

Protein or No Protein? Opportunities for DNA-Based Detection of Allergenic Foods

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ABSTRACT: In food allergy, a common immunological disease with a potentially severe outcome, a causative cure is not available. Correct ingredient labeling and risk assessment of unlabeled allergen cross-contact is a prerequisite for effective allergen avoidance. Specific and sensitive analytical methods, which allow for unequivocal identification and accurate quantification of allergenic components, are important tools in allergen risk management. Both protein- and DNA-based methods are in place and reveal pros and cons depending upon the application and individual analytical question. This perspective highlights relevant molecular aspects and discusses, especially, opportunities for the application of DNA-based methods for the detection of allergenic foods.

KEYWORDS: allergen detection, labeling, PCR, DNA, protein

INTRODUCTION

Food allergy is a global health burden and challenge and affects approximately 1–10% of the general population.¹ It constitutes an immunological disease of individuals that are sensitized against usually harmless food proteins, which are often of dietary value for the general population. With regard to health risk, the so-called immediate-type allergic reaction that is mediated by immunoglobulin E (IgE) isotype antibodies directed against food proteins is most prominent. After food ingestion, the onset of symptoms usually occurs within a few minutes and even severe, occasionally life-threatening reactions may result. Thus far, causative immunotherapy treatment is unavailable in a clinical routine, majorly because of potentially severe side effects and a lack of safe and efficacious immunotherapeutic reagents. Hence, strict avoidance of the offending food remains the only effective option to avoid an allergic reaction.² This requires the clear identification of the allergenic food ingredient and, thus, accurate labeling of allergenic ingredients. The latter is mandatory for a range of the most frequent and most severe food allergens used as ingredients in many countries around the globe. In the European Union (EU), 14 allergenic foods or groups of foods have to be obligatory labeled when used as an ingredient.³ Although this is beneficial for the allergenic ingredient identification, cross-contact of allergenic components may still occur, for example, as a result of shared equipment in food manufacture, which is not covered by mandatory labeling in the EU, and results in the presence of so-called “hidden” allergens. Hidden allergens may be present in spurious but also greater amounts, as homogenates or particulates, posing an unpredictable health risk to allergic individuals. Hidden allergens may be eliminated or minimized by allergen risk management, supported by effective allergen sanitation or cleaning procedures that are validated and monitored on a regular basis.⁴ Thus, analytical methods to detect and quantify allergenic components of foods are valuable tools to verify compliance with labeling requirements and the effectiveness of allergen sanitation plans in food manufacture. These methods

need to meet high analytical demands: detection specificity is required to avoid false-positive results that may cause unfounded food recalls; test sensitivity must reflect the clinical sensitivity of allergic subjects; quantitative features are the basis to enable decision making in risk management; and method performance should be robust with regard to the impact of the food matrix, processing, and range of biological variation of allergenic ingredients and components. Hence, the selection of appropriate target molecules plays a key role to meet the high analytical demands in food allergen detection. Because proteins are the elicitors of allergic reactions to foods, targeted detection of allergenic proteins appears to be the primary choice with regard to basic allergen risk assessment. However, with regard to an everyday allergen risk management, several more options may arise for a robust, specific, and sensitive detection of the allergenic food in question, when using any qualified target protein, peptide(s) thereof, or the underlying genetic code, the deoxyribonucleic acid (DNA). For the verification of labeling requirements, identification of hidden allergens, and verification of allergen sanitation plans, is it really a question of “protein or no protein”? Normally, it should not be a question of relevance from an analytical point of view but can be a matter of debate for those countries where the regulation explicitly requires the detection of allergenic proteins or the protein fraction of the allergenic food.

Hence, this perspective will discuss relevant molecular aspects to consider and highlight the potential of DNA-based methods for allergenic food detection with regard to specificity, sensitivity, and quantitative performance. Direct comparison of polymerase chain reaction (PCR) with enzyme-linked immunosorbent assay (ELISA) and mass spectrometry (MS) methods intends to support a detailed understanding of similarities and differences of allergen detection methods and

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Table 1. Reference Doses (mg of Protein) for 11 Allergenic Foods According to VITAL and Conversion to Allergenic Food Applying Published Conversion Factors¹²

allergen (reference food ^a)	eliciting dose (ED)	protein reference dose (mg)	conversion factor (protein to food) ^a	converted reference dose food (mg)	concentration of allergenic food in 100 g serving (mg/kg)
peanut (whole peanut)	ED01	0.20	4.0	0.80	8.0
milk (non-fat dry milk)	ED01	0.10	2.8	0.28	2.8
egg (dried whole egg)	ED01 and ED05 (95% LCI ^b)	0.03	2.2	0.066	0.66
hazelnut (hazelnut flour)	ED01 and ED05 (95% LCI)	0.10	6.4	0.64	6.4
soy (whole soybean)	ED05 (95% LCI)	1.00	2.5	2.50	25
wheat (raw or cooked flour)	ED05 (95% LCI)	1.00	10.0	10.00	100
cashew (cashew flour)	ED05 (95% LCI)	2.00 (provisional)	5.3	10.60	106
mustard (mustard seed)	ED05 (95% LCI)	0.05	3.8	0.19	1.9
lupin (yellow lupin flour)	ED05 (95% LCI)	4.00	2.5	10.00	100
sesame (crushed seeds)	ED05 (95% LCI)	0.20	5.9	1.18	11.8
shrimp (whole cooked)	ED05 (95% LCI)	10.0	4.4	44.00	440

^aExamples of reference food and conversion factor to convert from total protein of allergenic food to total allergenic food, according to Table 2 from Taylor et al.¹² ^bLCI = lower confidence interval.

opportunities for the use of PCR in allergen detection. In this perspective, the term “allergen detection” is meant as a brief for the detection of allergenic foods. It should not be mistaken for the detection of a single allergenic protein molecule.

■ ANALYTICAL TARGETS AND METHODS FOR ALLERGEN DETECTION

Historically, ELISA kits were the first to allow for specific detection of proteins or allergens of the allergenic food in a quantitative and potentially high-throughput manner. ELISA is still the most used method in the detection of allergenic foods because of its ease of use and limited technical requirements. ELISA tests make use of either mono- or polyclonal antibodies. For both types of antibodies, the first step is the immunization of adequate host animals with single purified (allergenic) proteins, total protein extracts, or peptide molecules. With regard to specificity toward single allergenic proteins, ideally monoclonal antibodies (mab) would be raised. Normally, mab recognize only one binding site of the allergen molecule, and specificity can be as high as to differentiate between isoallergens, as was shown for the isoforms Dau c 1.01 and Dau c 1.02 of the major carrot allergen Dau c 1.⁵ However, the availability of a single binding site is likely impacted by food processing, such as thermal degradation, but also masking as a result of matrix effects. Polyclonal antibodies display a higher diversity of binding sites. However, in turn, specificity for the allergenic protein(s) may be negatively affected, at least partly, if proteins other than the relevant allergens are detected. In the literature, especially polyclonal antibodies developed against crude total protein or single protein fractions have been described.^{6–8} Only few have been characterized in detail with regard to their allergen specificity. Similarly, commercial ELISA kits make use of immunological reagents that may detect one or more allergens but likely other proteins as well. As was shown for peanut, different ELISA kits have differing specificities with regard to recognized allergenic proteins, and

only few allergen components are detected simultaneously or equally.⁹ Also, other methods based on antibodies exist for allergen detection, such as rapid lateral flow devices (LFDs) or surface plasmon resonance (SPR), but will not be further discussed in this perspective.

Recently, detection of allergenic proteins by mass spectrometry (MS) has evolved, and several publications indicate the potential of MS for a sensitive and specific detection. Detection is usually performed “bottom-up” on the basis of peptides obtained after proteolytic digestion of the study sample.^{8,10} Again, usually only few target allergens are detected. For example, when considering the detection of peanut allergens, it has to be kept in mind that usually only a very limited number of allergens are detected, whereas 17 peanut allergens have been described to date (www.allergen.org). Taken together, both protein-specific methods, ELISA and MS, usually detect a limited number of target proteins or allergens. In the case of MS, usually small peptides are detected that are likely not allergenic.

The third option for the detection of allergenic foods is based on the detection of a part of the DNA that is specific for the target allergenic food. The target DNA may be part of a gene that is specific for a relevant allergenic protein but can be any part of the DNA as long as it allows for specific detection of the allergenic food. DNA is a fairly stable molecule. However, food processing may also truncate DNA, which is why mainly short stretches of, for example, less than 200 base pairs in length are targeted. Independent of whether protein or DNA is detected, recovery of target molecule(s) may be negatively affected by the food matrix and potentially even worse by food processing, such as thermal treatment.⁸ If possible, appropriate factors of correction should be identified and applied for quantitative readouts. Usually, PCR is performed to specifically amplify DNA stretches, and sequence specificity is additionally verified in real time if performed with sequence-specific probes in real-time PCR, also known as

quantitative polymerase chain reaction (qPCR). PCR is an indirect method with regard to the presence of proteins. It generally allows for the detection of the presence of specific DNA as a surrogate target molecule and the quantification of this specific DNA to extrapolate for the quantity of source material or total protein.¹¹ Further characteristics of allergen detection using PCR in comparison to ELISA and MS are discussed below.

METHOD READOUT AND COMPARISON TO CLINICAL PROTEIN THRESHOLD DATA

Method sensitivity needs to be compared to clinical sensitivity of allergic individuals that experience an allergic reaction, which is obtained in clinical threshold studies.¹² Individual clinical threshold data have been used to establish reference doses for allergenic food residues as a part of the Voluntary Incidental Trace Allergen Labelling (VITAL) program of the Allergen Bureau of Australia & New Zealand. The obtained reference doses, in terms of milligrams of total protein of the allergenic food, were derived from statistical dose distribution models and were based on eliciting dose (ED), either ED01, ED05, or both, for a range of allergenic foods (Table 1). ED refers to the dose at which a certain percentage of allergic individuals (01 stands for 1%) would react to the allergenic food according to the dose distribution model and applying a 95% confidence interval. Individual thresholds in the underlying clinical studies have been calculated on the basis of the allergenic food or total protein of the allergenic food but not individual allergenic proteins. To model dose distribution curves, challenge doses were normalized to milligrams of total protein from the allergenic food using conversion factors.¹² As a consequence, sensitivity of analytical methods needs to be determined against the total protein of the allergenic food, which includes allergens and non-allergenic proteins alike. On the basis of the detection of a subgroup of proteins or allergens in both ELISA and MS, the results need to be extrapolated for total protein of the allergenic food. However, differences in quantitative results as a result of the use of different ELISA tests have frequently been reported.⁸ The major reasons for this are the use of different detection antibodies and calibrants. Accordingly, appropriate conversion factors need to be identified, because conversion from target to total protein is influenced by the selection of the target protein (allergen). Several aspects need to be taken into account, such as average protein content of allergenic source material, target protein content, range of biological variation, response rate of the method to target protein, and product/processing types of the most relevant allergenic ingredient. If calibration is performed against total protein of a selected comparator allergenic food, conversion factors are already empirically included but may vary between methods, depending upon their target response and quantitative target distribution. Thus, the final quantitative readout in total protein of the allergenic food varies with the calibrator used and the response rate of the method. Moreover, the method readout with regard to allergen risk assessment is limited to the detected allergens or peptides thereof in comparison to the complete qualitative and quantitative allergen distribution in the study sample. Hence, several assumptions need to be made, and some degree of uncertainty persists. With these limitations in mind, it appears not justified to describe ELISA or MS methods for allergenic foods as completely direct methods for the purpose of allergen detection and allergen risk assessment. Using PCR to detect

DNA instead of protein of the allergenic food, quantification of total protein of the allergenic food can be performed similarly to ELISA or MS based on calculation if appropriate factors of conversion are applied. In PCR, direct calculation from quantified specific DNA to the allergenic food or total protein thereof is limited by the fact that no universal factor of conversion for target molecule(s) to a total protein readout can be applied. This is somehow similar to ELISA and MS methods, as described above. Accordingly, calibration can be performed against the allergenic food itself. Again, a factor of conversion is already included in this calibration, and this may be prone to biological variation too. Moreover, a second conversion factor needs to be applied to calculate for total protein of the allergenic food based on tabular values for the protein content, such as the values used by Taylor et al. to normalize the outcome of clinical threshold studies.¹² This of course may also add to uncertainty. As an example, in Table 1, the published VITAL protein reference doses for 11 allergenic foods were converted to reference doses of the allergenic foods, applying selected conversion factors related to certain reference foods. To ensure suitable method sensitivity, DNA-based methods, which are calibrated against the reference foods, would need to be as sensitive to allow for detection of this amount of reference food in a relevant serving size. The last column of Table 1 depicts the corresponding concentration that would need to be detectable to verify VITAL protein reference doses in a serving of 100 g.

Allergen detection using PCR is based on the assumption that a positive DNA result correlates directly with the presence of protein of the allergenic food. In the great majority of allergenic foods, this has not been a limitation because of a good correlation between the presence of detectable DNA and protein of the allergenic food.¹¹ It should be kept in mind that PCR cannot specifically detect milk or egg, because DNA may also originate from other beef or chicken tissue, nor can PCR detect a single protein, such as gluten. The intended primary use of gluten-specific methods is to increase food safety for consumers with celiac disease. For application of gluten-specific methods in the field of food allergy, the conversion of readout with regard to allergy against wheat-containing cereals is required but not directly apparent. ELISA tests may directly detect gluten but cannot fully differentiate the gluten-containing cereals, which require mandatory ingredient labeling in various countries. In contrast, PCR can detect and differentiate between the gluten-containing cereals, and the quantitative conversion to total protein and to gluten can be performed with appropriate conversion factors, as described above. Thus, PCR methods for the indirect detection and quantification of gluten have both limitations and advantages similar to a protein-based detection.

In summary, the correctness of allergen analysis using ELISA, MS, and PCR is influenced by several variables. As a consequence, a certain measurement inaccuracy would always need to be accepted, as long as one cannot calibrate on the present allergenic food. To overcome these limitations as best as possible, generally accepted reference materials are needed to reach better comparability of method results when detecting allergens with unknown characteristics.

Similar to protein-based ELISA and MS methods, DNA-based methods offer valuable benefits for the detection of allergenic foods that are further discussed below. Thus, PCR may serve as a confirmatory method or even as the only

applicable method depending upon the allergenic food to detect.

■ PCR FOR ALLERGEN DETECTION

ELISA methods depend upon specific antibodies, usually of polyclonal nature, with an unknown range of epitope specificities. Antibody characteristics and other relevant reagent details are usually undisclosed in commercial ELISA kits that are frequently applied. Even if disclosed, use of such detail is limited, because each immunization is unique, and even consistent use of protocols may lead to different antibody specificities and sensitivities. Nonetheless, the application of ready-to-use ELISA kits is convenient and does not require sophisticated instrumentation. In contrast, MS methods for proteins do require pricy and sophisticated instrumentation and extensive expertise. A big advantage of MS methods for allergen detection is the possibility to disclose all method details, which is important to regulatory bodies. Likewise, PCR chemistry and protocols can be fully disclosed, and because qPCR instrumentation has become affordable for routine laboratories, it is considered a standard laboratory method, like ELISA. For example, in Germany and Japan, PCR has become a standard method for allergen detection performed by governmental food control laboratories. In Japan, PCR analysis is performed as a confirmatory test for wheat, buckwheat, peanut, shrimp, prawn, and crab.¹³ In Germany, PCR has been an official method according to food and feed law for a range of allergenic foods, such as wheat, rye, peanut, soybean, lupine, hazelnut, almond, celery, mustard, and sesame, and the range of application specificities is further expanded.

One major advantage of PCR is its exceptional specificity that may even surpass the level of single amino acid specificity. In fact, more molecular differences are available on the nucleotide level, because for most amino acids, more than one triple nucleotide codon exists and non-coding sequences are available in addition to the protein-coding sequence.¹¹ This allows for specific differentiation of allergenic foods, even in the presence of other components with close phylogenetic relation that may result in false-positive detection at the protein level. One such example is the highly specific PCR-based detection of celery (*Apium graveolens*),¹⁴ which requires mandatory ingredient labeling in the EU. However, celery cannot be differentiated from other plant foods of the Apiaceae family, such as carrots, using antibody-based ELISA.¹⁵ Also, using commercial ELISA, almond (*Prunus dulcis*) cannot be detected specifically in comparison to other kernels of the genus *Prunus*, such as apricot (*Prunus armeniaca*).¹⁶ In Europe, both almond and apricot are used for the manufacture of sweets, such as marzipan and persipan, but almond is the allergenic ingredient that requires mandatory labeling. Using qPCR, almond was detectable in chocolate and cookies at a level of sensitivity similar to that of commercial ELISA kits. In addition, the specificity of qPCR for almond was up to 10 000 times higher than that of the commercial ELISA tests and allowed to discriminate almond from other *Prunus* species, such as apricot, peach, cherry, plum, or nectarine.¹⁶ In 2015, several spice products were withdrawn from the international food market because of the suspected presence of almond as detected using ELISA methods. As described above, the immunoassays showed significant cross-reactivity with other species within the *Prunus* genus. A novel qPCR assay was capable of identifying *Prunus mahaleb* as the species causing

false positives in almond immunoassay analysis of the spice products.¹⁷

In addition to high specificity, PCR also allows for detection of allergenic foods with high sensitivity that is comparable to that of immunoassays, such as ELISA. Generally, PCR sensitivity depends upon the target gene(s) used for the specific detection and the ratio of target gene(s) to total source material. Usually, this ratio is not critical; however, in the case of egg, milk, and products thereof, the amount of amplifiable DNA is limited, which does not allow for developing PCR applications that are sensitive enough for allergen detection in foods. With the limited tissue specificity of PCR also taken into account, the detection of allergenic milk and egg ingredients using PCR is not recommended. Apart from these two, the vast majority of allergenic foods that need to be labeled according to European regulation³ can be traced by PCR tests. Many PCR applications have been based on so-called single-copy genes and allow for detection of allergenic ingredients at a level of approximately 10–50 mg/kg of food or below.^{18–21} The related level of total protein of the allergenic ingredient is accordingly lower, applying conversion factors as suggested by Taylor et al.¹² Making use of multicopy genes, such as mitochondrial or ribosomal genes, sensitivities in the range of 0.1–1 mg/kg of food can be achieved.^{22,23} With such low levels of detectability, verification of relevant clinical protein reference doses,¹² even in a large serving size of 500 g, can be accomplished. In some cases, such as soy detection, PCR analysis appears even more robust than analysis based on immunoassays. In a survey of proficiency testing performed within 6 years, PCR-based detection of soy was much more reliable than ELISA-based soy detection. Blank sausage meat and pastry matrices were fortified with thermally treated soy flour or granulate at levels between 184 and 5500 mg of soy protein/kg of matrix. Blank matrix samples were detected negative, with a high average confirmation rate above 90% using both ELISA and PCR. However, matrix samples having soy protein were detected positive, with an average confirmation rate of 95 and 67% using PCR and ELISA, respectively.²⁴

Using qPCR, the amount of an allergenic food in a composed food can be quantified.

As in allergen detection using ELISA or MS,⁸ food matrix and food processing may negatively impact the recovery of the specific target DNA. While the effect of processing is usually difficult to tackle, matrix effects on allergen recovery can be normalized using PCR. On the one hand, quality and quantity of extractable DNA may depend on differences in food matrix composition. On the other hand, differences in the quality of extracted DNA affect amplification efficiency, and because PCR is a cyclic method, differences in amplification efficiency are propagated by each cycle of repetitive amplification. To compensate for error propagation, several strategies for quantification of allergenic foods using qPCR have been described, such as matrix-adopted standards,²⁵ the classic standard addition method,²⁶ and non-competitive^{27,21} and competitive²⁸ normalization. The latter strategy provides several advantages, including the avoidance of external standards and a relevant reduction of technical sample replicates. To minimize matrix effects during both DNA extraction and amplification, an artificial competitive DNA is added at a predetermined amount to the sample prior to DNA extraction, co-extracted, and co-amplified with the target DNA. The ratio of co-amplified competitor and target DNA allows

for the calculation of the amount of target allergenic food. Competitor DNA serves as an internal calibrator and controls for inhibition effects. This competitive qPCR, which is based on a single-copy gene, allowed for matrix-normalized quantification of peanut in the range of 10–1000 mg/kg of food, using only four replicates per sample. Recovery in chocolate, vanilla ice cream, cookie dough, cookie, and muesli was 87% in comparison to 199% obtained by three commercial ELISA kits.²⁸ This principle, applied to mitochondrial multicopy genes, allowed for matrix normalization and even more sensitive soybean and peanut quantification in various matrixes (sausage, cookie, hollandaise sauce, and skim milk powder) at a level of 1–100 mg/kg of food.²³ When published conversion factors are applied,¹² this corresponds to 0.25–40 mg of protein of peanut and soybean per kilogram of food, respectively. Thus, the method sensitivity and quantitative capacity would allow for verification of VITAL reference doses, even in a large serving size of 500 g. Similarly, multicopy-based PCR allowed for a highly sensitive detection of allergenic species of the animal kingdom, namely, penaeid shrimp and blue crab, and demonstrated that cooking had little effect on assay performance.²⁹ As shown for MS analysis,^{8,10} DNA-based detection of allergenic foods offers, in principle, multiplexing capability¹⁹ and the option to develop simple and rapid screening tools, comparable to antibody-based lateral flow devices.³⁰ For example, celery was successfully detected in the range of food items using loop-mediated isothermal amplification,³¹ which, after DNA extraction, does, in principle, only need a water bath and visual inspection of the resulting color change after 30–60 min of isothermal incubation of the reaction mixture.

CONCLUDING REMARKS

PCR applications for the detection of allergenic foods have been established and applied successfully for nearly 2 decades. For the vast majority of allergenic foods that require mandatory ingredient labeling and voluntary control for cross-contact, DNA-based detection using PCR has been proven useful and efficient. Sensitivity of multicopy-based detection is comparable to that of immunoassays, and PCR offers extraordinary specificity in comparison to immunoassays. Instrumentation and other required resources have become affordable for routine analytical laboratories, and optimized protocols may allow for rapid PCR detection of allergenic foods. Many publications provide evidence of robust DNA-based detection and good correlation between DNA- and protein-based analysis of allergenic foods. PCR has been accepted and applied by several governmental laboratories. Accurate quantification using qPCR with matrix normalization has been shown. More emerging developments, such as digital PCR, are explored for the quantification of allergenic foods. Taken together, DNA-based detection and quantification offer many beneficial options for allergenic food detection. No individual method exists that combines all advantages for affordable, robust, rapid, and unequivocal identification and quantification of all relevant allergenic food components. Thus, the most appropriate application should be chosen, and for some analytical questions, the use of more than one technique might be necessary. To compare analytical results to clinical protein reference doses, similar to protein-based detection using immunoassays or MS, quantitative results obtained by qPCR are based on conversion factors that, in turn, present uncertainty to some extent. Future work needs to describe and

limit the range of uncertainties for both DNA- and protein-based detection methods for allergenic foods. Immunoassays will remain a major pillar in the detection of allergenic foods because of easy use and quantitative readout, despite evident antibody limitations that potentially lead to unspecific cross-reactivity or processing-related false-negative results. PCR may overcome these limitations but remains an indirect method for protein detection. However, not using PCR for allergen detection, as either a complementary or primary method, deprives the analytical laboratory from valuable options. Thus, the principal question of “protein or no protein?” should be amended to “protein and DNA!”. Independently, each individual application, be it ELISA, MS, or PCR, must prove its fitness for purpose with regard to sensitivity, specificity, quantitative capability, and situation-oriented applicability, to effectively support allergen risk management for the food allergic population.

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Notes

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REFERENCES

- (1) Nwaru, B. I.; Hickstein, L.; Panesar, S. S.; Muraro, A.; Werfel, T.; Cardona, V.; Dubois, A. E. J.; Halken, S.; Hoffmann-Sommergruber, K.; Poulsen, L. K.; Roberts, G.; van Ree, R.; Vlieg-Boerstra, B. J.; Sheikh, A. The epidemiology of food allergy in Europe: A systematic review and meta-analysis. *Allergy* **2014**, *69*, 62–75.
- (2) Burks, A. W.; Tang, M.; Sicherer, S.; Muraro, A.; Eigenmann, P. A.; Ebisawa, M.; Fiocchi, A.; Chiang, W.; Beyer, K.; Wood, R.; Hourihane, J.; Jones, S. M.; Lack, G.; Sampson, H. A. ICON: Food allergy. *J. Allergy Clin. Immunol.* **2012**, *129*, 906–920.
- (3) European Union (EU). Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. *Off. J. Eur. Union, L* **2011**, *54*, 18–63.
- (4) Roder, M.; Baltruweit, I.; Gruyters, H.; Ibach, A.; Mucke, I.; Matissek, R.; Vieths, S.; Holzhauser, T. Allergen sanitation in the food industry: A systematic industrial scale approach to reduce hazelnut cross-contamination of cookies. *J. Food Prot.* **2010**, *73*, 1671–1679.
- (5) Foetisch, K.; Dahl, L.; Jansen, B.; Becker, W.-M.; Lidholm, J.; van Ree, R.; Broll, H.; Kaul, S.; Vieths, S.; Holzhauser, T. Development and in-house validation of allergen-specific ELISA tests for the quantification of Dau c 1.01, Dau c 1.02 and Dau c 4 in carrot extracts (*Daucus carota*). *Anal. Bioanal. Chem.* **2011**, *399*, 935–943.
- (6) Holzhauser, T.; Vieths, S. Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrixes. *J. Agric. Food Chem.* **1999**, *47*, 4209–4218.
- (7) Morishita, N.; Kamiya, K.; Matsumoto, T.; Sakai, S.; Teshima, R.; Urisu, A.; Moriyama, T.; Ogawa, T.; Akiyama, H.; Morimatsu, F. Reliable enzyme-linked immunosorbent assay for the determination of soybean proteins in processed foods. *J. Agric. Food Chem.* **2008**, *56*, 6818–6824.

- (8) Parker, C. H.; Khuda, S. E.; Pereira, M.; Ross, M. M.; Fu, T. J.; Fan, X.; Wu, Y.; Williams, K. M.; DeVries, J.; Pulvermacher, B.; Bedford, B.; Zhang, X.; Jackson, S. L. Multi-allergen quantification and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography–tandem mass spectrometry. *J. Agric. Food Chem.* **2015**, *63*, 10669–10680.
- (9) Jayasena, S.; Smits, M.; Fiechter, D.; de Jong, A.; Nordlee, J.; Baumert, J.; Taylor, S. L.; Pieters, R. H.; Koppelman, S. J. Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. *J. Agric. Food Chem.* **2015**, *63*, 1849–1855.
- (10) Brockmeyer, J. Novel Approaches for the MS-Based Detection of Food Allergens. High Resolution, MS³, and Beyond. *J. AOAC Int.* **2018**, *101*, 124–131.
- (11) Holzhauser, T.; Roder, M. Polymerase chain reaction (PCR) methods for detecting allergens in foods. In *Handbook of Food Allergen Detection and Control*; Flanagan, S., Ed.; Woodhead Publishing: Sawston, U.K., 2015; Chapter 13, pp 245–263, DOI: 10.1533/9781782420217.2.245.
- (12) Taylor, S. L.; Baumert, J. L.; Kruizinga, A. G.; Remington, B. C.; Crevel, R. W. R.; Brooke-Taylor, S.; Allen, K. J.; Houben, G. Establishment of Reference Doses for residues of allergenic foods: Report of the VITAL Expert Panel. *Food Chem. Toxicol.* **2014**, *63*, 9–17.
- (13) Sakai, S.; Adachi, R.; Akiyama, H.; Teshima, R. Validation of quantitative and qualitative methods for detecting allergenic ingredients in processed foods in Japan. *J. Agric. Food Chem.* **2013**, *61*, 5675–5680.
- (14) Hupfer, C.; Waiblinger, H.-U.; Busch, U. Development and validation of a real-time PCR detection method for celery in food. *Eur. Food Res. Technol.* **2007**, *225*, 329–335.
- (15) Faeste, C. K.; Jonscher, K. R.; Sit, L.; Klawitter, J.; Løvberg, K. E.; Moen, L. H. Differentiating cross-reacting allergens in the immunological analysis of celery (*Apium graveolens*) by mass spectrometry. *J. AOAC Int.* **2010**, *93*, 451–461.
- (16) Roder, M.; Vieths, S.; Holzhauser, T. Sensitive and specific detection of potentially allergenic almond (*Prunus dulcis*) in complex food matrices by Taqman® real-time polymerase chain reaction in comparison to commercially available protein-based enzyme-linked immunosorbent assay. *Anal. Chim. Acta* **2011**, *685*, 74–83.
- (17) Burns, M.; Walker, M.; Wilkes, T.; Hall, L.; Gray, K.; Nixon, G. Development of a Real-Time PCR Approach for the Specific Detection of *Prunus mahaleb*. *Food Nutr. Sci.* **2016**, *07*, 703–710.
- (18) Roder, M.; Filbert, H.; Holzhauser, T. A novel, sensitive and specific real-time PCR for the detection of traces of allergenic Brazil nut (*Bertholletia excelsa*) in processed foods. *Anal. Bioanal. Chem.* **2010**, *398*, 2279–2288.
- (19) Köppel, R.; Dvorak, V.; Zimmerli, F.; Breitenmoser, A.; Eugster, A.; Waiblinger, H.-U. Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur. Food Res. Technol.* **2010**, *230*, 367–374.
- (20) Platteau, C.; De Loose, M.; De Meulenaer, B.; Taverniers, I. Detection of allergenic ingredients using real-time PCR. A case study on hazelnut (*Corylus avellana*) and soy (*Glycine max*). *J. Agric. Food Chem.* **2011**, *59*, 10803–10814.
- (21) Costa, J.; Amaral, J. S.; Grazina, L.; Oliveira, M. B. P. P.; Mafra, I. Matrix-normalised real-time PCR approach to quantify soybean as a potential food allergen as affected by thermal processing. *Food Chem.* **2017**, *221*, 1843–1850.
- (22) López-Calleja, I. M.; de la Cruz, S.; Pegels, N.; González, I.; García, T.; Martín, R. High resolution TaqMan real-time PCR approach to detect hazelnut DNA encoding for ITS rDNA in foods. *Food Chem.* **2013**, *141*, 1872–1880.
- (23) Ladenburger, E.-M.; Dehmer, M.; Grünberg, R.; Waiblinger, H.-U.; Stoll, D.; Bergemann, J. Highly Sensitive Matrix-Independent Quantification of Major Food Allergens Peanut and Soy by Competitive Real-Time PCR Targeting Mitochondrial DNA. *J. AOAC Int.* **2018**, *101*, 170–184.
- (24) Scharf, A.; Kasel, U.; Wichmann, G.; Besler, M. Performance of ELISA and PCR methods for the determination of allergens in food: An evaluation of six years of proficiency testing for soy (*Glycine max* L.) and wheat gluten (*Triticum aestivum* L.). *J. Agric. Food Chem.* **2013**, *61*, 10261–10272.
- (25) Siegel, M.; Schnur, K.; Boernsen, B.; Pietsch, K.; Waiblinger, H.-U. First ring-trial validation of real-time PCR methods for the quantification of allergenic food ingredients. *Eur. Food Res. Technol.* **2012**, *235*, 619–630.
- (26) Eugster, A. Alternative quantitative Bestimmung von Allergenen in Lebensmitteln mittels real-time PCR (Alternative determination allergens in food by real-time PCR). *Dtsch. Lebensm.-Rundsch.* **2010**, *106*, 434–438.
- (27) Hirao, T.; Hiramoto, M.; Imai, S.; Kato, H. A novel PCR method for quantification of buckwheat by using a unique internal standard material. *J. Food Prot.* **2006**, *69*, 2478–2486.
- (28) Holzhauser, T.; Kleiner, K.; Janise, A.; Roder, M. Matrix-normalised quantification of species by threshold-calibrated competitive real-time PCR: Allergenic peanut in food as one example. *Food Chem.* **2014**, *163*, 68–76.
- (29) Eischeid, A. E.; Kim, B. H.; Kasko, S. M. Two quantitative real-time PCR assays for the detection of penaeid shrimp and blue crab, crustacean shellfish allergens. *J. Agric. Food Chem.* **2013**, *61*, 5669–5674.
- (30) Roder, M.; Vieths, S.; Holzhauser, T. Commercial lateral flow devices for rapid detection of peanut (*Arachis hypogaea*) and hazelnut (*Corylus avellana*) cross-contamination in the industrial production of cookies. *Anal. Bioanal. Chem.* **2009**, *395*, 103–109.
- (31) Zahradnik, C.; Martzy, R.; Mach, R. L.; Krska, R.; Farnleitner, A. H.; Brunner, K. Detection of the food allergen celery via loop-mediated isothermal amplification technique. *Anal. Bioanal. Chem.* **2014**, *406*, 6827–6833.