'$ chain of $\beta$-conglycinin (Gly m 5) as stable markers. Soybean genotype

and environmental influences on allergen and anti-nutritional proteins in soybean were evaluated in four varieties of non-genetically engineered soybeans grown in six geographically distinct regions [218, 219]. Absolute quantitation of eight soybean allergens by MRM using an isotopically labeled synthetic peptide standard demonstrated the effects of environment to be greater than breeding condition for most soy allergens.

## 4.4 DNA-Based Methods

Polymerase chain reaction (PCR) is a technique in which a particular segment of DNA is amplified using sequence-specific primers which flank the target region and a polymerase enzyme which synthesizes new DNA. In real-time PCR, an additional sequence-specific, fluorogenic probe is included within the target region. Specificity of a PCR-based method is controlled by the researcher: primers and probes for PCR can be designed using DNA sequences which are highly specific to a single target or allergenic food, or they can be less specific and detect a group of allergenic foods. The probes used in real-time PCR generate a fluorescent signal as new PCR products are created; this signal is recorded with each cycle of PCR, in real time. Use of probes in real-time PCR negates the need for post-PCR analysis and adds an additional level of sequence specificity. Real-time PCR results in an assay which is more rapid and more sensitive than conventional PCR, and can be used to quantitate targets through generation of a linear standard curve. The standard curve is analyzed with respect to linear range, statistical $R^2$ value, and slope; slope is used to determine reaction efficiency [220, 221]. The optimal real-time PCR reaction has a linear range spanning 6–8 orders of magnitude, an $R^2$ value of 0.98 or higher, and reaction efficiency of 100 ± 10%.

As PCR detects DNA, and the allergenic molecules in food are proteins, PCR does not detect allergens directly. The suitability of PCR-based detection therefore depends on the allergenic food. For some allergenic foods, such as eggs and milk, DNA content is inherently low. For other allergenic foods, such as wheat and soy, the protein fraction is commonly used in food products. DNA-based assays such as PCR are less appropriate for these foods. However, other allergenic foods contain high levels of DNA in conjunction with allergenic proteins, so DNA is a good indicator of the presence of allergenic proteins. These foods are good candidates for PCR-based detection and include fish, crustacean shellfish, peanut, and tree nuts. In cases for which PCR is appropriate, it has significant advantages over techniques which detect allergenic proteins directly. Protein-based

detection methods are dependent on knowledge of specific protein properties, yet many allergenic proteins have not been discovered, and many of those which have been discovered are not well characterized. Since it is a DNA-based method, PCR is more straightforward. The DNA of different allergenic foods and food matrices has variation in nucleotide sequence but not in the chemical properties which affect extraction, response to processing, or interactions between allergen and matrix. The same methods can therefore be used to extract DNA from a variety of allergenic foods in a variety of different food matrices. DNA is more stable than proteins, so it is better able to withstand both rigorous laboratory extraction methods and food processing methods. Important aspects of PCR-based allergen detection are DNA extraction, DNA target region, PCR product size, internal controls, and optimization of PCR conditions. Each of these is discussed in greater detail below.

An important early step in PCR-based allergen detection is DNA extraction, as samples used for PCR must be free of substances which may break down the DNA or interfere with PCR. The DNA should be extracted with high efficiency from a variety of food matrices in order to maximize sensitivity of the method; highly efficient extraction is especially important for quantitative methods based on real-time PCR. Numerous techniques have been used for DNA extraction in allergen detection methods, including both classical organic extraction using phenol-chloroform and commercial silica-column-based methods. DNA extraction based on protease digestion, guanidine hydrochloride treatment, and cleanup on a silica-based column provides excellent results and outperforms other DNA extraction methods [222–225]. An additional salt extraction step has also been used to isolate DNA from complex food matrices [226–228]. These techniques have been used successfully with both plant-based and animal-based allergenic foods and in a variety of food matrices.

Initial selection of an appropriate target region of the genome is an aspect of PCR assay design which has important implications for method performance. Genes which code for an allergenic protein are frequently used, however, these allergen genes may not necessarily be the best targets. The best target is one which provides optimal levels of specificity and sensitivity. The greatest sensitivity can be achieved by targeting genes or DNA regions which have many copies in the genome or cell of an organism. These may be high copy number targets from the nuclear genome or targets from the genomes of abundant organelles, such as chloroplasts and mitochondria. Design and *in-silico* cross-reactivity testing of PCR primers and probes are greatly facilitated by the use of genes or gene regions for which sequence data are available from a large number of species. Targets

for detection of allergenic foods have been located in both nuclear and organellar genomes, and have included genes that code for proteins, genes that code for ribosomal RNAs, and noncoding regions of the genome.

Numerous PCR-based allergen methods target the genes encoding allergenic proteins [223, 224, 229–235]. Allergen genes are nuclear. They are not often high copy number, and therefore do not yield the most sensitive assays. Nested PCR is a technique which has been used to improve the sensitivity of assays targeting allergen genes. During nested PCR, a first phase of PCR is used for initial amplification of a longer target, and it is followed by a second phase for amplification of a shorter target internal to the first. Sensitivity is improved because the first phase provides "pre-amplification" of the longer target, which is then used as a template for amplification of the shorter detection target in the second phase. Nested PCR can also yield improved specificity because it requires the use of two pairs of sequence-specific primers: one for the longer target, and one for the shorter target. In real-time PCR assays for tree nuts and peanuts, nested PCR improves sensitivity by 2–5 fold [236–239]. Among non-allergen nuclear genes, the most common high copy number target used in detection of allergenic foods has been the internal transcribed spacer region, or ITS-1. ITS-1 is a non-coding region of DNA located between the 18S and 5.8S ribosomal RNA genes in the nucleus. Since the ITS-1 region is known to be highly variable, it can also be used to distinguish closely related allergens. Targeting of ITS-1 has yielded highly successful conventional PCR assays for peanut, soy, and wheat, as well as real-time PCR assays for buckwheat and several tree nuts [225, 240–243]. Real-time PCR assays using ITS-1 have performed well, with linearity spanning 5–9 orders of magnitude and an LOD as low as 0.1 ppm.

In addition to nuclear targets, several different genes have been targeted in abundant organelles, such as mitochondria and chloroplasts, each containing their own genomes. While sufficient high-quality nuclear genome sequence data can be scarce for some species, in many cases high quality sequence data are readily available for the smaller, more manageable genomes of mitochondria and chloroplasts. Mitochondrial targets used in allergen detection have included the 12S and 16S ribosomal RNA genes, as well as the cytochrome b and cytochrome oxidase I protein coding genes for detection of fish and crustaceans [227, 228, 244–246]. The mitochondrial nad1 gene has been used for detection of hazelnut and the atpA gene for detection of soy [247, 248]. The chloroplast matK gene has been used for detection of walnut [249]. Assays targeting mitochondrial genes have achieved linearity over 6–8 orders of magnitude and an LOD as low as 0.1 ppm in complex food matrices. Direct comparisons of nuclear and

mitochondrial gene targets have shown that allergen detection using mitochondrial targets is 10–100 times more sensitive than detection using an allergen gene or commercial kit targeting nuclear DNA [227, 247].

The size of the PCR product produced is another relevant aspect of selecting an optimal target. In general, assays using smaller PCR products perform better. The role of PCR product size in assay performance becomes most salient during the analysis of processed foods in which DNA is likely to be degraded; PCR amplification of degraded DNA is more likely to be successful with small products of approximately 120 bp or less [250]. Rapid cycling, which is often preferred in real-time assays, also seems to be more successful with smaller PCR products: short cycling limits the amount of time available for primer binding and polymerase activity [251].

Internal controls for PCR-based detection assays can be designed to indicate the presence of inhibitors in the DNA sample or to determine suitability of extracted DNA for PCR amplification. Internal controls must amplify independently of the assay target and therefore do not share sequence similarity. Controls to detect PCR inhibition are based on detection of exogenous DNA, which is added directly to PCR reactions after DNA extraction. Exogenous template DNA can be cloned into a plasmid or obtained directly from a commercial supplier, and a published universal internal control based on exogenous DNA has been shown to work well in allergen detection assays [227, 228, 235, 252]. Controls used to confirm suitability of extracted DNA for PCR are based on amplification of a conserved region of endogenous DNA, which is expected to amplify regardless of whether the intended allergenic target is present. In allergen assays, such controls have targeted nuclear 18S, mitochondrial 16S, and plant chloroplast DNA [225, 235, 241, 245, 253]. In addition to these, a unique type of internal control has been based on the seeds of an ornamental plant, not likely to be found in food products, which were spiked into foods prior to DNA extraction [240].

Optimization of the reaction itself is an overlooked and underreported aspect of developing a successful PCR method. This includes determining the most favorable concentrations of all reaction components, including magnesium, primers, probes, deoxynucletides (dNTPs), and template DNA, as well as determining optimal cycling conditions. For real-time assays, thorough optimization of reaction components should be carried out not only to determine conditions which yield successful amplification for a given sample, but those which yield the best standard curve for samples across a wide range of concentrations. Several published studies have demonstrated the importance of optimizing the PCR protocol.

In conventional PCR, a specially designed high-$Mg^{2+}$ buffer containing 9 mM Mg and EGTA has been shown to improve sensitivity of hazelnut detection [248]. Excess amounts of template DNA can actually interfere with PCR, and this is especially relevant for real-time assays [222]. Cycling conditions also affect results: rapid cycling can have adverse effects on assay performance, and annealing temperature may affect cross-reactivity [227, 248, 251].

Any allergen detection method faces the significant challenges of detecting trace amounts of an allergenic food against a high background of a complex food matrix material, and must work well with processed foods in order to be useful in practice. Well-designed PCR-based methods have proven to be more than capable of meeting these demands. With respect to the eight major allergenic foods, the vast majority of work conducted on PCR-based allergen detection has been focused on crustacean shellfish and tree nuts.

### 4.4.1 Crustacean Shellfish

Crustacean shellfish—including shrimp, crab, and lobster—have been detected in complex food matrices using both conventional and real-time PCR. Real-time PCR assays for shrimp, lobster, and blue crab have achieved linearity over 6–8 orders of magnitude, high reaction efficiencies, and an LOD of 0.1 ppm for crustaceans spiked into soups, noodles, sauces, juices, and prepared seafood products [227, 228]. These assays have high specificity for the intended targets and have been unaffected by heat and pressure treatment, including baking, boiling, microwaving, and autoclaving. Cao *et al.* [244] also determined that heat treatment did not have an adverse effect on real-time PCR-based detection of shrimp. A notable exception occurs with the nearly complete loss of signal observed after heat treatment in an acidic food matrix [228]. This is likely a result of the accelerated degradation of DNA which has been shown to occur in acidic conditions and to affect PCR results [254, 255]. Conventional PCR has achieved a detection limit of 10 ppm for shrimp and crab spiked into soup mix, meat, rice, condiment paste, and a pastry/bread product [246]. Cross-reactivity analysis for this assay was carried out using PCR simulation software with sequences for over 70 species of crustaceans used for food. In one of very few multi-laboratory validation studies of PCR-based qualitative allergen detection methods, 100% of samples incurred at 10 ppm produced positive results from 9 participating laboratories using this assay [256].

### 4.4.2 Tree Nuts

Real-time PCR assays for detection of almond, cashew, and macadamia nuts in flour have achieved reaction efficiencies of 92–107%, linearity over 7 orders of magnitude, and lower LOD at 0.1 ppm [225, 242]. These assays were not adversely affected by roasting, showed high specificity for numerous species and cultivars of the target tree nuts, and did not cross-react with any other foods tested, including a wide variety of non-target tree nuts, legumes, fruits, vegetables, grains, and meat products. Detection of walnut in sponge cake has been reported with high reaction efficiency, linearity over 5 orders of magnitude, and a lower LOD at 5 ppm; assay performance was not adversely affected by baking [238]. Real-time PCR-based detection of pistachio has been reported in a pastry matrix with linearity over 7 orders of magnitude and a lower LOD of 4 ppm; this assay tested positive for 11 different cultivars of pistachio and did not cross-react with non-target tree nuts, peanuts and other legumes, fruits, grains, or meat [243]. Detection of hazelnut was successfully reported in chocolate at 10 ppm [248]. Other real-time PCR-based methods for detection of cashew, hazelnut, pecan, and walnut reported significantly higher LOD, near 100 ppm [229–231, 233, 234]. Differences in assay performance do not reflect fundamental differences between tree nuts, but rather differences in laboratory methods and assay design as discussed above. In particular, the more sensitive tree nut detection methods cited here employed high-copy targets such as ITS-1 or mitochondrial genes, or enhanced sensitivity through the use of nested PCR, while others targeted allergen genes.

## 4.5 Method Validation

Analytical method development should be followed by validation to assess the performance characteristics and reliability of the assay. A single-laboratory validation is generally conducted in-house to determine method parameters such as specificity, sensitivity, LOD, LOQ, quantitation range, robustness/ruggedness, accuracy, precision, and stability of the assay. A multi-laboratory validation involves multiple laboratories analyzing assay performance, especially accuracy and precision, under different work settings such as location and personnel. Among the methods developed for food allergen quantitation, only a few have been evaluated by multi-laboratory validation (Table 4.2). Most of these studies used ELISA as the method of analysis. Differences in the validation study design make it difficult to compare method performance when detecting a common allergen. The inherent difference in the ELISA-based allergen detection

**Table 4.2** Multi-laboratory validation of methods used in quantitation of major food allergens in foods.

| Allergen | Method | No. of labs | Spiking material | Spiked concentration (ppm)[a] | Matrix | Recovery, % | $RSD_r$[b] | $RSD_R$[c] | References |
|---|---|---|---|---|---|---|---|---|---|
| Milk | ELISA Faspek | 10 | milk freeze-dried | 10 | sausage, boiled beef, cookie, orange juice, jam | 89–137 | 3–7 | 12–17 | [38] |
| | ELISA Fastkit | 10 | milk freeze-dried | 10 | sausage, boiled beef, cookie, orange juice, jam | 49–89 | 2–5 | 7–14 | [38] |
| | ELISA Sedium RD | 6 | naturally +[d] | N/A[e] | soy dessert caramel, butter with plant oil | N/A | 6–13 | 26–36 | [266] |
| | | | UHT milk | 2 and 6.6 µl/g | rice long grain, wheat flour, chicken meat | N/A | 6–22 | 22–49 | |
| | ELISA Euroclone SpA | 10 | caseinate | 0, 1, 1.6, 2.2, 2.5, 3, 6.4, 6.5, 7 | white wine | N/A | 0.4–29 | 12–90 | [267] |
| | ELISA Ridascreen Fast casein | 18 | purified casein | 0, 1.1, 6.8, 10.5 | red wine | 81–93 | 12–24 | 19–36 | [268] |
| | | | | 0, 0.37, 1.1, 5.3, 7.2 | white wine | 32–76 | 9–35 | 14–54 | |
| | ELISA Allergeneye | 14 | defatted milk powder | 10 | rice gruel, sweet adzuki bean soup, orange juice, pork sausage, miso soup | 52–67 | 3–4 | 7–11 | [269] |
| Egg | ELISA Faspek | 10 | egg powder | 10 | sausage, boiled beef, cookie, orange juice, jam | 52–87 | 4–5 | 8–17 | [38] |
| | ELISA Fastkit | 10 | egg powder | 10 | sausage, boiled beef, cookie, orange juice, jam | 66–98 | 2–5 | 6–15 | [38] |

(*Continued*)

**Table 4.2** Cont.

| Allergen | Method | No. of labs | Spiking material | Spiked concentration (ppm)[a] | Matrix | Recovery, % | $RSD_r$[b] | $RSD_R$[c] | References |
|---|---|---|---|---|---|---|---|---|---|
| | ELISA Euroclone SpA | 11 | egg albumin | 0, 1.2, 1.5, 3.5, 4.5, 6.5, 7 | red wine | 86–102 | 13–28 | 32–158 | [270] |
| | ELISA Ridacreen Fast Ei/Egg | 18 | egg white powder | 0, 0.33, 1.13, 5.5, 7.6 | red wine | 79–101 | 10–56 | 13–68 | [268] |
| | | | | 0, 0.82, 5.1, 7.5 | white wine | 70–86 | 11–37 | 17–52 | |
| | ELISA Allergeneye | 14 | egg powder | 10 | rice gruel, sweet adzuki bean soup, orange juice, pork sausage, miso soup | 62–89 | 2–4 | 4–6 | [269] |
| Crustacean | ELISA Maruha | 10 | crustacean powder | 10 | fish sausage, freeze-dried egg soup, tomato sauce, creamy croquette, chicken ball | 82–103 | 4–10 | 18–21 | [271] |
| | ELISA Nissui | 10 | crustacean powder | 10 | fish sausage, freeze-dried egg soup, tomato sauce, creamy croquette, chicken ball | 65–86 | 4–5 | 4–8 | [271] |
| Peanut | ELISA Biokits | 14 | oil-roasted peanut flour | 0, 2, 5, 10 | biscuit | 109–126 | 21–48 | 28–57 | [272] |
| | | | dry-roasted peanut flour | 0, 2.5, 5, 10 | chocolate | 110–126 | 9–26 | 21–36 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ELISA Elisa Systems | 13 | oil-roasted peanut flour | 0, 2, 5, 10 | biscuit | 91–143 | 61–93 | 61–93 | [272] |
| | | | dry-roasted peanut flour | 0, 2.5, 5, 10 | chocolate | 76–82 | 9–36 | 42–70 | |
| | ELISA Prolisa | 12 | oil-roasted peanut flour | 0, 2, 5, 10 | biscuit | 147–221 | 32–62 | 38–62 | [272] |
| | | | dry-roasted peanut flour | 0, 2.5, 5, 10 | chocolate | 11–106 | 9–95 | 33–148 | |
| | ELISA R-Biopharm | 13 | oil-roasted peanut flour | 0, 2, 5, 10 | biscuit | 58–100 | 53–104 | 58–127 | [272] |
| | | | dry-roasted peanut flour | 0, 2.5, 5, 10 | chocolate | 33–55 | 22–33 | 42–102 | |
| | ELISA Veratox | 13 | oil-roasted peanut flour | 0, 2, 5, 10 | biscuit | 176–206 | 20–40 | 23–45 | [272] |
| | | | dry-roasted peanut flour | 0, 2.5, 5, 10 | chocolate | 138–161 | 8–27 | 20–27 | |
| | ELISA Faspek | 10 | peanut powder | 10 | sausage, boiled beef, tomato sauce, cookie, orange juice | 118–173 | 5–8 | 11–45 | [38] |
| | ELISA Fastkit | 10 | peanut powder | 10 | sausage, boiled beef, tomato sauce, cookie, orange juice | 82–116 | 4–7 | 9–34 | [38] |
| | ELISA Sedium RD | 7–10 | naturally + | N/A | deli nut bar, sponge cake with added peanut, nut filling, maized extruded snack with peanut, biscuit, filled wafer | N/A | 0.1–347 | 0.1–313 | [273] |

(*Continued*)

**Table 4.2** Cont.

| Allergen | Method | No. of labs | Spiking material | Spiked concentration (ppm)[a] | Matrix | Recovery, % | $RSD_r$[b] | $RSD_R$[c] | References |
|---|---|---|---|---|---|---|---|---|---|
| | ELISA competitive | 4 | peanut butter | 0, 100, 500, 1000, 2500, 5000, 10000, 25000 | biscuit sample | N/A | 16–44 | 30–111 | [28] |
| Walnut | ELISA Morinaga | 12 | defatted walnut flour | 10 | biscuit, bread, sponge cake, orange juice, jelly, chicken meatballs, rice gruel | 81–119 | 3–6 | 6–10 | [274] |
| Almond | PCR single system | 16 | almond | 0, 10, 20, 100, 400 | rice cookie | 90–105 | 19–44 | 28–49 | [275] |
| | | | | 123 | wheat cookie | 95 | 22 | 42 | |
| | | | | 100 | sauce hollandaise powder | 88 | 44 | 43 | |
| | PCR multiplex system | 16 | almond | 0, 10, 20, 100, 400 | rice cookie | 107–121 | 18–36 | 33–48 | [275] |
| | | | | 123 | wheat cookie | 98 | 16 | 33 | |
| | | | | 100 | sauce hollandaise powder | 94 | 36 | 43 | |
| Brazil nut | PCR single system | 16 | Brazil nut | 0, 10, 20, 100, 400 | rice cookie | 87–98 | 34–40 | 34–42 | [275] |
| | | | | 123 | wheat cookie | 66 | 26 | 36 | |
| | | | | 100 | sauce hollandaise powder | 43 | 28 | 40 | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PCR multiplex system | 16 | Brazil nut | 0, 10, 20, 100, 400 | rice cookie | 71–97 | 17–54 | 32–57 | [275] |
| | | | | 123 | wheat cookie | 62 | 16 | 36 | |
| | | | | 100 | sauce hollandaise powder | 48 | 34 | 38 | |
| Wheat/ Gluten | ELISA Ingezim | 20 | gliadin | 0, 79, 35, 168 | maize flour bread | 72–84 | 13–23 | 23–33 | [276] |
| | | | gliadin | 0, 41, 147 | rice dough | 70–93 | 21 | 27 | |
| | | | contaminated | <1.5, 12, 13, 14, 15 | contaminated samples | 73–131 | 19–25 | 29–47 | |
| | ELISA Ridascreen | 20 | gliadin | 0, 79, 35, 168 | maize flour bread | 82–102 | 11–21 | 23–33 | [276] |
| | | | gliadin | 0, 41, 147 | rice dough | 82–83 | 17–22 | 25–28 | |
| | | | contaminated | <1.5, 12, 13, 14, 15 | contaminated samples | 101–119 | 18–22 | 31–33 | |
| | ELISA Faspek | 10 | whole wheat flour | 10 | sausage, boiled beef, tomato sauce, orange juice, jam | 16–122 | 3–19 | 5–33 | [38] |
| | ELISA Fastkit | 10 | whole wheat flour | 10 | sausage, boiled beef, tomato sauce, orange juice, jam | 28–123 | 4–18 | 9–22 | [38] |
| | ELISA Gluten Tec | 12 | 5% wheat based baby food | 250, 500, 1000, 2000, 4000 | rice-based baby food | N/A | 6–15 | 11–34 | [277] |
| | | | wheat flour | 22.1, 44.2 | maize bread | N/A | 11–26 | 28–46 | |
| | | | gluten containing chocolate mix | 2500 | chocolate cake mix | N/A | 8 | 19 | |

(*Continued*)

**Table 4.2** Cont.

| Allergen | Method | No. of labs | Spiking material | Spiked concentration (ppm)[a] | Matrix | Recovery, % | $RSD_r$[b] | $RSD_R$[c] | References |
|---|---|---|---|---|---|---|---|---|---|
| | | | made from barley malt | N/A | beer | N/A | 12 | 18 | [277] |
| | ELISA AgraQuant | 18 | wheat flour | 0, 10, 20,100 | rice flour | 101–135 | 15–48 | 30–116 | [264] |
| | | | | 0, 10, 20,100 | chocolate cake | 62–66 | 8–2348 | 19–2348 | |
| | | | | 4.5, 15, 24, 102 | crisp bread | 91–111 | 10–46 | 29–69 | |
| | ELISA competitive Ridascreen | 13 | Peptic-Tryptic hordein digest | 0, 30, 100 | beer | 87–119 | 30–98 | 31–126 | [265] |
| | | | contaminated | 0, naturally + | starch syrup | N/A | 16–157 | 34–236 | |
| | | | dried rye sourdough | 70, 150 | sourdough | 69–97 | 20–23 | 26–28 | |
| Soy | PCR | 10 | soybean flour | 10, 20, 40, 100 | boiled sausage | 82–99 | 17–31 | 24–32 | [278] |

[a]The spiked concentration may be based upon the protein concentration and not necessarily represent the amount of spiking material used. Select spiked concentrations are converted from % to ppm, and mg/L is considered as ppm.
[b]$RSD_r$ = Repeatability relative standard deviation, %.
[c]$RSD_R$ = Reproducibility relative standard deviation, %.
[d]naturally + = naturally positive samples.
[e]N/A = not available.

methods (such as antibody, calibration standard, extraction methods and buffer) contributes partly to these differences. However, differences also arise due to study design-related variables such as choice of food matrix, number of participating laboratories, availability and choice of spiking material for recovery studies, and sample preparation (spiked vs. incurred). Harmonized guidelines and requirements to validate methods of analysis have been published [257–259] and can be adopted for validation of food allergen quantitation methods. Specific guidelines for validation of food allergen and gluten quantitation by ELISA have been published in recent years [260, 261]. Some of the key terms evaluated in the validation of methods for allergen quantitation are described below.

### 4.5.1 Specificity and Cross-Reactivity

In allergen detection methods, specificity may be sometimes referred to the allergen detected by the method. For example, a method detecting peanut may have specificity towards the Ara h 1 allergen. However, in validation studies, specificity refers to the response produced by the target allergen as compared to other matrix/sample components. This is in contrast to cross-reactivity, which refers to the signal/response produced by components other than target allergen that may be caused by nonspecific interactions. The matrix components selected for studying cross-reactivity varies with the allergen and primarily depends on the homology with the target allergen, and likelihood of the component to be present along with the target allergen in the food [260]. High specificity and no cross-reactivity are optimal assay characteristics for accurate allergen detection.

### 4.5.2 Robustness and Ruggedness

Robustness and ruggedness refers to the performance of method under minor changes in method parameters and sample type. These terms are generally used interchangeably and measured by assessing the effect of change in experimental conditions on the accuracy and precision of the method [262]. For food allergen and gluten detection by ELISA, the recommended variations to assess ruggedness include ± 5 to 10% for time and volume-related parameters and ± 3 to 5 °C for the temperature parameter [260, 261].

### 4.5.3 Sensitivity, LOD and LOQ

Sensitivity refers to the change in signal with respect to the change in allergen concentration. It can be measured by the slope of a calibration curve,

but is generally not used in validation studies [257, 262]. LOD and LOQ are the most commonly used terms when validating quantitative assay for food allergens. As the names suggest, the terms LOD and LOQ are the lowest amount of allergen that can be detected (LOD) and quantitated (LOQ) with defined certainty. For constant and normally distributed variances, the LOD and LOQ of an assay can be calculated from the standard deviation of the blank or zero concentration level, while an advanced calculation can be used where variance increases with an increase in the mean value [260, 261].

### 4.5.4 Accuracy and Trueness

Accuracy and trueness refers to the closeness of the measured amount to the actual or true amount of an allergen. Accuracy can be measured by calculating the percent recovery or from the slope of linear regression analysis of the straight line plot between the spiked and measured concentrations [263]. A recovery of 100% implies that the method is accurate, whereas values below or above 100% suggest under- and overestimation, respectively. A recovery of 80–120% is ideal, but due to the complexity of food matrices and processing conditions, a recovery of 50–150% may be considered as an acceptable range for ELISA [260]. Trueness refers to the bias and is measured as difference between the measured amount and the true amount [262]. Trueness or accuracy can be derived from measuring allergen amount in the spiked samples, certified reference material, or by comparing measured values with another reference method [258]. However, determining trueness of allergen may be challenging in the absence of a reference material and reference method. Since the actual or true value may vary depending on the allergen material used for spike-recovery studies by various detection methods, one should be cautious in interpreting the accuracy of the method or comparing accuracy between methods. Availability of a certified reference material and its use in validation studies may help towards achieving consistent accuracies that could be comparable between methods.

### 4.5.5 Precision

Precision refers to the closeness of measured values to each other at a given allergen concentration, and is measured by calculating the relative standard deviation (RSD) or coefficient of variation (CV) of the measured value. The RSD is independent of concentration and thus more suitable to measure the precision when comparing assay performance at various

allergen concentrations [258, 263]. In a multi-laboratory validation, the RSD is further characterized by repeatability RSD ($RSD_r$) and reproducibility RSD ($RSD_R$), which is the measure of variance associated within a laboratory and between laboratories, respectively. The $RSD_R$ tends to be greater than the $RSD_r$ as higher variability is associated between the laboratories as compared to within a laboratory (Table 4.2). Typically high RSDs have been observed for samples with zero or very low level of allergen content. For example, in Table 4.2, the $RSD_R$ of 2348% and 236% was associated with gluten-free chocolate cake [264] and gluten-free starch syrup [265], respectively. It is important to ensure the homogeneity of spiked samples in order to prevent high RSD associated with poor homogeneity. Though not used in validation studies, total variance can be divided into sampling and analytical variance, where the latter can give a better measure of analytical precision by eliminating the sample-related variations [263].

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