Chapter 2

Food Allergen Analysis: Detection, Quantification and Validation by Mass Spectrometry

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69361

Abstract

Worldwide, food-allergy-related diseases are a significant health problem. While the food industry works on managing cross-contaminations and while clinicians deal with treatment, laboratories must develop efficient analytical methods to ensure detection of hidden allergens that can cause severe adverse reactions. Over the past few years, huge progress has been made in mass spectrometry for the analysis of allergens in incurred and processed foodstuffs, especially as regards sample preparation and enrichment (solid phase extraction, protein precipitation and ultrafiltration). These achievements make it possible to meet the Allergen Bureau's Voluntary Incidental Trace Allergen Labelling (VITAL) sensitivity criteria. The present chapter details the different steps in the development of mass spectrometry methods, from peptide selection to the validation of qualitative and quantitative methods. The chapter focuses mainly on studies performed with incurred and processed food samples to ensure the applicability of the methods to allergen detection in real food products.

Keywords: allergens, advances, detection, quantification, challenges, mass spectrometry, UHPLC-MS/MS, validation

1. Introduction

Food allergies have increased significantly, affecting between 3 and 4% of adults and at least 6% of children [1]. According to the European Academy of Allergology and Clinical Immunology (EAACI), the prevalence of food allergy has doubled over the past 10 years [2]. After an adverse reaction to a foodstuff, which may range from mild to severe (e.g.

anaphylaxis) [3, 4], allergic patients have to exclude that foodstuff from their diet. Each year in the United States, some 100 deaths are caused by anaphylaxis due to food allergy [5], the main culprits being allergens from peanut, tree nuts, fish, shellfish and milk [6]. Currently, there exist no treatments for food allergy, but clinical trials have been performed to test subcutaneous immunotherapy and oral immunotherapy used to desensitize patients [7]. The high level of adverse reactions observed in these trials has led clinicians to find safer alternative therapies, such as sublingual and epicutaneous immunotherapy. These approaches consist, respectively, in placing allergens (drops or tablets) under the tongue or in using a skin patch to induce sustained protection against anaphylaxis [8]. Although they do not treat allergic disease, they improve considerably the quality of life of highly allergic patients and constitute a real hope for them [9, 10]. The number of potentially allergenic ingredients that must appear on food labels differs in different parts of the world [11]. In Europe, regulation (EU) 1169/2011 imposes indicating the following 14 ingredients: milk, peanut, egg, soybean, fish, crustaceans, cereals containing gluten, tree nuts, celery, lupin, mustard, sesame, molluscs and sulfur dioxide [12]. This regulation fails to take into account the accidental introduction of allergens during production, transportation or storage, even though allergens introduced in this manner can trigger severe reactions [13–15]. To protect food consumers, the industry has widely used precautionary allergen labelling (PAL) (i.e. statements such as 'may contain', 'may contain traces of…') [16]. Yet, the lack of correlation between the presence of allergens and precautionary labelling has led customers to lose trust in food labels [17–20]. In a study of food product recalls over a four-year period in the European Union, the United States, Canada, Hong Kong, Australia and New Zealand, 42–90% of the recalls, depending on the country, were justified by the presence of allergens not indicated on the label [21]. Between 2007 and 2012, the Food and Drug Administration (FDA) recalled 732 products because of allergen contaminations [22] and allergic reactions are due to five foods: milk, egg, peanuts, wheat and soybean (**Figure 1**).

The distribution of these recalls in the European Union, reported in **Figure 2**, shows that the products most commonly involved in food recalls are cereals and bakery products.

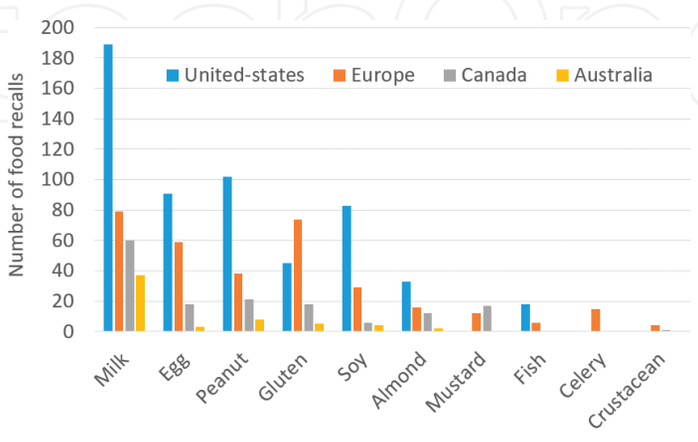

Figure 1. Number of food recalls per allergen category in the United States, Europe, Canada, and Australia between 2012 and 2015 [23–26].

http://dx.doi.org/10.5772/intechopen.69361

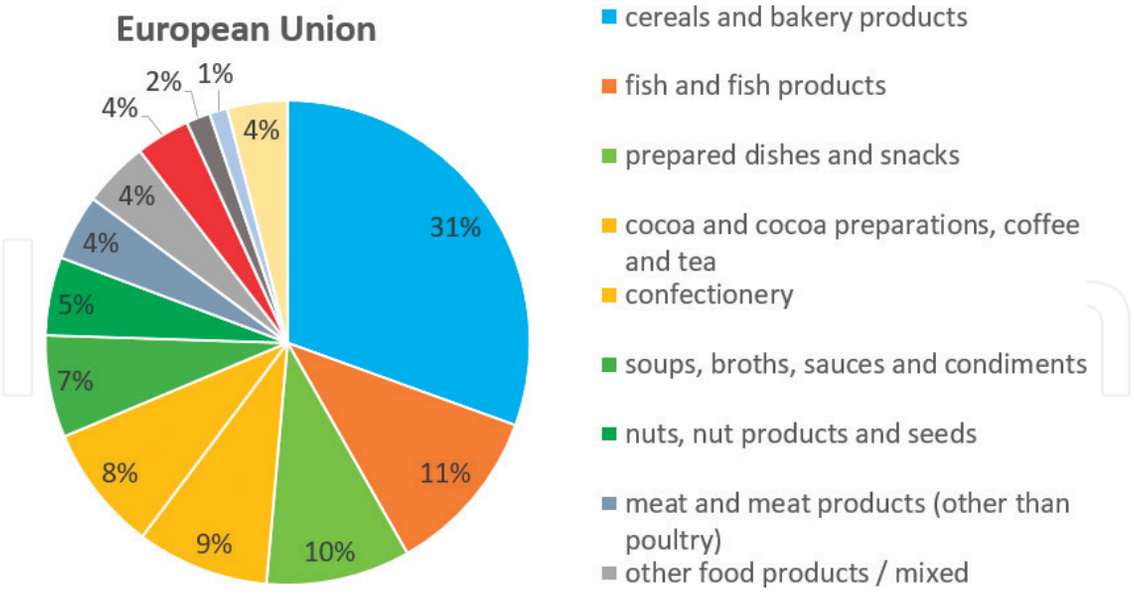

Figure 2. Percentage distribution of food allergen recalls in the European Union (according to the Rapid Alert System for Food and Feed) [24].

The widespread use of PAL can be explained by the lack of regulatory thresholds and the complexity of food allergen management through the supply chain. To counter this lack, the Voluntary Incidental Trace Allergen Labelling (VITAL) system has been developed in Australia and New Zealand to assist food producers in managing cross-contaminations during food production [27]. This system sets allergen thresholds, based on clinical studies, for the protection of 95–99% of the allergic population. Other referentials for allergen thresholds are the European Academy of Allergy and Clinical Immunology (EAACI) and the Netherlands Food and Consumer Product Safety Authority (NVWA) [28] (**Table 1**).

While the systems just mentioned have no regulatory value, food laboratories use them in evaluating method sensitivity. To obtain a concentration expressed in 'mg proteins per kilogram', a food portion size must be considered in order to compare the analytical method with VITAL thresholds (e.g. a portion size of 50 g, **Table 1**). Yet while VITAL thresholds are expressed in 'mg proteins', laboratories express their results in 'mg ingredients' [29, 30] or may refer either to soluble proteins [31, 32] or total proteins [33] per kg. To compare method performances, a conversion factor must thus be applied (e.g. 25% proteins in whole peanuts [34]). Moreover, VITAL action levels have been determined from clinical studies, mostly on the basis of the allergenicity of raw ingredients, although studies have demonstrated a major decrease in allergenicity in baked products. For example, 50–85% of allergic children are able to tolerate baked egg [35] and a study published in 2015 found 63% to tolerate 3.8 g egg-white protein in baked-egg products [36].

Nevertheless, the prevalence of baked product recalls confirms that laboratories must develop sensitive methods for detecting allergens in processed foodstuffs. The most widely used methods are based on the recognition of allergen proteins by antibodies, notably lateral flow device methods and enzyme-linked immunosorbent assays (ELISAs) [39]. DNA-based meth-

Food	Reference dose VITAL (mg of proteins) [27, 34, 37]	Reference dose ǀEAACI (mg of proteins) [38]	Reference dose NVWA (mg of proteins) [28]	Reference dose VITAL (mg of proteins per kg) Portion size: 50 g
Peanut	0.2	0.2	0.015	4
Cow milk	0.1	0.1	0.016	2
Egg	0.03	0.03	0.0043	0.6
Hazelnut	0.1	0.1	0.011	4
Soy	1.0	1.0	0.078	20
Wheat	1.0	1.0	0.14	20
Cashew	2.0	2.0	1.4	40
Mustard	0.05	0.05	0.022	1
Lupin	4.0	4.0	0.83	80
Sesame	0.2	0.2	0.10	4
Shrimp	10	10.0	3.7	200
Fish	/	0.1	/	/

Table 1. VITAL (http://allergenbureau.net/vital/), EAACI (http://www.eaaci.org/) and NVWA (https://www.nvwa.nl/) reference doses for different food allergens.

ods such as those exploiting the real-time polymerase chain reaction (PCR) [40] are also used to detect the presence of allergens. Currently, mass spectrometry is becoming an alternative to these methods, as heat-processing induces protein denaturation and structural modifications that might result in non-recognition of the target protein by conformational antibodies and thus in the case of ELISAs, lead to false negatives or at least major underestimation of allergen content [41–44]. Mass spectrometry has the advantage of permitting simultaneous analyses for several allergens in food, including processed food products, with high sensitivity and specificity.

This chapter highlights the important improvements made over the last 10 years in mass spectrometry applied to the development of allergen detection methods. It covers and discusses the mass spectrometry methods currently used to detect and quantify allergens in processed food products, including their validation.

2. Detecting food allergens

2.1. Selecting marker peptides

Food allergens (except sulfites) are proteins that need to be digested by enzymes (trypsin and chymotrypsin) so as to generate peptides suitable for routine mass spectrometry analysis. Identification and selection of robust peptides are generally done first on digested raw ingredients before analysis of digested processed ingredients in food matrices. This section

summarizes two approaches commonly used to select marker peptides (the instrumental approach and the *in silico* approach) and the specificity and sensitivity critera used.

2.1.1. Peptide selection

2.1.1.1. Instrumental peptide selection

The first approach is to identify abundant marker peptides by high resolution mass spectrometry (HRMS). Downstream from allergen analysis by HRMS, the generated data are transferred into an algorithm for assigning peptides to MS/MS spectra (MASCOT, X!Tandem, SEQUEST) [45]. For example, Sealey-Voyksner et al. analysed 12 tree nuts and peanut-raw and roasted (176.7°C, 30 min) by time of flight (q-TOF) (Agilent 6530) spectrometry and selected two abundant peptides per tree nut and four for peanut [46]. In a previous study, ice cream spiked with peanuts was analysed by q-TOF (Waters Micromass II) to identify peptides of the Ara h1 allergen [47]. In a 2012 study, Cucu et al. identified several soybean marker peptides by matrix-assisted laser desorption ionization (MALDI-TOF/MS) [48]. The main advantage of this approach is that global peptide and protein profiles can be analysed for the different samples.

2.1.1.2. In silico peptide selection

Another strategy for selecting marker peptides is to retrieve target protein sequences from a database, e.g. Uniprot (http://www.uniprot.org/), and to perform an *in silico* digestion with an open access software, e.g. Skyline or MRMaid [49, 50] (**Figure 3**).

In silico digestion with multiple reaction monitoring (MRM) involves generating a list of criteria that must be applied or set by the user as regards peptides, transitions and MS/MS

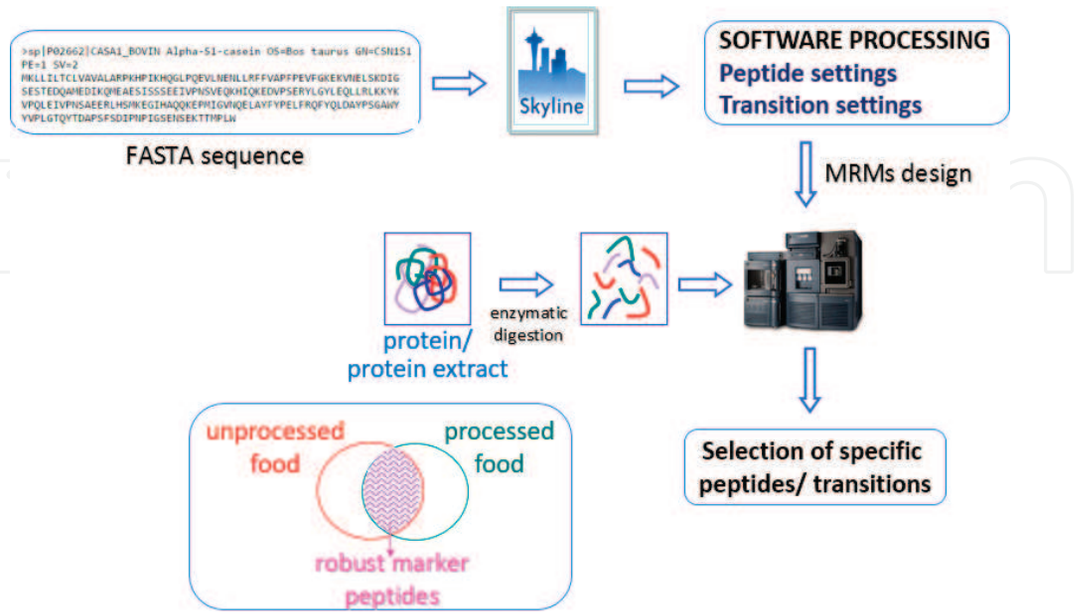

Figure 3. *In silico* peptide selection with the Uniprot database and Skyline software.

parameters (e.g. peptide length, charge states, fragmentation and enzyme). Then raw ingredients or incurred matrices can be analysed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). This approach allows identification of abundant peptides. It was used by Rogniaux et al. for the analysis of wheat varieties: several gluten peptides were identified with a linear ion trap quadrupole mass filter in tandem with an orbitrap (Thermo Fisher Scientific) [51].

An *in silico* approach also requires a complete database with available protein sequences. Uniprot inventories, however, can be too large (e.g. >145,000 proteins for the wheat species-*Triticum aestivum*), making it necessary to first select target proteins from the literature. Use of a routine UHPLC-MS/MS instrument is the main advantage of the *in silico* approach for laboratories unwilling to invest in a high-resolution mass spectrometer.

2.1.2. Specificity

BLAST: After this selection, blasting must be performed to guarantee the specificity of marker peptides. This step is mandatory but not always included in method development. In one study, for example, Hoofnagle et al. selected five peptides for the detection of β-casein in cookies: EMPFPK (6AA), VLPVPQK (7AA), AVPYPQR (7AA), GPFPIIV (7AA) and DMPIQAFLLYQEPVLGPVR (19AA) [52]. Only one of these peptides could be blasted, and this peptide is 100% homologous to goat, zebu, buffalo, yak and sheep β-casein (Uniprot). In proteomics, peptide blasting should be systematic, even though the international trade frequently introduces new food products and although some proteins can still be missing in the different databases.

The **specificity of selected fragments** is also paramount. To improve specificity, the mass-to-charge ratio (m/z) of the precursor should be lower than the m/z of the fragments. Too-small fragments should be avoided. At least, fragments of 1 to 2 amino acids (b1, b2, y1, y2) should be excluded, which is not always the case in published methods [53, 54].

Blanks: Matrices without allergens must also be analysed to ensure the specificity of the selected transitions of the target peptides. As databases do not cover all possible proteins and as new food products enter the food chain regularly, this experimental testing is crucial to proving method specificity.

2.1.3. Identifying marker peptides in incurred foodstuffs

The advantage of using mass spectrometry is detection of allergens in industrial food products. For such applications, only target peptides and proteins that will be detected in incurred and processed matrices, such as those listed in **Table 2**, need to be retained in the analytical methods. Some peptides are common to the majority of published methods: FFVAPFPEVFGK and YLGYLEQLLR (Casein αS1), and GGLEPINFQTAADQAR (ovalbumin), among others. Target peptides detected after different extraction and purification steps in several types of matrices constitute potential marker peptides for the detection of allergens in a wide variety of foodstuffs.

Authors	Matrix	Allergen	Protein	Peptide	Fragments
Heick et al. [53]	Bread (60 min, 200°C)	Milk	αS1-casein	YLGYLEQLLR	b2, y8
				FFVAPFPEVFGK	y8, y9
			αS2-casein	NAVPITPTLNR	b2, y8
				FALPQYLK	a1, y5
		Egg	Ovalbumin	HIATNAVLFFGR	a2, y10
				YPILPEYLQCVK	y6, y8
				DILNQITKPNDVYSFSLASR	a2, y8
				ELINSWVESQTNGIIR	y9, y10
		Soy	Glycinin	NLQGENEGEDKGAIVTVK	a2, b3
				VFDGELQEGR	a2, y8
				SQSDNFEYVSFK	y3, y10
				EAFGVNMQIVR	y6, y8
		Peanut	Ara h1	DLAFPGSGEQVEK	a3, y9
				GTGNLELVAVR	y5, y6
			Ara h3/4	RPFYSNAPQEIFIQQGR	y6, b7
				WLGLSAEYGNLYR	a2, y11
		Hazelnut	11S globulin	ADIYTEQVGR	y6, y7
				INTVNSNTLPVLR	y4, y9
				QGQVLTIPQNFAVAK	y8, y10
				ALPDDVLANAFQISR	y8, y9
		Walnut	Jug r1	DLPNECGISSQR	y4, y10
				QCCQQLSQMDEQCQCEGLR	y3, y10
				GEEMEEMVQSAR	y7, y8
		Almond	Prunin	GNLDFVQPPR	y3, y7
				GVLGAVFSGCPETFEESQQSSQQGR	y6, y7
				ALPDEVLANAYQISR	y8, y9
				NGLHLPSYSNAPQLIYIVQGR	y6, b11
Pilolli et al. 2016 [56]	Cookie (200°C, 12 min)	Milk	αS1-casein	FFVAPFPEVFGK	y8, y9, y10
				YLGYLEQLLR	y5, y6, y8
		Egg	Ovalbumin	GGLEPINFQTAADQAR	y7, y10, y12
				YPILPEYLQCVK	b4, y8, y9
		Peanut	Conarachin	VLLEENAGGEQEER	y7, y8, y12
				EGEQEWGTPGSEVR	y6, y8, y9

Authors	Matrix	Allergen	Protein	Peptide	Fragments
		Soy	Glycinin G1-G2	SQSDNFEYVSFK	y3, y10
				FYLAGNQEQEFLK	y9, y10, y11
		Hazelnut	11S globulin-like protein	ADIYTEQVGR	y6, y7
				ALPDDVLANAFQISR	y7, y8, y13
Lamberti et al. [57]	Cookie (180°C, 10 min)	Milk	αS1-casein	YLGYLEQLLR	y8, y9, y10
				FFVAPFPEVFGK	y8, y9, y10
				HQGLPQEVLNENLLR	y11, y12
Pedreschi et al. [58]	Cookie (180°C, 16 min)	Peanut	Ara h1	VLLEENAGGEQEER	y9, y8, y7, y6, y4, y2
				DLAFPGSGEQVEK	y10, y9, y8, b4, b3, b2
			Ara h2	CCNELNEFENNQR	y8, y6, y5, y4
				NLPQQCGLR	y7, y6, y5, a2
				CDLEVESGGR	y8, y6, y5, y4
				CMCEALQQIMENQSDR	y14, y11, y10, y8, y7, y6, y5, b2
			Ara h3	LNAQRPDNR	ymax, y8, y7, y5, b2
				SPDIYNPQAGSLK	ymax, y12, y9, y8, y7, y5, b3
				AHVQVVDSNGNR	b7, y6, b5
Huschek et al. [59]	Cookie (190°C, 13 min)	Soy	Gly m6	VFDGELQEGR	903.6/ 489.2/ 788.5
				LSAEFGLR	432.3/ 779.4/ 579.3
				LNALKPDNR	742.4/ 629.3/ 501.2
		Sesame	Ses i6	ISGAQPSLR	472.3/ 728.4/ 671.4
				AFYLAGGVPR	556.3/ 485.3/ 669.4
				SPLAGYTSVIR	795.4/ 866.5/ 575.4
		Lupine	β-conglutin	LLGFGINADENQR	846.4/661.3/ 797.4
				NTLEATFNTR	951.5/838.4/ 709.4
				NPYHFSSQR	761.4/ 624.3/ 477.2

Table 2a. List of target marker peptides used to detect several allergens in bread and cookies [55–59].

2.2. Developing mass spectrometry methods

After selection of marker peptides, the developed method must be able to detect traces of the allergen proteins in the 'mg allergen proteins per kg food product' range. The real chal-

Authors	Matrix	Allergen	Protein	Peptide	Fragments
Planque et al. [33, 60]	Cookie (180°C—18 min), sauce (95°C, 45 min), ice cream and chocolate	Milk	αS1-Casein	FFVAPFPEVFGK	y6, y8, y9
				HQGLPQEVLNENLLR	b4, y6, y7
				YLGYLEQLLR	y5, y6, y7
			αS2-casein	NAVPITPTLNR	b3, y8, y8
			β-lactoglobulin	VYVEELKPTPEGDLEILLQK	y11, y14, y16
				VLVLDTDYK	y5, y6, y7
				LSFNPTQLEEQCHI	y7, y10, y10
		Egg	Ovalbumin	GGLEPINFQTAADQAR	y10, y12, y12
				LTEWTSSNVM EER	y7, y8, y9
				ISQAVHAAHAEINEAGR	y9, y10, y11
			Vitellogenin	EALQPIHDLADEAISR	y6, y7, y12
				NIPFAEYPTYK	y4, y9, y9
				NIGELGVEK	y5, y6, y7
				YLLDLLPAAASHR	y7, y7, y11
			Apovitellenin	NFLINETAR	y5, y6, y7
		Peanut	Cupin	NTLEAAFNAEFNEIR	y7, y8, y9
				RPFYSNAPQEIFIQQGR	b7, y6, y10
				FNLAGNHEQEFLR	y5, y9, y10
				TANELNLLILR	y6, y7, y8
		Soy	Glycinin	ISTLNSLTLPALR	y7, y8, y9
				EAFGVNMQIVR	y5, y6, y7
				ELINLATMCR	y5, y6, y8
				LITLAIPVNKPGR	y7, y9, y11
Parker et al. [61]	Muffin (177°C, 48 min)	Egg	Lysozyme	FESNFNTQATNR	Not provided
				NTDGSTDYGILQINSR	
			Ovalbumin	ELINSWVESQTNGIIR	
				GGLEPINFQTAADQAR	
				HIATNAVLFFGR	
		Milk	αS1-casein	FFVAPFPEVFGK	
				HQGLPQEVLNENLLR	
				YLGYLEQLLR	
			β-lactoglobulin	LSFNPTQLEEQCHI	
				TPEVDDEALEK	
				VLVLDTDYK	

Authors	Matrix	Allergen	Protein	Peptide	Fragments
		Peanut	Ara h1	GTGNLELVAVR	
				NNPFYFPSR	
			Ara h2	CCNELNEFENNQR	
				CMCEALQQIMENQSDR	
				NLPQQCGLR	
			Ara h3	FNLAGNHEQEFLR	
				SPDIYNPQAGSLK	
				WLGLSAEYGNLYR	
Gomaa et al. 2014 [62]	Cookie (177°C, 12 min)	Milk	αS1-casein	HQGLPQEVLNENLLR	best transitions not selected
			αS2-casein	NAVPITPTLNR	
				LNFLK	
				ALNEINQFYQK	
			κ-casein	YIPIQYVLSR	
		Soy	Glycinin G1 precursor	HNIGQTSSPDIYNPQAGSVTTATSLDFPALSWLR	
				TNDTPMIGTLAGANSLLNALPEEVIQHTFNLK	
				VLIVPQNFVVAAR	
				HQQEEENEGGSILSGFTLEFLEHAFSVDK	
				EGDLIAVP…DQMPR	
			Glycinin G2 precursor	TNDRPSIGNLAGANSLLNALPEEVIQHTFNLK	
				QNIGQNSSPDIYNPQAGSITTATSLDFPALWLLK	
			Beta conglycinin alpha chain precursor	DLDIFLSIVDMNEGALLLPHFNSK	
				AIVILVINEGDANIELVGLK	
		Wheat	Alpha amylase trypsin inhibitor	YFIALPVPSQPVDPR	
				LLVAPGQCNLATIHNVR	
				LTAASITAVCR	
				LPIVVDASGDGAYVCK	
				SGNVGESGLIDLPGCPR	
				EMQWDFVR	
				DYVLQQTCGTFTPGSK	

Table 2b. List of target marker peptides used to detect several allergens in sauce, ice cream, chocolate, cookies and muffins [60–62].

lenge for laboratories is to achieve this sensitivity with processed foodstuffs. To reach this sensitivity, two factors must be considered: instrument sensitivity and optimization of sample preparation. The different strategies used to evaluate sensitivity are described below.

Instrument sensitivity: No comparison of the sensitivities of different instruments with the same peptide extract has yet been published for allergen analysis, although the sensitivity of the instrument is crucial to the sensitivity of the method, as in the case of other contaminants. One should bear in mind, however, that the most sensitive research-dedicated instrument might not be the best choice for routine analysis (automated injection and short analytical run).

Extraction and purification of proteins: The ideal sample preparation protocol should allow extraction of 100% of the target compounds, the final extract used for MS analysis being as pure as possible. Yet, the preparation of samples for food allergen analysis is difficult, because it should be applicable to a very broad range of food matrices and because the extractability of proteins might be altered in a processed food [63]. In addition, several modifications can occur, e.g. asparagine deamination, the Maillard reaction and several reactions of lysine. Such modifications cause a mass shift of tryptic peptides, resulting in non-recognition of several peptides by mass spectrometry [64–66]. To improve protein extraction, different parameters can be optimized: the composition of extraction buffers, the temperature, the sample-to-buffer ratio and the presence of detergents. Furthermore, the purification step is as important as extraction in order to concentrate proteins in and eliminate interferences from the supernatant. Purification usually involves solid phase extraction (SPE), protein precipitation, ultrafiltration and size exclusion chromatography (SEC), among others. Optimizing extraction and purification is a key step in developing sensitive methods for the detection of allergens by mass spectrometry (**Table 3**).

Determining the sensitivity: The sensitivity of food allergen analysis can be determined on spiked samples (obtained by incorporating extracted proteins into a matrix after processing), fortified samples (obtained by incorporating raw ingredients into a matrix after processing) or processed samples (obtained by incorporating raw ingredients into a matrix before processing). For spiked and fortified samples ('non-processed samples'), examples of the limit of quantification (LOQ) reached are 0.1 mg milk protein, 0.3 mg egg protein and 2 mg soy protein per kg cookies [67] and 0.1–1.3 mg tree nuts per kg biscuit [68]. Although these studies demonstrate the sensitivity of mass spectrometry, the real challenge is to reach this sensitivity in thermally processed samples. Important improvements have been made over the last 5 years in the detection of allergens in processed samples. Recently, developed methods allow reaching an LOQ near or below the VITAL threshold (**Table 1**), e.g. 0.5 mg for milk protein, 3.4 mg egg protein, 5 mg soy protein and 2.5 mg peanut protein per kg incurred cookie (180°C, 18 min, with SPE purification) [60]. In another study, the LOQs achieved were 30 mg egg (13.8 mg proteins), 20 mg milk (7.2 mg proteins), 19 mg soy (6.8 mg proteins), 20 mg hazelnut (3 mg proteins) and 40 mg peanut (10 mg proteins) per kg incurred cookie (200°C, 12 min, with SEC purification) [56].

As described above, the sensitivity reached for processed samples is lower than that obtained with spiked or fortified samples. The same applies to ELISAs, which can show up to 100-fold lower sensitivity when applied to processed food than when applied to raw food, as demonstrated by the poor performance of several ELISAs for egg detection in cookies after processing. In 2010, Dumont et al. showed that one ELISA kit was not even able to detect 1000 mg egg powder per kg baked cookie, and four others strongly underestimated the amount of egg in

Authors	Allergen	Matrix	Extraction	Purification	Digestion	Instrument	Sensitivity
Heick et al. (2010) [55]	Milk, egg, soy, peanut, hazelnut, walnut, almond	Bread (200°C, 60 min)	2 g/20 ml Buffer: TRIS-HCl pH 8.2 Agitation: 60°C for 3h	Ultrafiltration (Amilcon Ultra 15 mL, 5 kDa molecular weight cut-off) (Millipore)	Dilution: 1 mg of proteins by ml with NH4HCO3 (100 mM) Aliquot: 100 µl Reduction: 50 µl DTT (200 mM), 45 min Alkylation: 40 µl IA (1 M), 45 min in the dark 20 µl DTT (200 mM) + 50 µl NH4HCO3 (100 mM) Digestion: 10 µl trypsin (1 µg/µl) 12 h - 37°C 2 µl formic acid	LC: 1200 HPLC (Agilent) Column: Xbridge C18 3.5 µm (2.1×150 mm) (Waters) MS: API 4000QTrap (MDS Sciex)	LOD (S/N>3) 5 mg of soluble milk proteins by kg 42 mg of soluble egg proteins by kg 24 mg of soluble soy proteins by kg 11 mg of soluble peanut proteins by kg 5 mg of soluble hazelnut proteins by kg 70 mg of soluble walnut proteins by kg 3 mg of soluble almond proteins by kg
Pilolli et al. [56]	milk, egg,soy,peanut, hazelnut	Cookie (200°C, 12 min)	2.5 g/50 ml Buffer: 20 mM TRIS-HCl pH 8.2 Ultrasound: 30 min	1.2 µm acetate cellulose membrane, Size exclusion column (SEC) (G25 Sephadex column)	Elution SEC: 3.5 ml NH4HCO3 (50 mM) Aliquot: 300 µl Protein denaturation: 15 min at 95°C Reduction: 15 µl of 50 mM DTT 30 min at 60°C Alkylation: 30 µl of 100 mM IAA 30 min in the dark at room temperature	LC: - Column: Aeris Peptide XB-C18 (150 × 2.1 mm) (Phenomenex) MS: Dual pressure Linear Ion Trap Spectrometer Velos Pro (Thermo Fisher Scientic)	LOD (S/N>3) 7 mg of milk by kg 9 mg of egg by kg 6 mg of soy by kg 13 mg of peanut by kg

Authors	Allergen	Matrix	Extraction	Purification	Digestion	Instrument	Sensitivity
					Digestion:4 μl trypsin (1 μg/μl) 14h		7 mg of hazelnut by kg
					Acidification: 1M HCl		
					Filtration: 0.2 μm		
Lamberti et al. [57]	Milk	Cookie (180°C, 10 min)	10 mg /200 μl	Protein precipitation methanol/ chloroform	Pellet + 50 μl of 0.025M NH4HCO3 pH 8.0 RT	LC: HP 1100 HPLC (Agilent)	LOD: 1.3 mg of milk proteins per kg
			Buffer: NH4HCO3 / (NH4)2CO3 +1 % SDS buffer, pH 8.2		3 μl of trypisn (75 ng/μl) 37°C, 90 min	Column: ACE C18 300A (250 mm × 1 mm)	LOQ: 4 mg of milk proteins per kg
			Agitation: 20 min, 60°C		5 μl of 5% formic acid	MS: XCT-Plus Ion trap mass spectrometer (Agilent)	
Pedreschi et al. [58]	Peanut	Cookie (180°C, 16 min)	Buffer: 20 mM TRIS - 150 mM NaCl, pH 7.4	GE Healthcare kit	50 μg of protein / 50 μl of Rapigest in a 50 mM ammonium bicarbonate buffer	LC: nano Acquity UPLC (Waters)	>10 mg of peanut per kg
			Ultrasound: 4°C, 20 min	Cut-off filtration 3000 MWCO	2.5 μl of 50 mM DTT 30 min, 60°C	Column: nano Acquity BEH130 C18 1.7 μm (75 μm × 100 mm)	
					5 μl 100 mM IAA 30 min-dark	Column: nano Acquity UPLC Trap SymC18 5 μm (180 μm × 20 mm)	
					1 μl of 1μg/μm of trypsin 5h-37°C	MS: Q-Tof Ultima Global (Waters)	

Table 3a. Mass-spectrometry-based methods (extraction, purification, digestion, and analysis) for detecting allergens in processed food products [55–58].

http://dx.doi.org/10.5772/intechopen.69361

Author	Allergens	Matrices	Extraction	Purification	Digestion	Instrument	Sensitivity
Huschek et al. [59]	Soy, sesame, lupin	Wheat, cookie (190°C, 13 min), bread (220°C, 30 min)	1 g	SPE cardridge (LiChrolut RP-18 Merck Millipore)	Alkylation: IAA 20 min at 50°C	LC: Nexera XR UHPLC (Shimadzu)	LOQ (S/N > 10)
			Buffer: 100 mM NH4HCO3, 4M urea 5 mM DTT pH 8.2		Digestion: Trypsin formic acid 2%	Column: Aeris Peptide XB-C18 (100 × 2.1 mm, 1.7 μm) (Phenomenex)	10–20 mg of soy per kg 10–50 mg of sesame per kg
			Agitation 30 min RT			MS: Qtrap 5500 MS/MS (Sciex)	10–50 mg of lupine per kg
Planque et al. [33, 60]	Milk, egg, soy, peanut	Tomato sauce (95°C, 45 min), cookie (180°C, 18 min), ice cream, chocolate	2 g / 20 ml	Sep-Pack tC18 6cc (Waters)	10 ml extract + 10 ml NH4HCO3 (200 mM)	LC: UPLC Acquity (Waters)	LOQ (S/N > 10)
			Buffer: 200 mM TRIS-HCl pH 9.2, 2M urea		Reduction: 1 ml DTT (400 mM), 45 min	Column: BEH130 (2.1 × 150 mm) (Waters)	0.5 mg of milk proteins by kg
					Alkylation: 2 ml IAA (500 mM), 45 min in the dark	MS: Xevo TQS (Waters)	3.4 mg of egg proteins by kg
			Agitation:30 min Ultrasound: 15 min		Digestion: Ratio protein:trypsin 1:20 16 h, 37°C		5 mg of soy proteins by kg
					300 μl formic acid 20%		2.5 mg of peanut proteins by kg
Parker et al. [61]	Milk, egg, peanut	Muffin (177°C, 48 min)	Buffer: 2 M urea, 50 mM TRIS Ph 8.0, 25 mM DTT	Microcentrifuge tubes	Filter-aided sample preparation (FASP) sample cocentration and digestion protocol	LC: nano Acquity UPLC (Waters)	/
				Amicon Ultra 0.5 ml	Reduction: 10 mM DTT	Column: nano Acquity BEH130 C18 1.7 μm (100 μm × 100 mm)	
			vortex: 5 min at 1400 rpm	Utracel-10 membrane	Alkylation:25 mM IAA	MS:6500 QTRAP (Sciex)	
			Ultrasound: 10 min at 4°C		Digestion: Ratio protein:trypsin 1:100 16 h -37°C		
					0.1% trifluoroacetic acid and 2% acetonitrile		

Author	Allergens	Matrices	Extraction	Purification	Digestion	Instrument	Sensitivity
Gomaa et al. [62]	Milk, soy, wheat	Cookie (177°C, 12 min)	1 g/9 ml	OMIX C18/ tip (Varian)	Protein extract: 100 µl at 2 mg/ml	LC: nano Acquity UPLC (Waters)	10 mg of casein per kg
			Buffer: 50 mM NH4HCO3		Reduction: 1µl of DTT (0.5 M) 56°C, 20 min	Column: nano Acquity BEH130 C18 1.7 µm (75 µm × 100 mm)	10 mg of soy proteins per kg
					Alkylation: 2.7 µl IAA (0.55 M, 15 min)	nano Acquity UPLC Trap SymC18 5 µm (180 µm × 20 mm)	100 mg of gluten per kg
					Digestion: ratio enzyme:subtrate 1:20 3h-37°C	MS: Q-Tof Synapt MS (Waters)	
					5 µl trifluoroacetic acid (2.5%)		

Table 3b. Mass-spectrometry-based methods (extraction, purification, digestion, and analysis) for detecting allergens in processed chocolate, sauce, ice cream, muffins and cookies [59–62].

http://dx.doi.org/10.5772/intechopen.69361

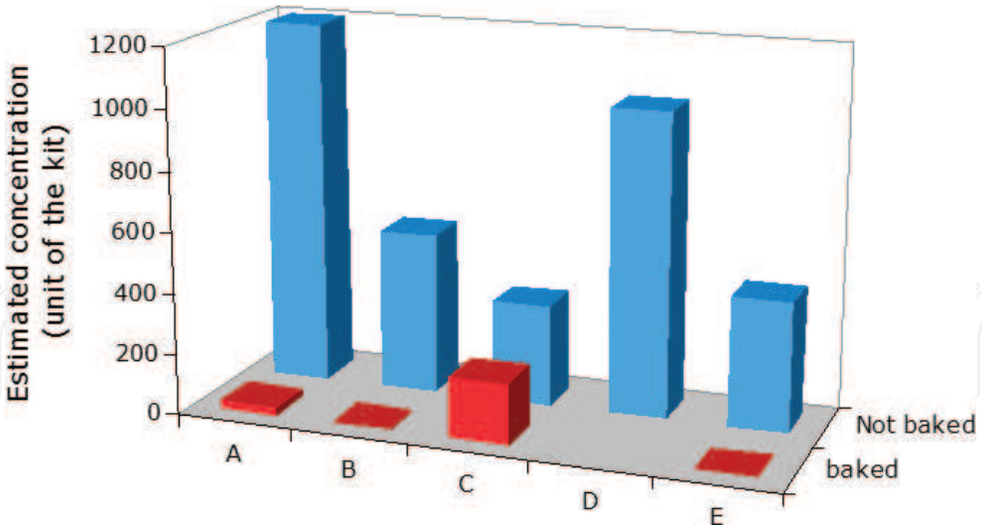

Figure 4. Analytical results for 1000 mg spray-dried whole egg powder (National Institute of Standards and Technology RM 8445) per kg incurred cookies, obtained with the different enzyme-linked immunosorbent assay test kits for egg detection (A–E) (from Ref. [69]).

the samples (**Figure 4** of Ref. [69]). While mass spectrometry and ELISAs show comparable sensitivities when applied to unbaked products, mass spectrometry seems to be the method of choice for the analysis of allergens in baked food products.

3. Quantifying food allergens

Detecting hidden allergens in food products is essential to protecting the food-allergic population. For full transparency of allergen labelling, laboratories should also be able to quantify allergens in order to help food manufacturers manage cross-contamination during food production [70]. However, significant signal suppressions have been observed in various food matrices, and the level of suppression depends on the matrix considered. In one study, for example, high-protein-content food products showed greater suppression of the peptide signal than ones with a low protein content: the determined LOQ values were 20 mg skim milk powder per kg for high-protein foods and 5 mg skim milk powder per kg for low-protein foods [71]. The food protein content is not the only parameter to be considered in relation to suppression of the peptide signal obtained by mass spectrometry: factors such as the type of process, the fat content and the presence of tannins also have an important influence on food allergen detection and must be taken into account.

While detecting allergens in various food products is difficult, quantifying them is even worse. In recent years, mass spectrometry techniques have been used for quantitation in proteomic analysis. Two approaches have emerged as the most relevant for food allergen quantification: label-free quantification and the use of stable-isotope-labelled peptides or proteins [70, 72, 73]. The two strategies are compared in **Table 4** (target peptides, internal standards and calibration curves) and discussed in relation to the AOAC guideline 2016.002 method performance requirements for the quantification of allergens in food products, specifying a recovery between 60 and 120% and intra-day and inter-day coefficients of variation lower than 20 and 30%, respectively [74] (**Table 5**).

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Careri et al. [76]	Rice crispy/ chocolate snacks	Peanut	Ara h2	CCNELNEFENNQR	Q-TOF Micro (Waters)	No internal standard	Rice crispy/ chocolate snacks were spiked with peanut proteins
				CMCEALQQIMENQSDR			
			Ara h3/4	AHVQVVDSNGDR			
				SPDIYNPQAGSLK			
Monaci et al. [75]	Fruit juices	Milk	α-lactalbumin	/	Ultima triple quadrupole mass spectrometer (Waters)	No internal standard	Fruit juices were spiked with milk proteins
			β-lactoglobulin A				
			β-lactoglobulin B				
Korte et al. [88]	Bread matrix, ice cream, chocolate, muesli with fruit and berry	Almond	Pru du 6.0101	GNLDFVQPPR	QTRAP 6500 (Sciex)	No internal standard	Matrices were spiked with allergen proteins
			Pru du 6.0201	VQGQLDFVSPFSR			
				ALPDEVLQNAFR			
		Cashew	Ana o2	ADIYTPEVGR			
				EGQMLVVPQNFAVVK			
				LTTLNSLNLPILK			
		Hazelnut	Cor a 9	LNALEPTNR			
				VQVVDDNGNTVFDDELR			
				QGQVLTIPQNFAVAK			
		Peanut	Ara h3	FNLAGNHEQEFLR			
				WLGLSAEYGNLYR			
				TANDLNLLILR			

http://dx.doi.org/10.5772/intechopen.69361

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
		Pistachio	Pis v 5	AMISPLAGSTSVLR			
				ITSLNSLNLPILK			
				GFESEEESEYER			
		Walnut	Jug r 2	FFDQQEQR			
				ATLTLVSQETR			
			Jug r4	ALPEEVLATAFQIPR			
Mattarozzi et al. [77]	Pasta, biscuit	Lupin	β-conglutin	IVEFQSKPNTLILPK	LTQ XL linear ion trap (Thermo)	No internal standard	Pasta and biscuits were fortified with lupin proteins
Zhang et al. [78]	Infant formulas and whey proteins	Milk	α-lactalbumin	VGINYWLAHK	Xevo TQ triple quadrupole (Waters)	KILDKVGINNYWLAHKALCSE	Matrices were spiked with synthetic peptide VGINYWLAHK
Posada-Ayala et al. [79]	Sauces and salty biscuit	Mustard	Sin a1	ACQQWLHK	6460 triple quadrupole (Agilent technologies)	Purified protein Sin a 1	Standard addition of mustard in sauces and salty biscuits
				IYQTATHLPK			
				EFQQAQHLR			

Table 4a. Quantification of food allergens in different food products by mass spectrometry using label-free quantification with an (1) external calibration curve [75–79, 88], (2) unlabelled modified synthetic peptide [78], and (3) standard addition [79].

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Newsome and Scholl [82]	Cookie (180°C, 16 min)	Milk	α-s1 casein	HQGLPQEVLNENLLR	Hybrid triple-quadrupole 4000 QTRAP (AB Sciex)	HQGLPQEVLNENLLR[13C, 15N]	Cookies were spiked with isotope labelled peptides
				YLGYLEQLLR		YLGYLEQLLR[13C6, 15N]	
				FFVAPFPEVFGK		FFVAPFPEVF[13C6, 15N]GK	
						15N-α-s1 casein	
Parker et al. [61]	Cereal bar (177°C, 30 min) Muffin (177°C, 48 min)	Egg	Lysozyme	NTDGSTDYGILQINSR	6500 Qtrap (AB Sciex)	Heavy isotope [13C, 15N] labelled peptides / labelled amino acid: R or K	Cereal and muffin were spiked with isotope labelled peptides
			Ovalbumin	GGLEPINFQTAADQAR			
		Milk	α-s1 casein	YLGYLEQLLR			
			β-lactoglobulin	LSFNPTQLEEQCHI			
		Peanut	Ara h1	NNPFYFPSR			
			Ara h2	NLPQQCGLR			
			Ara h3	SPDIYNPQAGSLK			
Huschek et al. [59]	Wheat, cookies (190°C, 13 min), soft bread (220°C-	Soy	Gly m6	VFDGELQEGR	QTRAP 5500 (Sciex)	VFDGELQEGR[13C6, 15N4]	Wheat and cookies were spiked with allergen proteins
		Sesame	Ses i6	ISGAQPSLR		ISGAQPSLR[13C6, 15N4]	
		Lupin	β-conglutin	LLGFGINADENQR		LLGFGINADENQR[13C6, 15N4]	
Lutter et al. (2011) [71]	Baby food soy-based formula, infant cereals, breakfast cereals, rince water	Milk	β-casein	AVPYPQR	6460 triple quadrupole (Agilent technologies)	AVPYPQR [13C6, 15N4]	0.1% formic acid were spiked with proteins
			α-s2 casein	ALNEINQFYQK		ALNEINQFYQK[13C6, 15N2]	
			α-s2 casein	FALPQYLK		FALPQYLK[13C6, 15N2]	
			κ-casein	YIPIQYVLSR		YIPIQYVLSR[13C6, 15N2]	
			β-lactoglobulin	TPEVDDEALEK		TPEVDDEALEK[13C6, 15N2]	
				VLVLDTDYK		VLVLDTDYK[13C6, 15N4]	
Monaci et al. [75]	White wine	Milk	α-s1 casein	FFVAPFPEVFGK	Extractive ESI Orbitrap (Thermo Electron)	FFV[15N]APFPEV[15N]FGK	White wine were spiked with milk and egg proteins
		Egg	Ovalbumin	LTEWTSSNVMEER		LTEWTSSNV[15N]MEER	

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Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Yi-Shun et al. (2017) [84]	Beer, wine, chips, flour, cookies…	Gluten	α-glyadin γ-Hordein	LWQIPEQSR QQCCQQLANINEQSR	6490 triple quad (Agilent)	LWQIPEQSR[13C, 15N] QQCCQQLANINEQSR [13C, 15N]	Matrices were spiked with gluten proteins
Ippoushi et al. [89]	Sweet cherry fruit	Cherry	Pru av2	TGCCAMSTDASGK	Xevo TQD Zspray ion source (Waters)	TGCCAMSTDASGK[13C6,15N2]	Sweet cherry fruit were spiekd with isotope labelled peptides
Rahman et al. (2012) [90]	/	Shrimp	Tropomyosine Arginine kinase	SEEEVFGLQK QQLVDDHFLFVSGDR	Micro mass Quattro Ultima (Waters)	SEEEV[D8]VFGLQK QQLV[D8]VDDHFLFV[D8]SGDR	Solvent were spiked with shrimp proteins
Chen et al. (2014) [83]	Baked food (170°C, 25 min)	Milk	β-casein	VLPVPQR	TOF-MS Synapt G2 HDMS (Waters)	VL[13C6, 15N]PV[13C5, 15N]PQK (IS1) QSVLSLSQSKVL[13C6, 15N]PV[13C5, 15N]PQKAVPYPQRQ (IS2) Human β-casein (IS3)	Solvent were spiked with milk proteins

Table 4b. Quantification of food allergens in different food products by mass spectrometry using stable isotope labelling quantification with an (1) isotope-labelled protein [82], (2) isotope-labelled peptide [59, 61, 71, 75, 84, 89, 90] or (3) long isotope-labelled peptide [83].

Parameter	Target allergen			
	Whole egg	**Milk**	**Peanut**	**Hazelnut**
Analytical range, ppm	10–1000	10–1000	10–1000	10–1000
MLQ, ppm	≤5	≤10	≤10	≤10
MDL, ppm	≤1.65	≤3	≤3	≤3
Recovery %	60–120	60–120	60–120	60–120
RSD_r %	≤20	≤20	≤20	≤20
RSD_R %	≤30	≤30	≤30	≤30

Reported as ppm of the target allergen in food commodity i.e. 25 ppm of 'whole egg' in cookies.

Table 5. Method performance requirements from the AOAC guideline SMPR 2016.002 for egg, milk, peanut and hazelnut allergens in terms of analytical range, method quantification limit, recovery and intra-day and inter-day coefficients of variation (table from Paez et al. [74]).

3.1. Label-free quantification

The label-free quantification strategy is based on comparing the peptide signal intensities of different samples (**Table 4a**). Three label-free quantification possibilities are described below.

External calibration: Monaci et al. used this approach to quantify milk proteins in fruit juice. Using a calibration curve obtained by spiking fruit juice with extracted milk proteins, they found recoveries between 68 and 79% [75]. This strategy was also used to quantify peanut proteins in rice crispy/chocolate snacks [76]. A significant suppression effect, ranging from 30 to 50%, was observed for the Ara h2 peptide signal, while suppression of the Ara h3/4 peptide signal was less than 10%. A more recent study by Mattarozzi et al. obtained recoveries between 95 and 118% for lupin β-conglutin peptide in spiked biscuits [77]. Although less expensive than other approaches, this approach requires a calibration curve for each matrix.

Modified synthetic peptide approach: Zhang et al. introduced an internal standard peptide (KILDKVGINNYWLAHKALCSE) with an added asparagine residue (N) in the β-casein peptide VGINYWLAHK. They obtained recoveries between 98.8 and 100.6% [78]. The use of an internal standard allows better recovery, but adding an amino acid can change the retention time and modify the ionization of target peptides.

Standard addition: This label-free quantification strategy consists in adding standards to the matrices. It was used by Posada-Ayala et al. for the quantification of commercial food products [79]. This approach consists in adding different known quantities of extracted allergen proteins directly to the sample to be analysed before digestion and in quantifying the target allergens with the resulting calibration curve. The recovery was not specified, but this approach allows correcting at least for digestion and matrix effects. However, the theoretical level of contamination in the samples must be known in order to adapt the quantities of standards to be added.

3.2. Stable isotope labelling quantification

This strategy is based on the use of isotope-labelled (^{13}C-, ^{15}N-, D-labelled) peptides or proteins [80] (**Table 4b**). It is recommend to use a 6-Da mass difference with respect to the amino acid for doubly charged precursors and an 8–10-Da mass difference for triply charged precursors [52]. Although more expensive than the strategies described above, this approach has the advantage that the unlabelled and isotope-labelled peptides show similar ionization and similar mass spectrometry response signals. For allergen quantification, three kinds of isotope-labelled standards exist [81]: proteins [82], concatemers [83] (or long isotope-labelled peptides) and Aqua peptides [61, 71, 75, 84] (isotope-labelled peptides) (**Figure 5**).

Isotope-labelled proteins: The principle of this approach is to add a labelled protein to the sample before extraction. Newsome et al. studied the recovery of the milk allergen α-S1 casein in baked cookies using a labelled internal α-S1 casein, and obtained recoveries ranging from 60 to 80% [82]. Use of an internal standard allows correcting for the matrix effect and for effects linked to different steps in the sample preparation protocol (protein extraction and enzymatic digestion). It thus allows accurate determination of the recovery and precision for processed samples. This 'gold standard' approach is really expensive, however, making its use unrealistic for the vast majority of routine laboratories.

Isotope-labelled peptides: The principle is to add labelled peptides to the sample after digestion and before the purification steps. This approach is less expensive than the use of isotope-labelled proteins, and synthetic labelled peptides can easily be commercially obtained. Huschek et al. used isotope-labelled peptides to quantify soy, lupin and sesame allergens [59]. They determined the recovery of their method by spiking wheat, cookie and bread with the labelled peptides and obtained results between 69.4 and 112.9%. One could argue, however, that very similar matrices were used in this study (wheat-based products) and that this type of study should be extended to other matrices in order to validate the ability of the isotope-labelled peptide to correct for matrix effects.

Lutter et al. quantified milk proteins in baby food, infant cereals, breakfast cereals and rinsing water, using a calibration curve obtained by spiking 0.1% formic acid with milk protein. The estimated recovery rates were between 16 and 66% [71] Lutter et al. In this study, the isotope-labelled peptides were used to correct for effects related to different steps of the analysis. While using a single calibration curve can be useful in the routine laboratory context, the relatively low recoveries obtained in this study reveal the inability of an isotope-labelled peptide to correct for sample-preparation-related effects. We have compared the areas of milk, egg, peanut and soy peptide peaks for three matrices with and without isotope labelled peptides. Our results clearly show that an isotope-labelled peptide is able to correct for matrix effects but not for effects linked to the extraction and digestion steps [85] planque et al.

Isotope-labelled concatemers/long isotope-labelled peptides: The isotope-labelled concatemer used in this technique is a chimeric protein containing all the labelled target peptides.

http://dx.doi.org/10.5772/intechopen.69361

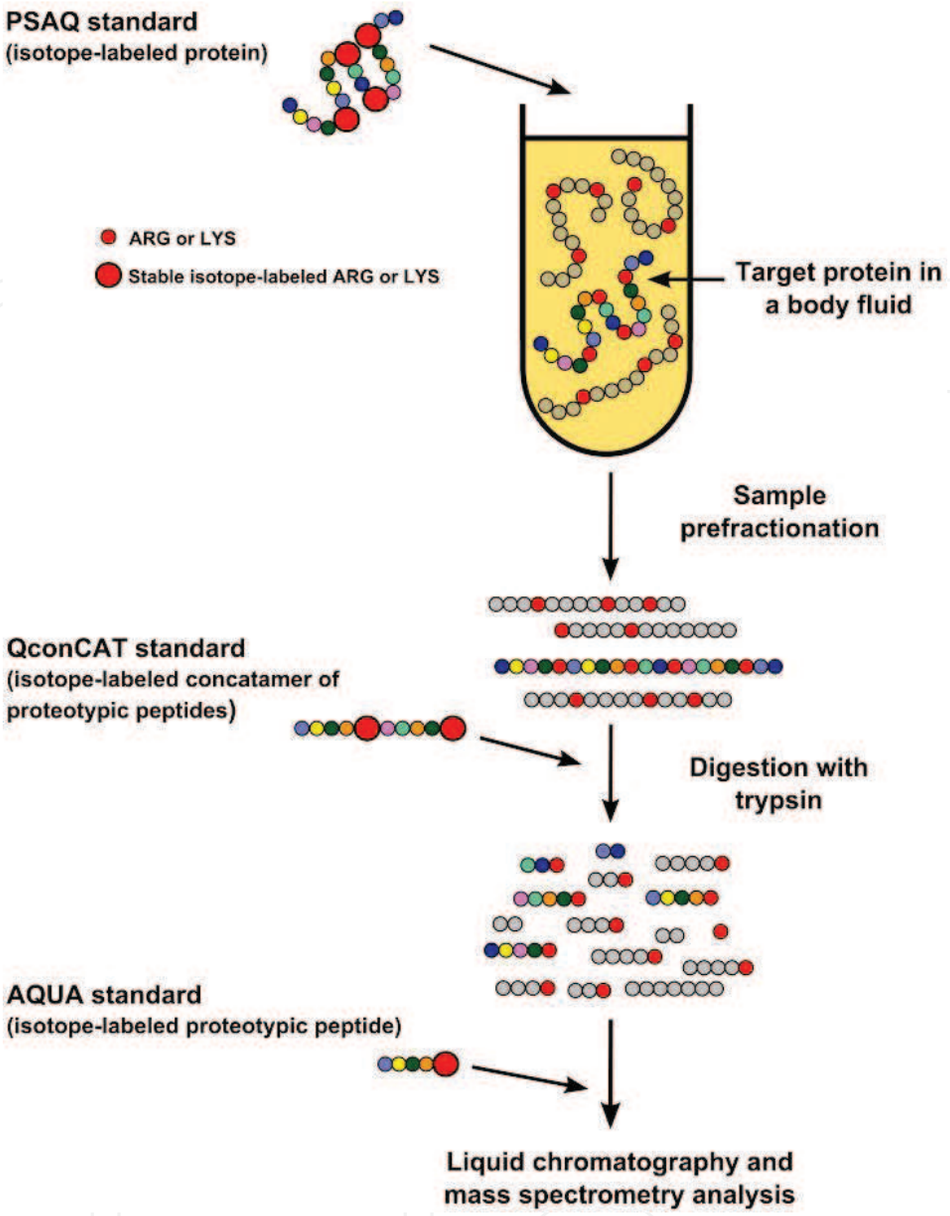

Figure 5. Three types of internal standards are used for the quantification of proteins by mass spectrometry (1) isotope-labelled protein (2) Isotope-labelled concatemers or long isotope-labelled peptides (3) isotope-labelled peptide (from Ref. [81]).

This internal standard is added to the sample before enzymatic digestion. The advantage of this method is that a single concatemer can contain peptides belonging to different proteins or allergens. This strategy has been used in proteomics, but it is not yet used for food allergen quantification [86]. An emerging alternative to use of a concatemer is use of a so-called 'long isotope-labelled peptide'. Chen et al. compared the use of three types of internal standard: human β-casein, isotope-labelled peptide VL [$^{13}C_6$, ^{15}N] PV[$^{13}C_5$, ^{15}N]PQK and a long isotope-labelled peptide QSVLSLSQSKVL[$^{13}C_6$,^{15}N] PV[$^{13}C_5$,^{15}N]PQKAVPYPQRQ [83]. The long isotope-labelled peptide provided better recovery, due to correction for digestion-step-related effects. The recovery based on spiked materials was between 98.8 and 106.7%. In 2016, it was

shown that long isotope-labelled peptides allow recoveries of 97.2–102.5% for α-lactalbumin and 99.5–100.3% for β-casein in the quantification of human milk [87]. This strategy is a good compromise between isotope-labelled proteins and peptides. It allows correcting both for the matrix effect and for digestion-step effects, unlike the use of isotope-labelled peptides.

In conclusion, these studies show that using an isotope-labelled protein or a long isotope-labelled peptide provides better recovery than the isotope-labelled peptide approach. As explained below in the section devoted to result validation, the recovery must be determined with allergen-spiked samples and processed matrices in order to meet AOAC specifications. Published methods, however, do not always meet the AOAC requirements, even with spiked samples. For instance, Careri et al. [76] observed a suppression effect between 30 and 50% for the Ara h2 peptide signal, and Monaci et al. [75] obtained recoveries ranging from 68 to 79% for α-lactalbumin and β lactoglobulin. Altogether, these works show that internal standards are needed for the quantification of allergens in food matrices. Currently, furthermore, the use of a calibration curve for each type of sample is the best way to respect the AOAC guideline requiring a recovery between 60 and 120%.

Future studies should thus still be done to improve the quantification of allergens from a single calibration curve with a good recovery.

4. Validating food allergen methods

While mass spectrometry methods are increasingly sensitive, there remains room for improvement. Furthermore, there subsist obstacles to the harmonization of allergen detection methods in food laboratories [85]. In April 2016, the AOAC SMPR 2016.002 guideline 'Standard method requirements for the detection and quantification of selected food allergens' was published. This guideline is the first to specify target limits for sensitivity and range of linearity, target matrices and reference materials for the analysis of allergens (egg, milk, peanut and hazelnut) in food matrices by mass spectrometry (**Table 5**).

To obtain comparable results among laboratories, it is crucial to adopt validation guidelines. The AOAC guideline, however, is not sufficiently detailed, and each laboratory tends to apply its own rules. In what follows, we compare this guideline with published methods in terms of sensitivity, range of linearity, recovery and precision.

Sensitivity: In the AOAC guideline, the method quantitation limit (MQL) is defined as MQL = average (blank) + 10 x s_0 (blank). Laboratories, however, often use other strategies to determine the limit of quantification (LOQ), such as determining a signal-to-noise (S/N) ratio which should be higher than 10 [56, 60] or estimating an LOD and an LOQ as 3s/slope and 10s/slope, respectively, where s is the standard deviation of the blank signal (n = 10) [57]. On the other hand, the sensitivity can differ from one matrix to another. For example, in a study where cookie, ice cream and sauce were spiked with 0.5 mg milk proteins per kg, the observed S/N ratio was 26 for the cookie matrix, 83 for ice cream and 228 for sauce [85]. This also highlights the importance of a 'fit-for-purpose' description of

an analytical method. Moreover, the sensitivities of developed methods should be determined on the same reference materials (MoniQa, LGC) to ensure (1) their capacity to reach the sensitivity set by the AOAC guideline and (2) an appropriate comparison of method performances.

Linearity: The range of linearity is set as 0.001 to 0.1% allergen contamination (10 mg to 1000 mg) of allergenic ingredients per kg) and thus does not always include the MQL (e. g. an MLQ_{egg} of 5 mg per kg). In the case of high-sensitivity methods, the coefficient of regression is determined using a lower range of concentrations [57, 71].

Recovery: Recovery must range from 60 to 120%. Such recovery values are hard to reach for the detection of allergens in processed samples, and recovery can only be determined by spiking food matrices with allergens. Focusing on egg, milk and peanut in spiked and incurred muffin and cereal bars, Parker et al. constructed calibration curves by spiking the matrices with allergen proteins [61]. In the case of spiked muffin, the determined recovery was 98.6% for egg peptide (GGLEPINFQTAADQAR), 87.7% for milk peptide (YLGYLEQLLR) and 100.2% for peanut peptide (SPDIYNPQAGSLK). When the muffins were baked for 48 min at 177°C, the recoveries were dramatically lower: respectively 45.2%, 75.2% and 70.2%.

Inter- and intra-day coefficients of variation: According to AOAC SPMR, three unknown samples should be analyzed at least seven times to determine the reproducibility of the method. Lamberti et al. determined an intra-day coefficient of variation between 5 and 20% by performing three independent extractions at two different concentrations and three injections per extract [57].

Guidelines for the validation of mass-spectrometry-based methods for allergen analysis should be more precise, like the guidelines 2002/657/EC 'Validation of residues in products of animal origin' and SANCO/12574/2013 'Residues in products of animal origin method validation procedures for pesticide residues analysis in food and feed'. In SMPR 2016.002, several details are missing:

- The number of target peptides that a method should include to confirm the presence of an allergen, as well as fragment ion number and/or type.
- Criteria for the relative retention time, the ion ratio and the specificity of the method (blast, analysis of different blank and matrices), the level of spiking for determining precision and accuracy (for example, the LOQ, action limit and upper limit).

5. Conclusion

The major increase of the allergic population has prompted the development of numerous allergen detection methods. Over the past few years, improvements in the detection of allergens by mass spectrometry have been impressive, allowing detection of processed allergens with high sensitivity (a few mg of proteins per kg of food). Optimization of extraction and purification steps has notably played a key role in the improvement of analytical methods. Allergen quantification is performed mainly with labelled internal standards.

The best approach involves the use of labelled proteins, allowing correction for effects occurring throughout the sample preparation protocol. The high cost of labelled proteins, however, has promoted the use of other strategies, such as methods based on long isotope-labelled peptides and standard addition of allergens.

The validation of qualitative and quantitative MS-based methods for routine detection of allergens is still very recent. The AOAC guideline is a first step towards the development of methods that will allow procedure harmonization, making it possible to compare results between laboratories. These methods should be both improved and extended to other allergens in order to demonstrate their validity and robustness.

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