Guidance on how to analyse unintended food allergen presence

Considerations for selection of allergen ELISA and DNA kits to analyse unintended food allergen presence

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Nathalie Smits[1], Marleen Voorhuijzen - Harink[1], Stella Cochrane[2], Piotr Robouch[3], Elena Cubero Leon[3], Federica Cattapan[4], Marjan van Ravenhorst[5], Isabel Taverniers[6], Nicola Bortoletto[7], Charlene Taylor[8], Christine Bruggeman[1], Winnie Tao[1], Andries Koops[1], Elise Hoek - van den Hil[1]

[1] Wageningen Food Safety Research, Wageningen University & Research, Akkermaalsbos 2, 6708 WB Wageningen, the Netherlands
[2] Unilever SEAC, Colworth Science Park, Bedfordshire, UK
[3] European Commission, Joint Research Centre, Retieseweg 111, 2440 Geel, Belgium
[4] Mérieux NutriSciences Italy, Via Fratta 25, 31023 Resana (TV), Italy
[5] Allergenen Consultancy B.V., Oosteinde 85d, 3925 LB Scherpenzeel, the Netherlands
[6] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Burg. Van Gansberghelaan 115, 9820 Merelbeke, Belgium
[7] Generon S.p.A., Via San Geminiano 4, 41030 San Prospero (MO), Italy
[8] Neogen Corporation, 620 Lesher Place, Lansing MI 48912, USA

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P.O. Box 230, 6700 AE Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.wfsr@wur.nl, www.wur.eu/food-safety-research. WFSR is part of Wageningen University & Research.

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Those who have contributed to the conceptualization of this document or to the underlying work are:

Nathalie Smits	Wageningen Food Safety Research	nathalie.smits@wur.nl
Elise Hoek	Wageningen Food Safety Research	elise.hoek@wur.nl
Marleen Voorhuijzen	Wageningen Food Safety Research	marleen.voorhuijzen@wur.nl
Andries Koops	Wageningen Food Safety Research	andries.koops@wur.nl
Christine Bruggeman	Wageningen Food Safety Research	christine.bruggeman@wur.nl
Winnie Tao	Wageningen Food Safety Research	winnie.tao@wur.nl
Stella Cochrane	Unilever	stella.A.Cochrane@unilever.com
Samantha Strand	Unilever	samantha.Strand@unilever.com
Marjan van Ravenhorst	Allergenen Consultancy	m.vanravenhorst@allergenenconsultancy.nl
Jason Robotham	BioFront Technologies	robotham@biofronttech.com
Jeyran Akhoundzadeh	Danone	jeyran.akhoundzadeh@danone.com
Anastasia Meimaridou	Danone	anastasia.meimaridou@danone.com
Nicola Bortoletto	Generon	nicola.bortoletto@generon.it
Federica Cattapan	Mérieux NutriSciences	federica.cattapan@mxns.com
Emilliano Dominicis	Mérieux NutriSciences	emiliano.de.dominicis@mxns.com
Samim Saner	Mérieux NutriSciences	samim.saner@mxns.com
Masayoshi Tomiki	Morinaga	m-tomiki-aj@morinaga.co.jp
Pauline Titchener	Neogen	PTitchener@neogen.com
Gabriela Lopez	Neogen	GLopez@neogen.com
Jonathan Walsh	Neogen	JWalsh4@neogen.com
Pieter Vos	Nutrilab	p.vos@nutrilab.nl
Bart van der Nagel	Nutrilab	bvdNagel@nutrilab.nl
Sander Tomei	Nutrilab	s.tomei@nutrilab.nl
Bénédicte Renaville	Progenus	renaville.b@progenus.be
Celine Muys	Progenus	muys.c@progenus.be
Martin Candia	Romer Labs	martin.candia@dsm-firmenich.com
Elena Cubero Leon	Joint Research Centre of the European Commission (EC-JRC)	elena.cubero-leon@ec.europa.eu
Piotr Robouch	Joint Research Centre of the European Commission (EC-JRC)	piotr.robouch@ec.europa,eu
Marc de Loose	Instituut voor Landbouw, visserij- en Voedingsonderzoek (ILVO)	marc.deloose@ilvo.vlaanderen.be
Isabel Taverniers	ILVO	isabel.taverniers@ilvo.vlaanderen.be

List of abbreviations

Ab	Antibody
ANZ	Australia and New Zealand
AOAC	Association of Official Analytical Collaboration
CCFH	Codex Committee on Food Hygiene
CCFL	Codex Committee on Food Labelling
CE	Capillary Electrophoresis
CEN	European Committee for Standardization
CT	Cycle Treshold
cdPCR	chamber digital PCR
DNA	Deoxyribonucleic Acid
ddPCR	droplet digital PCR
dPCR	digital PCR
ED	Eliciting Dose
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization
EU	European Union
FAO	Food and Agriculture Organisation
FARRP	Food Allergy Research and Research Program
FN	False Negative
FSA	Food Standards Agency
GSLPF	General Standard for the Labelling of Pre-packaged Foods
ILSI	International Life Sciences Institute
ISO	International Organization for Standardization
(EC-)JRC	Joint Research Centre of the European Commission
LC-MS	Liquid Chromatography – Mass Spectrometry
LFD	Lateral Flow Devices
LFIA	Lateral Flow Immunoassays
LOD	Limit of Detection
LOQ	Limit of Quantification
MU	Measurement Uncertainty
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
PAL	Precautionary Allergen Labelling
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
qPCR	quantitative PCR
RfD	Reference Dose
SPE	Solid Phase Extraction
UAP	unintentional allergen presence
UK	United Kingdom
USA	United States of America
VITAL	Voluntary Incidental Trace Allergen Labelling
WHO	World Health Organisation

Preface

We have compared the performance of commercial allergen ELISA and PCR kits for the detection of egg, milk, soy and celery in different food matrices within the project AL-kits. The results can be used by food industry and commercial and control laboratories to select the kits suitable for the matrix of interest. Most of these results have been published or will be published soon in peer -review journals [2, 3, 4].

During the project a lot of experience was gained regarding allergen testing with different types of allergen kits in different food matrixes. Furthermore, we experienced that a guidance on how to select the best fit for purpose allergen test for analyzing the presence of unintended food allergens was missing.

Therefore, we have built together with other allergen experts involved in the project from WFSR, EC-JRC, ILVO, Unilever, Allergenen Consultancy, Neogen, BioFront Technologies, Danone, Generon, Merieux NutriSciences, Morinaga, Nutrilab, Progenus and Romer Labs this guidance document.

We hope this document can guide you and help you in selecting the best fit for purpose allergen test.

Best regards,

Nathalie G.E. Smits and Elise F. Hoek - van den Hil

1 Introduction

Approximately 220 million people worldwide are affected by food allergies [5]. The majority of allergic reactions to foods are however caused by a limited number of food allergens. In most countries, there are regulations requiring clear labelling on the packaging of a prioritized list of allergens when they are deliberately added to foods as ingredients. In addition to food allergens intentionally added as ingredients, there is a potential risk to the allergic individual from food allergens whose presence in food products is unintended but may be a consequence of accidental cross-contact along the production chain. There continues to be a lack of regulation or detailed guidance on how to perform the risk assessment for the unintentional allergen presence (UAP) and when to apply 'precautionary allergen labelling' (PAL, i.e. "may contain" labelling) to communicate the risk to consumers. This means, that although there has been great progress in understanding allergic reactions to food allergens in allergic individuals and also in provision of industry guidance on risk assessment and risk management of UAP, PAL is not applied on a consistent basis generating confusion among allergic consumers and food industry and reducing the credibility of such labels.

To date, no legal threshold doses exist at an EU level that indicate the amount of UAP needed to trigger use of PAL. Most recently however, the Netherlands have implemented a new allergen policy (as of January 1st 2024) with a two year transition period. It requires that PAL is based on a risk assessment and indicates that PAL should be triggered by the ED05 based Reference Doses (RfDs), as recommended by an FAO/WHO Expert Committee (detailed below) [6]. At the time of writing however, detailed guidance for industry implementation and enforcement had not been published. The threshold or Reference Dose (RfD) is a dose of allergenic protein below which a defined proportion of allergic people are likely to experience an adverse reaction. The RfD could be based on the Eliciting Dose (ED), i.e. the ED_{01}, which is the dose that is estimated to elicit a reaction in 1% of individuals within the allergic population. The use of RfDs as a basis for PAL risk assessment was originally proposed by the Voluntary Incidental Trace Allergen Labelling (VITAL) Scientific Expert Panel from the Australia-New Zealand Allergen Bureau [7]. The first set of VITAL recommended RfDs was released in 2012. Whilst the VITAL approach and proposed RfDs were well received, and many food companies and authorities (ANSES, FSA) have endorsed their use, there has been a continued lack of consensus regarding regulation of PAL by authorities. Most recently the Codex Committee on Food Labelling (CCFL) began reviewing provisions relevant to allergen labelling, including developing guidance on the use of PAL. The Food and Agriculture Organisation (FAO) of the United Nations and the World Health Organisation (WHO) established an ad hoc Expert Consultation to address a range of questions on Risk Assessment of Food Allergens in response to the CCFL requesting scientific advice to support this. One of the tasks of the Expert Consultation was to establish threshold levels in foods for the selected priority allergens. The Expert Consultation also recommended use of RfDs for PAL risk assessment [8]. The science-based establishment of RfDs was a break-through in food allergen management. If the use of RfDs can be successfully harmonised and applied in a standardised way across the food industry it will allow the implementation of minimum limits of detection and quantification for analytical methods and ensure that PAL is applied in a consistent, appropriate manner across different countries.

In the near future, changes in CODEX ALIMENTARIUS, and potentially regional and national regulations may occur in which RfDs will be introduced. Irrespective of the establishment of harmonised RfDs, food producers need to have robust food allergen risk assessment tools and have their risk management in place, followed by controlling the risk at the lowest practicable level. To do so, the use of reliable analytical techniques, able to detect and quantify relevant allergens at the required level, is fundamental. There are many challenges that come with analyzing allergens in the wide range of food matrices that exist. There is not one technique that is able to detect all food allergens. Also, the type of matrix greatly influences the detection capacity.
This document gives guidance on how to analyze for the presence of unintended food allergens and how to select the best fit for purpose allergen test.

Unintentional allergen presence as a result of cross contact, can for example occur as a result of agricultural crop comingling, or through the use of shared lines in factories. For how to prevent cross-contact is outside the scope of this document, also out of scope is detailed coverage of when and how to undertake a quantitative risk assessment. We would like to refer the readers to guidance documents on food allergen management, such as from Codex [9], ILSI [10], FooddrinkEurope [11] and Allergen Bureau [12]. We would also like to refer the readers to ILSI [10] for guidance regarding sampling strategy, such as where to sample, how much to sample and how many samples to take. The present guidance document focuses on how to select a suitable test and generate robust, fit for purpose, test data for a given sample when it has been deemed that analytical data is required. Three different main methods are available for allergen detection, namely ELISA (an antibody-based method), PCR (a DNA-based method) and LC-MS (a chromatography-based mass spectrometric method). In this document we mainly focus on ELISA and PCR, as at the moment these are most widely applied by industry and governmental parties.

For further reading on:

- How to prevent cross-contact and on allergen management: guidance documents from Codex [9], ILSI [10], FooddrinkEurope [11] and Allergen Bureau [12].
- Sampling strategy: guidance document from ILSI [10].

The present guidance document covers food proteins that can trigger allergies or other immune-mediated reactions [13]. This means that gluten (proteins present in several cereals, which are the trigger in coeliac disease) are included, but sulphites and lactose (which is related to a failure to properly digest lactose due to a lack of lactase, referred to as lactose intolerance) are not. Regarding lactose, as a milk derived ingredient that contains milk proteins it does still require labelling and management, but this is in relation to IgE mediated allergy and not lactose intolerance.

Food allergen management is a shared responsibility and requires implementation and provision of accurate information on food allergens along the entire supply chain. Analytical methods play an important role in this respect. such that target groups of this guidance document are ingredient suppliers, food manufacturers and commercial and governmental laboratories that execute analyses which are used to make allergen management decisions.

It is recommended that a dialogue is initiated with the selected analytical laboratory and/or kit supplier. A good laboratory and/or kit supplier should offer a confidential service and welcome early discussion of any proposed testing and associated study aims. Thereby providing advice on correct test selection and study design, covering the concepts covered in this guidance document.

This guidance document is structured as follows. Chapter 2 provides basic information on food allergens and describes definitions used. In Chapter 3, the technical aspects regarding detection of allergens in food matrices are discussed. As is the main focus of the chapter: the pros and cons for a particular method, as it is shown that the performance of a given method for a given target is strongly dependent on the matrix analyzed. Chapter 4 presents considerations to select the best fit for purpose allergen detection kit and how to properly execute it to obtain reliable data. Finally in Chapter 5, the guidance continues with interpretation and reporting of results and provides a number of examples to illustrate technical challenges.

The aim of this document is to give guidance on how to analyze for the presence of unintended food allergens and how to select the best fit for purpose allergen test.

2 Food allergen prioritisation, definition and source

2.1 Food allergen prioritisation

A food allergen is for most consumers a normal harmless food ingredient, but it can cause an (immediate) allergic reaction in susceptible persons [14]. In this document, under the term food allergens the compounds that trigger an (IgE-mediated) immune response (e.g. egg, soya and peanut) and gluten (harmful to people with coeliac disease) are considered. Food allergens included are proteins that can trigger allergies or other immune- mediated reactions and therefore sulphites or lactose will not be addressed and neither will their specific methodological challenges. Allergens can be present in food products as ingredients (intentional) or as a result of cross-contact (unintentional). Eight priority food allergens are currently defined by CODEX ALIMENTARIUS in the General Standard for the Labelling of Pre-packaged Foods (GSLPF) [15] (Figure 1). Many countries adopt the food allergens listed by the Codex in their legislative framework, but differences exist. For example, the EU also includes celery, mustard, sesame, lupin and molluscs (according to Annex II of (EU) 1169/2011 [16]), while these are not included in the US, most countries in South-America and China for example. The priority allergen families for the main regions worldwide are the allergens that are deemed to pose a public health risk such that these are also the allergens that require risk assessment and management when present unintentionally. As regional prioritization is subject to amendments, it is recommended to consult (governmental) websites on national food allergen labelling requirements for up to date information on food allergen labelling requirements (Table 1).

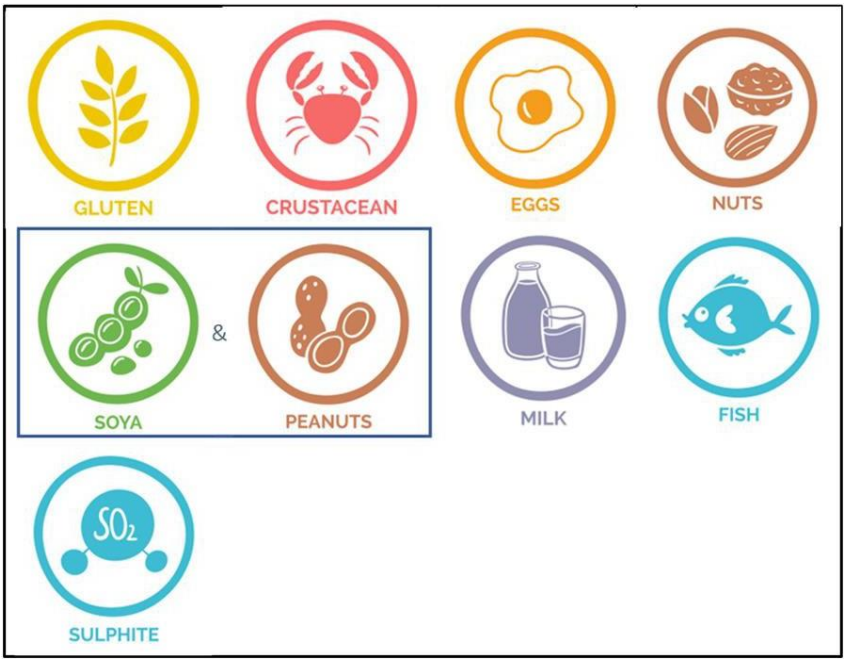


Figure 1 *The eight priority food allergens as defined by CODEX ALIMENTARIUS.*

Prioritization of allergens can change continuously as a result of new insights from clinical studies and regional differences in food preferences and consequential sensitization. Moreover, it is expected that the FAO/WHO recommendation to revise the global priority allergens [17] will be adopted by the Codex, and that regional and national authorities can add food allergens for mandatory or voluntary labelling as seen fit to protect allergic consumers in their region. To manage (unintentional) allergens in food resulting from cross-contact, food business operators should follow good manufacturing practices such as the code of practice developed by Codex Committee on Food Hygiene (CCFH), or the risk assessment tool developed by the Allergen Bureau [7].

In Europe, Regulation (EU) No 1169/2011 requires that allergen information is provided on both prepacked and non-prepacked foods when allergens are intentionally incorporated in foods irrespective of the amount. The required labelling is also irrespective of the form, i.e. the original form or as a product derived from it, and labelling is mandated regardless of the processing of the ingredient, e.g. heating, hydrolysis, fermentation or fractionation. However, some ingredients are exempted from allergen labelling (for the EU, Table 2) because they have undergone extensive processing that reduces the causative protein responsible for the allergy, combined with considerations of how they are used (which may be restricted in some cases) such that they ultimately pose a low risk to allergic consumers. The list of exemptions may differ per global region, for example, a fish protein produced by yeast (an ice structuring protein) is exempted by the FDA [18].

Table 1 *Links to websites that provide up to date information on national food allergen labelling requirements. Also included are links to the original food law or governmental guidance, with the notion that these links will probably not be updated once new law or guidance is published. Links have been accessed at December 6, 2023.*

Region	Website
Australia & New Zealand	Food Standards Code | Food Standards Australia New Zealand
Canada	https://foodallergycanada.ca/food-allergy-basics/
Europe	https://europa.eu/youreurope/business/product-requirements/food-labelling/general-rules/index_en.htm https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:304:0018:0063:en:PDF
Japan	https://www.caa.go.jp/en/policy/food_labeling/
USA	https://www.fda.gov/food/food-labeling-nutrition/food-allergieshttps://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance- industry-questions-and-answers-regarding-food-allergen-labeling-edition-5
Overview	https://farrp.unl.edu/IRChart

Table 2 *Exemptions on food allergen labelling according to Annex II (EU) 1169/2011. Not all foods exempted in the EU are exempted in other regions.*

Food allergen	Exemptions
Cereals containing gluten, including wheat, rye, barley, oats	• Glucose syrups from wheat and barley, including dextrose • Wheat based maltodextrins • Distillates made from cereals
Milk	• Whey used for making alcoholic distillates including ethyl alcohol of • agricultural origin • Lactitol
Soy	• Fully refined soybean oil • Tocopherols and derivatives (from oil) • Phytosterol(esters) and stanol esters from oil
Fish	• Fish gelatine used as carrier for vitamin or carotenoid preparations • Fish gelatine or Isinglass used as fining agent in beer and wine
Tree nuts	• Nuts used in distillates for spirits

2.2 Food allergen definitions

Different definitions for allergens or related terms could be used by food manufacturers or clinicians. To harmonise the wordings throughout this document, the most used and thus relevant definitions are listed in Table 3. In addition, we briefly introduce words that are used to describe allergens from the biological perspective: *allergen*, *protein*, *peptide*, *epitope*. The definitions emphasize either the legislative, food production, analytical or clinical perspective.

The term *allergen* as such is not used in Regulation (EU) No 1169/2011, but Annex II [19] lists 14 *substances or products causing allergies or intolerances*. For the purpose of the clarity of this document we name the Annex II substances and the food ingredients as listed by Codex *Food allergens* (Table 3). Allergenicity of food allergens is caused by *proteins* from the source of the allergenic food. Food allergens are

proteins with the ability to trigger a reaction in sensitive consumer, thereby a patient, mediated by the patient's immune system. The protein(s) that elicits a response in a patient is commonly called *allergenic protein* (Table 3).

Table 3 *Definitions of allergens used in the document.*

Definitions	Description	Examples	Reference
Food Allergen	A substance or product causing allergies that require mandatory labelling as described in a regional food law	Such as the lists in the Codex or Annex II of (EU) 1169/2011.	Annex II, REGULATION (EU) No 1169/2011 [16]
Allergenic ingredient[20]	A substance or product causing allergies intentionally added to a food product	Substances listed in e.g. Annex II, 1169/2011, but seen as ingredient added to a food product, e.g. hazelnuts added to chocolate; sesame seeds to bread, celery to spreads	RASFF SOPS for food and feed [21]
Total protein of the allergenic ingredient in food	All proteins representing the allergenic ingredient, where the allergenic ingredient is analysed by a method to detect one or more of its proteins and where the protein quantity is expressed as mass of the total protein of the allergenic ingredient per mass of food expressed in mg kg^{-1}	As previous described in the column left, where the total protein fraction of the allergenic ingredient is expressed as the total amount of allergenic ingredient protein per total amount of food product	JRC technical report [22, 23], FAO/WHO Risk assessment of food allergens - part 3 [24]
Fractionated allergenic protein	Proteins or peptides fractionated from a food allergen, to capitalize on its physiochemical properties	Wheat gluten, wheat glutenins, milk casein, cow milk hydrolysate, soy hydrolysate	
Protein family	A group of evolutionarily related proteins, reflected by their related function and amino acid sequence similarity	Gliadin (wheat), parvalbumin (fish), tropomyosin (crustaceans and molluscs)	EMBL [25]
Allergenic protein, peptide or epitope	Proteins or peptides derived from a food allergen triggering an allergenic response in a patient, e.g. IgE mediated allergy, coeliac disease, etc.	Allergenic proteins: gluten, gliadin, γ-gliadin (Tri a 20). Example of one epitope from Tri a 20: IQPQQPAQYEVIR	

2.3 Occurrence and source of food allergens

Food allergens as listed in legislation encompass a broad range of food and processing aids, which do not always bear the name of the original allergenic ingredient. Sometimes the name highlights the function of the protein fraction and lacks the association with the original allergen. Taking milk as an example, the adverse response could be induced by: a) the whole, unprocessed source material, such as cow milk; b) curd and whey derived from milk by coagulation; c) fractionated allergenic proteins as casein or lactoperoxidase. None of these products are exempt from allergen labelling. These protein fractions should also be considered in managing possible cross-contacts.

A more detailed overview of unexpected allergens is published by the Allergen Bureau.[26] To assess the potential presence of an allergen in a food matrix, and to select a fit for purpose test kit and interpret its result, insights in the original source, degree of fractionation or degree of processing of the food is necessary.

Examples of unexpected allergens in food

- *Food additives.* Proteins provide excellent technical and nutritional functionality. Especially proteins from large food commodities such as cereals, legumes, milk or egg can be found in an endless number of food products. Their use as e.g. foam enhancer, emulsifier or nutritional supplement, can cause a loss of association with the original food allergen.
- *Allergenic proteins as processing aid*. Casein purified from cow milk, or gelatine fractionated from fish waste, may be used for clarification, decolouration or deodorization of beers and wines. Commercial milk ELISA's may detect either total milk proteins, total whey proteins, caseins or β-lactoglobulins, or any combination thereof [27]. Furthermore, only a selection of available fish ELISA tests will be fit for purpose since most fish ELISA's target parvalbumin.
- *Hydrolysed proteins.* Proteins hydrolysates are produced from protein isolates or processing by-products from milk, whey, beef, fish, legumes and cereals. Proteolytic digestion result in peptide products that are used to improve food's nutritional value or its physiochemical properties such as flavour, viscosity or foaming. They can also be used as a processing aid to prevent e.g. coagulation and starch aging. Proteolytic hydrolysis does not remove allergenicity, which is shown for extensively hydrolysed caseins, that are still capable of eliciting allergic responses in milk-allergic individuals [28]. Therefore, hydrolysed milk formulas are not exempt from labelling in the EU and US. In order to select a fit for purpose test kit, at least two questions need to be addressed in this case: i) what is the source protein used to produce the hydrolysate, especially from hydrolysed vegetable proteins, and ii) what is the type of protease, and does it break down the protein targeted by the test kit antibody.

2.4 Similarity between allergens, phylogenetic relationships and cross-reactivity

Cross-reactivity issues are more common in ELISA, designed to recognize a food allergen ingredient, and will give a false positive reading with another food commodity that does not contain any of the targeted food allergen ingredients. Antibodies (used in ELISA) raised against a target food allergen ingredient may show cross-reactivity between proteins with homologous amino acid sequences. Homologous amino acid sequences can for instance be seen in food ingredients derived from phylogenetically related species. How foods are phylogenetically related can be found in the National Center for Biotechnology Information (NCBI) taxonomy database [29]. As an example, all beans belong to the *Fabacea* family (pea, faba bean, chickpea etc.), from which only three species (soy, peanut, lupin) are listed as food allergens. This may indicate that cross-reactivity in antibody-based test kits can occur equally well: ELISAs for soy, lupin and peanut need to be checked for cross- reactivity with other legumes. A basic understanding of phylogenetic relationships and cross-reactivity may be needed to select the best fit for purpose kit and to anticipate on possible misinterpretations from a test result.

Questions that should be raised are:

- *Does my product contain ingredients that can cross-react in this kit?*
- *For which food commodities is the test kit validated?*

A recent AOAC guidance on the validation of allergen ELISA[30] cross reactivity-testing gives insights for kit users on kit selection criteria to minimize the risk for cross-reactivity. Cross-reactivity can occur dependent on target, the matrix and the processing. In selecting a kit, awareness on possible cross-reactivities is needed.

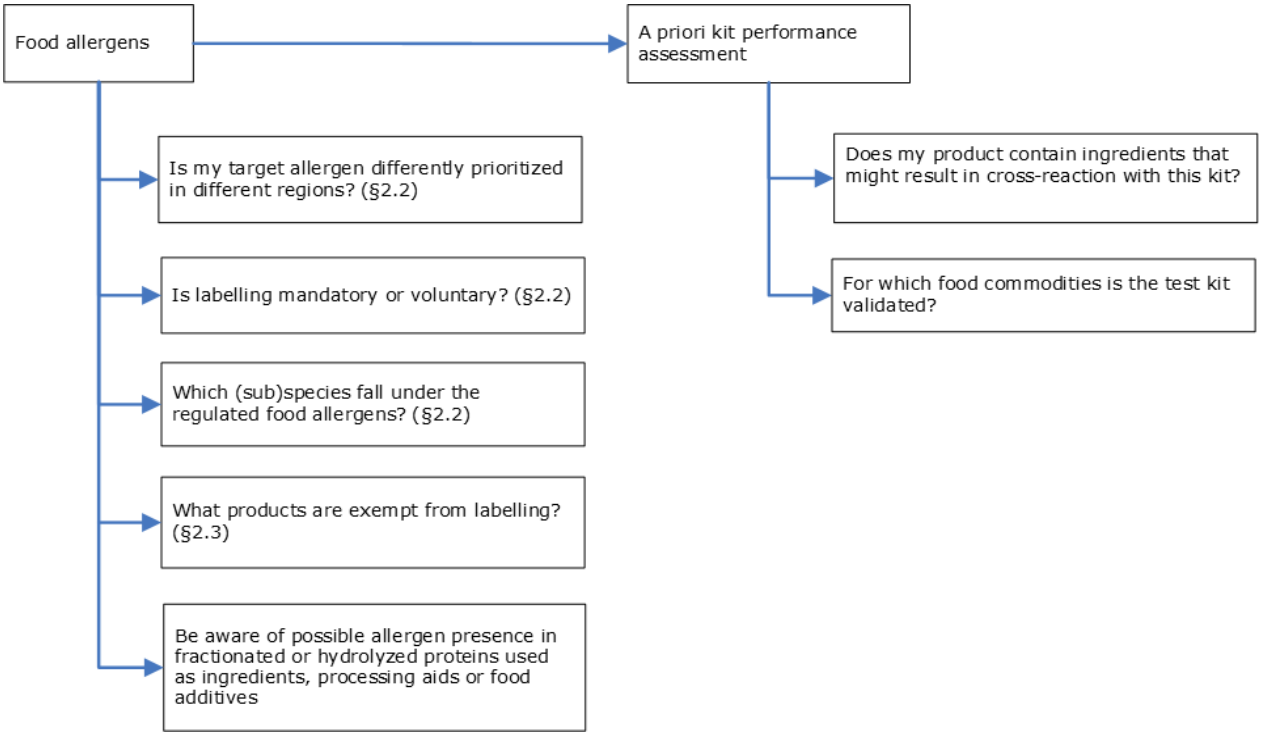
Examples of cross-reactivity issues in the food production chain

Cross-reactivity can limit kit selection or even lead to unwanted discussions with food safety authorities, e.g. when cross-reactivity confuses a species not listed as food allergen with an allergenic relative (false positive). A few examples on cross-reactivity cases:

- *Apiaceae and* celery. Celery is a typical example how cross-reactivity limits kit selection. To our knowledge only one ELISA is available for celery, because most antibodies developed so far for the detection of celery cross-react with carrot, parsley and fennel, an ELISA with these antibodies will lead to false positives, leaving DNA or LC-MS based methods the better option for celery detection.
- *Rosaceae, almond and mahaleb*. In 2015, the UK FSA authorised to recall a batch of ground cumin which was tested positive for almond, which was not declared on the label. However, additional testing revealed that the cumin was contaminated with mahaleb (*Prunus mahaleb*), a close relative of almond (*Prunus amygdaloides*), but not a food allergen, therefore the recall was unnecessary. Mahaleb is used as a spice in the Middle- East and is used as an ingredient in spice blends.
- *Brassicaceae, mustard and rapeseed.* This example is also addressed in §5.4.1. The Brassicaceae offer a particularly wide range of (non)food crops ranging from cabbage, kale, turnip to wasabi, mustard and rapeseed. Rapeseed (*Brassica rapa*) and canola (*B. napus*) are widely used for spice oil production. Moreover, the protein-rich cake left after oil extraction from both food and non-food oils is used as feed. Recently, several industry initiatives have been launched to market rapeseed protein as novel food [31, 32, 33, 34]. For example, hydrolysed rape seed protein is used as flavour enhancer in products ranging from soups to spices. The oil seed Brassicas are closely related to mustard, which is listed as a food allergen in the EU and Canada (Table 1). 'Mustard' covers different species including white or yellow (*Sinapis alba*), brown (*B. juncea*) and black mustard (*B. nigra*), which are generally grown in different regions. In Europe, *S. alba* is used as cover crop. Common weeds are *B. napus* and *B. rapa* and the wild *S. arvensis* (wild mustard). Cover crops and weeds can easily contaminate crops that are harvested early in the growing season (e.g. winter wheat). Cross-contact at farm level in combination with ELISA cross- reactivity may pose a problem for food producers [35]. In a recent case, mustard was allegedly found in wheat flour in Italy [36], which led to a product recall request by the food safety authorities. However, ELISA cannot discriminate different *Brassica* and *Sinapis* species. In this case, DNA analysis by PCR revealed that rapeseed was the cross- contacting agent and the recall was wrong.

2.5 Summary

Detection and quantification are essential for the management of allergens in the supply chain. First, it is important to be familiar with regional differences in regulation and prioritization of allergens. Allergens can end up in food products due to factors that are not foreseen, such as cross-contact or fraud earlier in the production chain. The presence of allergens can be missed due to insufficient knowledge about unexpected sources, and accurate detection and/or quantification can be impacted by matrix effects (more in Chapter 3), intensive processing (Chapter 4) or misinterpretation of the results (Chapter 5), possibly leading to false negative results, false positive results, or over and under reporting. Proteins from non-regulated species, mostly but not necessarily, closely related to the food allergen can also result in false positive results or impact accurate quantification. Some initial information to know or obtain from the kit supplier is summarized in the checklist below, and is built upon in the following chapters.



3 Technical aspects and matrices

3.1 Brief introduction of the methods

3.1.1 Antibody-based assays

The enzyme-linked immunosorbent assay (ELISA) is currently the most widely used technique to determine the presence of allergens in food. Reasons for its wide acceptance are the relative low costs, ease of implementation in the laboratory and the ability to semi-quantify allergenic protein ingredients in food matrices within hours. Still, this apparent ease of use must be balanced against the technology's inherent limitations.

ELISA tests are based on the direct detection of a protein of interest using antibodies that specifically recognizes an antigenic determinant (epitope), however not necessarily the specific allergenic epitope. Once the protein is bound to the antibody, the antibody/antigen complex can be measured through an enzymatic reaction that allow a semi-quantification, i.e. a good estimate of the concentration of these target proteins in the sample. For technical details regarding ELISA methods see Annex 1.

A number of commercial kits are currently available to identify various allergenic targets, usually to be applied in dedicated laboratories. In addition to conventional ELISAs, lateral flow immunoassays (LFIAs) as readout systems are also applied. Although LFIAs are currently mainly used in allergen management to identify small amounts of allergens on surfaces or in washing waters, they can also be applied to detect trace allergens in food. LFIAs are faster and easier to perform than conventional ELISA tests, as they do not require instruments or specialized areas like laboratories, thereby offering a simple and quick alternative to traditional methods of detecting food allergens and allow applications at site, i.e. allow on-site testing. Although their reliability is often questioned due to the lack of quantitative information, LFIAs can be used after proper validation. However, it is important to consider the composition of the sample material. For detailed information on ELISA and LFIA/LFD see Annex 1.

3.1.2 PCR

DNA-based methods provide a number of advantages in the allergen field, for instance the higher thermal stability of DNA compared to proteins, the ability to discriminate between closely related species, and the ability for an untargeted approach. Polymerase Chain Reaction (PCR), a technique in which a specific DNA sequence is amplified, is the most applied and straightforward DNA-based method. Next to the mostly used real-time or quantitative PCR (qPCR), digital PCR (dPCR) which enables absolute quantification without the need for calibrators or the setting up of a standard curve and (next-generation) sequencing for untargeted allergen identification, can be applied. In this document the term PCR includes qPCR and dPCR.

Despite these advantages, quantification of allergens using DNA-based methods is challenging. Although all methods used for allergen detection require a conversion to convert the detected marker to total protein content, as allergen thresholds are preferably expressed in mg of total protein of the allergenic food ingredient [37], for DNA-based method an additional conversion is required, namely the conversion of the amount of DNA to the amount of protein. For detailed information on PCR see Annex 2.

3.1.3 LC-MS

A third method applied for allergen monitoring is LC-MS, which is an analytical technique that combines separation of components via liquid chromatography (LC) and detection via mass spectrometry (MS). This document focusses on ELISA and PCR methods as these are mostly applied by industry and governmental parties. For detailed information on LC-MS see Annex 3.

3.1.4 ELISA, PCR and LC-MS for allergen monitoring

The choice for a method greatly depends on the target and the matrix, however, all methods have their specific advantages and limitations, either general or target/matrix specific. It can be stated that none of the methods is able to detect all priority allergens in all relevant matrices. For instance, PCR cannot differentiate milk or eggs from the tissues of their corresponding species, cow and chicken respectively, thereby not able to distinguish between non-allergenic (e.g. meat) or allergenic (milk/eggs) components. On the other hand, some ELISAs are not able to differentiate celery from carrot and other members of the Apiaceae that are not regulated allergens, as they show extensive cross-reactivity. These examples stress the importance of careful selection of a method, or even methods, taking into account the following considerations. More information on when to use ELISA or PCR is provided in §4.1.3.

General considerations for method selection

- None of the methods (ELISA, PCR and LC-MS) detects the eliciting factor (allergenic epitopes). Instead, all methods target a marker to indirectly prove the presence of an allergenic epitope by detecting an alternative species-specific peptide-, epitope of a protein- or DNA-marker.
- Quantification is challenging with all methods as in a proper reference measurement system or food allergen quantification, the food allergen must be expressed in mg total protein of an allergenic food ingredient [22]. The amount of detected marker by either ELISA, PCR or LC- MS therefore needs to be converted to total protein content, which is particularly challenging for PCR (see Annex 2 of this report for detailed information).
- All matrices contain interfering compounds that may or may not affect the analysis/outcome. See §3.2 for further details on matrix contribution.

Notes

- High cross-reactivity with antibody-based assays (ELISA, LFIA/LFD): celery.
- Not possible with PCR: egg, milk.

3.2 Food matrices

3.2.1 Matrix definition

The general term "matrix" in this document refers to an aggregate group including single food ingredients (e.g. corn flour), food components (e.g. ice cream flavouring fruit compote), semi-finished food (e.g. creme-caramel preparation), finished food ready-to-eat (e.g. sandwich) or ready-to-reheat (e.g. frozen pizza). Food supplements are included as a distinct item given the peculiar composition. With the general term "process" we are here referring to any manipulation step of the matrix.

3.2.2 Matrix characteristics

To understand how matrices interfere with detection it is useful to divide matrix components in their main food constituents: carbohydrates, fats, proteins, inorganics (salts), metabolites (includes many interfering compounds), natural additives (colorants, emulsifiers, preservatives) and synthetic additives (dyes). These constituents can influence analysis in multiple ways; hence it is recommended to evaluate matrix composition before selecting the appropriate method of analysis.

Examples of the effect of processing [38, 39]:

- Processed starches can gel when immersed in extraction buffers and heated. This in turn will limit the bioavailability of protein and DNA for downstream analysis.
- Lipids can encapsulate the allergenic protein ingredients masking them to antibody detection.
- Emulsifiers, thickening agents and gluten networks can seize water from extraction buffer impairing allergen solubilization and DNA extraction.
- Sugars and insoluble proteins can generate positive results by sticking to antibodies a- specifically.
- Products from the Maillard reaction can mask targeted epitopes from the allergenic ingredient impairing antibody binding.
- pH and bivalent cations can interfere in enzyme activity and antibody affinity to the epitope from the allergenic ingredient and to non-target proteins.
- Dyes and pigments can inhibit enzymes or cause non-specific signals in instrument analysis.

The food journey from farm to fork normally includes multiple manipulation steps. Raw plant materials need to be harvested and transported, and they are often sieved, sorted, stored and packed. Animals need to be slaughtered, cut and then refrigerated or frozen. As food harvesting and processing equipment generally processes multiple foods, these manipulations can be a primary cause of cross-contact with food allergens. In addition, when entering industrial food production, vegetal and animal raw materials undergo different treatments which can impact the capacity to detect the presence of the allergens.

Any of the industrial treatments may affect protein and DNA integrity in multiple ways. High or low temperature, change in pH, oxidization, and shear stress may denature the protein or modify the tertiary structure thereby impairing the capacity of antibodies to bind the targeted epitopes or creating sites for non- specific binding.

Examples of industrial treatments:

- Hermal treatments, such as drying, pasteurization, cooking, autoclaving, roasting, baking, grilling, frying, where temperature, pressure, humidity and duration are the process variables.
- Non thermal treatments, as high-pressure, pulsed light, cold plasma, ultrasound, pulsed electric field, and enzyme hydrolysis.
- Mechanical, like stirring, spraying, grinding and homogenization.
- Physical, like extraction, isolation and purification.
- Biochemical, like fermentation, leavening, and enzymatic hydrolysis.

Examples of effects of industrial treatments

- Chemical reactions with other components (e.g. Maillard reactions, or heating proteins in the presence of glucose and/or wheat proteins) can create large molecular complexes resulting in masking the allergenic protein because they are not accessible by antibodies or by trypsin digestion and overcome the size of exclusion systems used in sample preparation steps for MS detection [40].
- Part of the proteins can be selectively removed from the product or specifically modified/degraded by biochemical reactions; as a consequence, they cannot be traced anymore [41].
- DNA can be oxidized and fragmented (or removed as well) impairing the possibility to be detected using PCR based technologies [42, 43].

3.2.3 Matrix effects: stacking layers

All in all, each matrix is the result of stacking layers of unique characteristics: matrix composition, the effect of the three macroconstituents, interfering microconstituents, and processing (Figure 2). This together translates into the so-called **matrix effect** that can be defined as the way and the level in which the matrix influences the outcome of the experiments. We must remember that any experiment is the sum of three steps:

1. Sampling ➔ 2. Extraction ➔ 3. Detection

The matrix can affect all steps and thereby biasing the results qualitatively and quantitatively.

The effect on sampling is mainly connected to the *homogeneity* of the matrix; if the allergen is suspected to segregate in niches or present in hot spots in the processing line (e.g. dead spots in machinery), or in the sample itself (e.g. precipitation of fractionation into a biochemical niche like an oil droplet). This must be taken into account either by increasing the amount of ground sample or, when possible, segregating the niches analyzing them separately. Also, matrices difficult to homogenize (e.g. dry raisins) might represent a serious issue in sampling bias. Details on sampling are described in the ILSI Guidance [10].

Extraction and detection can be biased by the *composition* of the matrix (the sum of the stacking layers). Here we define analytical interferences as all the factors that impact the steps 2 and 3 and thus the ultimate measurement, analysis, or detection outcome. The matrix can affect the analytical outcome because of multiple analytical interferences influenced by three fundamental parameters:

1. The quantity of the sample: amount of proteins/DNA that can be extracted
2. The quality of the sample: antigenicity of proteins/DNA amplifiability
3. The purity of the sample: substances which are co-extracted with proteins/DNA interfering with enzymatic reactions

Many of the interferents were already mentioned in this chapter. However, it is noteworthy that for DNA based analysis the integrity of the DNA extracted from the sample can be qualified in terms of quality, quantity and purity, providing a certain degree of safety, while for proteins on regular basis it is too time consuming to check for their quality and quantity in the extracts prepared, but it is possible to assess for protein purity. In addition, see Table 5, §5.3.1 on how to deal with false negative outcomes.

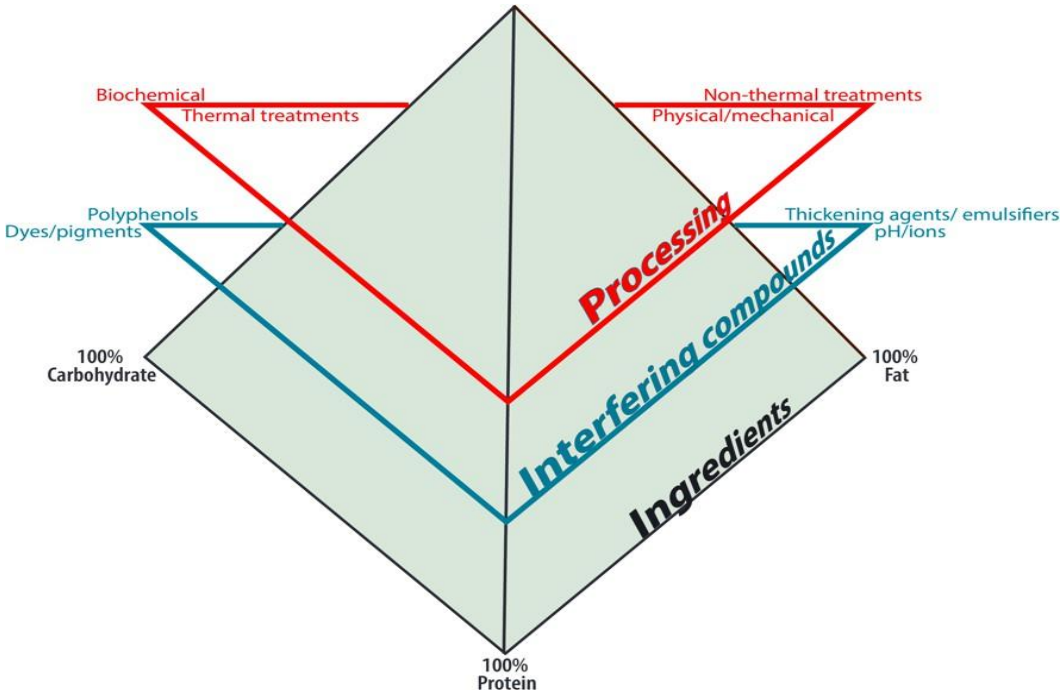


Figure 2 *Stacking layers of unique characteristics of the matrix composition, the effect of the three macroconstituents (green), interfering microconstituents (blue) and processing (brown).*

3.2.4 Interfering compounds

There is no generic rule defining what is interfering in immunochemical and molecular methods for the detection of food contaminants. Food is a complex and ever-changing matrix in comparison to e.g. plasma, blood, or urine samples. The most recent ISO norms for food testing using molecular methods (i.e. ISO 20224) emphasize the necessity to run many controls in each experimental session when evaluating a new matrix.

Interfering compounds might lead to either false positive or false negative results in both immunochemical and DNA methods. ELISA/LFD assays are normally more exposed to this risk, because mostly samples are directly tested, i.e. no sample target extraction or purification before ELISA/LFD analysis. Therefore the experimental sample might still contain:

- Denaturing agents (pH, oxidizing compounds)
  - Effect: modification in epitope hampering recognition by antibodies
- Affinity modifier (salts, pH)
  - Effect: modification of affinity of antibodies for epitopes
- Crosslinker
  - Effect: A not specific binding of antibodies
- Dyes and pigments
  - Effect: increasing background noise, i.e. reducing sensitivity and the dynamic range
- Masking (physical and chemical)
  - Effect: modification in epitope recognition by antibodies, and/or bioavailability of targets

DNA based analysis requires extraction and purification of the DNA using dedicated procedures or kits, resulting in the presence of less interfering compounds affecting the PCR detection methods. However, despite the extraction procedure, interfering compounds as dyes, lipids and polyphenols known to impact PCR can still be present in the DNA extracts. Therefore, controls to check for possible PCR inhibition are of utmost importance.

3.2.5 Timing of cross-contact

Cross-contact is defined as the unintentional incorporation of a residue or other trace amount of a food allergen into another food and can occur at different stages of processing: (i) pre-processing, (ii) during-processing, and (iii) post-processing (see Figure 3) [44,45].

Examples of cross-contact

- Ice creams can contain small amounts of pistachio because unintended residues of these nuts were present in flavouring compotes (pre/during processing), or ice-cream containers (during/post-processing).
- Wheat kernels can contain traces of mustard due to co-mingling in the field (pre- processing), during harvest (processing) or in the storage (post-processing).
- Wheat flour can contain traces of lupine due to cross-contact in the bakery (during- processing).

The moment of this possible cross-contact can not only affect the allergenicity of the compound, it also has an effect on its detectability. If cross-contact occurs during pre-processing, for instance during transport from farm to food producing facility, the allergenic compound will be subjected to the processing procedure of the final food product, possibly resulting in the protein or DNA of the allergenic species to be affected. On the other hand, if cross-contact occurs during post-processing, the proteins and DNA will not be affected but will be present in their native conformation. Possible cross-contact moments are depicted in Figure 3.

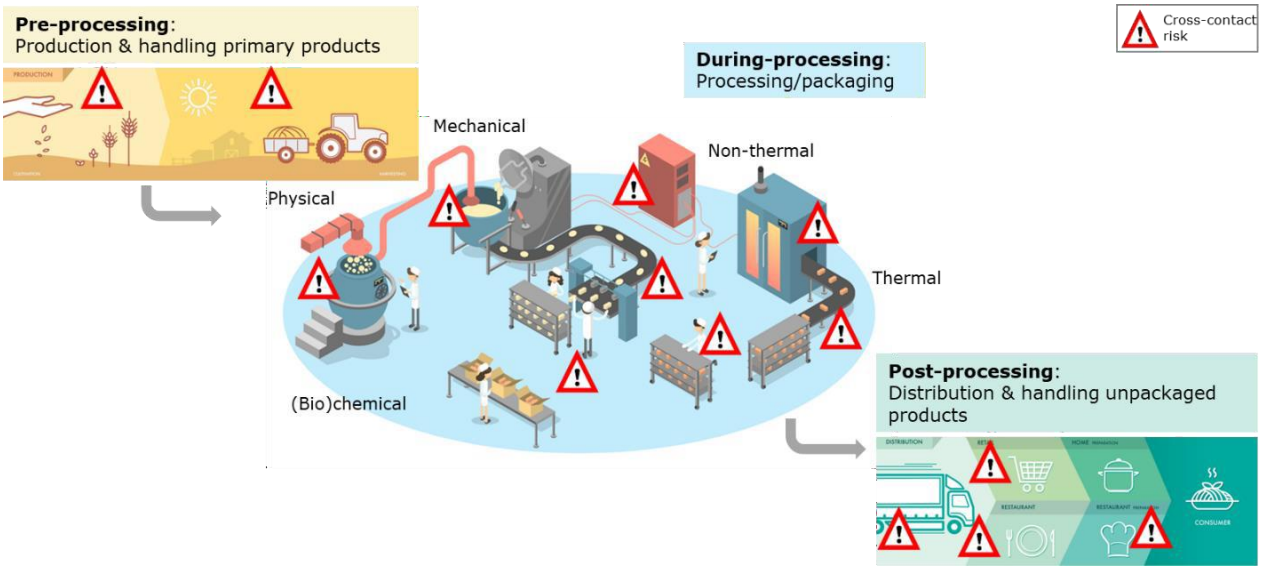
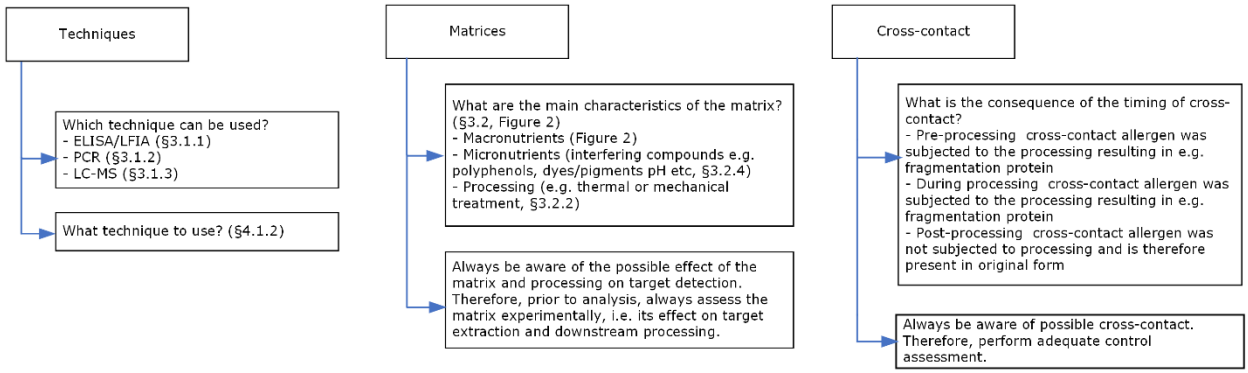


Figure 3 *Possible entry points for food allergen cross-contact in the food chain from farm to fork.*

As processing can change the form of the allergen and consequently affect the recovery of the protein or DNA, it is important to understand where and how in the food chain the matrices came in contact with the allergen.

3.3 Summary

In the majority of cases ELISA or PCR can be applied for allergen detection, both with their own advantages and disadvantages (for detailed information on ELISA see Annex 1, for PCR Annex 2). In order to make a well-informed choice on what technique to use, it is important to know what target will be tested, but also what is the matrix in which it will be tested and if and how the matrix was processed. Thus next to the composition of the matrix, also the processing can have an effect on the target form, its subsequent extractability and detectability (§3.2). The timing of possible cross-contact is also important, as this moment can determine whether the possible cross-contact allergen will be subjected to processing or not (§3.2.5).



4 Kit selection and use

4.1 Kit selection

4.1.1 When to aim for qualitative or quantitative results?

An important question to answer, in order to select the most suitable method for allergen analysis, is whether to aim for a qualitative or quantitative result. A qualitative result expresses the presence or absence of a clearly defined allergen in the matrix of interest with a known selectivity and limit of detection (LOD). It is of crucial importance that the LOD is low enough to meet safe threshold limits for allergic individuals. A qualitative result can for example be used when food manufacturers examine their equipment surfaces for the presence of allergens after cleaning. In this situation it is not needed to determine the exact amount of the allergen: a simple 'below the detection limit' is sufficient.

The reading can be converted into quantitative units, preferably mg total protein from the allergenic ingredient per kg of food, which is the recommended measurand to perform quantitative risk assessments for the application of PAL. A quantitative result is a result expressing the numeric value of a clearly defined allergen ingredient with a known selectivity and limit of quantification (LOQ) within matrix-group of interest. A quantitative result is for example required to assess possible cross-contact in a final product, to determine if PAL labelling is needed. The FAO/WHO expert committee recommends (FAO/WHO 2022 part 2. section 8.2 [8]) that test methods should be able to report results three times below the action levels, i.e. concentrations of UAP above which action is required (based on the RfDs presented in the same report) [46].

The three techniques available for allergen detection (ELISA, PCR and LC-MS) do not directly determine the total allergenic protein content in the food commodity, as they target individual or mix of proteins and epitopes, DNA, or peptides, respectively. In order to report quantification results in the units recommended by the FAO/WHO Expert Committee and required for quantitative risk assessment using reference doses, the result should be expressed in mg total protein of the allergenic ingredient/kg product, therefore in all cases requiring conversion. How to do that and to make different methods comparable is outlined in Cubero-Leon et al., 2023 [37].

4.1.2 When to use ELISA and when to use PCR?

A number of commercially available methods exist for allergen analysis, and the majority of these are either an ELISA or PCR method. When considering which methodology is appropriate, ELISA or PCR, it is important for users to understand the nature and format of the allergenic ingredient to be detected and the benefits and/or limitations of the analytical method. An overview of allergenic ingredients and possible detection methods can be found in Table 4.

ELISA methods detect epitopes from specific proteins, however in many cases the epitopes targeted with ELISA are not necessarily the epitopes that elicit the allergic response in a patient. A number of commercial kits and formats provide quantitative, semi-quantitative or qualitative results.

PCR detects the presence of (traces of) DNA belonging to the species eliciting the allergic response. It provides indirect information on the potential presence of the allergens. PCR expresses its result in the DNA copy number of a specific species in a total DNA sample, but there is no generally applicable method to convert the copy number to the amount of total protein of the allergenic ingredient. However, the advantage of PCR over ELISA is that it provides information on the allergen source on family, genus or species level, and can discriminate closely related species as mustard and rapeseed, or celery and parsley, which is not always possible with an ELISA.

The techniques used may provide complementary information, but not all allergenic ingredients can be detected with each method. For example, DNA methods are not appropriate for milk and egg allergen analysis as it detects the species (bovine or ovine). Moreover, egg yolk contains a single copy DNA, while egg white (powders) is generally considered to contain no DNA (new insights however suggest otherwise [47]).

As previously described, for some ELISA assays, there can be challenges in cross-reactivity with similar or closely related species, for example mustard and rapeseed, or celery and carrot. Whilst it is common for a food manufacturer to use one method for routine analysis, a combination of the available methods is strongly recommended in situations where unexpected or disputable results are observed. Note that for ELISA the use of different brands of kits can lead to different (and incomparable) results [48], therefore the combination of multiple techniques (e.g. ELISA and PCR) is preferred over the combination of multiple ELISA kits.

Important considerations for kit selection

- Is (semi-)quantification requested?
- Is the target of interest singular or a group (e.g. nuts)?
- What is the matrix?
- For what matrices and/or processing is the kit validated?
- Is the target prone to cross-reactivity?
- Is cross-reactivity specified in the manual?
- What is the specificity and sensitivity of the kit?

A good kit manufacturer defines at least the following in the manual [49]

- Sensitivity.
- Specificity.
- Cross-reactivity.
- Validation report specifying the ingredients and matrices investigated.
- Reporting unit and conversion factor to total protein of the allergenic ingredient.
- Calculation model and worksheet or software.
- Calibrants composition (correlates with the possibility of calculating the conversion factor.

Table 4 *Overview of allergenic ingredients and possible detection methods.*

Technique			
Allergenic ingredient	**ELISA**	**PCR**	**LC-MS**
Cereals containing gluten	•	•	•
Crustacean	•	•	•
Egg	•	x	•
Fish	•	•	•
Peanut	•	•	•
Soy	•	•	•
Milk	•	x	•
Tree nuts	•	•	•
Celery	◇	•	•
Mustard	◇	•	•
Sesame	•	•	•
Lupin	•	•	•
Molluscs	•	•	•

• = detection is possible with this technique, ◇ = commercially available ELISAs typically have antibodies that show cross-reactivity, x = the species will be detected (cow, chicken) thereby not differentiating between the non- allergenic tissue (meat) from the allergenic tissue (milk, eggs).

The next paragraph describes how to check if a kit is fit for intended use, based on information provided by the supplier. If the supplied information is insufficiently clear, it is of paramount importance to always contact the manufacturer prior to purchase/testing.

4.1.3 How to check whether commercial methods are fit for intended use?

The information presented by commercial kit providers with respect to protocols and validation reports, should be carefully reviewed to understand the scope of the method, its specificity, sensitivity, and potential required additional method validation.

Working range. The test kit protocol should include LOD/LOQ of the method, and for quantitative analysis, the method should also state the working range, the LOQ to the upper limit of detectable concentration, in assessed matrices. It is important to check in which matrix the LOD/LOQ is determined (i.e. in buffer or in a relevant food matrix) as this will affect the LOD/LOQ. When the quantitative result will be used for risk based PAL, the Expert Committee of FAO/WHO recommends the LOQ to be 3-fold lower than the action level for that food to account for performance variability and to assure that the analytical result is truly at or below the action level.

Specificity and cross-reactivity. The test kit protocol should include assessed specificity and determined cross-reactivity. However, possible cross-reactivities of the supplied antibodies are not always extensively reported by a kit manufacturer.

Note

In some cases the kit description does not provide details about the protein target used to raise the antibodies. In other cases the target protein is specified, but the protein composition of a food product is unknown/unclear declared, leading to a possible wrong kit selection. For example, an ELISA targeting casein will allow the detection of milk and casein used for fining of beverages, but will most probably not recognize whey protein, or α-lactalbumin or lactoferrin isolates, which like casein are also used as processing aids in beverages.

Matrix and ingredient validation. Different allergen-containing ingredients may respond differently on an assay, e.g. a caseinate tested on an ELISA assay calibrated for skimmed milk powder will respond differently as the caseinate will have different binding affinity compared to the calibrant and so conversion factors in this instance would not be appropriate. Therefore, always check for which matrices the kit was validated/tested.

Additional verification. The kit protocol should outline step by step the procedure for extraction and testing of the sample for allergen analysis, what materials or equipment is required, what materials are provided, and how to analyze data (e.g. standard curve interpolation models), possibly supplying a worksheet or software for data analysis. It is important to note that if the kit operator plans to use materials/equipment, extraction or testing procedures other than presented by the kit protocol, additional verification by the laboratory prior to routine use is required.

4.2 How to generate reliable data

Next step is to generate robust data on the presence of allergen food ingredients with the selected kit. Next to the optimal execution of the kit by trained personnel, obtaining robust data already starts at the sampling phase as discussed in §4.2.1. The performance realized by the kit provider in their laboratory is more likely to be reached when the operator's laboratory meets a set of minimal requirements and is addressed in §4.2.2. The requirements to interpret and interpolate the raw data will be discussed in §4.2.3.

4.2.1 Sampling

Most errors associated with food testing can be attributed to how the original sample was taken and/or handled. Measuring allergen concentration is an essential step in quantitative risk assessment as it serves to decide whether the level is below or above a certain threshold. Sampling is the gateway to a reliable result: "Does the sample represent the food (batch) under consideration?", and "Does the measurement reflect the actual allergen content?"

Appropriate sampling for ingredient quantification, or for the assessment of UAP presence, comprises careful selection of sample location, number and size of samples. Considerations in sampling relate to the nature of the allergen: is it homogenously distributed in the sample (e.g. fluid allergenic ingredients), or is it particulate (e.g. seeds)? Furthermore, the composition of the product: is the product a composed food product or a composite food such as ready-to-eat meals that may need to be split into several components? Moreover, regarding a possible UAP presence, as a result of the product change in the processing line, the first products after the product change may have a higher risk of contamination, and the first amounts may then be higher and then decreasing in time. Several ISO Sampling Guidelines [50] and the ILSI Guidance document [10] have specific sections addressing adequate food sampling. Note that sampling size and frequency should be based on a risk-based model and should take into account the risk profile of the food to be tested.

In addition to sampling, the subsequent sample handling requires careful attention as well. Sample handling includes amongst others the determination of the amount of sample to be homogenized, and techniques to obtain a homogeneous sample for solid products as well as liquids, e.g. stirring is not sufficient for lecithin analysis, or how to deal with phase separation while heating margarines. Guidance for sample handling is to some extend described in the ILSI Guidance [10].

4.2.2 What are the minimal laboratory requirements for ELISA and PCR methods

The test kit protocols for ELISA and PCR manufacturers should outline the materials provided in the assay and the material and/or equipment that should be provided by the operator. Wherever possible, the user should follow the protocol, or consult with the kit provider on how to run the test on equipment specific for the operator's laboratory. Deviations must be validated to ensure the method is still fit for purpose and meets the expected performance criteria. For example, if the method states a requirement of a shaker water bath, using a static water bath may not yield similar results.

The laboratory should have a quality management system and procedures in place. In particular for allergen analysis, the laboratory area must be clean, and procedures must be put in place to reduce the risk of cross-contamination.

For both methods (ELISA and PCR), homogenization of the sample may be challenging (e.g. high viscosity, grinding) as only a very small quantity of sample (few mg) will be analyzed. Decontamination of the homogenizer are key to avoid cross contamination in the laboratory.

The user should ensure that all operators of the method have been trained to the appropriate level. The use of internal quality control samples and the regular participation to proficiency testing round(s) will confirm the competence of the laboratory and highlight any potential issues.

4.2.3 Reference materials

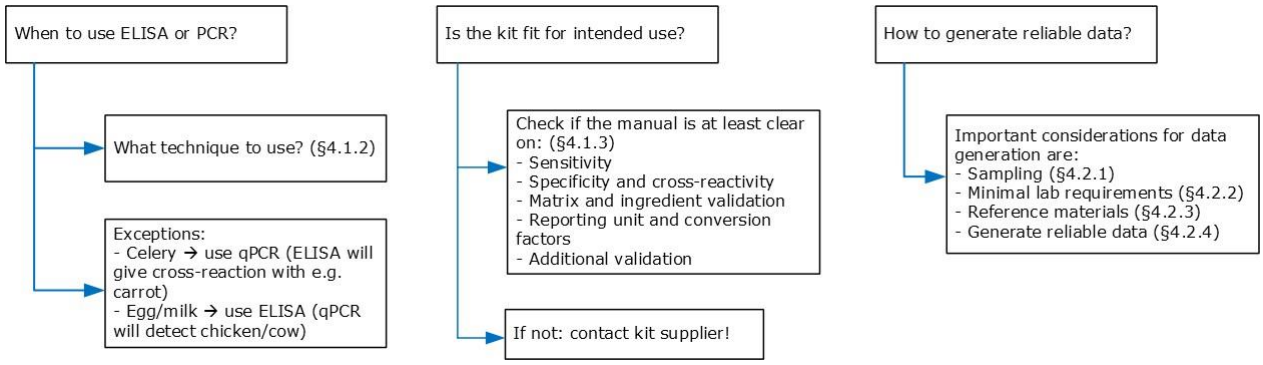
To improve comparability and harmonization of quantitative food allergen measurements between methods and laboratories, steps have been taken to develop and provide well characterized calibrants and reference materials [37, 51]. Whilst several reference materials (RMs) have been developed, they are not available for all the regulated allergens, nor has there been any consensus on which ones to use. Despite this, there are some available and therefore considerations should be given to the use of these RMs, whilst recognizing there is a need for further development of them and harmonization of their use, in both method development and application.

4.2.4 Generate reliable data

The raw data from the method should be analyzed according to manufacturer instructions, for example with method specific software or consulting the manufacturer on how to construct calibration curves. Manufacturers should provide clear instruction on data analysis and supply validated worksheets or software for calculation. The results should be reported in line with the units of measurement and conversion factors provided in the test kit protocol.

4.3 Summary

In order to select the best technique and kit to use it is important to know the details of the target to be analyzed, the question(s) that needs to be answered (e.g. quantitative or qualitative result), and the validation parameters of the kit. Both ELISA and PCR have their advantages and disadvantages that differ dependent on these questions. Key for correct and meaningful allergen detection and reporting is proper preparation: know what needs to be analyzed, in what form/matrix, and what to do with the results (see also Chapter 5). In case of doubts: always contact the kit supplier prior to practical assessment. A good kit supplier should offer a confidential service, welcome early discussion of any proposed testing and associated study, and aims providing advice on correct test selection and study design, covering the concepts presented in the previous and following Chapters.



5 Interpretation and reporting of results

5.1 Importance of interpretation and reporting

Effective reporting extends beyond the simple presentation of quantitative measurements. It requires a rigorous execution assay process, presented in the previous chapters, to address the client's inquiries and to enable quantitative risk assessment. Currently, no harmonised reporting standard or guideline is available. Several recommendations were recently published by the AOAC [27] and ILSI [10]. This chapter will further investigate the criteria for sound reporting and offer insights on result interpretation. Furthermore, it will present three practical (real-case) examples of challenges encountered in the interpretation, understanding, and reporting of results of food allergen analysis.

Customers, like for instance food business operators, patient organizations etc., requesting food allergen analysis may have varying levels of knowledge and understanding. Some customers may lack the expertise to specify their requirements, highlighting the need for education and guidance (as provided by this document) and dialogue between customer and testing laboratory. It cannot be stressed enough that the customer requesting the analysis needs to start with thoroughly overseeing the purpose of the allergen analysis, and the nature and possible entry points of UAP whenever this information is available. Especially when addressing new cases or when using the test for the first time, communications between the customer, laboratory performing the analysis, and the developer/ kit manufacturer are of utmost importance. Moreover, right interpretation and reporting of food allergen ingredients data will aid towards reliable quantitative risk assessment.

5.2 Reporting requirements

The reporting requirements are customer driven. They derive from a dialogue between the laboratory and the customer requesting the analysis who must define the reporting expectations. The list below presents the basic reporting requirements we recommend.

Reporting requirements

- Why the analysis request is made.
- Laboratory name.
- Customers.
- Sample ID.
- Scope of the analysis (*determination of X in matrix Y*).
- Short description of the sample received and analysed (including storage conditions).
- State, when applicable, "Analysis performed in the frame of the scope of the accredited method".
- Dates of sample reception and sample analysis.
- Measurement procedure used (*ELISA, PCR, LC-MS, other*).
- Name of the test kit used (when relevant).
- Targeted allergen or protein(s).
- Test manufacturer.
- Specification of the measurand (e.g. "*mass fraction of a particular protein in a food commodity*" or "*mass of total protein of the allergenic food ingredient per mass of food",* expressed in mg kg^{-1}).
- Short description of the principles of the method. Specify which standard/reference method was used (if any). Include relevant experimental details (modifications/deviations/adjustments; additional observations).
- Specification of the calibrant/calibrator.
- Any relevant known cross reactivity details.
- Reporting of the measurement result.
- Reporting of the associated measurement uncertainty (specify the coverage factor, if the expanded uncertainty is reported).

Reporting requirements

- Reporting unit (preferably as mg of total protein of the allergenic ingredient per kg food.
- Recovery and if the results are corrected for the recovery.
- Correction / conversion factor (including relevant literature reference).
- Specify the limits of detection and quantification (LOD/LOQ) and the matrices investigated.
- Specification of the working range (lower and upper limit).

5.3 Interpretation of results

In order to interpret the results effectively, it is crucial to have a clear understanding of the customer's request at hand. In other words: 'Do the provided results directly address the customer's request?'

5.3.1 Analysis request

The selection of the analytical marker for the food allergen to be tested and the choice of the analytical platform must be aligned with the request of the costumer. This purpose can range from verifying "free from" claims and cross contact, characterizing raw materials, monitoring changes within a facility including cleaning efficiency, PAL risk assessment, or serving law enforcement needs. In some cases, multiple methods may be required to obtain meaningful results. Then assess whether the chosen method/assay is appropriate and whether the kit has been validated for the specific matrix to be tested.

Surprising or unforeseen results should be discussed between the laboratory and the customer. Unexpected findings and potential underlying factors that may require additional laboratory steps (in consultation with the customer) are presented in Table 5.

Table 5 *Illustration of issues observed or foreseen in obtained results and the action or questions that needs to be addressed accordingly.*

Issue observed or foreseen	Action/Question to be asked
Expected result below LOQ or action levels below LOQ	Can LOQ be lowered? (how for a test kit?) ➔ Optimise/modify the assay. ➔ Consider selecting another method.
Expected result above the upper limit of quantification which can be quantified	Was an appropriate sample dilution taken along? ➔ Additional sample dilutions required.
Large measurement uncertainty expected (which may impact claims)	Is the measurement uncertainty (MU) overestimated? ➔ Review the measurement uncertainty calculation. Can the MU be reduced? ➔ Additional experiments may be required. Is sufficient time available?
False negative results	Was the appropriate method selected? Is the analytical target protein appropriate? For example, choosing an ELISA test kit targeting "egg white" proteins will give false negative (FN) results for products containing "egg yolk" proteins ➔ Consider selecting another method.
False positive results	Check for matrix interferences (§3.2.3 and §3.2.4) The method may have insufficient specificity and/or selectivity. ➔ Consider selecting another method

Addressing the methodological complexity at hand demands not only supplementary approaches that offer diverse perspectives to comprehend reality, but also necessitates requirements in terms of standardization, harmonization, and alignment concerning language, analytical methodologies, units of result expression, performance standards, acceptability criteria, and validation protocols.

5.4 Measurand and conversion factors

The recommended measurand for quantitative risk assessment is the *mass of total protein of the allergenic food ingredient per mass of food* (JRC 2017) [22], as is used in VITAL and clinical reporting regarding thresholds of allergenic reactions. Unfortunately, none of the current analytical platforms used for the quantification of the food allergen in food can directly measure the total protein content, and additionally the different analytical platforms may report in different units.

Whenever there is a difference in the reporting unit between the requested or agreed *quantity intended to be measured* (measurand) and what is *actually measured* (quantity of an analytical marker targeted by a specific measurement procedure) conversion factors must be used. These conversion factors should be normally provided by the test kit manufacturer or calculated by the laboratory. In any case, details about the used conversion factors and calibrators should be given to the test kit user.

The ILSI "Practical guidance [10] on the application of food allergen quantitative risk assessment" identified the following cases:

- The content of the requested "measurand" is equal to the reported content.
- The results are stated as quantity of the allergenic food (i.e. mg of total protein of the allergenic food ingredient per kg of food).
- The results are stated as quantity of a "specific protein" of the allergenic ingredient (i.e. mg of total parvalbumin or total casein in the sample).

Different scenarios should be considered:

- A food manufacturer intends to monitor his production line. The food operator asks the laboratory to **quantify the content of a specific "protein marker"** in the product (measurand) at regular intervals to identify any trends. The derived control chart based on the quantified content of the same "protein marker" (analyte) is fit-for-purpose. No further calculations - using conversion factors - are required.
- An official control authority requests the laboratory to quantify the **mass fraction of total protein from the allergic ingredient in food**.
  i. The laboratory uses a test kit targeting a "specific protein" (analyte) that provides the results as "total protein" content (measurand). This indicates that the manufacturer imbedded a conversion factor in the calibration curve. While the observed results can be reported *as such* (no further calculations required), the certificate/report of analysis must ideally specify (i) the calibrant/calibrator used, and (ii) the value of the conversion factor "implemented" by the manufacturer.
  ii. The laboratory uses a test kit targeting a "specific protein" that provides the results for the "specific protein" content. The laboratory must then apply a conversion factor to calculate the content measurand value (*measurand content = conversion factor * analyte content*). The certificate/report of analysis should then specify the value of the conversion factor used. This applies only when the ratio of the specific protein to the total protein of the allergenic ingredient present in the sample is known.

The conversion of "specific protein(s)" results to "total protein" values used for risk assessment requires prior knowledge of the ratio of the specific protein to the total protein of the potential source(s) of cross contact. The ILSI guidance provides a table of commonly used sub-proteins for allergen analysis and their ratios to total protein from the allergic ingredient in food.

Example of extra considerations: egg in pasta

The scenarios described above need to be considered with due care. The samples to be analysed must be properly described before analysis. For instance cross-contact is of concern for egg in pasta production. When pasta production is transferred from egg containing pasta to egg-free pasta, although lines are cleaned, egg stays present for over an hour which correlates to 2000- 4000 kg pasta according to Marengo et al. [52]. To detect if egg is still present in the pasta, it is important to realize the ratio egg yolk/albumin in pasta. This ratio is optimized and not per se similar to ratios which can be found in whole egg [53]. The determination of "total egg protein" in pasta therefore requires results obtained by a test kit targeting ovalbumin (analyte) to be converted using the appropriate/adapted conversion factor. The use of the traditional conversion factor for total egg protein is inappropriate in this case and may lead to a wrongly estimated result.

5.5 Examples to illustrate possible methodological challenges

5.5.1 Cross-reactivity: mustard

What is the challenge?

- Due to a shift towards promoting biodiversity by flowering fields, crops like *Brassicaceae* and edge habitats, canola (*Brassica napus*), rapeseed (*Brassica rapa*) and charlock (*Sinapis arvensis*) are commonly found in agricultural fields and products (see also section 2.4).
- These species tend to interfere with the mustard analysis performed using standard methods such as enzyme-linked immunosorbent assay or ELISA. The resulting mustard content is likely to lead to false positive or overestimated results.
- Several RASFF alerts of 2019 triggered the recall of wheat and flax seeds seemingly containing mustard due to the use of ELISA assays.

What methods can be used to determine the presence and amount of mustard?

- Visual examination in and of agricultural crops.
- qPCR or ddPCR can be used for the quantification of the amount of mustard DNA present in the DNA extract.
- DNA-metabarcoding (NGS). It enables the simultaneous detection/identification of multiple species in complex samples in one analysis [3, 53].
- LC-MS: method development is ongoing and the accurate determination of closely related species/varieties is difficult, due to the lack of appropriate reference materials.

What are the learnings?

- It is important to select the right method and carefully interpret the data.
- Be aware of possible cross contamination.
- Mustard contamination can consist of whole mustard seeds and their fragments, resulting in a heterogeneous, particulate contamination. Variations in the amount of product ground during sample preparation can influence the concentration.

5.5.2 Overestimate results: detection of gluten in barley

Why is analysis needed?

- Gluten is a protein fraction from many cereals that are toxic to individuals with coeliac disease (CD).
- Recent reports of overestimations when using the R5 and G12 antibodies in barley-based products, in part due to the calibrator which may be less suitable for barley due to its distinct protein composition compared to wheat.
- This potentially impacts on gluten-free or very low gluten claims.

What regulation exists for the amount of gluten present in food products?

- The CODEX Alimentarius has established a threshold of 20 mg/kg for gluten in the final product, which has been adopted by Commission Implementing Regulation (EU) No 828/2014 for the labelling of gluten-free foods.
- Foods containing less than 100 mg/kg of gluten can be labelled as 'very low gluten.' These regulations are applicable to both naturally gluten-free ingredients and processed foods with reduced gluten content.

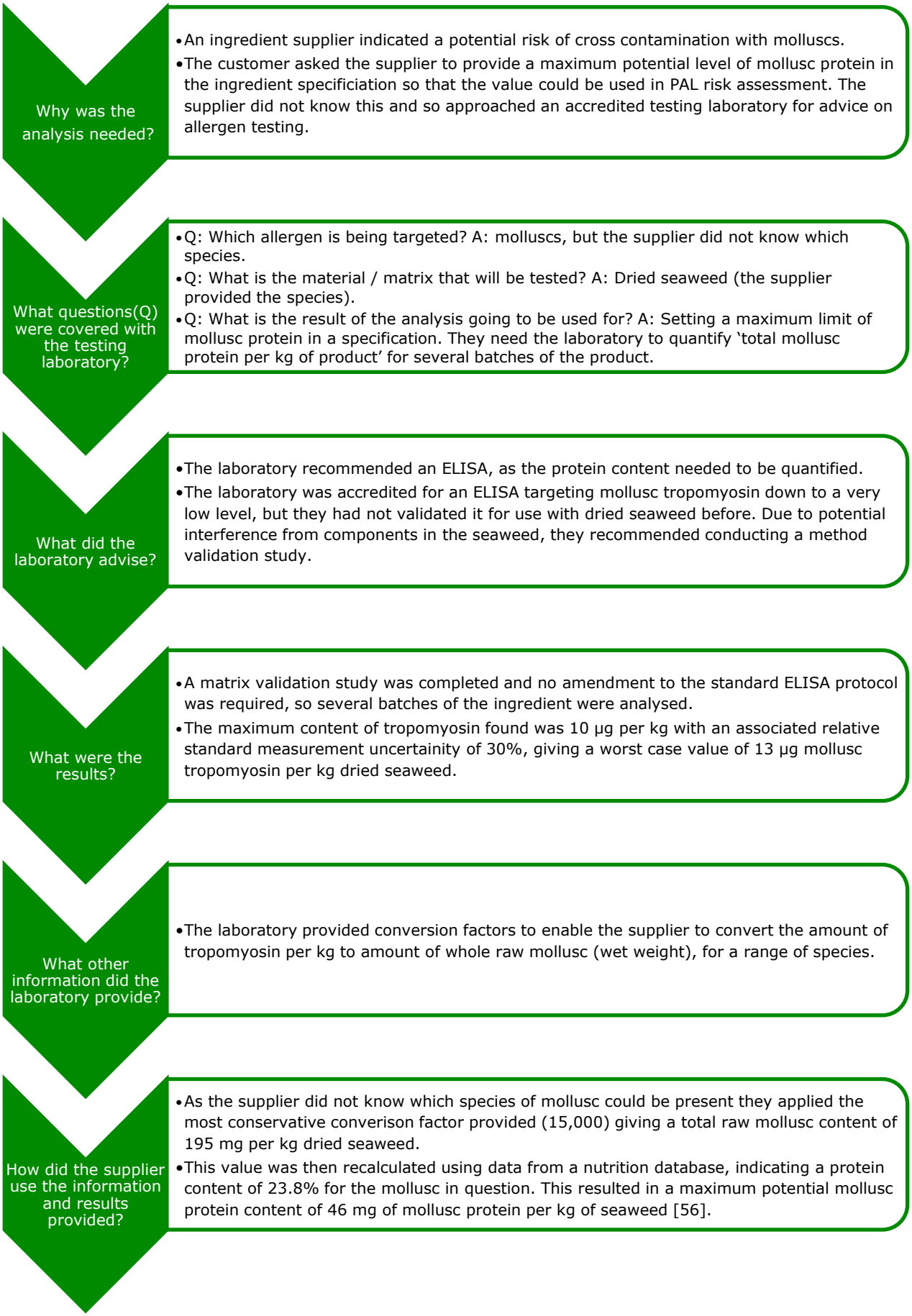
What methods are currently used for gluten detection?

- A wide range of commercially available ELISA test kits exists for gluten detection. They are based on the Skerrit, R5, G12 and α20 monoclonal antibodies (mAbs). The R5 Mendez ELISA is the established method for gluten- free quality control.

How can methods for gluten detection be optimized?

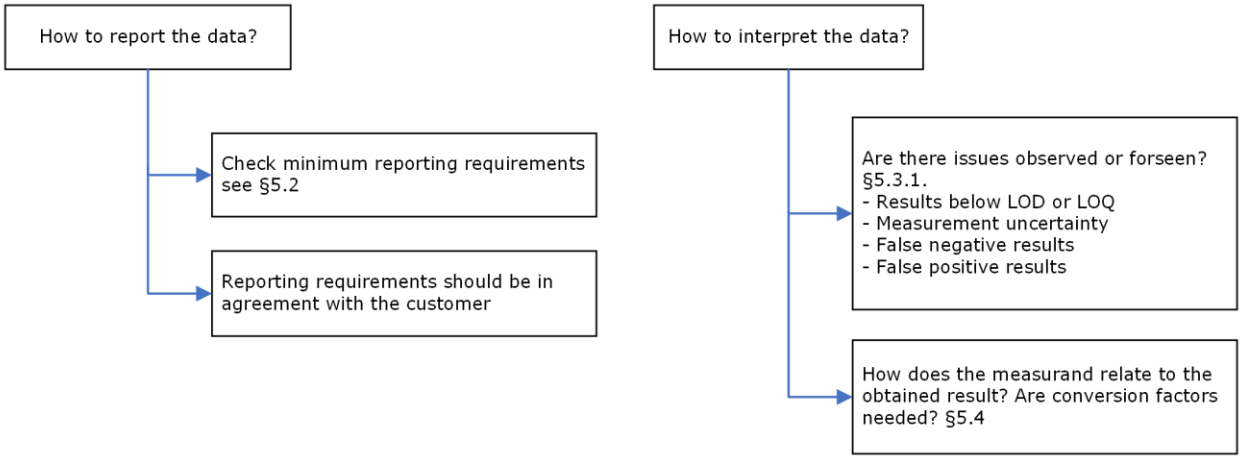
- The conversion factor for barley prolamins to gluten should be adjusted from 2 to 1.6 [54].
- This will only solve partially the problem as a higher reactivity of the prolamin fraction of barley has been observed in different ELISA test kits (including the R5 mAb) [55].
- A reference material for barley may improve the between-assay variability but will not correct variation due to differences in selectivity of the assays [56].
- Mass spectrometry methods can help with determining the source of gluten if unknown [57].

5.5.3 Quantification of protein content: conversion factor for molluscs



5.6 Summary

For interpretation and reporting of results it is crucial to have a clear understanding of the customer's request at hand. With this information the proper kit can be selected as described in Chapter 4. Results need to be properly reported, a set of minimum reporting requirements is summed up in §5.1 and §5.2. Results obtained need to be questioned for issues observed or foreseen to exclude "wrong" results. For result reporting in the proper measurand and unit (§5.3) the right conversion factors need to be applied (§5.4).



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Annex 1 ELISA

There are basically two different types of antibodies: monoclonal and polyclonal. Monoclonal antibodies are used to recognize a specific antigen based on a single epitope (unique protein recognition motif), while polyclonal antibodies recognize multiple epitopes distributed on one or more proteins. There are significant differences in the complexity of production of these two classes of antibodies. Polyclonal antibodies are derived from the sera of immunized animals and usually consist of a complex mixture of antibodies against common antigens that recognize different epitopes. Monoclonal antibodies are the result of multiple isolation, replication, and purification steps that result in homogeneous antibody production from a single immune cell (hybridoma immune cell, or monoclonal) that targets one or two specific epitopes.

Table 1 summarizes the pros and cons of these 2 types of antibodies.

Table 1 *Evaluation of pros and cons of polyclonal and monoclonal antibodies.*

Polyclonal: Heterogeneous Antibody Population	**Monoclonal**: Homogeneous Antibody Population
PROS	**PROS**
Less expensive to produce.	Batch-to-batch reproducibility (**high homogeneity**).
Higher overall **antibody affinity** against the antigen due to the recognition of multiple epitopes.	Possibility to produce large quantities of **identical antibody**
Have a **high sensitivity** for detecting low-quantity proteins.	**High specificity** to a single epitope reflected in **low cross-reactivity.**
Superior for use in detecting a **native protein**.	**More sensitive** in assays requiring quantitation of the protein levels.
	Low background noise.
	Reduction of animal experiments
CONS	**CONS**
Batch-to-batch variability as produced in different animals at different times.	**More expensive and longer** to produce.
High chance of cross-reactivity due to a recognition of multiple epitopes	**Narrower target specificity** which may result in result variation.
Need for animal experiments	

There are also two main types of ELISA strategies: the so-called "sandwich ELISA" or "competitive ELISA". The main difference between the "sandwich" strategy and the "competition" strategy is the setup of the assay. In both cases, the amount of protein detected was quantified by interpolating the colorimetric or chemiluminescence signal into the calibration curve. The standards required to obtain the curves are included in the kit provided by the manufacturer.

Sandwich ELISA

This is the most commonly used ELISA method. The target protein is bound by two different antibodies (Abs): a capture Ab and a recognition Ab, forming a "sandwich" pattern. The capture antibodies are bound to a surface and the recognition/Detection antibodies are usually chemically conjugated to the enzyme and quantification is based on the level of enzymatic reaction with the appropriate substrate. The analysis can be summarized as follows:

Step 1: Immobilize specific antibodies, the so-called capture antibodies, on a solid surface (e.g. microwell).

Step 2: Antigens present in the test sample are captured by these specific antibodies.

Step 3: The complex reacts with a second analyte-specific antibody, the detection antibody, to form a sandwich structure. This detection antibody is conjugated to an enzyme.

Step 4: The enzyme reacts with a specific substrate and colour is produced. The presence of the antigen is measured by the absorbance of the coloured product. The colour is directly proportional to the amount of allergen present in the sample.

Competitive ELISA

Competitive ELISAs follow a different strategy: there is competition for specific antibody binding between the target protein in the sample and the target protein bound to a surface or labelled to an enzyme. Unlike sandwich ELISA, in the competitive ELISA the allergen concentration in the test sample is inversely proportional to the signal. Depending on the use of labelled antibodies or labelled antigens, there are various ways to design these competitive ELISAs. However, to simplify the explanation, the mechanism can be divided into the following steps. An example workflow for an immobilized antigen competition kit is summarized below:

Step 1: Immobilize the specific antigen (target antigen) in the well.

Step 2: Add the primary specific antibody to the sample extract to bind to the target antigen present in the sample (food allergen ingredient to be tested). The target antigen in the sample binds to the specific antibody and reduces the amount of reactive ("free") antibody in solution.

Step 3: Add the sample - antibody mixture to the wells. Unbound Ab from step 2 binds to immobilized specific antigen from step 1. The antigen-Ab complex formed in step 2 remains in solution.

Step 4: Wash to remove unbound antibody and antigen-Ab complexes. After this step, only antibodies attached to the immobilized antigen remain.

Step 5: An enzyme labelled secondary antibody recognizing and binding to the primary antibody is added. When substrate is added, colour or chemiluminescent signal is produced. The concentration of antigen in the sample is inversely proportional to the amount of signal.

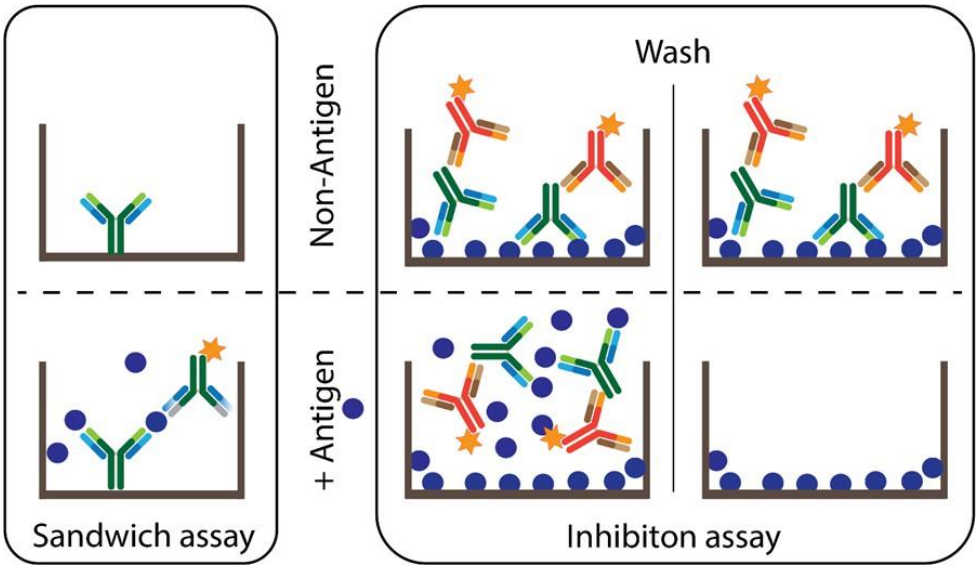

Figure 1 *Infographic of a sandwich- (left) and competitive (right) ELISA. In the sandwich ELISA the capture antibody immobilized on the plate surface captures the target allergen protein which will subsequently bind to the detection antibody. In the competitive ELISA the target allergen protein is immobilized on the plate surface and the target allergen protein present in the sample competes for the antibody binding.*

Table 2 summarizes the pros and cons of these 2 types of ELISA mechanisms.

Table 2 *Evaluation of pros and cons of the Sandwich ELISA and Competitive ELISA strategy.*

SANDWICH: more presence of targeted protein = **more signal** **TWO points of linkage** in the targeted protein are requested to interact with the 2 antibodies Higher sensitivity than competitive ELISA	**COMPETITIVE**: more presence of targeted protein = **less signal** **ONE unique point of linkage** is requested in the targeted protein Lower sensitivity than sandwich ELISA
PROS **More selectivity** due to its double binding mechanism	**PROS** **Easiness of application** due to its **single binding** mechanism **Possibility to detect hydrolyzed** or denatured proteins = binding sites easier to target
CONS **Difficult** to apply **if the protein has been hydrolyzed** or has changed its spatial conformation **Higher variations at lower levels**	**CONS** **Possibility of interferences:** similar proteins can interact easily with antibody (cross-reactivity) **Higher variations at lower levels**

Sandwich and Competitive ELISA assay are usually performed in dedicated laboratory and supported in specifically 48- or 96-well microplates. Another kind of support is used in the Lateral flow immunoassay: LFIA.

LFIA

Allergen-specific lateral-flow devices are qualitative or semi-quantitative immunochromatographic tests, based on the ELISA technique. The concept underlying the LFA is straightforward: a liquid specimen (or its extract) that contains the target substance of interest flows through different sections of polymeric strips via capillary action, without the need for external forces. These strips are equipped with molecules that can interact with the target substance. A typical lateral flow test strip comprises overlapping membranes that are affixed to a backing card to enhance stability and ease of handling. At one end of the strip, the sample is applied onto the adsorbent sample pad, which contains reagents that prepare the sample for interaction with the detection

system. The sample pad ensures that the target substance in the sample can bind to the capture reagents of conjugates on the membrane.

The sample that has been treated moves across the conjugate release pad. This pad contains antibodies that are tailored to the specific target analyte. These antibodies are linked to particles that have colour or fluorescence, with colloidal gold and latex microspheres being the most frequently used.

As the sample moves along the strip, it travels alongside the conjugated antibody that is attached to the target analyte. Within the detection zone, there exists a permeable membrane typically made of nitrocellulose: it contains specific biological elements, primarily antibodies, that are immobilized in lines. Their purpose is to interact with the target that is bound to the conjugated antibody. When the target is recognized, a corresponding reaction occurs on the test line (with this remark that very high target concentrations may trigger a hook effect, diminishing the test line). A response on the control line indicates that the liquid has flowed properly through the strip. The results, displayed as lines of varying intensity, can be evaluated visually or with the aid of a dedicated reader. The movement of liquid across the device occurs due to the capillary force of the strip material. To facilitate this flow, an absorbent pad is present at the end of the strip. The absorbent pad (named reservoir) serves to draw out excess reagents and prevent any liquid from flowing backwards. As for ELISA, there are two distinct formats of the LFIA: direct and competitive.

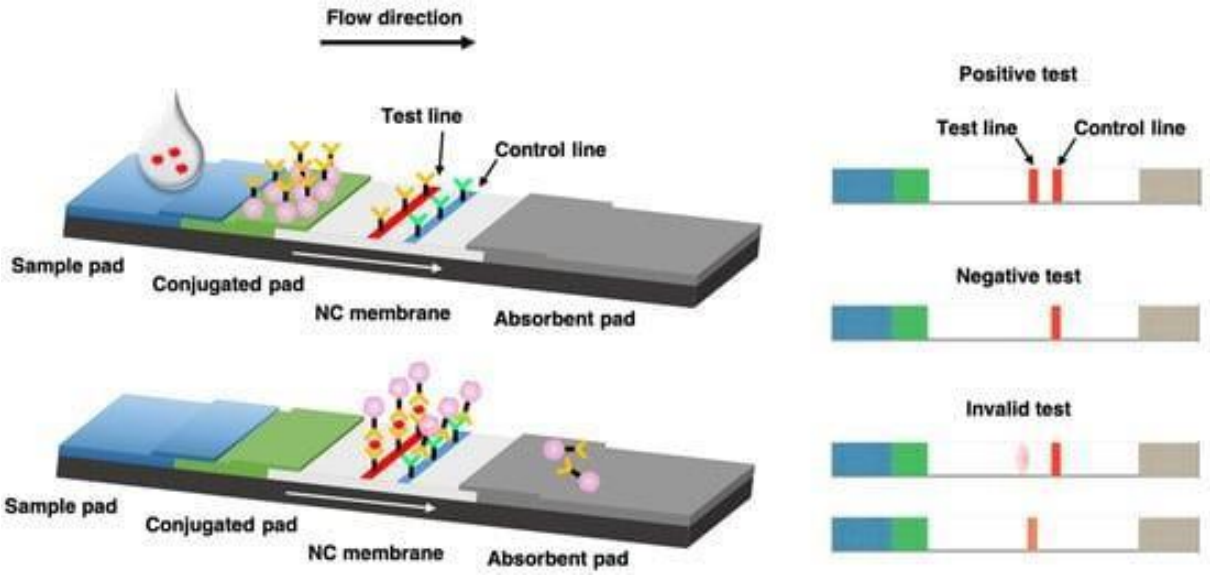

Figure 2 *Schematic illustration of an antigen detection-based LFIA test. From: Biosensors | Free Full-Text | Recent Advances in Novel Lateral Flow Technologies for Detection of COVID-19 (mdpi.com).*

Annex 2 PCR

In Polymerase Chain Reaction (PCR), a specific DNA sequence is amplified in a reaction tube containing the target DNA, called template; specific short oligonucleotide sequences recognizing and binding to the template DNA sequence, called primers; Taq polymerase enzyme which can build in nucleotide bases one by one based on a specific high temperature and very fast; and the oligonucleotide bases as such, called dNTPs. The reaction mixture undergoes a thermocycling program, consisting of an initial denaturation stage - typically 5-10 minutes at ~95 °C - followed by a number of repeated 'cycles' - typically 35 up to 45 - and finalized with an extra extension stage, typically 5-10 minutes extra at 72 °C. Each PCR 'cycle' consists of denaturation (e.g. 1 minute at 95 °C), annealing (e.g. 1 minute at 60 °C) and extension of the primers (e.g. 1 minute at 72 °C). With each PCR cycle, the template gets doubled. In this way, after running the whole thermocycling program with n cycles, the template DNA sequence has been exponentially amplified to give 2n or thus several million copies (where '2' is the most optimal case i.e. it is supposed that each of the present template DNA copies is 'doubled' after each cycle).

After the PCR program is run, the generated amplicons need to be visualized. Typically, in the first generation of PCR techniques, this is done by loading on agarose gel and thus manual visualisation and interpretation of the results. The second generation of PCR techniques is real-time PCR, where the amplification process can be followed online, a threshold cycle number (CT value) is obtained for the unknown sample, and quantification of the template DNA sequence becomes possible through the use of standards or calibrants and comparison between the calibrants CT values and that of the unknown sample (CT comparison). Thus 2nd generation PCRs are quantitative (qPCR). Quantification can be done absolutely, by comparing CT values, or relatively, by comparing delta Ct values (e.g. difference with an internal control such as an endogenous DNA sequence target that is always present in any sample). In real-time PCR (equal to qPCR, quantitative PCR), visualisation of the generated amplicons is done online with specific software that makes automatic data processing and interpretation possible.

The third and most recent generation of PCR techniques is digital PCR (dPCR). While 2nd generation PCR techniques are real-time detections of a specific target in the reaction tube as a whole, 3rd generation PCRs are end-point detections (yes/no answer) however based on a large number of partitions wherein the reaction takes place. In each of the partitions, 0 (no) - 1 (yes) or maximally a few copies of the target sequence is/are present, therefrom comes the yes/no answer (present or not) and the term 'digital' (0/1). The basic principle of dPCR is "divide and conquer" by partitioning - amplifying - counting. The partitioning or compartmentalization - i.e. the generation of thousands to ten-thousands of individual compartments - can be done into chambers (chamber digital PCR or cdPCR) or into droplets (droplet digital PCR or ddPCR). Several technologies by different companies already exist for both cdPCR and ddPCR. While cdPCR is based on microfluidics technologies producing reaction chambers within specially designed chips or plates, ddPCR is an 'emulsion PCR', which is PCR taking place in individual droplets in a water-oil emulsion, followed by flow cytometric counting of the positive reactions.

Pros and cons of different PCR techniques

The specific advantage of digital PCR compared to real-time and conventional PCR, is that absolute quantification is possible without the need for calibrators or the setting up of a standard curve. Also, dPCR is less sensitive to PCR inhibitors, and generally reaches higher precision at low target copy concentrations. A broad dynamic range (between 0,1 up to several hundred thousand of target copies in the reaction tube) is possible, allowing for both high resolution and high sensitivity, compared to conventional PCR techniques. Moreover, multiplexing possibilities would be higher with dPCR than with real-time PCR. Disadvantages of dPCR are the relatively limited throughput, extensive hands-on time at least if working with manual steps in the dPCR flow. Time/efforts and consumables are mainly needed for compartmentalization e.g. droplet generation and the careful handlings that are required for representative compartmentalization; this drawback can be overcome by working with automatization e.g. an automated droplet generator. The working cost per sample might also be higher in dPCR compared to qPCR.

Stepwise approach in qPCR and dPCR analysis - Importance of DNA extraction

In general PCR-based analysis methods consist of sample preparation (homogenization by mixing and e.g. milling of the raw sample), eventual reduction of the sample by subsampling (taking a subsample that is representative for the original laboratory sample), DNA extraction from 1 or ideally 2 duplicate sample test portions, DNA concentration and quality measurement, and finally the PCR amplification. As such, many pre-PCR steps proceed the actual PCR and will determine the final outcome in PCR. After analytical milling of the laboratory sample into a fine powder, small test portions - typically 100 up to several hundreds of milligrams of the fine powder are considered as being 'representative' for the original sample. The DNA extraction that follows disrupts or opens cell membranes (cell lysis), uses nucleases to set free the DNA, adds purification buffers for further hydrolysis, and finally dilutes the DNA into a water-based buffer. A good DNA extraction protocol should yield sufficient amounts of DNA (in ng of µg of DNA per g of kg of sample) with sufficiently high integrity (low fragmentation or degradation) as well as purity (no PCR inhibitors present). It should also be applicable to a wide range of product matrices and be practicable, including making use of buffers and reagents that are safe to humans and for the environment. After the DNA extraction, DNA quantification i.e. concentration measurements can be done to know more about the DNA yield; tests for inhibition e.g. amplifiability check based on generic DNA target sequence amplification to check for DNA purity; and tests for degradation e.g. loading on agarose gel or onto a capillary electrophoresis (CE) instrument to size the (fragmented or not) DNA to check for DNA integrity.

Influence of PCR inhibitors

Heating and mechanical treatment, both from food processing as well as the DNA extraction procedure, may affect the DNA quality and further detection/quantification of the allergen target in PCR. More in particular such processes are known to lead to more fragmented DNA. Food processing and product composition may also affect the PCR in terms of adding polyphenols or polysaccharides e.g. from plant materials, to the reaction, or by denaturing or precipitating the DNA that needs to be amplified. Below are some steps that can prevent PCR inhibition or low amplifiability:

- Add polyvinylpyrrolidone (PVP) to remove polyphenols
- Add non-ionic detergents (Tween 20, Triton X100) to remove polysaccharides
- Removal or denaturation of proteinase K during DNA extraction
- Improve extraction efficiency or facilitate extraction
- Washing to removing salts, sugars, spices
- Defatting fat-rich matrices
- Alfa-amylase treatment of starch-rich matrices
- Performing additional DNA Cleanup to remove PCR inhibitors
- Dilution of the DNA to dilute also possible inhibitors, provided that DNA quantity is sufficient

Similar to other food allergen detection techniques, such as ELISA and also LC-MS, PCR does not allow the direct detection of the allergenic protein or epitope as such. PCR allows for indirect detection of an allergenic ingredient, through the selection of endogenous gene-specific sequences as targets. PCR methods for allergens might be more specific than ELISA, taking into account the possibility of cross-reactivities which is more likely with ELISA. Also, DNA is a highly stable analyte in comparison to proteins and other metabolites. Although PCR is sensitive to PCR inhibitors (e.g. polyphenols, polysaccharides; see above), it is relatively stable at high temperatures.

Quantification

Quantification of DNA targets is performed by qPCR using an internal standard or standard curve(qPCR), or with dPCR without the need for an internal standard.[1]

An internal standard material is added to the samples before the DNA extraction and amplification, and can be used to overcome the limitation of the lack of an endogenous reference gene. A standard curve is constructed by in parallel amplifying known amounts of DNA of a known target in decreasing quantities. Using a standard curve absolute quantification can be reached, provided that the DNA derived from the allergenic species is amplified with the same efficiency as the DNA from the known target.

For qPCR as well as dPCR the analytical results must be converted into mg total protein for performing quantitative risk assessments to enable decisions on the use of PAL.

[1] Mayer, W., et al. "Quantification of the allergen soy (Glycine max) in food using digital droplet PCR (ddPCR)." European Food Research and Technology 245 (2019): 499-509.

Annex 3 LC-MS

The most frequently used separation of peptides or proteins by liquid chromatography is reversed phase liquid chromatography (RP-LC). RP-LC uses a column with a hydrophobic stationary phase, and a mobile phase to separate the components based on their polarity. In the mobile phase the percentage organic solvents is increased during a run and depending on the interaction between the stationary and mobile phase components will exit the column at different times. There are different types of RP-LC columns, and the choice of the RP-LC column depends on the components you want to separate. When components exit the LC column they can be detected my mass spectrometry. For the detection to take place the components must be ionized. There are different interfaces to ionize molecules, but electrospray ionisation (ESI) is commonly used for proteins as it is compatible for polar and even very polar molecules such as peptides amongst others. After ionization the components are transferred to the high vacuum chamber of the mass analyzer of the system. The mass spectrometer (MS) then measures mass to charge ratios m/z of the charged ions. For detection of allergenic ingredients, the targeted components are proteins. As proteins in general are large molecules different approaches for separation and detection are possible. First there is the top-down approach. Here the proteins of interest are extracted from the sample, separated by RP-LC and measured as intact proteins by MS. The sensitivity of this top-down approach however, is limited due to the high complexity of the obtained data. As multiple charge states per target proteins are obtained and moreover, from different proteins present, these are often overlying (Monaci et la. 2018). The second approach is the bottom-up approach, here the extracted proteins are digested to smaller molecules, the so called peptides. When the mass of the allergenic ingredient specific peptide is known, targeted measurement is possible. Measuring the mass of the peptide and consequently the mass of its fragments by MS/MS enables identification of the peptide by databases. For this the MS is operated in positive ion ESI-MS mode. In the first quadropole (Q1) analyzer the m/z values of the protonated peptides are selected. If further determination of the peptides is required a triple quadrupole is used. After measurement of the m/z ratios of the peptides, the peptides of interest are selected, fragmented (Q2) and fragments are measured in the third quadrupole (Q3) as shown in Figure 3.

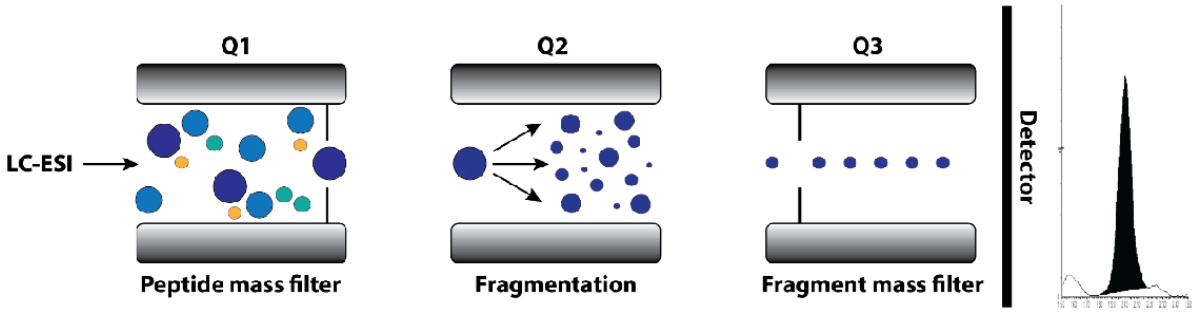

Figure 3 *Infographic of a triple quadrupol MS/MS measurement. The peptides of the enzyme digested allergen sample are ionized by electrospray ionization (ESI). In the first quadrupole (Q1) the peptides are mass/charge selected, then the second quadrupole (Q2) functions as a collision cell peptides are fragmented. These fragments are then mass/charge analyzed in the third quadrupole (Q3).*

Points of consideration:

- Qualitative/quantitative
  - Reference materials are needed for quantification (especially for screening methods of multiple allergens).
  - LC-MS screening can provide qualitative information as multiple allergens can be analysed in one single run.
  - LC-MS can distinguish between related species within an allergenic group (e.g. fish, crustaceans, or mustard subspecies).
  - Validation of LC-MS method is needed to provide more information on the measuring range and matrix effects.
  - Quantitative methods are still not widely used because of the lack of reference materials for certain allergens, and the costly process of validation. Also matrix effects can be very challenging. Standard addition can be used to minimalize matrix effects.
- Calibrants
- Isotope labelled full ingredient most optimal -> Gavage et al. [2]
- Cost and time to execute this method
  - Large investment
  - Highly trained personnel needed, also for data analysis
  - Limited reference methods
  - Sample preparation is very time consuming and challenging; long digestion times (1 hour to overnight)
  - Not all detergents applicable due to incompatibility with the LC-MS system (e.g. ion-pairing on the LC columns causing shifting retention times)
  - Salts will affect the ionization process; sample clean-up procedures are essential (e.g. ultrafiltration or solid phase extraction (SPE))
- Throughput
  - Typical runtimes are 20 to 60 minutes for an allergen screening method with LC-MS. Sample preparation approx. 2 days.

[2] Gavage, M., Van Vlierberghe, K., Dieu, M., Renard, P., Arnould, T., Gevaert, K., ... & Gillard, N. (2023). Multi-Allergen Quantification in Food Using Concatemer-Based Isotope Dilution Mass Spectrometry: An Interlaboratory Study. Journal of AOAC International, 106(4), 886-898.has a good alternative.

Wageningen Food Safety Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
wur.eu/food-safety-research

WFSR Report 2024.007

The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 7,700 employees (7,000 fte), 2,500 PhD and EngD candidates, 13,100 students and over 150,000 participants to WUR's Life Long Learning, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.

To explore the potential of nature to improve the quality of life

Wageningen Food Safety Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0) 317 48 02 56
wur.eu/food-safety-research

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