



Review

Are current analytical methods suitable to verify VITAL® 2.0/3.0 allergen reference doses for EU allergens in foods?



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ABSTRACT

Food allergy affects up to 6% of Europeans. Allergen identification is important for the risk assessment and management of the inadvertent presence of allergens in foods. The VITAL® initiative for voluntary incidental trace allergen labeling suggests protein reference doses, based on clinical reactivity in food challenge studies, at or below which voluntary labelling is unnecessary.

Here, we investigated if current analytical methodology could verify the published VITAL® 2.0 doses, that were available during this analysis, in serving sizes between 5 and 500 g. Available data on published and commercial ELISA, PCR and mass spectrometry methods, especially for the detection of peanuts, soy, hazelnut, wheat, cow's milk and hen's egg were reviewed in detail. Limit of detection, quantitative capability, matrix compatibility, and specificity were assessed. Implications by the recently published VITAL® 3.0 doses were also considered. We conclude that available analytical methods are capable of reasonably robust detection of peanut, soy, hazelnut and wheat allergens for levels at or below the VITAL® 2.0 and also 3.0 doses, with some methods even capable of achieving this in a large 500 g serving size. Cow's milk and hen's egg are more problematic, largely due to matrix/processing incompatibility. An unmet need remains for harmonized reporting units, available reference materials, and method ring-trials to enable validation and the provision of comparable measurement results.

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Abbreviations

ELISA	Enzyme-linked immunosorbent assay
MS	Mass spectrometry
PAL	Precautionary allergen labelling
PCR	Polymerase chain reaction
VITAL® 2.0/3.0	Voluntary Incidental Trace Allergen Labelling; Program Version 2.0/3.0
LoD	Limit of detection
LoQ	Limit of quantification

1. Introduction

Food allergy is a common immunological disorder involving otherwise harmless food proteins and affects children and adults worldwide. Based on a meta-analysis of published food challenge data, cow's milk, tree nuts, soy, hen's egg, peanut, wheat, fish, and shellfish were the most common elicitors of food allergy in Europe with an estimated accumulated prevalence of 2.1% (1.2–3.3%) of the population. The lifetime self-reported prevalence of allergy to common foods in Europe ranged between 0.1 and 6% (Nwaru et al., 2014). Currently, there are no accessible cure for food allergy. Strict allergen avoidance remains the most efficient allergy management to avoid an allergic reaction, which can be severe and occasionally life-threatening (Muraro et al., 2014a). Successful allergen avoidance depends on the identification of the relevant food allergen. In order to enable consumers to immediately identify relevant allergens, the European Union (EU) food information regulation (Regulation EU 1169/2011, 2011) requires the mandatory labelling of 14 allergenic foods or food groups, including the above mentioned allergens, when used as ingredients in the manufacturing of foods. However, non-ingredient allergenic components that may result from cross-contact, e.g. during manufacturing or packing, are not covered by this regulation. In case of the potential presence of unintended allergens, food manufacturers may apply voluntary precautionary allergen labelling (PAL) in order to inform and protect consumers with allergies (Muraro et al., 2014a and 2014b). Unfortunately, mislabelling and an inconsistent use of PAL that is not based on quantitative allergen risk assessment may lead to discrepancy between labelling and the presence of allergens (Allen et al., 2014b; Crotty and Taylor, 2010; Remington et al., 2015), and thus pose significant risks to allergic consumers and reduce their range of product choice. Indeed, unintended allergic reactions to foods have been frequently reported (Blom et al., 2018). Against a backdrop of allergen labelling regulations that differ widely around the world, the issue of risk assessment and management of unintended allergen cross-contact also presents a big challenge for food producers. There is still uncertainty in the industry about how best to manage allergens, as evidenced by the fact that allergen-related issues still account for a significant proportion of all recalls and food alerts in North America, Australia and New Zealand (ANZ), and EU member states (Bucchini et al., 2016). A key challenge faced by industry is in deciding whether the risk of unintended allergen presence warrants the use of PAL on a food product. A major reason cited for the proliferation of PAL is the fact that we currently have no internationally recognised reference doses for allergenic foods. Therefore, manufacturers are defaulting to the use of PAL even where they perceive low levels of risk.

Voluntary Incidental Trace Allergen Labelling (VITAL) was developed by the Allergen Bureau in ANZ (Taylor et al., 2018) to provide a risk based methodology for food producers to use in assessing the impact of allergen cross-contact and provide appropriate PAL (<http://allergen.bureau.net/vital/>). Application of this approach aims to avoid unnecessary use of precautionary labelling and thereby preserve its value as a risk assessment and management tool. VITAL® therefore aims to minimise risk while communicating effectively to allergic consumers.

VITAL® 2.0, the program version 2.0 of VITAL, uses a grid consisting of one action level to assist in determining if the presence of residual protein from allergenic substances through unavoidable cross-contact requires a PAL statement. Very recently, VITAL® 2.0 was updated to VITAL® 3.0 with regard to reference doses (Bureau, 2019). Both VITAL® 2.0 (Allen et al., 2014a; Taylor et al., 2014) and VITAL® 3.0 (Remington et al., 2020) reference doses were published under peer-review.

Recent activities by the European Academy of Allergy and Clinical Immunology (EAACI) clearly demonstrate the growing number of EU consumers needing accurate risk communication regarding allergen avoidance and the current tools to support this are not sufficiently capable. Reliable analytical methods are a pivotal requirement for the introduction and adoption of such reference doses in the EU and beyond. Reference doses must be verifiable and it is therefore critical that allergens can be detected and quantified by existing analytical technology in relevant food matrices with appropriate sensitivity, specificity and accuracy (Muraro et al., 2014a and 2014b).

The main aim of this project was to assess if the limits of detection (LoD) or quantification (LoQ) of current analytical methods are capable of detecting or quantifying the presence of allergenic foods in various serving sizes according to the proposed VITAL® 2.0/3.0 total protein reference doses (Allen et al., 2014a; Remington et al., 2020; Taylor et al., 2014) and associated with the VITAL® tool. Hence, we investigated if current analytical methods are designed to ensure the level of unintended allergens is below the safety limits as proposed by VITAL® 2.0/3.0. We collected data from published and commercially available allergen detection methods from the mid 1990's until 2018 prior to detailed analysis. At this point, the peer-reviewed VITAL® 2.0 total protein reference doses were available for comparative analysis with available allergen detection methods. The focal point of this activity included allergenic foods for which (1) ingredient labelling is mandatory in the EU, (2) VITAL® 2.0 total protein reference doses are published (Taylor et al., 2014), (3) prioritisation with respect to public health relevance has been investigated (Houben et al., 2016), and (4) several specific detection methods are available. Considering the vast number of method publications in the literature, special attention was given to the detection of cow's milk, hazelnut, soy, hen's egg, peanut and wheat, all of which are frequent elicitors of allergic reactions in Europe (Nwaru et al., 2014).

Thus, the expert group first prepared collected data pertaining to current analytical technologies, and to their applicability to verification of VITAL® 2.0 total protein reference doses in relevant serving sizes of various food matrices. This data ideally included estimation of sensitivity, specificity, unit of measurement, expression of results and measurement uncertainty. Reference to existing standards for allergen detection and analytical method validation was included. The goal of this expert group review was not to provide recommendation of preferred methods or protocols, but to assess whether methodology exists that can provide appropriate sensitivity, specificity, and quantitative characteristics. These methods should therefore, in principle, allow for verification of VITAL® 2.0 reference doses and allow effective management of the unintended presence of allergens. Moreover, findings regarding method suitability to verify VITAL® 2.0 reference doses were extrapolated based on change in reference doses by the VITAL® 3.0 update, in order to understand the implications that the very recent update may have on method suitability. Finally, this work aimed at identifying gaps in current technology and knowledge and provide recommendations for further research in order to improve methods and overcome current limitations.

The creation of a common understanding regarding the capability of current analytical methods, the need for accurate, precise and reliable allergen determination in the near future, and the requirements for agreed reference materials and calibration standards will support better allergen management.

2. Analytical methods for allergen detection

2.1. Target molecules and reporting units

ELISA, PCR, and MS methods were selected for review because of their potential quantitative response, and prominent use in comparison to other methods for allergen detection.

Understanding the strengths and limitations of these analytical methods helps analysts select the most appropriate method or combination of methods to meet their analytical needs and provide the risk assessors and risk managers the necessary information to make decisions to manage risk on a public health level. VITAL® uses protein reference doses that are based on total protein of an allergenic food, e.g. total peanut protein (Table 1). For direct comparison with and verification of VITAL® protein reference doses in foodstuff, the reporting unit ideally is the total protein of the allergenic foodstuff and the methods ideally should measure total protein of the specific allergenic food. However, no such method exists for the specific detection and quantification of total protein of an allergenic food in complex food matrices. By contrast, marker molecules (analytical targets or determinants thereof) are specifically measured and are often converted to the amount of total protein (Fig. 1). In the total protein fraction of an allergenic food, only a subset of proteins (allergenic proteins) constitutes a risk to allergic consumers. The quantitative composition of which is unknown for many foods. ELISA and MS methods both detect the protein component of the food, by measuring either epitopes or peptides from selected proteins, respectively. These epitopes or peptides are not necessarily the allergenic IgE-binding structures of the allergenic proteins. PCR detects the allergenic food by measuring DNA sequences that are specific to the allergenic food in question. Many allergen detection methods report in units of either allergenic food or, rarely, total protein from allergenic food depending on how they are calibrated and converted into reporting units. This reporting unit relies on assumptions about the composition of protein or DNA in the food which may be different in native foods or changed in processed or fractionated foods. Accordingly, the selection of the calibration standard material and its related factor of conversion into appropriate reporting units, i.e. total allergenic foodstuff or total protein thereof, will strongly influence the measurement results. Depending on the selected calibration standard, these factors of conversion are already included in the measurement result or need to be applied after calibration. Any reporting unit that refers to the target molecule(s), i.e. DNA, peptide or single protein or protein fraction, without providing validated conversion factor(s) for total protein of the allergenic food cannot be compared with the total protein based VITAL® 2.0/3.0 reference dose. Taking this critical discussion into account, the accuracy of measurement results of methods for allergen detection should always be interpreted with care. The risk molecules (intact functional allergenic proteins) are in most cases not directly analysed and risk can only be inferred from the presence of the allergenic foodstuff or its total protein

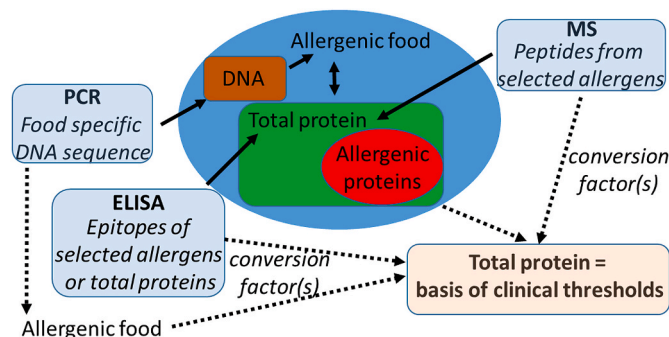


Fig. 1. The molecular composition of an allergenic food with special regard to the targeted molecules or determinants (selected epitopes of proteins, selected peptides of proteins, selected stretches of DNA), and the necessity of conversion to total protein, which is the basis of clinical thresholds.

component (Fig. 1). Each type of detection method requires different considerations for conversion to total allergenic food protein (detailed below).

2.2. Enzyme-linked immunosorbent assay (ELISA)

ELISA remains the most heavily used technique for allergen detection and quantification. One reason for this is that ELISA targets proteins, of which the subset of allergenic proteins are the main elicitors of allergy to food. ELISA is relatively simple and can be performed by trained laboratory personnel using relatively inexpensive equipment. Analysis can be completed within a few hours. There are numerous commercially available ELISA-based allergen detection kits available covering most major food allergens. Data analysis is relatively simplistic. ELISA employs antibodies, usually raised in animals (e.g. rabbits, mice or sheep) which have been immunised with an extracted protein fraction. This fraction may have not been characterized with respect to protein composition. A calibration standard containing known quantities of allergenic food, or allergenic food protein or target protein is used to create a standard curve. This calibration standard already includes a factor of conversion for total protein if the reporting unit refers to total protein of the allergenic food or total allergenic food. ELISA, relying on the interaction between antibody and allergen, is very reliant on the exact reagents used. Antibodies, particularly polyclonal antibodies, are subject to batch-to-batch variation, as is the calibration standard used to create the standard curve. Antibodies and calibration standards vary between manufacturers – often resulting in different results being obtained from different kits even if the same reporting units are used. Performance and quantification can vary on samples from different food sources due to interference from matrix effects and susceptibility of the detected proteins to denaturing effects of food processing.

Table 1

Protein action levels calculated from VITAL® 2.0 protein reference doses (Allen et al., 2014a; Taylor et al., 2014) in relation to various serving sizes. (LCI, lower confidence interval).

Allergen	Vital® 2.0 reference dose (mg protein)	quantitative risk	quality of database	Action levels as per serving size between 5 and 500 g (action levels in mg.kg ⁻¹ protein per food matrix)			
				5	50	250	500
Peanut	0.2	ED01	excellent	40	4	0.8	0.4
Cow's milk	0.1	ED01	excellent	20	2	0.4	0.2
Hen's eggs	0.03	ED01 and ED05 95% LCI	excellent	6	0.6	0.12	0.06
Hazelnut	0.1	ED01 and ED05 95% LCI	Good	20	2	0.4	0.2
Soy	1	ED05 95% LCI	sufficient	200	20	4	2
Wheat	1	ED05 95% LCI	sufficient	200	20	4	2
Mustard	0.05	ED05 95% LCI	sufficient	10	1	0.2	0.1
Lupine	4	ED05 95% LCI	sufficient	800	80	16	8
Sesame	0.2	ED05 95% LCI	marginally sufficient	40	4	0.8	0.4
Shrimp	10	ED05 95% LCI	marginally sufficient	2000	200	40	20

2.3. Polymerase chain reaction (PCR)

The PCR allows specific amplification and detection of selected deoxyribonucleic acid (DNA) stretches from species or groups of species that are used in food manufacturing but does not directly detect proteins or allergenic proteins. Nevertheless, PCR allows highly specific and sensitive detection and quantification of a target allergenic foodstuff. PCR sensitivity and specificity strongly depend on the selected DNA stretch of the targeted allergenic food. Using PCR, the time required to obtain results is often comparable to that required for ELISA but shorter than that for MS analysis. Some limitations exist in the use of PCR for the detection of allergenic foods, especially for milk and egg, which will be discussed in their specific chapters. In the case of state-of-the-art quantitative real-time PCR (qPCR), quantification of the target allergenic food and expression into reporting units of total allergenic food or total protein thereof (Fig. 1) can be achieved by calibration against the allergenic food commodity and the use of a suitable factor of conversion (examples in Table 2) from total allergenic food into total protein (Holzhauser and Röder, 2015). Several strategies for successful quantification of food allergens using qPCR have been shown (Eugster, 2010; Hirao et al., 2006; Holzhauser et al., 2014; Siegel et al., 2013). By contrast, methods that report DNA mass or copy numbers cannot be used for quantification of total food commodity or total protein thereof because of a lack of universal conversion factors for DNA.

Nucleic acid sequence provides more possible targets than do protein methods due to degeneracy (the presence of more than one codon for each amino acid) and additional non-translated intron positions. This advantage is particularly useful in the case of highly conserved protein sequences where specific peptides (MS) or antigens (ELISA) are lacking or may be difficult to identify. Moreover, PCR methods can be developed and optimized systematically based on available DNA sequences, and simultaneous detection of multiple (usually 4 to 6) DNA stretches, and thus multiplex allergen detection, can be achieved. PCR is based on common principles of synthetic chemistry, allowing use of open access analytical protocols. For example, in Germany and Japan, PCR methods have been established as official analytical methods for allergen detection. Further general aspects on PCR methods for detecting allergens in foods are detailed elsewhere (Holzhauser, 2018).

2.4. Mass spectrometry (MS)

A mass spectrometer separates ions in the gas phase according to their mass to charge ratio (m/z). For the analysis of proteins and peptides, MS is often coupled with reversed phase high performance liquid chromatography (RP-HPLC) via an electrospray ionisation source (ESI). Due to the superior sensitivity exhibited by peptides, these are now the markers of choice for the MS detection and quantification of food

allergens. To release the marker peptides, food proteins are first extracted from the matrix and then broken into representative peptides using a selective protease. Guidelines that aim to help users in the selection of target food allergen peptides, that act as good quantitative markers, have been developed (Johnson et al., 2011). However, this is still an area of research and considerations concerning the stability of peptides during food processing are necessary. The extraction and digestion steps required for accurate quantification can take hours and can differ greatly depending on the sample type/matrix. However, these procedures may be automated to improve sample throughput. The LC separation of the peptide digest normally takes between 5 and 30 min, depending on the number of peptides and anticipated complexity of the background matrix. However, the selectivity of MS/MS enables the sequential detection and quantification of tens or hundreds of peptides as they elute from the chromatographic column. This enables the detection of multiple proteins per allergenic food and multiple allergenic foods in one analysis. This multiplexing capability along with the possibility of detecting highly degraded proteins, via their protein specific peptides, are considered major advantages of MS methods for allergen detection.

Another advantage of MS, when used for quantifying allergen proteins, is in the choice of calibration standards available. Chemically pure stable peptides, which can be prepared gravimetrically, offer unrivalled traceability to the mole. Isotopic labelling (typically ^{13}C and ^{15}N) of these standards means they may be included in sample runs, decreasing run to run variability. Additional levels of calibration based on the use of purified proteins or the allergenic commodity itself are common. The use of matrix match calibration standards can alleviate differences in signal between background matrices.

The application of MS to allergen quantification is relatively new, and common practices are not well established. In particular, linking the MS signal to the amount of total allergenic food protein present is key to accurate quantification and is a process which would benefit from standardization.

3. Literature review and criteria for data selection

3.1. Available VITAL® reference doses and selection of reviewed allergenic foods

In 2018, 2019, at the time of analysis of collected data, VITAL® 2.0 protein reference doses were available as peer-reviewed work for peanut, milk, egg, hazelnut, soy, wheat, cashew, mustard, lupine, sesame seed, and shrimp (Allen et al., 2014a; Taylor et al., 2014) (Table 1).

In this work we mainly focussed on the detection of peanut, hen's eggs, cow's milk, hazelnut, soy, and wheat. These allergens were selected based on VITAL® 2.0 data quality (Allen et al., 2014a; Taylor

Table 2

Allergenic food action levels calculated from VITAL® 2.0 protein reference doses (Allen et al., 2014a; Taylor et al., 2014) in relation to various serving sizes (Table 1), and based on correlation factors as suggested by German official food control laboratories (Waublinger and Schulze, 2018). (LCI, lower confidence interval; NFDM, non-fat dry milk).

Allergen	Vital® 2.0 reference dose (mg protein)	conversion to	conversion factor to allergenic food	Action levels as per serving size between 5 and 500 g (action levels in mg.kg^{-1} allergenic food per food matrix)			
				5	50	250	500
Peanut	0.2	whole peanut	4	160	16	3.2	1.6
Cow's milk	0.1	NFDM	2.8	56	5.6	1.12	0.56
Hen's eggs	0.03	dried whole egg	2.2	13.2	1.32	0.264	0.132
Hazelnut	0.1	hazelnut flour	6.4	128	12.8	2.56	1.28
Soy	1	whole soybean	2.5	500	50	10	5
Wheat	1	raw or cooked wheat flour	10	2000	200	40	20
Mustard	0.05	mustard seed	3.8	38	3.8	0.76	0.38
Lupine	4	yellow lupin flour	2.5	2000	200	40	20
Sesame	0.2	crushed sesame seeds	5.9	236	23.6	4.72	2.36
Shrimp	10	whole cooked shrimp	4.4	8800	880	176	88

et al., 2014, 2018), and because they represent the most frequent elicitors of allergic reactions in Europe (Nwaru et al., 2014).

The Expert Group limited the ‘milk’ method review to cow’s (*Bos taurus*) milk, in spite of the fact that current EU legislation (Regulation EU 1169/2011, 2011) considers other types of milk (e.g. sheep or goat milk). This was due to the fact that there is limited information available on the performance of milk analytical methods for the detection of other types of milk, and that VITAL® 2.0 does not address the clinical reactivity of milk other than that of cow. Similarly, the review on methods for “egg” detection was limited to hen’s (*Gallus gallus domesticus*) egg due to our inability to address potential differences in analytical method performance to other farmed bird’s egg.

We identified a second group of allergenic foods as second priority for consideration. Shrimp (crustaceans), mustard, lupine, and sesame detection methods were summarized but not detailed. We do not address cashew, celery, fish (finfish), and walnut as these were first considered by VITAL® with the 3.0 update, which was not available during our data mining until 2018.

3.2. Calculation of required method sensitivity for verification of VITAL® 2.0 reference doses

Calculation of action levels from clinical reference doses in relation to serving sizes would be equivalent to the estimation of the tolerable risk for allergic consumers. Assuming scenarios such as “spoonful”, “scoopful”, “birthday cake”, and “beverage”, available VITAL® 2.0 protein reference doses were used to calculate action levels (concentration) on the basis of 5, 50, 250 and 500 g serving size (Table 1). For conversion of total foodstuff protein to total allergenic food, and vice versa, conversion factors, as recently summarized and also applied for VITAL® 2.0 reference doses (Taylor et al., 2014), were selected in agreement to a recommendation (Waiblinger and Schulze, 2018) of the German official food control laboratories (Table 2). Conversion of protein reference doses, e.g. peanut protein, to total allergenic food, e.g. peanut, was necessary to allow for comparison of methods calibrated against total protein and total allergenic foodstuff alike.

3.3. Criteria for data selection and comparative analysis

The literature review was restricted to the allergen detection methods ELISA, PCR, and MS, and encompassed the past two and a half decades, considering that first ELISA methods for allergen detection were published in the mid 1990’s. The literature review was closed in February 2018 to allow for a comprehensive analysis of data, to perform necessary conversions for data comparability, and to allow for several rounds of data verification. Even though some very recent publications may not be covered, the general picture obtained, and conclusions drawn are those obtained from an exhaustive review.

In brief, publicly available databases, including PubMed and Google Scholar, were searched. Additional literature databases were searched depending on authors’ access, such as SciSearch, Scopus, Web of Science, Science Direct, or ESBCO.

Search terms included the allergenic food (e.g. peanut, soy, hazelnut, wheat, cow’s milk, and hen’s egg), the target molecules (protein, DNA, gluten, casein, lactoglobulin, whey, egg white), and the methods (ELISA, immunoassay, PCR, MS, mass spectro*). Initial number of hits usually ranged from several hundred to several thousand. Further selection was performed according to relevance for allergen detection. Review articles, duplicates and literature not written in English language were removed. The final selection of publications ($n = 235$) is displayed in the Supplementary Table according to allergenic food and detection method. For the methods ELISA, PCR, and MS, 78, 100, and 57 publications were identified for a more detailed interrogation, respectively. In addition to peer reviewed published methods, commercially available methods were considered for further analysis, if manufacturer validation reports were publicly available, such as from company websites.

Identified methods were recorded together with additional available validation data in the Supplementary Table. Data were collected from published methods (p), commercial kits (c), and publications based on the use of commercial kits (p/c). The Supplementary Table summarises information about the target molecules, i.e. protein targets (protein fractions, allergen components such as peanut Ara h 2, peptides) and DNA targets (DNA sequences encoding allergens such as peanut Ara h 2 or non-allergenic proteins such as hazelnut heat-shock protein). It reports on the analysis of specificity, general quantitative response of the method, and matrix in which limit of detection (LoD) or lowest level of target analysis was performed. If reported, the LoD was directly recorded as mg.kg^{-1} total protein of the allergenic food per matrix or converted as such from mg.kg^{-1} allergenic food per matrix, using the conversion factors as displayed in Table 2. If provided by the authors or kit manufacturers, their reported conversion factors were preferred. In the case of wheat, cow’s milk and hen’s egg specific methods, we suggested conversion factors to account for the many methods that reported in individual protein components or protein fractions. Otherwise, these methods could not have been evaluated. Further details are given in the specific chapters and the Supplementary Table.

Other reporting units, such as picogram (pg) of DNA or ng.ml^{-1} target protein were additionally recorded but were unable to be converted into the above reporting units required for direct comparison with the calculated action levels (Tables 1 and 2). Original data (that reported in publication or reports of kit manufacturers) are highlighted in yellow. Finally, the reported LoD was compared to the calculated action levels in relation to different serving sizes (Tables 1 and 2). The largest possible serving size [g], in which the reference dose was detectable according to reported LoD, was stated.

Papers and commercial kits, which did not report LoD, or did not report LoD in units readily convertible to our standard unit, i.e. total foodstuff protein or total allergenic food, that can be compared to VITAL® protein reference doses, were excluded from further analysis. For each allergen a graphical overview of the recorded methods’ LoD, related to the type of method and in comparison, to the 4 action levels, related to 5–500 g serving size, was generated (Fig. 2). Reported LoD ranges were not considered in Fig. 2 as these cannot be plotted, and they cannot be assigned to individual matrices. They were essentially treated as if an LoD was not determined.

4. Food allergens and specific methods

For the allergens, peanut, soybean (soy), hazelnut, wheat, cow’s milk, and hen’s egg, a graphical overview of the recorded methods’ LoD, related to the type of method and in comparison to the 4 action levels, related to 5, 50, 250 and 500 g serving size, was generated (Fig. 2). The dotted lines represent the action levels, i.e. the allergen specific VITAL® 2.0 protein reference dose in relation to the defined serving size of 5–500 g. Thus, the required LoD of a method would need to be below the dotted line in order to allow for verification of the protein reference dose in the particular serving size. In the following allergen specific sub-chapters, methods with quantitative characteristics were reviewed in more detail if the depicted sensitivity would basically allow verification of VITAL® 2.0 reference doses in a large serving size of 250–500 g, or if quantitative determination in various food matrices and serving sizes of 50 g or greater was validated. Multi-allergen methods were reported for the individual allergen in the dedicated chapters.

4.1. Peanut

4.1.1. ELISA

4.1.1.1. Published methods (not kits). Of the key papers identified (Supplementary Table) the majority refer to commercially available ELISA kits. Two papers described non-commercial peanut ELISA

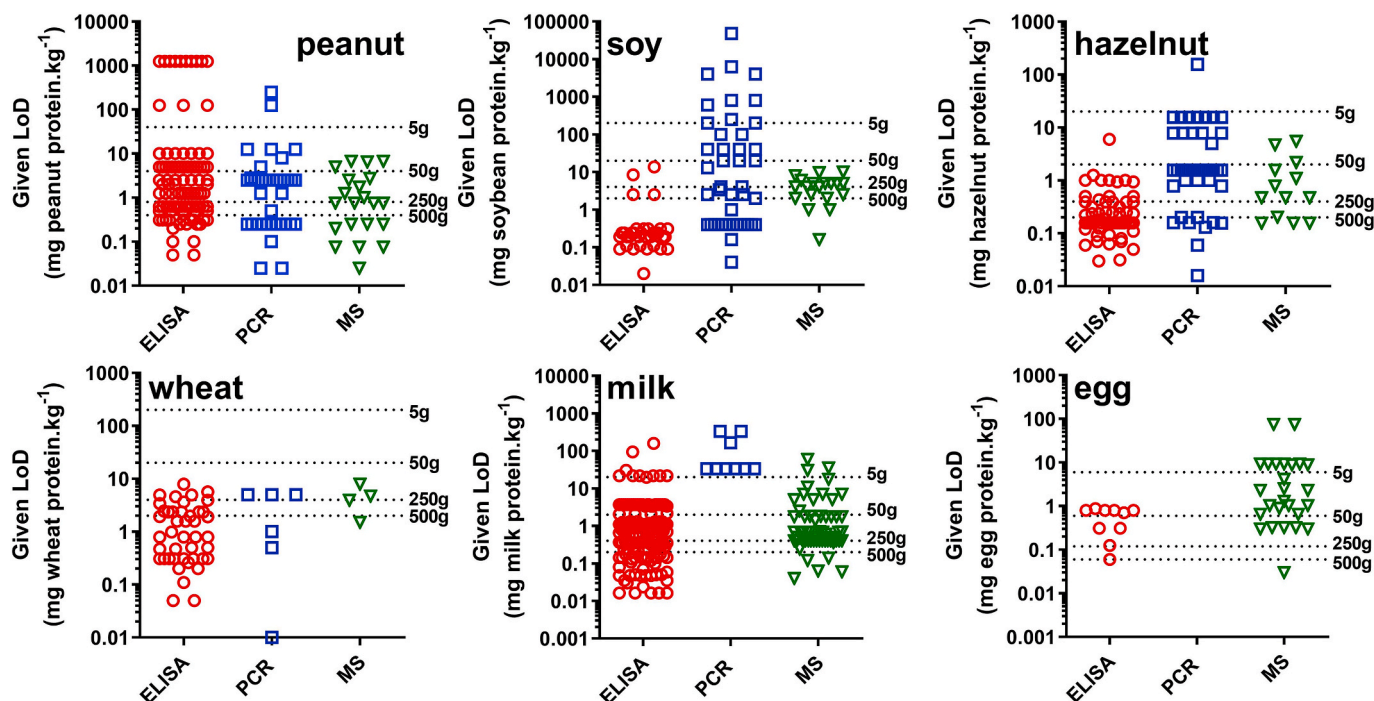


Fig. 2. Limits of detection for all collected methods for which LoD was available in standardized units ($\text{mg allergenic food protein.kg}^{-1}$, mg.kg^{-1}) or convertible to standardized units. For some methods, more than one LoD was reported from collected literature. In these instances, all reported LoD determinations are presented. Subfigures for each allergenic food are presented, with data for method basis (ELISA, PCR, MS) separated. For each allergenic food, the concentration of allergenic food protein yielding the VITAL® 2.0 reference dose for serving sizes of 5, 50, 250 and 500 g was calculated and is shown as a horizontal dotted line.

methods with lower limits of detection of 2 mg kg^{-1} within a variety of food matrices (Holzhauser and Vieths, 1999) and 0.2 mg kg^{-1} in cookies and in dark chocolate, 0.4 mg kg^{-1} in cereals and in vanilla ice cream, and 0.8 mg kg^{-1} in milk chocolate (Kiening et al., 2005). LoD at this range suggest that the measurement of VITAL® 2.0 reference doses even at the largest portion size may be possible. However, in these papers spike recovery experiments to demonstrate the performance of the assays were only performed to as low as 10 mg kg^{-1} (Holzhauser and Vieths, 1999) and 1 mg kg^{-1} (Kiening et al., 2005). At this level of detection, VITAL reference doses could only be applied to portion sizes of 5 and possibly 50 g.

4.1.1.2. Commercially available kits. For commercially available peanut allergen ELISA kits, the LoD declared by the manufacturers typically ranged between 2.5 mg kg^{-1} and 0.1 mg kg^{-1} total peanut. To allow for comparison of each kit to the VITAL® 2.0 reference doses, reporting units were converted to peanut protein from total peanut where necessary. The conversion was based on previous data showing that 25% of the total weight of peanut is protein (Supplementary Table).

Information provided by ELISA kit manufacturers and peer reviewed assessments of commercially available ELISA kits (Jayasena et al., 2015; Khuda et al., 2015; Koch et al., 2003; Park et al., 2005; Parker et al., 2015; Poms et al., 2005; Whitaker et al., 2005) suggest that most of the commercially available ELISA kits that were reviewed are able to detect peanut protein to the levels required to measure the VITAL 2.0 reference dose at the smallest portion size of 5 g, which equates to a 40 mg kg^{-1} of peanut protein per food. In support of this, a number of peer reviewed publications using allergen incurred matrices have demonstrated the detection of peanut at 5 mg kg^{-1} (Khuda et al., 2012b; Whitaker et al., 2005) and at 0.625 mg kg^{-1} (Poms et al., 2005). Similarly, it seems likely that most kits can detect levels of peanut required for the largest portion size (500 g portion, which equates to 0.4 mg kg^{-1} of peanut protein per food). However, there is a lack of peer reviewed data including recovery experiments to confirm whether the kits are able to quantify peanut within food at the lowest levels of detection, which

would be required to confirm the presence or absence of peanut allergen within the largest portions sizes of 250 and 500 g.

4.1.2. PCR

4.1.2.1. Published methods (not kits). Following the search criteria, several peer-reviewed publications have been identified for peanut detection using PCR of which the great majority are based on single copy target genes. Eight studies were not evaluated, as the reporting unit was based on DNA instead of peanut. Several methods have demonstrated utility in one or various matrices such as chocolate, ice cream, baked goods, or cereals. Only one of these single copy methods, a semi-quantitative PCR array published by Tortajada-Genaro et al. (2011) appeared to allow for a sensitive verification of the peanut equivalent to VITAL® 2.0 peanut protein reference dose in a large serving size of 500 g. Another two out of four multi copy based PCR methods appear to provide a comparably high sensitivity, allowing for verification of peanut according to VITAL® 2.0 in a large 500 g serving size (Ladenburger et al., 2018; López-Calleja et al., 2013b).

The qPCR method from López-Calleja et al. (2013b) targets ribosomal DNA and was calibrated on the basis of peanut López-Calleja et al., 2013b. Replicates of binary mixtures of raw and heated (160°C , 13 min) peanut in wheat flour and cocoa at levels between 0.1 and 10,000 mg.kg^{-1} were used as external standards and successfully detected. LoD or LoQ have not been determined, but detection of 0.1 mg kg^{-1} peanut in food was shown. Quantitative estimations of peanut in 123 retail samples were carried out based on simultaneous amplification of the peanut specific target DNA versus a universal eukaryotic control gene. In 27/73 samples without peanut ingredient labelling or PAL, detectable peanut was estimated between 0.5 and greater than 50 mg kg^{-1} . Despite demonstrating general quantitative characteristics, detailed quantitative validation of accuracy and precision of peanut determination is lacking.

The study from Ladenburger et al. (2018) describes a qPCR method specific for peanut and based on a mitochondrial multi copy target gene.

It utilizes a competitive amplification of the target gene versus an internal standard DNA for peanut quantification and for normalisation of matrix effects during DNA preparation and subsequent amplification. This strategy, first described for peanut quantification by Holzhauser et al. (2014), now combined with a multi copy gene target allowed for a sensitive quantification of peanut equivalent to the VITAL® 2.0 protein reference dose in a large serving size of 500 g. The method by Ladenburger et al. (2018) allowed for a matrix-normalized quantification in sausage, rice cookie, hollandaise sauce and skimmed milk powder between 1 and 100 mg kg⁻¹ peanut and achieved a mean accuracy of 123% (range 70–360%). Precision at the level of 10 mg kg⁻¹ peanut in the sausage, milk powder and rice cookie matrices ranged between 10 and 20% coefficient of variation.

4.1.2.2. Commercially available kits. Six commercial PCR kits have been identified for the detection of peanut. Two kits describe the sensitivity of the method based on DNA and thus were excluded from further evaluation. One kit reported an LoD of 10 mg kg⁻¹ and would allow for the detection of VITAL® 2.0 peanut protein reference dose within a sample size of 50 g. Three kits describe LoDs below 1.6 mg kg⁻¹ peanut that corresponds to <0.4 mg kg⁻¹ peanut protein (R-Biopharm AG, Darmstadt, Germany; Biotecon Diagnostics GmbH, Potsdam, Germany). Validation reports of commercial qPCR kits were either missing or limited in specific details, which did not allow for describing quantitative characteristics.

4.1.3. MS

The proteome of the peanut seed is dominated by major cupin and prolamin storage proteins. Of the cupin family, the 11S globulins (Ara h 3) and 7S albumins (Ara h 1) are most abundant, while the major prolamin proteins are 2S albumins (Ara h 2, 6 and 7). Quantitative detection of peanut in foodstuffs has focused on the use of these major proteins, also major allergens, as analytical proxies. Of the studies identified for this review, peptides derived from Ara h 1, Ara h 2 and Ara h 3 were used.

Quantitative methods for peanut have been developed and demonstrated to work in various matrices (chocolate, baked goods, cereals). Four studies identified here describe LoDs which are below the 0.4 mg kg⁻¹ representing VITAL® 2.0 levels in a 500 g serving size, though only three of these were determined in a food matrix, namely chocolate (Bignardi et al., 2013), cookie (Careri et al., 2008) and biscuit, ice cream or milk chocolate (Korte et al., 2016a). Interestingly, none of these studies utilized nano-liquid chromatography, thought to be required for the most sensitive methodology. It would appear that the most sensitive methods described to date have the capacity to meet the analytical challenges posed by VITAL® 2.0 reference doses. However, none of the methods described validation in sufficient numbers of matrices to provide comprehensive analysis of food groups in which peanut contamination is commonly analysed.

4.2. Soy

4.2.1. ELISA

4.2.1.1. Published methods (not kits). Four publications were identified for the ELISA analysis of soy as an allergenic ingredient in foods (Supplementary Table) (Jiang et al., 2015; Morishita et al., 2008, 2014; Pedersen et al., 2008). Pedersen et al. (2008) described a sandwich ELISA with a LoD of 0.05 mg kg⁻¹ of soy flake in food, which should enable verifying the VITAL® 2.0 reference dose of this ingredient at a 500 g portion. However, it is important to stress that there was insufficient information presented to allow an assessment of method reliability and the impact of matrix interference and processing on soy detection. Morishita et al. (2008) describe a sandwich ELISA with a LoD and a LoQ of 0.19 and 0.38 mg kg⁻¹ of soy protein in food, which suggests the

method has the potential to verifying the VITAL® 2.0 soy protein reference dose in foods at the 500 g portion. According to the authors, the major drawback of this ELISA concerned the inability to detect soy in fermented products. In order to overcome this disadvantage, the same authors combined the proposed ELISA system with an optimized extraction protocol that further enable the detection of soy in fermented products (Morishita et al., 2014).

4.2.1.2. Commercially available kits. There are several commercial ELISA kits for the detection of soy in a wide variety of foodstuffs. Soy soluble proteins, 7S globulin fraction (soy major protein component) and soybean trypsin inhibitor (STI) are frequently used targets for ELISA kits using polyclonal antibodies. Most kits declare high specificity (none or minor cross-reactivity with other species), and sensitivity, allowing detection of soy proteins with low LoD and LoQ (<1 mg kg⁻¹). According to the information provided by manufacturers, most of the ELISA kits would be able to detect soy at the 250 g portion, and some in the largest portion (500 g). Some of the kits fail to provide detailed information regarding the specificity or potential cross-reactivity of the antibodies, and the conversion factors that should be used to correctly quantify the amount of soy.

In the literature, some reports evaluate the capacity of ELISA kits to detect/quantify soy in foods, as well as the effect of matrix and the influence of food processing on their performance. In most cases, the kits described allow detection of soy in a wide variety of commercial foods or incurred/spiked model mixtures, reporting low LoD/LoQ that normally confirm the limits declared by the kits' manufacturers.

4.2.2. PCR

4.2.2.1. Published methods (not kits). Several peer-reviewed publications and commercial kits describe soy detection using PCR. The great majority of PCR methods were based on single copy targeting genes. At least five of these PCR methods have been developed for the detection of fraudulent addition of soy in meat or crop samples. Consequently, the authors did not validate their methods in the low mg.kg⁻¹ range. Those methods that have been developed for soy allergen detection demonstrated to work in one or various matrices like sausage, baked goods, or spices. Two studies could not be evaluated, because the sensitivity was described based on DNA determination as the read-out. Only one publication based on single copy detection accomplishes satisfactorily the required sensitivity to verify the equivalent VITAL® 2.0 soy protein reference dose in a large serving size of 500 g in all studied matrices. This method has been already described in the peanut section - a multiplex DNA micro assay based on a digital versatile disc (DVD) published by Tortajada-Genaro et al. (2011). A spike level of 1 mg kg⁻¹ soy in different food matrices, such as cookie, jam and others, was detectable.

Seven published methods were based on multi copy target genes, including ribosomal DNA (Hirao et al., 2009), mitochondrial DNA (Bauer et al., 2011; Galan et al., 2011; Ladenburger et al., 2018) or a sequence entitled as "interspersed repetitive element 1" (Yamakawa et al., 2007). Hirao et al. (2009) described the sensitivity on the basis of soy DNA. The studies from López-Calleja et al. (2017) and Yamakawa et al. (2007) only included food samples above 10 mg kg⁻¹ soy which was successfully detected in all samples and might therefore have the potential to detect lower levels.

The principle of the study from Ladenburger et al. (2018) has already been described in the peanut section. The method allowed for a matrix-normalized quantification of soy in sausage, rice cookie, hollandaise sauce and skimmed milk powder between 1 and 100 mg kg⁻¹, with an average recovery of 174% (range 114–290%). Thus, it allowed for a quantitative verification of soy in serving sizes of 500 g and above.

In the study of Bauer et al. (2011), LoD has been determined with soy spiked into a model spice to mimic concentrations from 0.1 to 10,000

mg kg⁻¹. With a demonstrated detectability of 1 mg kg⁻¹ soy in spice food, this published PCR method would allow detection of soy according to the VITAL® 2.0 reference dose in a large 500 g serving size. However, cross-reactivity was observed with several foods up to 0.0252%, and the method is further lacking quantitative validation for the use in different food matrices.

In the study from Galan et al. (2011) a duplex PCR for soy and lupin was developed. General quantitative characteristics were shown on the basis of serially diluted soy DNA for use in calibration curve. However, quantitative characterization is lacking. The LoD of 2.5 mg kg⁻¹ has been determined with replicate measurements of DNA extracts from cookies spiked with 1–10 mg kg⁻¹ soy flour, which were baked for 10 min at 180 °C. Detectability decreased with increasing baking time. Barley and buckwheat caused cross-reactive signals of 7.8% and 6.6%, respectively.

4.2.2.2. Commercially available kits. Of the identified commercial kits for soy detection, four describe the sensitivity of the method on the basis of DNA and thus have been excluded from further evaluation. Three kits describe LoDs below 5 mg kg⁻¹ soy for verification of soy equivalent to VITAL® 2.0 reference dose in a large serving of 500 g (R-Biopharm AG, Darmstadt, Germany; Biotec Diagnostics GmbH, Potsdam, Germany). Validation reports of commercial qPCR kits are missing or limited in specific details, which does not allow clear description of method characteristics. Apart from data on specificity, limitations are identified regarding quantitative validation.

4.2.3. MS

In soybean seeds, proteins belonging to three super-families prolamins, profilins and cupins, account for most of the seed proteins. They show considerable stability and are thus suitable candidates for reporter peptides in LC-MS/MS analysis. It so happens that the same soy proteins, which have been identified as major allergens, are also used as analytes in most of the published LC-MS/MS methods for the detection of soy in food commodities. Unique reporter peptides are mainly derived from different subunits of the soy conglycinin (Gly m 5; β -conglycinin), and soy glycinin (Gly m 6; glycinin G2). In addition, peptides from the prolamin proteins soy 2S albumin (Gly m 2S albumin) and lipid transfer protein (Gly m LTP) as well as Kunitz-type trypsin inhibitor (Gly m TI) have been used for LC-MS/MS detection in several studies.

The analysis of soy proteins by LC-MS/MS has been described in more than 35 method articles since 1992; however, the majority have focussed on profiling the soybean proteome by untargeted screening. More recent references have used targeted analysis of a few characteristic reporter proteins with high signal intensities for quantification purposes, mostly using parent ion-to-product ion transitions for specific identification (Supplementary Table). Some methods used matched stable-isotope labelled peptides as internal standards (Croote and Quake, 2016; Huscsek et al., 2016), one used a synthetic peptide with a replaced amino acid (Leu to Val) (Hoffmann et al., 2017) and some are multi-allergen analyses (Gomaa and Boye, 2015; Gu et al., 2018; Heick et al., 2011a, 2011b; Hoffmann et al., 2017; Huscsek et al., 2016; Monaci et al., 2014, 2016; Pilolli et al., 2017b; Planque et al., 2016, 2017).

Several of the newest quantification methods referred to the VITAL® 2.0 reference dose for soy (1 mg soy protein). Verification of soy in different matrices in a serving size of 50 g (equivalent to 20 mg kg⁻¹ soy protein or 50 mg kg⁻¹ whole soybean in food), considering a protein content of about 40% in soybean seeds (Huscsek et al., 2016), should be possible by all methods that have been reported since 2011. Some methods (Gu et al., 2018; Huscsek et al., 2016; Monaci et al., 2014, 2016; Planque et al., 2016, 2017) were theoretically also sensitive enough to verify soy in a 500 g portion at the respective protein level (2 mg kg⁻¹) (Supplementary Table). Four methods (Gu et al., 2018; Hoffmann et al., 2017; Huscsek et al., 2016; Planque et al., 2017) had been

validated by analyzing precision (% CV) and recovery, while the reported performance data of the other methods were restricted to the variance of the linear regression ($R^2 > 99\%$) of the calibration curves.

4.3. Hazelnut

4.3.1. ELISA

4.3.1.1. Published methods. Most published methods for ELISA detection of hazelnut describe non-commercial methods. Of these, a range of different LoDs were reported from 0.12 to 1 mg kg⁻¹ hazelnut protein in food. A number of papers using polyclonal antibodies accomplished the required sensitivity to detect the equivalent VITAL® 2.0 hazelnut protein reference dose in the larger portions of 250–500 g (0.4–0.2 mg kg⁻¹). Polyclonal antibodies isolated from antisera were able to detect both native and roasted hazelnut protein at levels down to 0.2 mg kg⁻¹ in practice (Holzhauser et al., 2002; Holzhauser and Vieths, 1999), however a LoD of 1 mg kg⁻¹ was employed to minimise false positives. A sandwich ELISA recognised raw and roasted hazelnut at levels of 1 mg kg⁻¹ total hazelnut (Koppelman et al., 1999) equivalent to 0.15 mg kg⁻¹ hazelnut protein in food based on hazelnut containing 15% protein as reported previously (Taylor et al., 2014). A method utilising both poly- and monoclonal antibodies was able to detect hazelnut protein in chocolate matrix at 1 mg kg⁻¹ (Costa et al., 2014, 2015) therefore was not sensitive enough to detect hazelnut protein in the larger portion sizes. Others were able to detect 0.12 mg kg⁻¹ in cake mix, 0.25 mg kg⁻¹ in milk chocolate, cereal and chocolate ice cream, 0.5 mg kg⁻¹ in oatmeal and 1 mg kg⁻¹ in granola bar (Blais and Phillippe, 2001). Another assay, using a monoclonal capture and polyclonal detection antibody, reported a matrix free LoD of 0.12 mg kg⁻¹. However, when tested in different matrices it was able to detect 0.2 mg kg⁻¹ in ice cream, 0.4 mg kg⁻¹ in cookie, 0.6 mg kg⁻¹ in cereal, 0.8 mg kg⁻¹ in dark chocolate and 1.2 mg kg⁻¹ in milk chocolate (Kiening et al., 2005).

4.3.1.2. Commercially available kits. For commercially available hazelnut immunoassays, the LoD declared by the manufacturers were as follows: Veratox (Neogen) 2.5 mg kg⁻¹ total hazelnut, Ridascreen (R-Biopharm) 2.5 mg kg⁻¹ total hazelnut and ELISA systems 0.5 mg kg⁻¹ hazelnut protein. When possible, results were converted to hazelnut protein as described above. This altered Veratox and Ridascreen LoDs to 0.375 mg kg⁻¹ hazelnut protein, based on manufactures protocols. This suggests that these commercially available kits are theoretically able to detect hazelnut protein at the 50 g (ELISA systems) and 250 g (Veratox and Ridascreen), but not the 500 g serving size.

As mentioned, many published papers utilising commercially available kits tested for recovery of spiked samples. Spiked samples (2.5 mg kg⁻¹) of whole hazelnut in different matrices were used in one such study. The recovery of the kits for the 2.5 mg kg⁻¹ spike varied greatly between 1.1 and 38 mg kg⁻¹ in oatmeal, chocolate and muffin matrices (Garber and Perry, 2010). Similarly, in results found by Platteau et al. (2011a), all commercial kits tested both over and underestimated spiked hazelnut in wheat flour, cookie dough and cookie matrices. Processing also affected the kit recovery of hazelnut (Cucu et al., 2011; Platteau et al., 2013). Unfortunately, none of the publications assessed the LoD of the kits in matrix free or matrix conditions, therefore it is difficult to determine if the kits can be used to reliably detect hazelnut protein at the levels required by VITAL® 2.0.

4.3.2. PCR

4.3.2.1. Published methods (not kits). Following the search criteria, a range of peer-reviewed publications about hazelnut detection using PCR were identified. The great majority of these methods have been based on single copy targeting genes. Most of the identified PCR methods have been developed and demonstrated to work in one or various matrices

such as chocolate, ice cream, baked goods, or cereals, but only a dozen publications presented quantitative data or a quantitative response. Several studies were excluded from further evaluation because the sensitivity was not described based on total hazelnut commodity. Only four publications described methods with appropriate sensitivity of $\leq 1.28 \text{ mg kg}^{-1}$ (Table 2) to detect total hazelnut, equivalent to the VITAL® 2.0 hazelnut protein reference dose of $\leq 0.1 \text{ mg hazelnut protein}$ (Table 1) in the largest 500 g serving size (Iniesto et al., 2013; López-Calleja et al., 2013a; Platteau et al., 2011a, 2011b). Despite the quantitative features of qPCR, Iniesto et al. (2013) did not present quantitative data, or read-outs were given as DNA masses in picograms (Platteau et al., 2011a, 2011b). Accordingly, the only sensitive, quantitative method with read-out of total hazelnut commodity is described by López-Calleja et al. (2013a) that is based on the specific amplification of a multi copy gene. Specificity was demonstrated against 31 species with a positive response to only hazelnut. For calibration, a binary mixture of raw and heated hazelnut spiked between 0.1 and 100,000 mg kg^{-1} into wheat flour was used. The LoD was reported as 0.1 mg kg^{-1} hazelnut in wheat flour. In 179 commercial retail samples, 40 food products, including chocolate, bonbon, biscuit, beverage, rice cake, nut bar, ice cream, bread and meat product, were detected positive between 0.003 and $> 100 \text{ mg kg}^{-1}$ hazelnut. Only four products exceeded 1.28 mg kg^{-1} hazelnut (Table 2), equivalent to $0.1 \text{ mg hazelnut protein}$ (Table 1) in a 500 g serving size. Despite quantitative calibration data in wheat matrix, studies on the accuracy (recovery) of hazelnut quantification were done in either the spiked wheat flour matrix or in the retail samples of various matrix types. Hence, detailed quantitative characterisation of accuracy and precision in real-life samples is lacking. Ring-trial data using this method were not identified.

4.3.2.2. Commercially available kits. Nine commercial kits for the detection of hazelnut were identified. Three kits describe the sensitivity of the method based on DNA, and two kit manufacturers have not provided further details. Of the remaining four kits, hazelnut LoDs are described below $0.4\text{--}1 \text{ mg kg}^{-1}$ (two kits of R-Biopharm AG, Darmstadt, Germany), around 0.8 mg kg^{-1} (Biotecon Diagnostics GmbH, Potsdam, Germany), and 100 mg kg^{-1} (Gen-ial GmbH, Troisdorf, Germany). When applying the hazelnut conversion factor of Table 2, the hazelnut LoDs of the three most sensitive kits (R-Biopharm AG, Biotecon Diagnostics GmbH) would correspond to hazelnut protein between 0.063 and 0.156 mg kg^{-1} . Accordingly, all three kits would in principle allow detecting the VITAL® 2.0 hazelnut protein reference dose even in a 500 g serving size. According to currently available validation reports, the qPCR kits were tested for specificity in a range of food commodities. Applicability in various food matrices, as well as quantitative data was described, but detailed quantitative data on accuracy and precision versus various matrices were missing.

4.3.3. MS

The major proteins of hazelnut are the 7S and 11S globulins, and the 2S seed storage albumins, known as Cor a 11, 9 and 14 respectively. These proteins have been demonstrated to bind IgE from hazelnut allergic individuals and are considered major allergens. Bet v 1 homologues (Cor a 1), non-specific lipid transfer proteins (Cor a 8) and profilins (Cor a 2) have also been identified as allergens in hazelnut, but these are of lesser abundance and clinical significance appears regional.

We identified five studies as providing quantitative information for MS detection of hazelnut. These publications focus on using peptides of Cor a 9 as analytical targets. The matrices used to validate these methods were chocolate, biscuit, bread, breakfast cereal and ice cream. The study by Korte et al. (2016a) is notable in that it provides independent LoDs for three matrices. However, only one transition capable of detection at the lowest VITAL® 2.0 level was described, although it should be noted that lower LoDs may be achievable but were not tested. The use of MS³ in this study was shown to considerably (>10 -fold) increase sensitivity

over more traditional multiple reaction monitoring (MRM)-based methods. Although a range of matrices have been evaluated, the most sensitive MS methods have not been demonstrated to achieve the sensitivity required for detection of VITAL® 2.0 levels of hazelnut in the largest (500 g) serving size. Detection of relevant VITAL® 2.0 levels of hazelnut in a 250 g serving has been adequately demonstrated (Fig. 3).

4.4. Wheat

4.4.1. Special considerations

The discussion of wheat immunoassays (and mass spectrometry) in terms of their compliance with VITAL® 2.0 reference doses was complicated by the fact that most published methods in this field focuses on gluten detection. However, as the VITAL Expert Panel stated that “with a reference dose of 1 mg of wheat protein, individuals with IgE mediated wheat allergy would be largely protected when selecting gluten-free products manufactured in conformity to Codex guidance” (Taylor et al., 2014), these methods will be discussed in subsequent sections.

Direct comparison of method performance to VITAL® 2.0 reference doses was very rarely possible because most methods report their results in mg.kg^{-1} gliadin or mg.kg^{-1} gluten units. When mg.kg^{-1} wheat protein units were available, they were used to assess compliance to VITAL®. For methods with gliadin reporting units, LoDs were recalculated based on a recent study of Hajas and co-workers (Hajas et al., 2018) which analysed 23 wheat varieties from all around the globe for their protein composition. This study found that the mean gliadin content of these varieties within the total extractable protein content (defined as the sum of the Osborne fractions) is 63.5% (5.1% CV). Thus, gliadin reporting unit values were divided by 0.635 to obtain wheat protein units. In case of methods with gluten reporting units, LoD values were firstly divided by 2 to obtain gliadin concentrations as recommended by the Codex Alimentarius (Codex, 2008) and several assay manufacturers.

4.4.2. ELISA

4.4.2.1. Published methods (not kits). Our database search left only eight publications that could be considered appropriate for comparison to VITAL® 2.0. These assays targeted either a mixture of prolamins (e.g. prolamins extracted from wheat, rye, barley and oats (Sorell et al., 1998)), or in many cases a specific prolamin peptide, e.g. a 19 amino acid-long toxic A-gliadin peptide (Bermudo Redondo et al., 2005; Ellis et al., 1998), the QQPFP epitope (Hernando et al., 2008; Valdes et al., 2003), an α -2-gliadin 33-mer peptide (Morón et al., 2008) or a DQ2.5-glia- α 3 peptide (Sajic et al., 2017). One method (Sajic et al., 2017) was able to detect the VITAL® 2.0 reference dose in the 50 g portion size, while the other methods were in compliance with VITAL® 2.0 even in the 250 g or 500 g portion sizes. It is very important to highlight that the current EU legislation requires the labelling of gluten-containing cereals including oats. Some of these methods are not suitable for analyzing products for wheat-allergic consumers because they do not cross-react with oats (Hernando et al., 2008; Sajic et al., 2017; Valdes et al., 2003).

4.4.2.2. Commercially available kits. Various commercial ELISA kits were identified that were capable of analysis of wheat residues. These methods, with no exception, were intended to detect gluten in a range of unprocessed or processed food matrices. All assays targeted prolamin, predominantly gliadin epitopes. Several methods did not specify their target epitopes, while other methods were known to apply the QQPFP epitope (R5 antibody, endorsed by Codex as a Type I method for gluten analysis), or a celiac-toxic 33-mer gliadin peptide. LoDs of the methods varied within a range of $0.14\text{--}5 \text{ mg kg}^{-1}$ gluten, which made all assays able to detect the VITAL® 2.0 reference dose in the 50 g portion size,

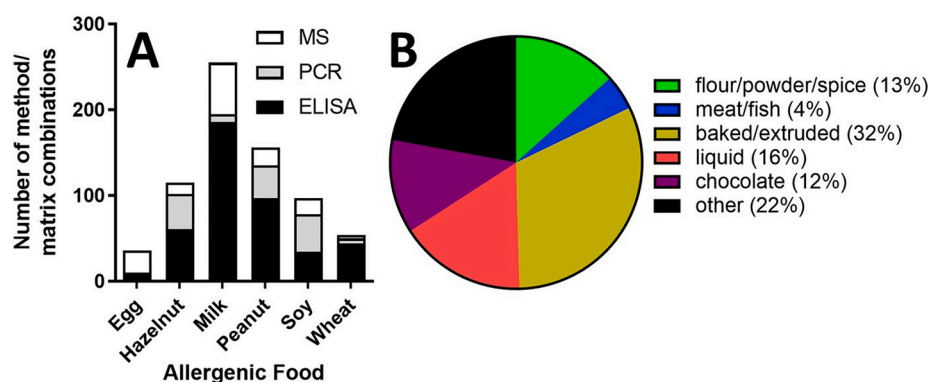


Fig. 3. A) Number of LoD determinations collected for each allergenic food. For some methods, more than one LoD was reported from collected literature, typically resulting from determinations in more than one food matrix. In these instances, all reported LoD determinations were included in the count. For each allergenic food, the methods are subdivided by method type (ELISA, PCR, MS). B) Distribution of food matrices used for LoD determination. For each method where matrix information was available, the matrix was grouped into one category of: flour/powder/spice; meat/fish; baked/extruded; liquid; chocolate; other/not given. The distribution of LoD determinations in each category is shown, along with percentage of the total number of LoD determinations assigned to a matrix. No matrix information was available for 131 LoD determinations.

while most methods were capable of detecting the reference amount in portions as large as 250 g or 500 g. Although the methods were reported to cross-react with several gluten-containing cereals, the problem of cross-reactivity with oats mentioned in section 4.4.1.2 persists. Several the commercial assays are reported to not cross-react with oats (including the Codex Type I R5 method), and cross-reactivity with other cereals are usually lower than reactivity to wheat.

A few major limitations of the methodology have been extensively investigated. Numerous studies reported that results of different kits very often show considerable variations (Alvarez and Boye, 2014; Bugyi et al., 2013; Geng et al., 2008; Scharf et al., 2013; Scherf, 2016). Major causes of variability include differences in antibody affinity (Allred and Ritter, 2010; Lexhaller et al., 2016, 2017; Panda et al., 2017), the effects of processing and the matrix (Bugyi et al., 2013; Gomaa and Boye, 2013, 2015; Panda et al., 2015) and the genetic and environmental variability of proteins (Hajas et al., 2018; Pahlavan et al., 2016). These issues demonstrate an urgent need of harmonization in this field.

4.4.3. PCR

4.4.3.1. Published methods (not kits). Following our search criteria, three peer-reviewed publications have been identified for wheat detection using PCR. Of these publications, two were based on multiplex methods detecting multiple DNA targets simultaneously and one based on qualitative detection using ligation-dependent probe amplification. Three papers provided sufficient accessible data to enable an assessment of applicability for use in this study. One study (Cheng et al., 2016) was a decaplex PCR combined with capillary electrophoresis for the simultaneous detection of ten food allergens; one of which was wheat. The relative LoD for wheat in a complex mixture was determined as 50 mg kg⁻¹ wheat which allows verification of the equivalent VITAL® 2.0 wheat protein reference dose in serving sizes of 5 and 50 g. However, no quantitative response was shown. One published qPCR (Martín-Fernández et al., 2016a) stated LoDs of 5, 10, and 50 mg kg⁻¹ wheat in rice, maize and soya, respectively. This method would allow for verification of the VITAL® 2.0 wheat protein reference dose in serving sizes of 50 and 500 g. Using a different wheat encoding sequence, the same authors reported a different method allowing the detection of wheat down to 20 mg kg⁻¹ in rice flour, which would enable the verification of reference dose in serving size of 500 g (Martín-Fernández et al., 2016b).

4.4.3.2. Commercially available kits. Three identified commercial qPCR kits that detect DNA from wheat were designed for detection of DNA from cereals that contain gluten rather than specifically for the detection of wheat. One kit reports the LoD in gene copies. The BIOTECN kit reports an LoD of 0.1–1 mg kg⁻¹ of cereal in spiked rice flour matrix for wheat, rye and barley. The LoQ was set at 0.8 mg kg⁻¹. This method would allow verification of the equivalent VITAL® 2.0 wheat protein reference dose in serving sizes of 5–500 g, but the sensitivity varied

depending on the sample type. The commercial Congen/R-Biopharm qPCR kit reported an LoD and LoQ of 0.4 and 1 mg kg⁻¹ gluten in corn flour. This would correspond to 0.31 and 0.78 mg kg⁻¹ wheat protein, which would allow detection of the VITAL® 2.0 protein reference dose in a serving size of 500 g. Despite general quantitative characteristics, further validation data on accuracy or precision are missing.

4.4.4. MS

The literature search resulted in 17 publications dealing with the quantification of wheat or wheat gluten. As with ELISA, most methods addressed the quantification of gluten. When analytical figures of merit for gliadin or gluten have been reported, these were converted to wheat protein using the same conversion factors used for the ELISA studies. Interestingly, most gluten/wheat publications addressed the issue of gluten alone and did not form part of a multi-allergen method.

Many of the publications addressed the relative quantification of the major wheat proteins in wheat flours and therefore did not address trace level detection (Fallahbaghery et al., 2017; Fekecssova et al., 2015; Flodrova et al., 2015; Mamone et al., 2000; Martinez-Esteso et al., 2016; Uvackova et al., 2013). However, such studies may be essential in linking the relative levels of individual wheat proteins or peptides to the quantity of total wheat protein. Another commonly reported complication was the similarity in sequence homology between the different gluten containing cereals. Therefore, the identification of selective peptide targets for wheat has proven a challenge. The use of MS for the detection of hydrolysed gluten peptides in beer (Colgrave et al., 2014; Fiedler et al., 2018) and wine (Simonato et al., 2011) has been reported as an alternative to ELISA. Whilst wheat gluten peptides were detected, no analytical figures of merit were reported in these studies.

Preliminary studies on the quantification of wheat gluten peptides in raw ingredients and processed foods suggested a set of six peptides that could be used for the quantification of wheat gluten. While excellent recoveries and LoDs were reported using MRM based approaches, the spiking studies were performed using peptide standards and the results were expressed in pg of target peptide per mg of sample (Sealey-Voyksner et al., 2010). More recent studies have focused on the quantification of a single “toxic” peptide from wheat gliadin using stable isotope labelled internal standards. Schalk et al. (2017) developed a method for the quantification of a 33-mer toxic gliadin fragment in digested flour samples via MRM. The LoD for the peptide spiked into rye flour was reported as 13.1 mg kg⁻¹. The reported content of the peptide in wheat cultivars varied between 90.9 and 602.6 mg kg⁻¹. Vatansever et al. (2017) reported a method using a stable isotope internal standard and MRM for the quantification of a 30-mer toxic peptide from gliadin. However, the variability of the marker in different wheat cultivars and the reporting of the LoQ in ng/μl make the assessment of this method for trace level wheat analysis difficult.

Colgrave et al. (2015) investigated four peptide markers for the quantification of wheat in cereal grains. A LoD of 15 mg kg⁻¹ wheat was

reported by gravimetrically spiking wheat flour into wheat free cereals. Manfredi et al. (2015) developed an LC-MS method for the quantification of prolamins in gluten free products. The gliadin content was first assigned using ELISA. While the LoDs were not provided for each matrix, a LoQ of 3–5 mg kg⁻¹ gliadin (5–8 mg kg⁻¹ wheat protein) was reported. A similar approach, incorporating the use of an isotopically labelled internal standard, was adopted by Liao et al. (2017). The reported LoD of 2.5 mg kg⁻¹ gliadin (4 mg kg⁻¹ wheat protein) suggests that these methods are close to achieving the required performance level for a 250 g serving VITAL® 2.0 action level.

One of the disadvantages of the LC-MS methods outlined above is the lack of knowledge concerning the observed peptide mass fraction and its conversion to gluten or total wheat protein. Schalk et al. (2018) took the first steps in quantifying the mass fraction of target peptides in wheat proteins and gluten. Conversion factors were reported for 16 wheat marker peptides (Schalk et al., 2018).

4.5. Cow's milk

4.5.1. Special considerations

Comparison of protein-based methods for milk detection, i.e. ELISA and MS, with VITAL® 2.0 milk protein reference doses was complicated by the fact that most identified methods (Supplementary Table) were not calibrated against total milk protein or milk. Accordingly, these methods could not have been assessed for compatibility with VITAL® 2.0 milk protein reference doses. Reporting units of methods with calibration against skim milk powder (SMP), in other words non-fat dry milk (NFDM), were converted to total milk protein using a factor of 0.351 that was used by Taylor et al. (2014). Reporting units that referred to caseins, alpha-lactalbumin (ALA), beta-lactoglobulin (BLG), bovine serum albumin (BSA), immunoglobulin and lactoferrin, respectively, were converted using factors as explained in the Supplementary Table.

4.5.2. ELISA

4.5.2.1. Published methods. Among the 27 peer-reviewed publications identified, approximately half of them provided an overview of commercialized kits. These latter are discussed separately in the next section. Of the 13 non-commercial peer-reviewed published methods, seven methods describe beta-lactoglobulin (BLG, Bos d 5)-based analytical detection, five published Bos d 8-based casein identification while the last one targets BLG, casein and alpha-lactalbumin (Bos d 4).

Within the eight publications describing BLG detection methods, only five of them provide LoD/LoQ in a food. Pelaez-Lorenzo et al. (2010) reported the lowest LoQ with 0.036 mg kg⁻¹ BLG detected in meat-based baby food, would still not be sufficient to cover the VITAL® 2.0 reference dose of 0.02 mg BLG in a 500 g portion size.

Among the seven publications describing casein detection ELISAs, only four provide limits of detection or quantification in foods. Three of them provide only LoD/LoQ reached in the assay, this information could not be further processed to enable method comparison.

The lowest LoD reached by ELISA-based casein detection methods is 0.1517 mg kg⁻¹ caseinate as reported by Restani et al. (2013). The method aimed at detecting the presence of milk based fining agents used in wine. This was the only method able to reach the detection limits of the most stringent VITAL® 2.0 reference dose of 0.17 mg caseins in a 500 g portion size.

4.5.2.2. Commercially available kits. Forty commercial kits were identified as being suitable for the detection of milk. Most of the methods target either caseins, or BLG, nine kits detect milk proteins and one targeted BSA. Most kit manufacturers disclose LoDs, but some provide LoQs only.

Seventeen kits describe LoDs or LoQs below 0.2 mg kg⁻¹ milk proteins that correspond to <0.16 mg kg⁻¹ caseins or 0.02 mg kg⁻¹ BLG. A

validation report is available for one of the kits, which has also been used and described in a multi-laboratory study (Johnson et al., 2014).

Commercial kits seem to be able to detect milk proteins at the most stringent VITAL® 2.0 reference dose of 0.2 mg milk protein in a 500 g portion size, but robust and peer-reviewed validation data is missing for most of them. Effectively, within the fourteen publications describing an overview of commercial kits, Diaz-Amigo (2009) shows that only two of six kits tested were able to detect 4 mg kg⁻¹ of NIST SRM #1549 material (whole milk powder). Downs and Taylor (2010) investigated the effect of processing (boiling, baking, frying) on the detection potential of three different commercial kits with the lowest incurred material prepared at 13.6 mg kg⁻¹ total caseins or 0.19 mg kg⁻¹ BLG. Johnson et al. (2014) showed that only one of five milk (casein) kits accurately determined milk at 6 and 15 mg kg⁻¹, but did not evaluate kits LoDs in food samples.

Lacorn et al. (2014) present two collaborative tests conducted using commercial ELISA kits to quantify casein in wines suggesting a LoQ of 0.18 mg kg⁻¹ milk protein may be achievable. A second collaborative study by Matsuda et al. (2006) shows that kits can detect the presence of 10 mg milk proteins/kg in meat-based products, biscuits, fruit juice and jam, although different recoveries were reported. Parker et al. (2015) finally compares ELISA detection with LC-MS and evaluates the impact of processing on milk detection, however no LoDs were reported.

A major issue is the quantitative accuracy of the kits which has been shown to be failing (Ivens et al., 2016; Khuda et al., 2012b) and seems to be due to the lack of reference materials, as well as the use of different antibodies in the development of commercial kits.

4.5.3. PCR

4.5.3.1. Published methods (not kits). DNA-based methods, namely PCR, are among the less used techniques for the detection of milk in foods. Some of the reports are based on conventional PCR, others make use of qPCR, either targeting single copy or multi copy genes, and some propose semi-quantitative methods for the specific detection of cow's and ovine (goat, ewe and sheep) milks (Kotowicz et al., 2007; López-Calleja et al., 2004; Mafrá et al., 2004; Maudet and Taberlet, 2001; Rodrigues et al., 2012). More recently, three qPCR methods were evaluated for the detection of cow's milk. These target mitochondrial genes (multi copy) and the alpha-lactalbumin gene (single copy gene) (Cottenet et al., 2011; Mayer et al., 2012; Xiao et al., 2016). Most of the publications reported the detection of cow's milk as an adulterant in value-added products, such as traditional cheeses, where milk from ovine source may be substituted by cow's milk for economic reasons. Accordingly, the sensitivities of the described methods are in the range of 1000–10,000 mg kg⁻¹ of cow's milk in cheese and other milks, which are considered adequate for authentication purposes but not for allergen analysis. Therefore, none of the proposed PCR-based methods presents the potential to detect cow's milk at a level required by the VITAL® 2.0 reference dose.

Despite the potential of PCR-based methods for the detection of several allergenic foods, the use of those techniques for cow's milk detection is controversial. Issues related to the fact that bovine DNA comes from somatic cells in milk (Mayer et al., 2012) and that it is difficult to differentiate the DNA from milk or meat cannot be ruled out, when developing a quantitative DNA-based method.

4.5.3.2. Commercially available kits. So far, there are no commercial kits for real-time PCR targeting cow's milk for allergen analysis, most likely because of the disadvantages of PCR for milk detection.

4.5.4. MS

Twenty publications dealing with the quantification of milk allergens in food were identified. Of these, nine publications focused solely on the detection of milk, while the remaining eleven publications attempted

the detection of multiple food allergen sources. Most papers focused on the detection of the presence of milk in baked goods. However, five papers specifically addressed the detection of milk-based fining agents in wines. For each publication the exact peptide target(s) and their proteins of origin are shown in the [Supplementary Table](#).

An early application using Q-TOF MS detected the presence of the intact proteins in fruit juice ([Monaci and van Hengel, 2007, 2008](#)). The reported LoD would protect consumers at the 5 g serving size. However, this would not be considered “fit for purpose” when considering the average size of a glass of fruit juice.

In wine production, the OIV (International Organisation of Vine and Wine) permit the use of milk, egg and fish proteins as fining agents. From the detection capability exhibited for standard peptides in solution, it was deduced that the LoD in wine would be in the region of 75 ng of milk protein per litre of wine ([Tolin et al., 2012](#)). However, due to difficulties in extraction and interferences, the observed LoD was 62 mg/L ([Monaci et al., 2010a](#)). Later applications targeted specific casein peptides. Studies report similar LoDs ranging from 0.125 mg/L to 0.375 mg/L of whole milk protein. These limits are useful for protecting affected consumers at the 250–500 g consumption rate ([De Angelis et al., 2017](#); [Mattarozzi et al., 2014](#); [Piolli et al., 2014](#)).

The first screening methods for baked food matrices also used Q-ToF mass spectrometers. Two peptides were observed in a matrix spiked with skimmed milk powder (SMP) and researchers reported an LoD of 5 mg kg⁻¹ of SMP ([Weber et al., 2006](#)). A similar approach reported a LoD for SMP of 100 mg kg⁻¹ in baked incurred cookies ([Monaci et al., 2010b](#)). To reduce the LoD a more targeted approach selecting specific stable milk peptides for analysis was adopted. However, LoDs for the individual peptides were expressed in ng/mL making the comparison with other methods difficult ([Ansari et al., 2011](#); [Ji et al., 2017](#)). A different approach for the preparation of standards was adopted by [Lamberti et al. \(2016\)](#). Here the researchers used a SMP to make a calibration curve in a dough matrix. Three marker peptides were monitored and a LoD of 1.3 mg kg⁻¹ milk powder was reported. The most comprehensive validation study to date for milk was performed on infant cereals. The authors used purified peptides and their isotopic analogues as internal standards for calibrating the MS response. A LoD between 1 and 5 mg kg⁻¹ of SMP was reported depending on the matrix studied. The LoD was based on the observation of at least 5 of the 6 marker peptides and is suitable for a VITAL® 2.0 serving size between 50 and 250 g ([Lutter et al., 2011](#)).

The major difference among the studies monitoring just milk and those monitoring more than one allergen is the number of milk marker peptides monitored. A LoD of 5 mg kg⁻¹ SMP was reported based on the most intense peptide signal observed from an incurred bread matrix ([Heick et al., 2011a, 2011b](#)) while a LoD of 0.12 mg kg⁻¹ milk protein was reported in a spiked biscuit material ([Monaci et al., 2014](#)). [Parker et al. \(2015\)](#) used a similar approach but incorporated the use of isotopically labelled peptides as internal standards for quantifying peptides. A multi-allergen method, that targeted two peptides, reported a LoD of 7 mg kg⁻¹ allergenic ingredient. The LoD was based on a raw cookie dough spiked with known mass fractions of a skimmed milk powder. However, a drop of greater than 80% in signal intensity was reported for the same milk peptides studied in same matrix when cooked ([Piolli et al., 2017b](#)). The final method used a triple quadrupole for the targeted quantification peptides. A LoD of 0.5 mg kg⁻¹ milk protein and 5 mg kg⁻¹ milk protein for a cookie material, based on casein and whey proteins respectively, was reported ([Planque et al., 2016](#)). However, the slopes for the target peptides differed greatly in the other matrices suggesting that these LoDs were not applicable to all matrices ([Planque et al., 2017](#)).

4.6. Hen's eggs

4.6.1. Special considerations

The comparison of protein based methods for hen's egg detection, i. e. ELISA and MS, with VITAL® 2.0 egg protein reference doses was

complicated by the fact that the majority of identified methods ([Supplementary Table](#)) were not calibrated against total hen's egg protein or hen's egg. Accordingly, these methods could not have been assessed for compatibility with VITAL® 2.0 milk protein reference doses. Thus, reporting units other than mg.kg⁻¹ egg or egg protein were converted on the basis of conversion factors as published by [Taylor et al. \(2014\)](#). Details are provided in the [Supplementary Table](#).

4.6.2. ELISA

4.6.2.1. Published methods (not kits). Following the search criteria, in total, 18 peer-reviewed publications were identified for hen's egg detection using ELISA. Of these 18 publications, only 3 reported sufficient data (LoD/LoQ) to enable an assessment of applicability for use in this study. The 3 assays were based on different antibody systems; the first assay was based on polyclonal antibodies raised against whole egg proteins ([Yeung et al., 2000](#)); the second assay used antibodies raised against purified ovalbumin (OVA) and dehydrated egg white solids ([Hefle et al., 2001](#)); the third assay utilized 2 monoclonal antibodies (mAb) recognizing different epitopes of ovomucoid ([Li et al., 2008](#)). Two of these immunoassays were demonstrated to work in one or various matrices including ice creams, noodles, pasta, and breads and other baked goods. The third assay had only been validated for use in assaying vaccines. Neither of the two methods validated for use with food matrices accomplished the required sensitivity to verify the equivalent VITAL® 2.0 egg protein reference dose in a larger serving size of 250–500 g. Both assays appeared to be able to detect at the VITAL® 2.0 reference dose in 5 g portions, but only one assay ([Yeung et al., 2000](#)) appeared suitable for detection of the VITAL® 2.0 reference dose in the 50 g serving size.

4.6.2.2. Commercially available kits. For hen's egg commercial immunoassays, the lower LoD stated by the manufacturers typically ranged from 0.3 to 2.5 mg kg⁻¹. As mentioned above, it is difficult to compare kits from different manufacturers. Often kits use different calibration standards to create a standard curve and there is a lack of uniformity in the reporting units. Similarly, peer review publications looking into the performance of kits do not always use the same criteria to compare the kits. For example, the recovery of whole egg powder or total egg protein. To allow for the comparison of each kit to the VITAL® 2.0 reference doses, when necessary reporting units were converted to total egg protein. Conversion to total egg protein resulted in a range of 0.06–0.88 mg kg⁻¹ ([Supplementary Table](#)). Another complicating factor is that some of the peer reviewed articles do not test the kits to the lowest level of detection as declared by the manufacturer or the lowest level of detection required to implement the VITAL® 2.0 reference doses. This leaves a paucity of peer reviewed data towards the lowest LoD.

Nevertheless, using the information provided by ELISA kit manufacturers (Neogen Biokits, Neogen Veratox, R-Biopharm, Romer, ELISA systems & Morinaga) and peer reviewed assessments of the ELISA kits, it suggests that all of the commercially available ELISA kits are theoretically able to detect hen's egg protein to the levels required to measure the VITAL® 2.0 reference dose at the smallest portion size (5 g portion, which equates to a 6 mg kg⁻¹ of total egg protein per food). Most commercial kits could theoretically detect egg protein in the 50 g serving size (0.6 mg kg⁻¹); and one kit could theoretically detect egg in a large portion size of 250–500 g (0.06 mg kg⁻¹).

Validation of assay performance criteria by the main commercial kit manufacturers does not appear to be harmonized either in terms of demonstrating recovery or in the range of sample matrices used to check for inhibition or cross-reactivity. It is therefore not possible to comment on the impact of matrix interference or food processing on the LoD of commercial assays and hence their suitability to detect at the suggested VITAL® 2.0 reference doses. [Khuda et al. \(2012a\)](#) reported a study assessing the role of heat treatment and detectability of a range of

different allergens including egg. The study included a range of commercial egg ELISA kits which were used to test both cookie dough and heat-treated cookies (190 °C for 25 & 30 min) prepared with NIST reference materials (spray-dried whole egg powder, NIST RM 8445). All kits showed a significant drop in recovery of egg between the uncooked and cooked samples tested (5–25% recovery in 30 min heat-treated cookie).

4.6.3. PCR

4.6.3.1. Published methods (not kits). Only two publications were identified (Köppel et al., 2010, 2012), based on tetraplex and hexaplex allergen detection, respectively. The authors were unable to detect egg in food products with declared egg ingredient and concluded that PCR was not suitable for egg detection.

The DNA-based PCR detection of hen's egg faces similar challenges as PCR based milk detection. PCR based on genomic DNA cannot distinguish between egg and chicken meat because it is not tissue specific. Moreover, the amount of target DNA is very low. Unfertilized eggs contain only a single haploid chromosome set in the egg yolk while the egg white, the portion that is high in allergenic proteins, is free from DNA. Further to this, the components egg white and egg yolk are often separated in foods. Hence, the probability of false negative results using PCR for the detection of egg components is high. Accordingly, this Expert Group does not consider PCR an appropriate method for the detection of egg as an allergen in food.

4.6.3.2. Commercially available kits. There are no commercial kits for real-time PCR targeting hen's (and other poultry) egg for allergen analysis available.

4.6.4. MS

We identified several papers addressing the quantitative determination of egg allergens by mass spectrometry (Supplementary Table). Among those, several strategies and applications of MS were proposed for the detection of egg allergen in different food matrices, often based on the analysis of the tryptic digest of ovalbumin and lysozyme, two major allergenic proteins in hen's eggs.

Two papers focused on the specific determination of the egg allergen. The first determines the presence of ovalbumin before and after thermal treatment. The results clearly show that detection in pasta is affected by the matrix and processing, which highlights the need for robust egg detection methodologies for processed foods. However, the MS method presented was limited to screening purposes (Azarnia et al., 2013). The second study, a MS method for the detection of egg allergens in wines, which uses immunoaffinity enrichment, reached a LoD, converted to 0.03 mg kg⁻¹ egg protein (Pilolli et al., 2017a), which is suitable for verifying the VITAL® 2.0 protein reference in a 50 g (50 mL) serving size.

Three papers address the multiple detection of egg and milk related proteins, typically employed in wines as fining agents, thus having special reporting criteria, i.e. often results are based on total caseinate or total egg white protein, but not reporting explicitly in total milk protein or egg protein (Mattarozzi et al., 2014; Monaci et al., 2013; Pilolli et al., 2014). After conversion into mg.kg⁻¹ total egg protein, the methods appear suitable to verify the VITAL® 2.0 egg protein reference dose in serving sizes between 5 and 50 g depending on the methods.

A MS method capable of simultaneous detection for seven allergens in unprocessed and processed samples (flour and bread) was reported. In that study, Tandem MS in SRM mode, was used to monitor four marker peptides for each allergenic food. The LoD for egg was estimated as 42 mg kg⁻¹ referred to the total soluble protein (determined by Bradford for each allergenic commodity). This approach was specific and suitable for monitoring the proteins of interest, but not sensitive enough for verifying VITAL® 2.0 protein reference doses at the smallest serving size of

5 g (Heick et al., 2011b).

In another example of multiplex MS/MS allergen detection in processed foods, calibration was performed using cookies prepared with known quantities of egg powder. The LoD was expressed in µg.g⁻¹ egg powder in cookie (Pilolli et al., 2017b). After conversion into egg protein, the method appears suitable for a 5 g serving size.

Only few studies report LoD or LoQ suitable for the direct comparison with VITAL® 2.0 reference doses. One microHPLC-ESI-MS/MS method (multi-target SRM) developed for the simultaneous detection of soy, egg and milk allergens in cookies reports a LoD of 0.3 mg kg⁻¹ egg protein, which allows verifying the VITAL® 2.0 egg protein reference dose in a 50 g portion (Monaci et al., 2014). In another MS method, the determined LoQ was expressed as 3.4 mg kg⁻¹ for egg protein in cookie, which is sensitive enough for verifying the VITAL® 2.0 reference dose for a 5 g serving size (Planque et al., 2016).

Finally, a targeted LC-MS/MS method was published that describes the detection and quantification of egg, milk, and peanut allergens in sugar cookies, at a level as low as 5 mg kg⁻¹ incurred allergen ingredient per matrix. This level of sensitivity would allow for verification of the VITAL® 2.0 egg protein reference dose up to a 5 g serving size (Boo et al., 2018).

4.7. Miscellaneous food allergens (mustard, lupine, sesame, crustaceans)

4.7.1. Crustaceans

4.7.1.1. ELISA. Crustacean ELISA methods are commercially available with stated LoQ below 1 mg kg⁻¹ of crustacean by weight. Compared to other food allergens, clinical data suggest that relatively large amounts of crustacean are required to provoke allergic reactions in sensitized individuals resulting in a detection limit of 20 mg kg⁻¹ shrimp protein for VITAL® 2.0 levels in a large 500 g food portion. Available commercial methods are viable to considerably below this limit. However, the diversity inherent in crustacean species results in quantification differences when testing various crustacean species. In many cases, cross-reactivity with insects and molluscs, a possible contaminant of food commodities, is observed most likely due to the frequent use of tropomyosin, a highly conserved protein among arthropods, as an analytical target.

4.7.1.2. PCR. Based on the reported LoDs and LoQs, PCR methods might be suitable for the detection at the high reference doses, resulting in action levels between 88 and 8800 mg kg⁻¹ shrimp or translated 20–2000 mg kg⁻¹ shrimp protein according to VITAL® 2.0 protein reference doses. Based on the reported sensitivity, LoDs and LoQs, PCR methods can be suitable to detect crustacean species within this allergen group. The real-time PCR system (targeting a multi copy gene) proposed by Fernandes et al. (2018) is an example of such methods, which allow the detection of shrimp in stuffing/filling down to 1 mg kg⁻¹, thus fulfilling the VITAL® 2.0 criterion for the 500 g serving size.

4.7.1.3. MS. The VITAL® 2.0 reference dose for shrimp is 10 mg protein. Verification in serving sizes of 5, 50 and 500 g would afford methods with detection limits of, respectively, 2000, 200 and 20 mg kg⁻¹ shrimp protein in food, which is equivalent to 8800, 880 and 88 mg kg⁻¹ total shrimp in food, considering a protein content of about 23% (Islam et al., 2017). Only one of the existing methods (Korte et al., 2016b) has measured crustacean proteins quantitatively in food matrix (salmon lasagne). The LoD using different reporter peptides in American lobster and Giant tiger prawn ranged from 100 to 1000 mg kg⁻¹ protein by MS² analysis and from 10 to 100 mg kg⁻¹ protein by MS³ analysis, meaning that the method would fulfil the VITAL® 2.0 criterion for the 50 g serving size.

4.7.2. Mustard

4.7.2.1. ELISA. Mustard ELISA methods are available commercially and usually detect thermostable 2S albumins (e.g. Sin a 1, Bra j 1) of the mustard family. Due to the need to detect three major mustard species (brown or Indian mustard/*Brassica juncea*, black mustard/*Brassica nigra* and yellow mustard/*Sinapis alba*) and high relatedness of other major crop plants (particularly rapeseed), cross-reactivity is often observed, and care must be taken in the interpretation of positive results, if presence of Brassica species is suspected. VITAL® 2.0 levels for mustard are low, with a LoD of 0.1 mg kg⁻¹ required to detect the reference dose in a 500 g serving size. No commercial ELISA kits claim detection at this level, with stated LoD of 1–2 mg kg⁻¹ being common.

4.7.2.2. PCR. Due to difficulty in distinguishing mustard from related and commonly grown Brassica crops using ELISA methods, PCR analysis is frequently used for mustard detection. Commercial PCR analysis of mustard with stated LoD of 0.4 mg kg⁻¹ mustard (equivalent to 0.1 mg kg⁻¹ mustard protein) are available, making detection of VITAL® 2.0 action levels for serving sizes of 5, 50 and 250 g achievable.

4.7.2.3. MS. The VITAL® 2.0 reference dose for mustard is as low as 0.05 mg protein. Verification in serving sizes of 5 g, 50 g and 500 g would afford methods with detection limits of, respectively, 10, 1 and 0.1 mg kg⁻¹ of mustard protein in food (equivalent to 22, 2.2 and 0.22 mg kg⁻¹ total mustard in food, considering a protein content of about 45%) (Marnoch and Diosady, 2006; Woods et al., 1991). Two MS-based methods for the detection of yellow mustard exist in the literature (Croote et al., 2017; Posada-Ayala et al., 2016), with stated LoDs of, 2.9 and 25 mg kg⁻¹ total mustard protein, respectively. These methods are thus capable of detecting action levels in the 5 g serving size. Both methods were validated by determining precision and recovery from food matrices. Despite current limitation in sensitivity, the possibility of a method capable of distinguishing mustard species from other common food crops suggests MS an attractive alternative for this analysis.

4.7.3. Lupine

4.7.3.1. ELISA. Lupine (genus *Lupinus*, particularly *L. albus*, *L. angustifolius* and *L. luteus*) is botanically related to peanut, and lupine allergy is related to peanut allergy. However, commercial ELISA test kits for lupine which do not cross-react in the presence of peanut are available, with stated LoD of less than 1 mg kg⁻¹ of lupine flour protein. The VITAL® 2.0 action levels for lupine in a 500 g serving indicate that detection at lower than 8.0 mg kg⁻¹ is required, and commercial ELISA kits are capable of this level of detection according to the information provided by the kit company.

4.7.3.2. PCR. Detection of lupine in various food matrices has been studied by Demmel et al. (2011), with an LoD in matrix of 1 mg kg⁻¹. This sensitivity is sufficient for detection of VITAL® 2.0 protein reference dose in all the food serving sizes considered in this review. The authors did, however, note considerable changes in detected levels of lupine according to matrix, storage and processing, making quantitative evaluation difficult without prior knowledge of the sample tested.

4.7.3.3. MS. Relatively few methods for the detection of lupine by mass spectrometry have been developed, and those few which exist are not readily commercially accessible. Mattarozzi et al. (2012) describe lupine detection in pasta and biscuits, with stated detection at around 1 mg kg⁻¹. This method would appear capable of detecting VITAL® 2.0 action levels in 5 and 50 g serving sizes only.

4.7.4. Sesame

4.7.4.1. ELISA. Sesame (genus *Sesamum*) seeds are often used for their oil but contain abundant seed storage proteins. Commercial ELISA kits targeting total extractable sesame protein or the 2S proteins (also known allergens) are available with stated LoD within the range 0.5–1 mg kg⁻¹ sesame seed protein. However, detection of sesame protein required by the VITAL® 2.0 protein reference dose for larger serving sizes (0.4 mg kg⁻¹ for a 500 g serving) indicate that current commercial ELISAs are not capable of meeting this level of sensitivity. Detection of the VITAL® 2.0 protein reference dose in 5 or 50 g serving sizes is, however, achievable.

4.7.4.2. PCR. Commercial PCR tests for sesame are available, with LoD of 0.4 mg kg⁻¹ sesame (equivalent to 0.07 mg kg⁻¹ sesame protein) achievable. At this detection level, action levels could be detected in the all the serving sizes (up to 500 g) considered in this review.

4.7.4.3. MS. Although researchers have applied mass spectrometry to the study of sesame as a food allergen, no methods meeting criteria for inclusion in this review have been published.

5. Discussion

5.1. Limitations of this study

The aim of this study was to gather information on commercial and research methods for allergen detection and evaluate whether they may be used to effectively detect EU allergens at the concentrations indicated by VITAL® 2.0 (Allen et al., 2014a; Taylor et al., 2014) at a range of likely serving sizes. Very recently, the reference doses were updated in VITAL® 3.0 (Remington et al., 2020), therefore, we also aimed to discuss our findings against these updated reference doses. As we analysed our data collection before VITAL® 3.0 protein reference doses were publicly available, we decided to base our assessment on the published peer-reviewed VITAL® 2.0 data, and subsequently to extrapolate our findings on the suitability of analytical methods according to the overall change that results from the updated VITAL® 3.0 reference doses. The literature regarding allergen detection is extensive and diverse. However, in the present study the need to convert analytical criteria (primarily LoD) into comparable units limited our methods to those for which this was possible. It is still the case, as has previously been noted, that there is little consensus on units in which analytical methods should report allergen content (Walker et al., 2016). We have performed unit conversions only between allergenic food weight and allergenic food protein weight and from single protein measurements where noted (e.g. converting gliadin to total wheat protein). All information regarding methods accepted for inclusion in this study is shown in tables in Supplemental Material. These tables are summaries of the information gathered from available sources (commercial method information, published research), and as such, relies entirely on the primary source of information for veracity.

Our primary indicator of method performance was LoD – the level at which an allergen containing sample may be distinguished from a sample not containing allergen. We employed this measure, rather than LoQ (the level at which quantity of allergen may be determined to predefined rigor) on the basis that accurate detection, but not quantification, at the reference dose is a minimum analytical requirement for ensuring compliance to implied action levels.

5.2. Reference doses, serving sizes and derived concentrations of target allergenic food (or total protein thereof)

In VITAL® 3.0, except for the hen's egg protein reference doses that increases by almost one order of magnitude, reference doses only

slightly increased (shrimp, milk) or decreased (lupine, soy, wheat, sesame) by two-to-three fold, or stay unchanged (peanut, hazelnut, mustard). Others have become available for the first time (cashew, celery, walnut, finfish) (Remington et al., 2020). Accordingly, the major conclusions drawn here for the six selected allergenic foods remained mostly unchanged, except for hen's egg where potentially more methods would be available to verify the VITAL® 3.0 instead of VITAL® 2.0 reference dose in serving sizes larger than 50 g (Table 3).

It should also be noted that VITAL is not a European initiative, and this being the case reference doses are not given for all allergens requiring labelling in the EU. In some cases, definitions used for certain allergenic foods in VITAL® version 2.0 and 3.0 may differ to those used within the EU (e.g. for milk), adding further complication.

Analytical methods are characterised by their ability to detect an amount of allergenic food in a quantity of sample (concentration or mass fraction). We therefore derive relative action levels using both protein reference dose and likely consumption amount in order to make analytical LoD comparable to protein reference doses in relation to a defined weight of serving size. The maximum serving size of 500 g used here is larger than would be applicable in most scenarios, meaning that more sensitivity is required for detection of VITAL reference doses than would otherwise be the case. This level was included largely to account for beverage consumption. For most non-beverages, we consider a serving size of 250 g to be adequate.

5.3. Method specific issues

5.3.1. Suitability of methodology for detection of allergens indicated by VITAL® 2.0 and 3.0 reference doses

Taking into account the discussion above, and the observations made from elsewhere in this manuscript, the Expert Group has formed opinions on the adequacy of the ability of currently available (commercial and published) methodology to detect cow's milk, hen's egg, peanut, hazelnut, wheat and soy as common allergens in food ingredients and foods. For ease and clarity, we present our opinions summarized in Table 3. However, we emphasize that distillation of the volume of information examined, and diversity of opinion within our expert group, necessitates the use of this information only in the context of the remainder of this manuscript.

The diversity of approaches used by developers has generated methods that are theoretically capable of detecting VITAL® 2.0 reference doses at serving sizes of 50–250 g or greater for most allergenic foods (Fig. 2, Table 3A).

For peanut, there are commercially available ELISA and PCR methods, which are suitably sensitive at all serving sizes examined. PCR, and MS methods not available in kit format, are also available and offer comparable or superior sensitivity. As the peanut protein reference dose did not change in VITAL® 3.0, the assessment remains identical (Table 3B). Similarly, for soy, there are commercial ELISA and PCR methods capable of detecting allergens at VITAL® 2.0 reference doses for all serving sizes, but with limited MS methods with sensitivity suitable for the largest 500 g dose size. The soy protein reference dose decreased by two-fold in VITAL® 3.0 against 2.0, but the assessment remains identical. Hazelnut could be detected at the highest serving size by multiple commercial methods and many method evaluations included a good range of food matrices. As the hazelnut protein reference dose did not change in VITAL® 3.0, the assessment remains identical.

Wheat detection is dominated by protein detection methods using especially ELISA methodology and gliadin as an analytical target. Although sensitivity is adequate for detection at all serving sizes examined, and matrix coverage is good, the questionable ability of current gluten detection methods to detect partially hydrolysed or fermented products is of concern. Additionally, the use of gluten as a target for nearly all methods means there is little diversity among available methods. The wheat protein reference dose slightly decreased in

Table 3

Opinions of the Expert Group on the suitability of examined methods for the detection of peanut, soy, hazelnut, wheat, milk and egg at VITAL® 2.0 (A) and VITAL® 3.0 (B) reference doses. For each food allergen the maximum serving size (g) in which allergen may be detected is given. The range of matrices in which methods are evaluated is shown (good = range of food types likely associated with adventitious allergen presence evaluated; moderate = most food types likely associated with adventitious allergen presence evaluated; poor = lack of good evaluation in key food types). Our opinion as to the overall suitability of current methods to detect VITAL® 2.0 Reference Doses is shown as good (detectable at maximum likely serving size in appropriate food matrices); moderate (less sensitive detection or lack of evaluation of some important food matrices) or poor (insufficient sensitivity and poor performance/no evaluation in key food matrices). The assessment of the suitability of methods for verification of VITAL® 3.0 reference doses was extrapolated from the assessment against VITAL® 2.0 reference doses by considering the change in reference doses against the methods' LoD as depicted in Fig. 2.

Allergen/Food Matrix	(A) Assessment versus VITAL® 2.0 reference doses				(B) Assessment versus VITAL® 3.0 reference doses (extrapolated from VITAL® 3.0 reference doses versus VITAL® 2.0 assessment and Fig. 2)			
	Range of matrices/ Allergen processing conditions	VITAL® 2.0 Reference dose (mg protein)	Compatible Serving Size of methods (g)/[majority of methods]	Suitability for verification of VITAL® 2.0 Reference Doses	VITAL® 3.0 Reference dose (mg protein)	Compatible Serving Size of methods (g)/[majority of methods]	Suitability for verification of VITAL® 3.0 Reference Doses	
Peanut	Good	0.2	up to 500 g [up to 50 g]	good	0.2	up to 500 g [up to 50 g]	good	
Soy	Moderate	1.0	up to 500 g [up to 250 g]	good	0.5	up to 500 g [up to 250 g]	good	
Hazelnut	Good	0.1	up to 500 g [up to 250 g]	good	0.1	up to 500 g [up to 250 g]	good	
Wheat	Moderate	1.0	up to 500 g (gluten) [up to 250 g]	moderate-good	0.7	up to 500 g (gluten) [up to 250 g]	moderate-good	
Milk	Moderate	0.1	up to 500 g (casein only) [up to 50 g]	poor-moderate	0.2	up to 500 g (casein only) [up to 50 g]	poor-moderate	
Egg	Poor	0.03	Up to 50 g [up to 5 g]	poor	0.2	Up to 250 g [up to 50 g]	poor-moderate	

VITAL® 3.0 over 2.0, however without change in the overall assessment of methods suitability to verify wheat reference dose.

Milk and especially egg methods were less frequently demonstrated as sufficient for detecting VITAL® 2.0 reference doses in a range of appropriate matrices. Known issues with recovering analytical targets after processing in matrix, scant PCR methods and low VITAL® 2.0 reference doses mean that egg is difficult to reliably detect with sufficient method robustness and sensitivity. However, several MS methods have been published which may allow for a sensitive detection of inadvertent milk and egg presence, respectively. The milk protein reference dose increased by two-fold in VITAL® 3.0 over 2.0, but the assessment remains identical. By contrast, the VITAL® 3.0 egg protein reference dose increased by almost seven-fold, which in turn allows more methods to verify the current reference dose. However, because the methods for egg protein lack good evaluation in key food types, the overall assessment of method suitability to verify VITAL® 3.0 reference dose only slightly improved from “poor” to “poor to moderate”.

It should be noted that the sensitivity of methods to detect allergens and verify VITAL® reference doses was done in the light of a final food product that is analysed and assessed for the allergen risk. Here, we concluded that a range of sensitive methods exist that would allow verifying VITAL® 2.0 and 3.0 reference doses, some even in a large 500 g serving size. Moreover, we should keep in mind that the methods are also applicable along the food manufacturing chain where verification of absence of protein below the reference doses would in principle allow using methods of less sensitivity, or if highly sensitive methods are used, to verify reference doses in ever larger serving sizes of a final product.

5.3.2. Identified gaps and needs, and recommendations for rectification

Another key goal of our study was to identify areas where lack of knowledge hindered our ability to evaluate methodological performance, which are summarized below. As will be apparent, many of these issues are related to lack of standardization or common practice.

5.3.2.1. Lack of appropriate reference materials. Reference materials allow the comparison of methodology (Lacorn and Immer, 2011). In many cases, no certified reference materials for allergenic foodstuffs are available. Further, incurred reference materials for the analysis of allergens in food matrices are desirable. We recommend increased urgency in the development and release of certified reference materials. An imperfect material is superior to no material at all. There are efforts underway by several groups to produce food allergen reference materials (e.g. Health Canada, Joint Research Centre - JRC, and National Institute of Standards and Technology - NIST). Although these have been slow to reach end-users, some reference materials have now become available for some allergens from the UK Laboratory of the Government Chemist (LGC) and from the MONIQA Association. Reference materials are clearly needed in the short-term.

5.3.2.2. Lack of standardized or exchangeable expression units for results. When possible, methods should report in one unit allowing ready comparison of methodology. This Expert Group recommends the use of **mg allergenic food protein.kg food matrix⁻¹** (mg.kg⁻¹ or mg/kg). The ability to interconvert units of expression is essential for method evaluation and comparison. Values of common unit conversions should be standardized, and we offer conversions between allergenic food (weight) and allergenic food protein (weight) as used in this manuscript as a starting point.

5.3.2.3. Lack of standardized calculation of method performance characteristics. Although the Association Of Analytical Collaboration (AOAC) provides standardized calculations for determination of LoD and LoQ for ELISA (Abbott et al., 2010), we note that most method developers do not follow these procedures. This is particularly the case for PCR and MS methods for which these standardized workflows may not be

appropriate. For PCR, other standards (e.g. MIQE) (Bustin et al., 2009) exist which are likely suitable for allergen analysis but require verification for use in the allergen field. For MS, it is likely that new standards developed through organisation efforts through analytical expert groups, such as AOAC, will be necessary.

5.3.2.4. Few determinations of method performance characteristics in multiple food matrices. The impact of the food matrix on detection has been addressed by many method developers, and is reflected in the number of methods, which were evaluated in the presence of one or more food matrices. However, few methods were characterised in more than one or two matrices, and in very few cases were analytical criteria (LoD, LoQ) evaluated in a greater variety of matrices (details in Supplementary Table).

AOAC Standard Method Performance (SMP) establish recommended matrices for a range of food allergen detection methods. However, there is no requirement for the determination of analytical criteria in different matrices, only recoveries of analyte are required. Additionally, relatively few methods use AOAC SMP criteria during method development. These factors mean that there are few methods for which evidence exists that detection limit determination occur in multiple conditions of matrix and processing. We appreciate the effort involved in producing performance characteristics in multiple matrices. However, such determinations are necessary to demonstrate the potential for successful application of methodology across a range of foodstuffs. We recommend that performance criteria (LoD, LoQ) be evaluated in matrices, which may be considered critical applications for the method in question. Such evaluation should preferentially be performed using allergen incurred into food matrix, given the limitations of spike/recovery experiments in accurately predicting method performance in foods (Taylor et al., 2009). Ring-trials on methods that use harmonized reporting units would allow for better comparison of the quantitative capabilities of the methods but are merely lacking.

5.3.2.5. Performance of analytical methods for detection of different forms of allergenic food. Method performance for the detection of different forms of allergenic food that are mentioned in regulation (e.g. egg from species other than *G. gallus*, milk from species other than *B. taurus*) is essentially unknown. If available methods are to be used to detect these allergens, characterisation of performance using these allergens is of obvious importance.

5.3.3. Summary and overall conclusions

Available analytical methods are capable of reasonably robust detection of peanut, soy, hazelnut and wheat allergens for levels at or below those dictated by VITAL® 2.0 reference doses, and as far as our extrapolation of data is acknowledged, also to the levels of VITAL® 3.0 reference doses. Milk and egg appear to be more problematic, largely due to the range of detected single proteins that demand for various factors of conversion but also matrix incompatibility of the method. The ability of methodology to detect foodborne risk in all eventualities is not a requirement of food safety regulation. If such were the case, we would have regulatory limits for the few of the many foodborne risks for which such limits currently exist. Although no guidance exists for capability of analytical methods to detect such risks before regulatory limits are enacted, we would suggest that the existence of analytical methods demonstrated to allow detection of these allergens in a plurality of circumstances, as demonstrated here at least for peanut, soy, hazelnut and wheat, is the current paradigm. Since VITAL® reference doses and the allergen detection methodology are work in progress, we aim to stimulate concerned stakeholders to use this work as a template to continuously update our findings, also for those allergens that have not been covered here.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

- Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., Van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S.L., Poms, R.E., Delahaut, P., 2010. Validation procedures for quantitative food allergen ELISA methods: community guidance and best practices. *J. AOAC Int.* 93, 442–450.
- Allen, K.J., Remington, B.C., Baumert, J.L., Crevel, R.W.R., Houben, G.F., Brooke-Taylor, S., Kruizinga, A.G., Taylor, S.L., 2014a. Allergen reference doses for precautionary labeling (VITAL 2.0): clinical implications. *J. Allergy Clin. Immunol.* 133, 156–164.
- Allen, K.J., Turner, P.J., Pawankar, R., Taylor, S., Sicherer, S., Lack, G., Rosario, N., Ebisawa, M., Wong, G., Mills, E.N.C., Beyer, K., Fiocchi, A., Sampson, H.A., 2014b. Precautionary labelling of foods for allergen content: are we ready for a global framework? *World Allergy Organ J.* 7, 10.
- Allred, L.K., Ritter, B.W., 2010. Recognition of gliadin and glutenin fractions in four commercial gluten assays. *J. AOAC Int.* 93, 190–196.
- Alvarez, P.A., Boye, J.L., 2014. Comparison of gluten recovery in gluten-incurred buckwheat flour using different commercial test kits. *Food Agric. Immunol.* 25, 200–208.
- Ansari, P., Stoppacher, N., Rudolf, J., Schuhmacher, R., Baumgartner, S., 2011. Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* 399, 1105–1115.
- Azarnia, S., Boye, J.L., Mongeon, V., Sabik, H., 2013. Detection of ovalbumin in egg white, whole egg and incurred pasta using LC-ESI-MS/MS and ELISA. *Food Res. Int.* 52, 526–534.
- Bauer, T., Kirschbaum, K., Panter, S., Kenk, M., Bergemann, J., 2011. Sensitive detection of soy (Glycine max) by real-time polymerase chain reaction targeting the mitochondrial atpA gene. *J. AOAC Int.* 94, 1863–1873.
- Bermudo Redondo, M.C., Griffin, P.B., Garzon Ransanz, M., Ellis, H.J., Ciclitira, P.J., O'Sullivan, C.K., 2005. Monoclonal antibody-based competitive assay for the sensitive detection of coeliac disease toxic prolamins. *Anal. Chim. Acta* 551, 105–114.
- Bignardi, C., Mattarozzi, M., Penna, A., Sidoli, S., Elviri, L., Careri, M., Mangia, A., 2013. A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by liquid chromatography-tandem mass spectrometry. *Food Anal. Methods* 6, 1144–1152.
- Blais, B.W., Phillippe, L., 2001. Detection of hazelnut proteins in foods by enzyme immunoassay using egg yolk antibodies. *J. Food Protect.* 64, 895–898.
- Blom, W.M., Michelsen-Huisman, A.D., van Os-Medendorp, H., van Duijn, G., de Zeeuw-Brouwer, M.L., Versluis, A., Castenmiller, J.J.M., Noteborn, H., Kruizinga, A.G., Knulst, A.C., Houben, G.F., 2018. Accidental food allergy reactions: products and undeclared ingredients. *J. Allergy Clin. Immunol.* 142, 865–875.
- Boo, C.C., Parker, C.H., Jackson, L.S., 2018. A targeted LC-MS/MS method for the simultaneous detection and quantitation of egg, milk, and peanut allergens in sugar cookies. *J. AOAC Int.* 101, 108–117.
- Buchini, L., Guzzon, A., Poms, R., Senyuva, H., 2016. Analysis and critical comparison of food allergen recalls from the European Union, USA, Canada, Hong Kong, Australia and New Zealand. *Food Addit. Contam.* 33, 760–771.
- Bugyi, Z., Török, K., Hajas, L., Adonyi, Z., Popping, B., Tömösközi, S., 2013. Comparative study of commercially available gluten ELISA kits using an incurred reference material. *Qual. Assur. Saf. Crop Foods* 5, 79–87.
- Bureau, A., 2019. VITAL Scientific Expert Panel 2019 Summary Recommendations – the New Allergen Reference Doses for VITAL® Program, Version 3.0.
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–622.
- Careri, M., Elviri, L., Lagos, J.B., Mangia, A., Speroni, F., Terenghi, M., 2008. Selective and rapid immunomagnetic bead-based sample treatment for the liquid chromatography-electrospray ion-trap mass spectrometry detection of Ara h3/4 peanut protein in foods. *J. Chromatogr. A* 1206, 89–94.
- Cheng, F., Wu, J., Zhang, J., Pan, A., Quan, S., Zhang, D., Kim, H., Li, X., Zhou, S., Yang, L., 2016. Development and inter-laboratory transfer of a decaplex polymerase chain reaction assay combined with capillary electrophoresis for the simultaneous detection of ten food allergens. *Food Chem.* 199, 799–808.
- Codex, 2008. In: FAO-WHO (Ed.), Codex Stan 118-1979 Rev. 2008, Codex Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten.
- Colgrave, M.L., Goswami, H., Blundell, M., Howitt, C.A., Tanner, G.J., 2014. Using mass spectrometry to detect hydrolysed gluten in beer that is responsible for false negatives by ELISA. *J. Chromatogr. A* 1370, 105–114.
- Colgrave, M.L., Goswami, H., Byrne, K., Blundell, M., Howitt, C.A., Tanner, G.J., 2015. Proteomic profiling of 16 cereal grains and the application of targeted proteomics to detect wheat contamination. *J. Proteome Res.* 14, 2659–2668.
- Costa, J., Ansari, P., Mafra, I., Oliveira, M.B.P.P., Baumgartner, S., 2014. Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR. *Anal. Bioanal. Chem.* 406, 2581–2590.
- Costa, J., Melo, V.S., Santos, C.G., Oliveira, M.B.P.P., Mafra, I., 2015. Tracing tree nut allergens in chocolate: a comparison of DNA extraction protocols. *Food Chem.* 187, 469–476.
- Cottenet, G., Blancpain, C., Golay, P.A., 2011. Simultaneous detection of cow and buffalo species in milk from China, India, and Pakistan using multiplex real-time PCR. *J. Dairy Sci.* 94, 3787–3793.
- Croote, D., Quake, S.R., 2016. Food allergen detection by mass spectrometry: the role of systems biology. *npj Syst. Biol. Appl.* 2.
- Croote, D., Braslavsky, I., Quake, S.R., 2017. Matrix-corrected mass spectrometry enables sensitive detection of food allergens and reveals widespread soy contamination of processed foods. *BioRxiv*. <https://doi.org/10.1101/231266>.
- Crotty, M.P., Taylor, S.L., 2010. Risks associated with foods having advisory milk labeling. *J. Allergy Clin. Immunol.* 125, 935–937.
- Cucu, T., Platteau, C., Taverniers, I., Devreese, B., de Loose, M., de Meulenaer, B., 2011. ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Addit. Contam.* 28, 1–10.
- De Angelis, E., Pilolli, R., Monaci, L., 2017. Coupling SPE on-line pre-enrichment with HPLC and MS/MS for the sensitive detection of multiple allergens in wine. *Food Contr.* 73, 814–820.
- Demmel, A., Hupfer, C., Busch, U., Engel, K.-H., 2011. Detection of lupine (*Lupinus* spp.) DNA in processed foods using real-time PCR. *Food Contr.* 22, 215–220.
- Diaz-Amigo, C., 2009. Towards a comprehensive validation of ELISA kits for food allergens. Case 2—milk. *Food Anal. Methods* 3, 351–356.
- Downs, M.L., Taylor, S.L., 2010. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *J. Agric. Food Chem.* 58, 10085–10091.
- Ellis, H.J., Rosen-Bronson, S., O'Reilly, N., Ciclitira, P.J., 1998. Measurement of gluten using a monoclonal antibody to a coeliac toxic peptide of A-gliadin. *Gut* 43, 190–195.
- Eugster, A., 2010. Alternative quantitative Bestimmung von Allergenen in Lebensmitteln mittels real-time PCR. *Dtsch. Lebensm.-Rundsch.* 106, 434–438.
- Fallahbaghery, A., Zou, W., Byrne, K., Howitt, C.A., Colgrave, M.L., 2017. Comparison of gluten extraction protocols assessed by LC-MS/MS analysis. *J. Agric. Food Chem.* 65, 2857–2866.
- Fekesova, S., Danchenko, M., Uvackova, L., Skultety, L., Hajdich, M., 2015. Using 7 cm immobilized pH gradient strips to determine levels of clinically relevant proteins in wheat grain extracts. *Front. Plant Sci.* 6, 433.
- Fernandes, T.J.R., Costa, J., Oliveira, M.B.P.P., Mafra, I., 2018. A new real-time PCR quantitative approach for the detection of shrimp crustaceans as potential allergens. *J. Food Compos. Anal.* 72, 7–14.

- Fiedler, K.L., Panda, R., Croley, T.R., 2018. Analysis of gluten in a wheat-gluten-incurred sorghum beer brewed in the presence of proline endopeptidase by LC/MS/MS. *Anal. Chem.* 90, 2111–2118.
- Flodrova, D., Benkova, D., Lastovickova, M., 2015. Study of quantitative changes of cereal allergenic proteins after food processing. *J. Sci. Food Agric.* 95, 983–990.
- Galan, A.M.G., Brohée, M., de Andrade Silva, E., van Hengel, A.J., Chassaigne, H., 2011. Development of a real-time PCR method for the simultaneous detection of soya and lupin mitochondrial DNA as markers for the presence of allergens in processed food. *Food Chem.* 127, 834–841.
- Garber, E.A.E., Perry, J., 2010. Detection of hazelnuts and almonds using commercial ELISA test kits. *Anal. Bioanal. Chem.* 396, 1939–1945.
- Geng, T., Westphal, C.D., Yeung, J.M., 2008. Detection of gluten by commercial test kits: effects of food matrices and extraction procedures, 1001, 462–475.
- Gomaa, A., Boye, J.I., 2013. Impact of thermal processing time and cookie size on the detection of casein, egg, gluten and soy allergens in food. *Food Res. Int.* 52, 483–489.
- Gomaa, A., Boye, J., 2015. Simultaneous detection of multi-allergens in an incurred food matrix using ELISA, multiplex flow cytometry and liquid chromatography mass spectrometry (LC-MS). *Food Chem.* 175, 585–592.
- Gu, S., Chen, N., Zhou, Y., Zhao, C., Zhan, L., Qu, L., Cao, C., Han, L., Deng, X., Ding, T., Song, C., Ding, Y., 2018. A rapid solid-phase extraction combined with liquid chromatography-tandem mass spectrometry for simultaneous screening of multiple allergens in chocolates. *Food Contr.* 84, 89–96.
- Hajas, L., Scherf, K.A., Török, K., Bugyi, Z., Schall, E., Poms, R.E., Koehler, P., Tömösközi, S., 2018. Variation in protein composition among wheat (*Triticum aestivum* L.) cultivars to identify cultivars suitable as reference material for wheat gluten analysis. *Food Chem.* 267, 387–394.
- Hefle, S.L., Jeannot, E., Taylor, S.L., 2001. Development of a sandwich enzyme-linked immunosorbent assay for the detection of egg residues in processed foodst. *J. Food Protect.* 64, 1812–1816.
- Heick, J., Fischer, M., Kerbach, S., Tamm, U., Popping, B., 2011a. Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. *J. AOAC Int.* 94, 1060–1068.
- Heick, J., Fischer, M., Popping, B., 2011b. First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J. Chromatogr. A* 1218, 938–943.
- Hernando, A., Mujico, J.R., Mena, M.C., Lombardia, M., Mendez, E., 2008. Measurement of wheat gluten and barley hordeins in contaminated oats from Europe, the United States and Canada by Sandwich R5 ELISA. *Eur. J. Gastroenterol. Hepatol.* 20, 545–554.
- Hirao, T., Hiramoto, M., Imai, S., Kato, H., 2006. A novel PCR method for quantification of buckwheat by using a unique internal standard material. *J. Food Protect.* 69, 2478–2486.
- Hirao, T., Watanabe, S., Temmei, Y., Hiramoto, M., Kato, H., 2009. Qualitative polymerase chain reaction methods for detecting major food allergens (peanut, soybean, and wheat) by using internal transcribed spacer region. *J. AOAC Int.* 92, 1464–1471.
- Hoffmann, B., Münch, S., Schwägele, F., Neusüß, C., Jira, W., 2017. A sensitive HPLC-MS/MS screening method for the simultaneous detection of lupine, pea, and soy proteins in meat products. *Food Contr.* 71, 200–209.
- Holzhauser, T., 2018. Protein or No protein? Opportunities for DNA-based detection of allergenic foods. *J. Agric. Food Chem.* 66, 9889–9894.
- Holzhauser, T., Röder, M., 2015. Polymerase Chain Reaction (PCR) Methods for Detecting Allergens in Foods, pp. 245–263.
- Holzhauser, T., Vieths, S., 1999. Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrices. *J. Agric. Food Chem.* 47 (10), 4209–4218.
- Holzhauser, T., Stephan, O., Vieths, S., 2002. Detection of potentially allergenic hazelnut (*corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *J. Agric. Food Chem.* 50, 5808–5815.
- Holzhauser, T., Kleiner, K., Janise, A., Roder, M., 2014. Matrix-normalised quantification of species by threshold-calibrated competitive real-time PCR: allergenic peanut in food as one example. *Food Chem.* 163, 68–76.
- Houben, G., Burney, P., Chan, C.H., Crevel, R., Dubois, A., Faludi, R., Klein Entink, R., Knulst, A., Taylor, S., Ronsmans, S., 2016. Prioritisation of allergenic foods with respect to public health relevance: report from an ILSI Europe food allergy task force expert group. *Food Chem. Toxicol.* 89, 8–18.
- Huschek, G., Bönick, J., Löwenstein, Y., Sievers, S., Rawel, H., 2016. Quantification of allergenic plant traces in baked products by targeted proteomics using isotope marked peptides. *LWT (Lebensm.-Wiss. & Technol.)* 74, 286–293.
- Iniesto, E., Jimenez, A., Prieto, N., Cabanillas, B., Burbano, C., Pedrosa, M.M., Rodriguez, J., Muzquiz, M., Crespo, J.F., Cuadrado, C., Linacero, R., 2013. Real Time PCR to detect hazelnut allergen coding sequences in processed foods. *Food Chem.* 138, 1976–1981.
- Islam, A., Mondal, S., Bhowmik, S., Islam, S., M, B., 2017. A comparative analysis of the proximate composition of wild and cultured prawn (*Macrobrachium rosenbergii*) and shrimp (*Penaeus monodon*). *Int. J. Fish. Aquat. Stud.* 5, 59–62.
- Ivens, K.O., Baumert, J.L., Taylor, S.L., 2016. Commercial milk enzyme-linked immunosorbent assay (ELISA) kit reactivities to purified milk proteins and milk-derived ingredients. *J. Food Sci.* 81, T1871–T1878.
- Jayasena, S., Smits, M., Fiechter, D., de Jong, A., Nordlee, J., Baumert, J., Taylor, S.L., Pieters, R.H., Koppelman, S.J., 2015. Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. *J. Agric. Food Chem.* 63, 1849–1855.
- Ji, J., Zhu, P., Pi, F., Sun, C., Sun, J., Jia, M., Ying, C., Zhang, Y., Sun, X., 2017. Development of a liquid chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens. *Food Contr.* 74, 79–88.
- Jiang, T.-L., Cai, Q.-F., Shen, J.-D., Huang, M.-J., Zhang, L.-J., Liu, G.-M., Cao, M.-J., 2015. Establishment of immunological methods for the detection of soybean proteins in surimi products. *LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.)* 64, 344–349.
- Johnson, P.E., Baumgartner, S., Aldick, T., Bessant, C., Giosafatto, V., Heick, J., Mamone, G., O'Connor, G., Poms, R., Popping, B., Reuter, A., Ulberth, F., Watson, A., Monaci, L., Mills, E.N., 2011. Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. *J. AOAC Int.* 94, 1026–1033.
- Johnson, P.E., Rigby, N.M., Dainty, J.R., Mackie, A.R., Immer, U.U., Rogers, A., Titchener, P., Shoji, M., Ryan, A., Mata, L., Brown, H., Holzhauser, T., Dumont, V., Wykes, J.A., Walker, M., Griffin, J., White, J., Taylor, G., Popping, B., Crevel, R., Miguel, S., Lutter, P., Gaskin, F., Koerner, T.B., Clarke, D., Sherlock, R., Flanagan, A., Chan, C.H., Mills, E.N., 2014. A multi-laboratory evaluation of a clinically-validated incurred quality control material for analysis of allergens in food. *Food Chem.* 148, 30–36.
- Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., Bigley 3rd, E. C., Whitaker, T., Williams, K.M., 2012a. Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: sugar cookies. *J. Agric. Food Chem.* 60, 4195–4203.
- Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., Bigley, E.C., Whitaker, T., Williams, K., 2012b. Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: dark chocolate. *J. Agric. Food Chem.* 60, 4204–4211.
- Khuda, S.E., Jackson, L.S., Fu, T.-J., Williams, K.M., 2015. Effects of processing on the recovery of food allergens from a model dark chocolate matrix. *Food Chem.* 168, 580–587.
- Kiening, M., Niessner, R., Drs, E., Baumgartner, S., Krska, R., Bremer, M., Tomkies, V., Reece, P., Danks, C., Immer, U., Weller, M.G., 2005. Sandwich immunoassays for the determination of peanut and hazelnut traces in foods. *J. Agric. Food Chem.* 53, 3321–3327.
- Koch, P., Schäppi, G.F., Poms, R.E., Wüthrich, B., Anklam, E., Battaglia, R., 2003. Comparison of commercially available ELISA kits with human sera-based detection methods for peanut allergens in foods. *Food Addit. Contam.* 20, 797–803.
- Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., Waiblinger, H.-U., 2010. Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. *Eur. Food Res. Technol.* 230, 367–374.
- Köppel, R., van Velsen-Zimmerli, F., Bucher, T., 2012. Two quantitative hexaplex real-time PCR systems for the detection and quantification of DNA from twelve allergens in food. *Eur. Food Res. Technol.* 235, 843–852.
- Koppelman, S.J., Knulst, A.C., Koers, W.J., Penninks, A.H., Peppelman, H., Vlooswijk, R., Pigmans, I., van Duijn, G., Hessing, M., 1999. Comparison of different immunochemical methods for the detection and quantification of hazelnut proteins in food products. *J. Immunol. Methods* 229, 107–120.
- Korte, R., Lepski, S., Brockmeyer, J., 2016a. Comprehensive peptide marker identification for the detection of multiple nut allergens using a non-targeted LC-HRMS multi-method. *Anal. Bioanal. Chem.* 408, 3059–3069.
- Korte, R., Monneuse, J.-M., Gemrot, E., Metton, I., Humpf, H.-U., Brockmeyer, J., 2016b. New high-performance liquid chromatography coupled mass spectrometry method for the detection of lobster and shrimp allergens in food samples via multiple reaction monitoring and multiple reaction monitoring cubed. *J. Agric. Food Chem.* 64, 6219–6227.
- Kotowicz, M., Adamczyk, E., Bania, J., 2007. Application of a duplex-PCR for detection of cows' milk in goats' milk. *Ann. Agric. Environ. Med.* 14, 215–218.
- Lacorn, M., Immer, U., 2011. Allergen determination in food: reference materials and traceability of results. *Accred. Qual. Assur.* 16, 449–452.
- Lacorn, M., Ristow, R., Weiss, T., Immer, U., 2014. Collaborative tests of ELISA methods for the determination of egg white protein and caseins used as fining agents in red and white wines. *Food Anal. Methods* 7, 417–429.
- Ladenburger, E.-M., Dehmer, M., Grünberg, R., Waiblinger, H.-U., Stoll, D., Bergemann, J., 2018. Highly sensitive matrix-independent quantification of major food allergens peanut and soy by competitive real-time PCR targeting mitochondrial DNA. *J. AOAC Int.* 101, 170–184.
- Lamberti, C., Acquadro, E., Corpiello, D., Giribaldi, M., Decastelli, L., Garino, C., Arlorio, M., Ricciardi, C., Cavallarin, L., Giuffrida, M.G., 2016. Validation of a mass spectrometry-based method for milk traces detection in baked food. *Food Chem.* 199, 119–127.
- Lexhaller, B., Tompos, C., Scherf, K.A., 2016. Comparative analysis of prolamin and glutenin fractions from wheat, rye, and barley with five sandwich ELISA test kits. *Anal. Bioanal. Chem.* 408, 6093–6104.
- Lexhaller, B., Tompos, C., Scherf, K.A., 2017. Fundamental study on reactivities of gluten protein types from wheat, rye and barley with five sandwich ELISA test kits. *Food Chem.* 237, 320–330.
- Li, Y., Song, C., Zhang, K., Wang, M., Yang, K., Yang, A., Jin, B., 2008. Establishment of a highly sensitive sandwich enzyme-linked immunosorbent assay specific for ovomucoid from hen's egg white. *J. Agric. Food Chem.* 56, 337–342.
- Liao, Y.-S., Kuo, J.-H., Chen, B.-L., Tsuei, H.-W., Lin, C.-Y., Lin, H.-Y., Cheng, H.-F., 2017. Development and validation of the detection method for wheat and barley gluteins using mass spectrometry in processed foods. *Food Anal. Methods* 10, 2839–2847.
- López-Calleja, I., González, I., Fajardo, V., Rodríguez, M.A., Hernández, P.E., García, T., Martín, R., 2004. Rapid detection of cows' milk in sheeps' and goats' milk by a species-specific polymerase chain reaction technique. *J. Dairy Sci.* 87, 2839–2845.

- López-Calleja, I.M., de la Cruz, S., Pegels, N., Gonzáles, I., García, T., Martín, R., 2013b. Development of a real time PCR assay for detection of allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *Food Control* 30 (2), 480–490.
- López-Calleja, I.M., de la Cruz, S., Pegels, N., Gonzáles, I., García, T., Martín, R., 2013a. High resolution TaqMan real-time PCR approach to detect hazelnut DNA encoding for ITS rDNA in foods. *Food Chem.* 141, 1872–1880.
- López-Calleja, I.M., García, A., Madrid, R., García, T., Martín, R., González, I., 2017. Multiplex ligation-dependent probe amplification (MLPA) for simultaneous detection of DNA from sunflower, poppy, flaxseed, sesame and soy allergenic ingredients in commercial food products. *Food Contr.* 71, 301–310.
- Lutter, P., Parisod, V., Weymuth, H., 2011. Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. *J. AOAC Int.* 94, 1043–1059.
- Mafra, I., Ferreira, Isabel M.P.L.V.O., Faria, M.A., Oliveira, B.P.P., 2004. A novel approach to the quantification of bovine milk in ovine cheeses using a duplex polymerase chain reaction method. *J. Agric. Food Chem.* 52, 4943–4947.
- Mamone, G., Ferranti, P., Chianese, L., Scafuri, L., Addeo, F., 2000. Qualitative and quantitative analysis of wheat gluten proteins by liquid chromatography and electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* 14, 897–904.
- Manfredi, A., Mattarozzi, M., Giannetto, M., Careri, M., 2015. Multiplex liquid chromatography-tandem mass spectrometry for the detection of wheat, oat, barley and rye prolamins towards the assessment of gluten-free product safety. *Anal. Chim. Acta* 895, 62–70.
- Marnoch, R., Diosady, L.L., 2006. Production of mustard protein isolates from oriental mustard seed (*Brassica juncea* L.). *J. Am. Oil Chem. Soc.* 83, 65–69.
- Martín-Fernández, B., Costa, J., Oliveira, M.B.P.P., López-Ruiz, B., Mafra, I., 2016a. Combined effects of matrix and gene marker on the real-time PCR detection of wheat. *Int. J. Food Sci. Technol.* 51, 1680–1688.
- Martín-Fernández, B., Costa, J., de los Santos-Álvarez, N., López-Ruiz, B., Oliveira, M.B. P.P., Mafra, I., 2016b. High resolution melting analysis as a new approach to discriminate gluten-containing cereals. *Food Chem.* 211, 383–391.
- Martínez-Esteso, M.J., Nørgaard, J., Brohee, M., Haraszi, R., Maquet, A., O'Connor, G., 2016. Defining the wheat gluten peptide fingerprint via a discovery and targeted proteomics approach. *J. Proteom.* 147, 156–168.
- Matsuda, R., Yoshioka, Y., Akiyama, H., Aburatani, K., Watanabe, Y., Matsumoto, T., Morishita, N., Sato, H., Mishima, T., Gamo, R., Kihira, Y., Maitani, T., 2006. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *J. AOAC Int.* 89, 1600–1608.
- Mattarozzi, M., Bignardi, C., Elviri, L., Careri, M., 2012. Rapid shotgun proteomic liquid chromatography–electrospray ionization–tandem mass spectrometry-based method for the lupin (*Lupinus albus* L.) multi-allergen determination in foods. *J. Agric. Food Chem.* 60, 5841–5846.
- Mattarozzi, M., Milioli, M., Bignardi, C., Elviri, L., Corradini, C., Careri, M., 2014. Investigation of different sample pre-treatment routes for liquid chromatography-tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine. *Food Contr.* 38, 82–87.
- Maudet, C., Taberlet, P., 2001. Detection of cows' milk in goats' cheeses inferred from mitochondrial DNA polymorphism. *J. Dairy Res.* 68, 229–235.
- Mayer, H.K., Bürger, J., Kaar, N., 2012. Quantification of cow's milk percentage in dairy products – a myth? *Anal. Bioanal. Chem.* 403, 3031–3040.
- Monaci, L., van Hengel, A.J., 2007. Effect of heat treatment on the detection of intact bovine beta-lactoglobulins by LC mass spectrometry. *J. Agric. Food Chem.* 55, 2985–2992.
- Monaci, L., van Hengel, A.J., 2008. Development of a method for the quantification of whey allergen traces in mixed-fruit juices based on liquid chromatography with mass spectrometric detection. *J. Chromatogr. A* 1192, 113–120.
- Monaci, L., Losito, I., Palmisano, F., Visconti, A., 2010a. Identification of allergenic milk proteins markers in fined white wines by capillary liquid chromatography-electrospray ionization-tandem mass spectrometry. *J. Chromatogr. A* 1217, 4300–4305.
- Monaci, L., Nørgaard, J.V., van Hengel, A.J., 2010b. Feasibility of a capillary LC/ESI-Q-TOF MS method for the detection of milk allergens in an incurred model food matrix. *Anal. Methods* 2, 967.
- Monaci, L., Losito, I., De Angelis, E., Pilolli, R., Visconti, A., 2013. Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry. *Rapid Commun. Mass Spectrom.* 27, 2009–2018.
- Monaci, L., Pilolli, R., De Angelis, E., Godula, M., Visconti, A., 2014. Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *J. Chromatogr. A* 1358, 136–144.
- Monaci, L., Pilolli, R., De Angelis, E., Carone, R., Pascale, M., 2016. LC-tandem mass spectrometry as a screening tool for multiple detection of allergenic ingredients in complex foods. *Acta Imeko* 5, 5.
- Morishita, N., Kamiya, K., Matsumoto, T., Sakai, S., Teshima, R., Urisu, A., Moriyama, T., Ogawa, T., Akiyama, H., Morimatsu, F., 2008. Reliable enzyme-linked immunosorbent assay for the determination of soybean proteins in processed foods. *J. Agric. Food Chem.* 56, 6818–6824.
- Morishita, N., Matsumoto, T., Morimatsu, F., Toyoda, M., 2014. Detection of soybean proteins in fermented soybean products by using heating extraction. *J. Food Sci.* 79, T1049–T1054.
- Morón, B., Cebolla, Á., Manyani, H., Álvarez-Maqueda, M., Megías, M., Thomas, M.d.C., López, M.C., Sousa, C., 2008. Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide. *Am. J. Clin. Nutr.* 87, 405–414.
- Muraro, A., Hoffmann-Sommergruber, K., Holzhauser, T., Poulsen, L.K., Gowland, M.H., Akdis, C.A., Mills, E.N., Papadopoulos, N., Roberts, G., Schnadt, S., van Ree, R., Sheikh, A., Vieths, S., EAACI Food Allergy and Anaphylaxis Guidelines Group, 2014b. EAACI Food Allergy and Anaphylaxis Guidelines. Protecting consumers with food allergies: understanding food consumption, meeting regulations and identifying unmet needs. *Allergy* 69 (11), 1464–1472.
- Muraro, A., Werfel, T., Hoffmann-Sommergruber, K., Roberts, G., Beyer, K., Bindslev-Jensen, C., Cardona, V., Dubois, A., duToit, G., Eigenmann, P., Fernandez Rivas, M., Halken, S., Hickstein, L., Host, A., Knol, E., Lack, G., Marchisotto, M.J., Niggemann, B., Nwaru, B.I., Papadopoulos, N.G., Poulsen, L.K., Santos, A.F., Skypala, I., Schoepfer, A., Van Ree, R., Venter, C., Worm, M., Vlieg-Boerstra, B., Panesar, S., de Silva, D., Soares-Weiser, K., Sheikh, A., Ballmer-Weber, B.K., Nilsson, C., de Jong, N.W., Akdis, C.A., 2014a. EAACI Food Allergy and Anaphylaxis Guidelines: diagnosis and management of food allergy. *Allergy* 69, 1008–1025.
- Nwaru, B.I., Hickstein, L., Panesar, S.S., Roberts, G., Muraro, A., Sheikh, A., 2014. Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy* 69, 992–1007.
- Pahlavan, A., Sharma, G.M., Pereira, M., Williams, K.M., 2016. Effects of grain species and cultivar, thermal processing, and enzymatic hydrolysis on gluten quantitation. *Food Chem.* 208, 264–271.
- Panda, R., Zoerb, H.F., Cho, C.Y., Jackson, L.S., Garber, E.A., 2015. Detection and quantification of gluten during the brewing and fermentation of beer using antibody-based technologies. *J. Food Protect.* 78, 1167–1177.
- Panda, R., Boyer, M., Garber, E.A.E., 2017. A multiplex competitive ELISA for the detection and characterization of gluten in fermented-hydrolyzed foods. *Anal. Bioanal. Chem.* 409, 6959–6973.
- Park, D.L., Coates, S., Brewer, V.A., Garber, E.A., Abouzied, M., Johnson, K., Ritter, B., McKenzie, D., 2005. Performance Tested Method multiple laboratory validation study of ELISA-based assays for the detection of peanuts in food. *J. AOAC Int.* 88, 156–160.
- 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, L.S., 2015. Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *J. Agric. Food Chem.* 63, 10669–10680.
- Pedersen, M.H., Holzhauser, T., Bisson, C., Conti, A., Jensen, L.B., Skov, P.S., Bindslev-Jensen, C., Brinch, D.S., Poulsen, L.K., 2008. Soybean allergen detection methods - a comparison study. *Mol. Nutr. Food Res.* 52, 1486–1496.
- Pelaez-Lorenzo, C., Diez-Masa, J.C., Vasallo, I., de Frutos, M., 2010. Development of an optimized ELISA and a sample preparation method for the detection of beta-lactoglobulin traces in baby foods. *J. Agric. Food Chem.* 58, 1664–1671.
- Pilolli, R., De Angelis, E., Godula, M., Visconti, A., Monaci, L., 2014. Orbitrap (TM) monostage MS versus hybrid linear ion trap MS: application to multi-allergen screening in wine. *J. Mass Spectrom.* 49, 1254–1263.
- Pilolli, R., Chaudhari, R., Palmisano, F., Monaci, L., 2017a. Development of a mass spectrometry immunoassay for unambiguous detection of egg allergen traces in wines. *Anal. Bioanal. Chem.* 409, 1581–1589.
- Pilolli, R., De Angelis, E., Monaci, L., 2017b. Streamlining the analytical workflow for multiplex MS/MS allergen detection in processed foods. *Food Chem.* 221, 1747–1753.
- Planque, M., Arnould, T., Dieu, M., Delahaut, P., Renard, P., Gillard, N., 2016. Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs. *J. Chromatogr. A* 1464, 115–123.
- Planque, M., Arnould, T., Renard, P., Delahaut, P., Dieu, M., Gillard, N., 2017. Highlight on bottlenecks in food allergen analysis: detection and quantification by mass spectrometry. *J. AOAC Int.* 100, 1126–1130.
- Platteau, C., De Loose, M., De Meulenaer, B., Taverniers, I., 2011a. Quantitative detection of hazelnut (*Corylus avellana*) in cookies: ELISA versus real-time PCR. *J. Agric. Food Chem.* 59, 11395–11402.
- Platteau, C., De Meulenaer, B., De Loose, M., Taverniers, I., 2011b. Detection of hazelnut in food by PCR. *Commun. Agric. Appl. Biol. Sci.* 76, 93–96.
- Platteau, C., Cucu, T., Taverniers, I., Devreese, B., De Loose, M., De Meulenaer, B., 2013. Effect of oxidation in the presence or absence of lipids on hazelnut and soybean protein detectability by commercial ELISA. *Food Agric. Immunol.* 24, 179–192.
- Poms, R.E., Agazzi, M.E., Bau, A., Brohee, M., Capelletti, C., Nørgaard, J.V., Anklam, E., 2005. Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. *Food Addit. Contam.* 22, 104–112.
- Posada-Ayala, M., Alvarez-Llamas, G., Maroto, A.S., Maes, X., Munoz-Garcia, E., Villalba, M., Rodriguez, R., Perez-Gordo, M., Vivanco, F., Pastor-Vargas, C., Cuesta-Herranz, J., 2016. Corrigendum to "Novel liquid chromatography-mass spectrometry method for sensitive determination of the mustard allergen Sin a 1 in food" [Food Chem. 183 (2015) 58–63]. *Food Chem.* 206, 291.
- Regulation Eu 1169/2011, 2011. 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 text with EEA relevance. In: Commission, E. (Ed.), Official Journal of the European Union, Official Journal of the European Union, p. 63.
- Remington, B.C., Baumert, J.L., Blom, W.M., Houben, G.F., Taylor, S.L., Kruizinga, A.G., 2015. Unintended allergens in precautionary labelled and unlabelled products pose significant risks to UK allergic consumers. *Allergy* 70, 813–819.

- Remington, B.C., Westerhout, J., Meima, M.Y., Blom, W.M., Kruizinga, A.G., Wheeler, M. W., Taylor, S.L., Houben, G.F., Baumert, J.L., 2020. Updated population minimal eliciting dose distributions for use in risk assessment of 14 priority food allergens. *Food Chem. Toxicol.* 139, 111259.
- Restani, P., Uberti, F., Tarantino, C., Ballabio, C., Gombac, F., Bastiani, E., Bolognini, L., Pavanello, F., Danzi, R., 2013. Collaborative interlaboratory studies for the validation of ELISA methods for the detection of allergenic fining agents used in wine according to the criteria of OIV resolution 427–2010 modified by OIV-comex 502–2012. *Food Anal. Methods* 7, 706–712.
- Rodrigues, N.P.A., Givisiez, P.E.N., Queiroga, R.C.R.E., Azevedo, P.S., Gebreyes, W.A., Oliveira, C.J.B., 2012. Milk adulteration: detection of bovine milk in bulk goat milk produced by smallholders in northeastern Brazil by a duplex PCR assay. *J. Dairy Sci.* 95, 2749–2752.
- Sajic, N., Oplatowska-Stachowiak, M., Streppel, L., Drijfhout, J.-W., Salden, M., Koning, F., 2017. Development and in-house validation of a competitive ELISA for the quantitative detection of gluten in food. *Food Contr.* 80, 401–410.
- Schalk, K., Lang, C., Wieser, H., Koehler, P., Scherf, K.A., 2017. Quantitation of the immunodominant 33-mer peptide from alpha-gliadin in wheat flours by liquid chromatography tandem mass spectrometry. *Sci. Rep.* 7, 45092.
- Schalk, K., Koehler, P., Scherf, K.A., 2018. Targeted liquid chromatography tandem mass spectrometry to quantitate wheat gluten using well-defined reference proteins. *PLoS One* 13, e0192804.
- Scharf, A., Kasel, U., Wichmann, G., Besler, M., 2013. 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.* 61, 10261–10272.
- Scherf, K.A., 2016. Gluten analysis of wheat starches with seven commercial ELISA test kits—up to six different values. *Food Anal. Methods* 10, 234–246.
- Sealey-Voyksner, J.A., Khosla, C., Voyksner, R.D., Jorgenson, J.W., 2010. Novel aspects of quantitation of immunogenic wheat gluten peptides by liquid chromatography-mass spectrometry/mass spectrometry. *J. Chromatogr. A* 1217, 4167–4183.
- Siegel, M., Mutschler, A., Boernsen, B., Pietsch, K., Waiblinger, H.-U., 2013. Food matrix standards for the quantification of allergenic food ingredients using real-time PCR. *Eur. Food Res. Technol.* 237, 185–197.
- Simonato, B., Mainente, F., Tolin, S., Pasini, G., 2011. Immunochemical and mass spectrometry detection of residual proteins in gluten fined red wine. *J. Agric. Food Chem.* 59, 3101–3110.
- Sorell, L., Lopez, J.A., Valdes, I., Alfonso, P., Camafeita, E., Acevedo, B., Chirido, F., Gavilondo, J., Mendez, E., 1998. An innovative sandwich ELISA system based on an antibody cocktail for gluten analysis. *FEBS Lett.* 439, 46–50.
- Taylor, S.L., Nordlee, J.A., Niemann, L.M., Lambrecht, D.M., 2009. Allergen immunoassays—considerations for use of naturally incurred standards. *Anal. Bioanal. Chem.* 395, 83–92.
- Taylor, S.L., Baumert, J.L., Kruizinga, A.G., Remington, B.C., Crevel, R.W., Brooke-Taylor, S., Allen, K.J., Allergen Bureau of, A., New, Z., Houben, G., 2014. Establishment of reference doses for residues of allergenic foods: report of the VITAL expert Panel. *Food Chem. Toxicol.* 63, 9–17.
- Taylor, S.B., Christensen, G., Grinter, K., Sherlock, R., Warren, L., 2018. The allergen Bureau VITAL program. *J. AOAC Int.* 101, 77–82.
- Tolin, S., Pasini, G., Simonato, B., Mainente, F., Arrigoni, G., 2012. Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens. *Food Contr.* 28, 321–326.
- Tortajada-Genaro, L.A., Santiago-Felipe, S., Morais, S., Gabaldón, J.A., Puchades, R., Maquieira, Á., 2011. Multiplex DNA detection of food allergens on a digital versatile disk. *J. Agric. Food Chem.* 60, 36–43.
- Uvackova, L., Skultety, L., Bekesova, S., McClain, S., Hajdúch, M., 2013. MS(E) based multiplex protein analysis quantified important allergenic proteins and detected relevant peptides carrying known epitopes in wheat grain extracts. *J. Proteome Res.* 12, 4862–4869.
- Valdes, I., Garcia, E., Llorente, M., Mendez, E., 2003. Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol. *Eur. J. Gastroenterol. Hepatol.* 15, 465–474.
- Vatansever, B., Munoz, A., Klein, C.L., Reinert, K., 2017. Development and optimisation of a generic micro LC-ESI-MS method for the qualitative and quantitative determination of 30-mer toxic gliadin peptides in wheat flour for food analysis. *Anal. Bioanal. Chem.* 409, 989–997.
- Waiblinger, H.-U., Schulze, G., 2018. Action levels for food allergens: an approach for official food control in Germany. *J. AOAC Int.* 101, 17–22.
- Walker, M.J., Burns, D.T., Elliott, C.T., Gowland, M.H., Mills, E.N., 2016. Is food allergen analysis flawed? Health and supply chain risks and a proposed framework to address urgent analytical needs. *Analyst* 141, 24–35.
- Weber, D., Raymond, P., Ben-Rejeb, S., Lau, B., 2006. Development of a liquid chromatography-tandem mass spectrometry method using capillary liquid chromatography and nanoelectrospray ionization-quadrupole time-of-flight hybrid mass spectrometer for the detection of milk allergens. *J. Agric. Food Chem.* 54, 1604–1610.
- Whitaker, T.B., Williams, K.M., Trucksess, M.W., Slate, A.B., 2005. Immunochemical analytical methods for the determination of peanut proteins in foods. *J. AOAC Int.* 88, 161–174.
- Woods, D.L., Capcara, J.J., Downey, R.K., 1991. The potential of mustard (*Brassica juncea* (L.) Coss) as an edible oil crop on the Canadian Prairies. *Can. J. Plant Sci.* 71, 195–198.
- Xiao, G., Qin, C., Wenju, Z., Qin, C., 2016. Development of a real-time quantitative PCR assay using a TaqMan minor groove binder probe for the detection of α -lactalbumin in food. *J. Dairy Sci.* 99, 1716–1724.
- Yamakawa, H., Hirose, T., Kuroda, M., Yamaguchi, T., 2007. Comprehensive expression profiling of rice grain filling-related genes under high temperature using DNA microarray. *Plant Physiol.* 144, 258–277.
- Yeung, J.M., Newsome, W.H., Abbott, M.A., 2000. Determination of egg proteins in food products by enzyme immunoassay. *J. AOAC Int.* 83, 139–143.