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

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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.

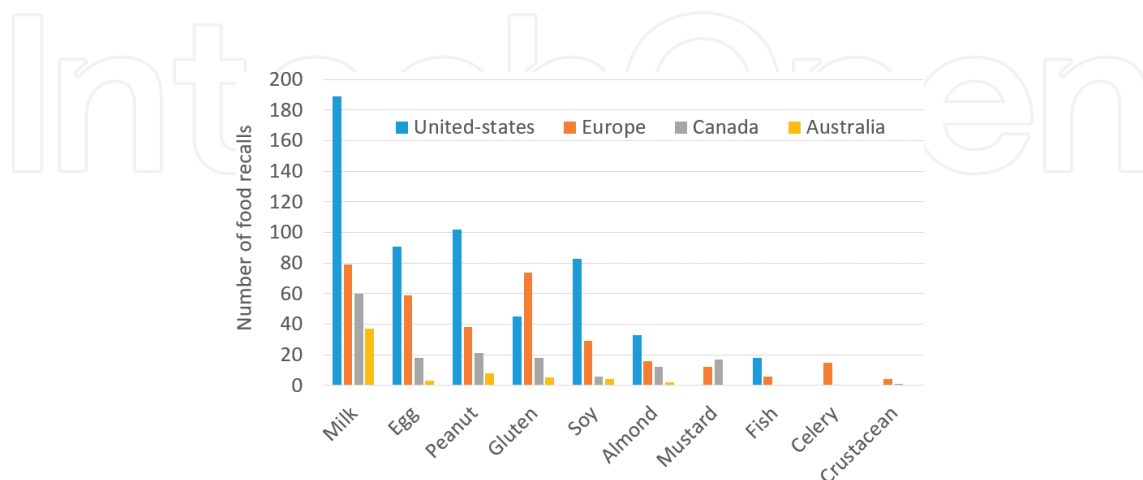


Figure 1. Number of food recalls per allergen category in the United States, Europe, Canada, and Australia between 2012 and 2015 [23–26].

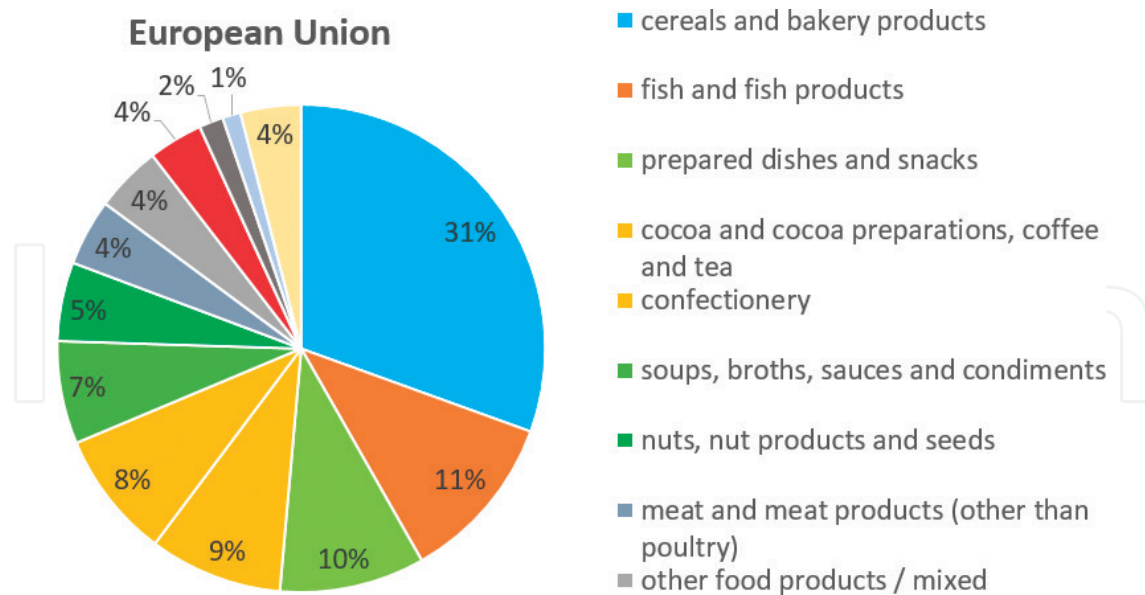


Figure 2. Percentage distribution of food allergen recalls in the European Union (according to the Rapid Alert System for Food and Feed) [24].

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 EAACI (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 criteria 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

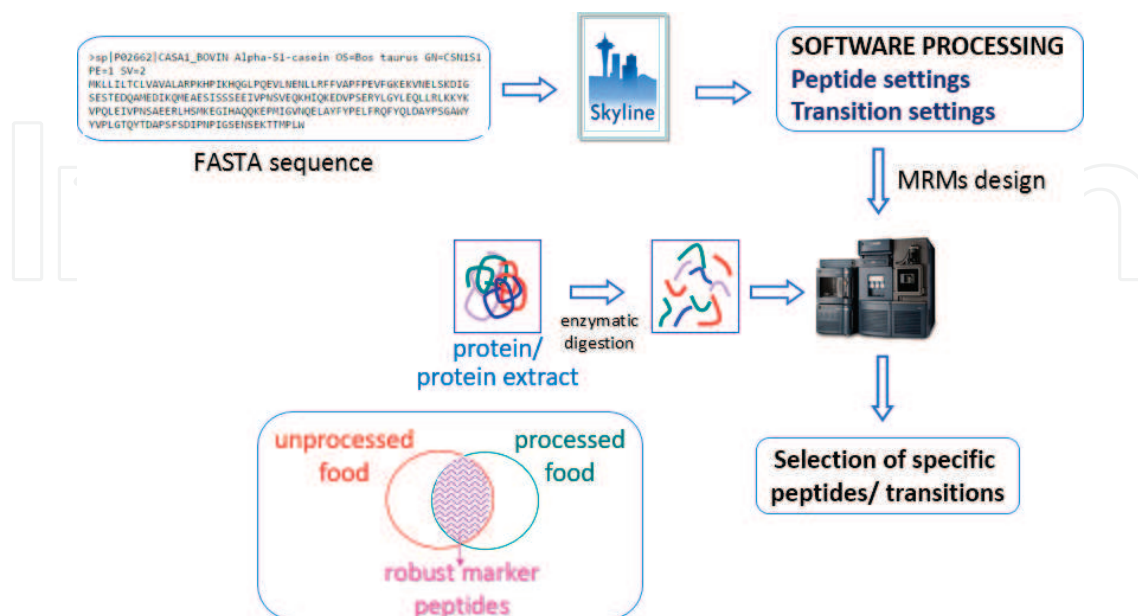


Figure 3. *In silico* peptide selection with the Uniprot database and Skyline software.

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), AVYPYQR (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 α S1), 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	α S1-casein	YLGYLEQLLR	b2, y8
				FFVAPFPEVFGK	y8, y9
			α S2-casein	NAVPIPTLNR	b2, y8
				FALPQYLK	a1, y5
		Egg	Ovalbumin	HIATNAVLFFGR	a2, y10
				YPILPEYLQCVK	y6, y8
				DILNQITKPNDVYSFLASR	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
				QGQVLTIPQNFVAVK	y8, y10
				ALPDDVLANAFQISR	y8, y9
		Walnut	Jug r1	DLPNECGISSQR	y4, y10
				QCCQQLSQMDEQCCEGLR	y3, y10
				GEEMEEMVQSAR	y7, y8
GNLDFVQPPR	y3, y7				
Almond	Prunin	GVLGAVFSGCPETFEESSQQQGR	y6, y7		
		ALPDEVLANAYQISR	y8, y9		
		NGLHLPSYSNAPQLIYVQGR	y6, b11		
Pilolli et al. 2016 [56]	Cookie (200°C, 12 min)	Milk	α S1-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			
Lamberti et al. [57]	Cookie (180°C, 10 min)	Soy	Glycinin G1-G2	SQSDNFEYVSFK	y3, y10			
				FYLAGNQEQEFLK	y9, y10, y11			
		Hazelnut	11S globulin-like protein	ADIYTEQVGR	y6, y7			
				ALPDDVLANAFQISR	y7, y8, y13			
		Milk	α S1-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	y _{max} , y8, y7, y5, b2			
				SPDIYNPQAGSLK	y _{max} , 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	α S1-Casein	FFVAPFPEVFGK	y6, y8, y9	
				HQGLPQEVLNENLLR	b4, y6, y7	
				YLGYLEQLLR	y5, y6, y7	
			α S2-casein	NAVPITPTLNR	b3, y8, y8	
				β -lactoglobulin	VYVEELKPTPEGDLEILLQK	y11, y14, y16
					VLVLDTDYK	y5, y6, y7
		Egg	Ovalbumin	LSFNPTQLEEQCHI	y7, y10, y10	
				GGLEPINFQTAADQAR	y10, y12, y12	
				LTEWTSSVM EER	y7, y8, y9	
			Vitellogenin	ISQAVHAAHAEINEAGR	y9, y10, y11	
				EALQPIHDLADEAISR	y6, y7, y12	
				NIPFAEYPTYK	y4, y9, y9	
				NIGELGVEK	y5, y6, y7	
			Apovitellenin	YLLDLLPAAASHR	y7, y7, y11	
				NFLINETAR	y5, y6, y7	
				Peanut	Cupin	NTLEAAFNAEFNEIR
		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					
LITLAIPVKNKPGR	y7, y9, y11					
Parker et al. [61]	Muffin (177°C, 48 min)			Egg	Lysozyme	FESNFNTQATNR
		NTDGSTDYGILQINSR				
		Ovalbumin	ELINSWVESQTNGIIR			
			GGLEPINFQTAADQAR			
			HIATNAVLFFGR			
			Milk	α S1-casein	FFVAPFPEVFGK	
		HQGLPQEVLNENLLR				
		β -lactoglobulin		YLGYLEQLLR		
				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	α S1-casein	HQGLPQEVLNENLLR	best transitions not selected
			α S2-casein	NAVPITPTLNR LNFLK ALNEINQFYQK	
			κ -casein	YIPIQYVLSR	
		Soy	Glycinin G1 precursor	HNIGQTSSPDIYNPQAGSVTTATSLDFPALSCLR TNDTPMIGTLAGANSLLNALPEEVIQHTFNLK VLIVPQNFVVAAR HQQEEENEGGSILSGFTLEFLEHAFSVDK EGDLIAP...DQMPR	
			Glycinin G2 precursor	TNDRPSIGNLAGANSLLNALPEEVIQHTFNLK QNIGQNSSPDIYNPQAGSITTATSLDFPALWLLK	
			Beta conglycinin alpha chain precursor	DLDIFLSIVDMNEGALLPHFNSK AIVILVINEGDANIELVGLK	
		Wheat	Alpha amylase trypsin inhibitor	YFIALPVPSQPVDPR LLVAPGQCENLATIHNVR 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	Ultrafiltration (Amilcon Ultra 15 mL, 5 kDa molecular weight cut-off) (Millipore)	Dilution: 1 mg of proteins by ml with NH ₄ HCO ₃ (100 mM)	LC: 1200 HPLC (Agilent)	LOD (S/N>3)
			Buffer: TRIS-HCl pH 8.2		Aliquot: 100 µl	Column: Xbridge C18 3.5 µm (2.1×150 mm) (Waters)	5 mg of soluble milk proteins by kg
			Agitation: 60°C for 3h		Reduction: 50 µl DTT (200 mM), 45 min	MS: API 4000QTrap (MDS Sciex)	42 mg of soluble egg proteins by kg
					Alkylation: 40 µl IA (1 M), 45 min in the dark		24 mg of soluble soy proteins by kg
					20 µl DTT (200 mM) + 50 µl NH ₄ HCO ₃ (100 mM)		11 mg of soluble peanut proteins by kg
					Digestion: 10 µl trypsin (1 µg/µl) 12 h - 37°C		5 mg of soluble hazelnut proteins by kg
Pilolli et al. [56]	milk, egg, soy, peanut, hazelnut	Cookie (200°C, 12 min)	2.5 g/50 ml	1.2 µm acetate cellulose membrane, Size exclusion column (SEC) (G25 Sephadex column)	Elution SEC: 3.5 ml NH ₄ HCO ₃ (50 mM)	LC: -	LOD (S/N>3)
			Buffer: 20 mM TRIS-HCl pH 8.2		Aliquot: 300 µl	Column: Aeris Peptide XB-C18 (150 × 2.1 mm) (Phenomenex)	7 mg of milk by kg
					Protein denaturation: 15 min at 95°C	MS: Dual pressure Linear Ion Trap Spectrometer Velos Pro (Thermo Fisher Scientific)	9 mg of egg by kg
			Ultrasound: 30 min		Reduction: 15 µl of 50 mM DTT 30 min at 60°C		6 mg of soy by kg
					Alkylation: 30 µl of 100 mM IAA 30 min in the dark at room temperature		13 mg of peanut by kg

Authors	Allergen	Matrix	Extraction	Purification	Digestion	Instrument	Sensitivity
Lamberti et al. [57]	Milk	Cookie (180°C, 10 min)	10 mg /200 µl Buffer: NH ₄ HCO ₃ / (NH ₄) ₂ CO ₃ +1 % SDS buffer, pH 8.2 Agitation: 20 min, 60°C	Protein precipitation methanol/ chloroform	Digestion: 4 µl trypsin (1 µg/µl) 14h Acidification: 1M HCl Filtration: 0.2 µm	LC: HP 1100 HPLC (Agilent) Column: ACE C18 300A (250 mm × 1 mm) MS: XCT-Plus Ion trap mass spectrometer (Agilent)	7 mg of hazelnut by kg
					Pellet + 50 µl of 0.025M NH ₄ HCO ₃ pH 8.0 RT 3 µl of trypsin (75 ng/µl) 37°C, 90 min		LOD: 1.3 mg of milk proteins per kg LOQ: 4 mg of milk proteins per kg
					5 µl of 5% formic acid		
Pedreschi et al. [58]	Peanut	Cookie (180°C, 16 min)	Buffer: 20 mM TRIS - 150 mM NaCl, pH 7.4 Ultrasound: 4°C, 20 min	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
				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 1 µl of 1µg/µm of trypsin 5h-37°C	Column: nano Acquity UPLC Trap SymC18 5 µm (180 µm × 20 mm) 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].

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 Buffer: 100 mM NH ₄ HCO ₃ , 4M urea 5 mM DTT pH 8.2 Agitation 30 min RT	SPE cartridge (LiChrolut RP-18 Merck Millipore)	Alkylation: IAA 20 min at 50°C Digestion: Trypsin formic acid 2%	LC: Nexera XR UHPLC (Shimadzu) Column: Aeris Peptide XB-C18 (100 × 2.1 mm, 1.7 μm) (Phenomenex) MS: Qtrap 5500 MS/MS (Sciex)	LOQ (S/N > 10) 10–20 mg of soy per kg 10–50 mg of sesame per kg 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 Buffer: 200 mM TRIS-HCl pH 9.2, 2M urea Agitation: 30 min Ultrasound: 15 min	Sep-Pack tC18 6cc (Waters)	10 ml extract + 10 ml NH ₄ HCO ₃ (200 mM) Reduction: 1 ml DTT (400 mM), 45 min Alkylation: 2 ml IAA (500 mM), 45 min in the dark Digestion: Ratio protein:trypsin 1:20 16 h, 37°C 300 μl formic acid 20%	LC: UPLC Acquity (Waters) Column: BEH130 (2.1 × 150 mm) (Waters) MS: Xevo TQS (Waters)	LOQ (S/N > 10) 0.5 mg of milk proteins by kg 3.4 mg of egg proteins by kg 5 mg of soy proteins by kg 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 vortex: 5 min at 1400 rpm Ultrasound: 10 min at 4°C	Microcentrifuge tubes Amicon Ultra 0.5 ml Utracel-10 membrane	Filter-aided sample preparation (FASP) sample cocentration and digestion protocol Reduction: 10 mM DTT Alkylation: 25 mM IAA Digestion: Ratio protein:trypsin 1:100 16 h -37°C 0.1% trifluoroacetic acid and 2% acetonitrile	LC: nano Acquity UPLC (Waters) Column: nano Acquity BEH130 C18 1.7 μm (100 μm × 100 mm) MS: 6500 QTRAP (Sciex)	/

Author	Allergens	Matrices	Extraction	Purification	Digestion	Instrument	Sensitivity
Gomaa et al. [62]	Milk, soy, wheat	Cookie (177°C, 12 min)	1 g/9 ml Buffer: 50 mM NH ₄ HCO ₃	OMIX C18/ tip (Varian)	Protein extract: 100 µl at 2 mg/ml Reduction: 1 µl of DTT (0.5 M) 56°C, 20 min Alkylation: 2.7 µl IAA (0.55 M, 15 min) Digestion: ratio enzyme:substrate 1:20 3h-37°C 5 µl trifluoroacetic acid (2.5%)	LC: nano Acquity UPLC (Waters) Column: nano Acquity BEH130 C18 1.7 µm (75 µm × 100 mm) nano Acquity UPLC Trap SymC18 5 µm (180 µm × 20 mm) MS: Q-ToF Synapt MS (Waters)	10 mg of casein per kg 10 mg of soy proteins per kg 100 mg of gluten per kg

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].

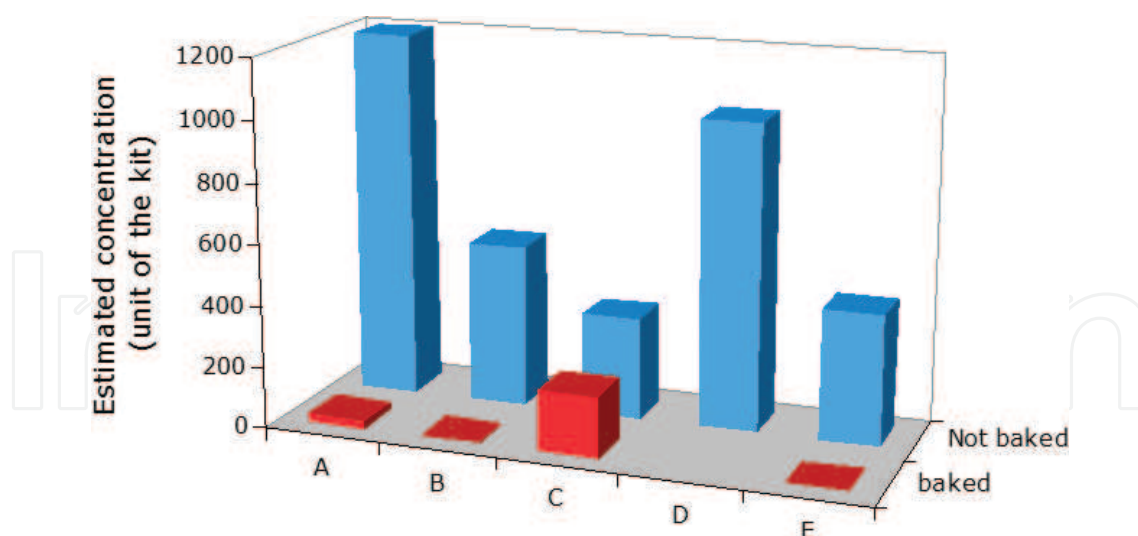


Figure 4. Analytical results for 1000 mg spray-dried whole egg powder (National Institute of Standards and Technology RM 8445) per kg incurred cookies, obtained with the different enzyme-linked immunosorbent assay test kits for egg detection (A–E) (from Ref. [69]).

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 CMCEALQQIMENQSDR	Q-TOF Micro (Waters)	No internal standard	Rice crispy/ chocolate snacks were spiked with peanut proteins		
			Ara h3/4	AHVQVVDSNGDR SPDIYNPQAGSLK					
Monaci et al. [75]	Fruit juices	Milk	α -lactalbumin β -lactoglobulin A β -lactoglobulin B	/	Ultima triple quadrupole mass spectrometer (Waters)	No internal standard	Fruit juices were spiked with milk proteins		
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 L TTLNSLNLPI LK					
				Hazelnut				Cor a 9	LNALEPTNR VQVDDNGNTVFDDEL R
									QGGVLTIPQNF A VAK
		Peanut	Ara h3	FNLAGNHEQEFLR WLGLSAEYGNLYR TANDL NLLIL R					

Authors	Matrix	Allergen	Protein	Peptide	Mass spectrometer	Internal standard	Calibration curve
Mattarozzi et al. [77]	Pasta, biscuit	Pistachio	Pis v 5	AMISPLAGSTSVLR	LTQ XL linear ion trap (Thermo)	No internal standard	Pasta and biscuits were fortified with lupin proteins
				ITSLNSLNLPIK			
				GFESSESEYER			
		Walnut	Jug r 2	FFDQQEQR			
				ATLTLVSQETR			
				Jug r4			
Lupin	β -conglutin	IVEFQSKPNTLILPK					
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 IYQTATHLPK EFQQAQHLR	6460 triple quadrupole (Agilent technologies)	Purified protein Sin a 1	Standard addition of mustard in sauces and salty biscuits

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	HQGLPQEVLENLLR	Hybrid triple-quadrupole 4000 QTRAP (AB Sciex)	HQGLPQEVLENLLR[13C, 15N]	Cookies were spiked with isotope labelled peptides
				YLGYLEQLLR		YLGYLEQLLR[13C6, 15N]	
				FFVAPFPEVFGK		FFVAPFPEVF[13C6, 15N]GK	
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	

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 spiked 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 recommended 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.

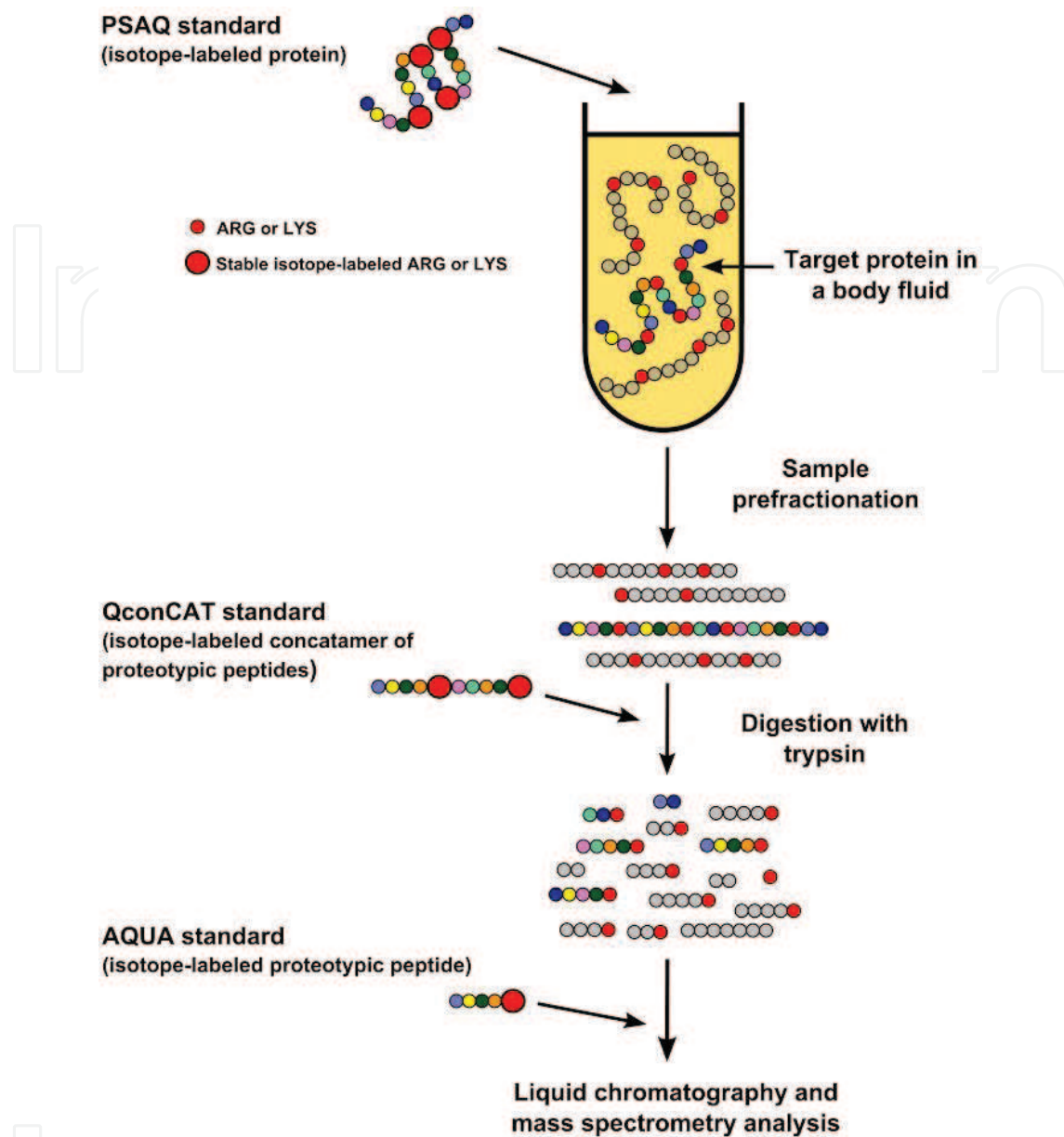


Figure 5. Three types of internal standards are used for the quantification of proteins by mass spectrometry (1) isotope-labelled protein (2) Isotope-labelled concatemers or long isotope-labelled peptides (3) isotope-labelled peptide (from Ref. [81]).

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 [$^{13}\text{C}_6$, ^{15}N] PV[$^{13}\text{C}_5$, ^{15}N]PQK and a long isotope-labelled peptide QSVLSLSQSKVL[$^{13}\text{C}_6$, ^{15}N] PV[$^{13}\text{C}_5$, ^{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 $MQL = \text{average}(\text{blank}) + 10 \times s_0(\text{blank})$. 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 $3s/\text{slope}$ and $10s/\text{slope}$, 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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References

- [1] Wang J, Sampson HA, Rona R, Branum A, Lukacs S, Sicherer S, et al. Food allergy. *Journal of Clinical Investigation* [Internet]. American Society for Clinical Investigation. 2011;**121**(3):827-835. Available from: <http://www.jci.org/articles/view/45434> [Accessed: 10 June 2016]
- [2] Manea I, Ailenei E, Deleanu D. Overview of food allergy diagnosis. *Clujul medical* (1957) [Internet]. 2016;**89**(1):5-10. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4777468&tool=pmcentrez&rendertype=abstract> [Accessed: 9 April 2016]
- [3] Tam JS. Cutaneous manifestation of food allergy. *Immunology and Allergy Clinics of North America* [Internet]. 2017;**37**(1):217-231. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0889856116300765> [Accessed: 23 March 2017]
- [4] Sampson HA. Update on food allergy ☆. *Journal of Allergy and Clinical Immunology* [Internet]. 2004;**113**(5):805-819. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15131561> [Accessed: 23 March 2017]
- [5] Ho MH-K, Wong WH-S, Chang C. Clinical spectrum of food allergies: A comprehensive review. *Clinical Reviews in Allergy & Immunology* [Internet]. USA: Springer. 2014;**46**(3):225-240. Available from: <http://link.springer.com/10.1007/s12016-012-8339-6> [Accessed: 10 June 2016]

- [6] Cianferoni A, Muraro A. Food-induced anaphylaxis. *Immunology and Allergy Clinics of North America* [Internet]. NIH Public Access. 2012;**32**(1):165-195. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22244239> [Accessed: 7 March 2017]
- [7] Sindher S, Fleischer DM, Spergel JM. Advances in the treatment of food allergy. *Immunology and Allergy Clinics of North America* [Internet]. 2016;**36**(1):39-54. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26617226> [Accessed: 15 March 2017]
- [8] Tordesillas L, Mondoulet L, Blazquez AB, Benhamou P-H, Sampson HA, Berin MC. Epicutaneous immunotherapy induces gastrointestinal LAP+ regulatory T cells and prevents food-induced anaphylaxis. *Journal of Allergy and Clinical Immunology* [Internet]. 2017;**139**(1):189-201.e4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27417020> [Accessed: 7 March 2017]
- [9] Ravid NL, Annunziato RA, Ambrose MA, Chuang K, Mullarkey C, Sicherer SH, et al. Mental health and quality-of-life concerns related to the burden of food allergy. *Psychiatric Clinics of North America* [Internet]. 2015;**38**(1):77-89. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0193953X14001014> [Accessed: 7 March 2017]
- [10] King RM, Knibb RC, Hourihane JO. Impact of peanut allergy on quality of life, stress and anxiety in the family. *Allergy* [Internet]. 2009;**64**(3):461-468. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19076542> [Accessed: 26 April 2016]
- [11] Gendel SM. Comparison of international food allergen labeling regulations. *Regulatory Toxicology and Pharmacology*. 2012;**63**:279-285
- [12] Regulation (EU) No 1169/2011 of the European parliament and of the council of 25 October 2011. 2011
- [13] Spanjersberg MQI, Knulst AC, Kruizinga AG, Van Duijn G, Houben GF. Concentrations of undeclared allergens in food products can reach levels that are relevant for public health. *Food Additives & Contaminants: Part A* [Internet]. Taylor & Francis Group. 2010;**27**(2):169-174. Available from: <http://www.tandfonline.com/doi/abs/10.1080/19440040903317513> [Accessed: 30 September 2016]
- [14] DZWOLAK, Waldemar. Assessment of food allergen management in small food facilities. *Food Control*, 2017;**73**:323-331
- [15] Hefle SL, Taylor SL. Food allergy and the food industry. *Current Allergy and Asthma Reports* [Internet]. Current Medicine Group. 2004;**4**(1):55-59. Available from: <http://link.springer.com/10.1007/s11882-004-0044-y> [Accessed: 22 November 2016]
- [16] WALKER, M. J., BURNS, D. T., ELLIOTT, C. T., et al. Is food allergen analysis flawed? Health and supply chain risks and a proposed framework to address urgent analytical needs. *Analyst*, 2016;**141**(1):24-35
- [17] DunnGalvin A, Chan C-H, Crevel R, Grimshaw K, Poms R, Schnadt S, et al. Precautionary allergen labelling: Perspectives from key stakeholder groups. *Allergy* [Internet]. 2015;**70**(9):1039-1051. Available from: <http://doi.wiley.com/10.1111/all.12614> [Accessed: 14 June 2016]

- [18] Zurzolo GA, Mathai ML, Koplin JJ, Allen KJ. Precautionary allergen labelling following new labelling practice in Australia. *Journal of Paediatrics and Child Health* [Internet]. 2013;**49**(4):E306-310. Available from: <http://doi.wiley.com/10.1111/jpc.12138> [Accessed: 22 November 2016]
- [19] Pele M, Brohée M, Anklam E, Van Hengel AJ. Peanut and hazelnut traces in cookies and chocolates: Relationship between analytical results and declaration of food allergens on product labels. *Food Additives and Contaminants* [Internet]. 2007;**24**(12):1334-1344. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17852393> [Accessed: 26 April 2016]
- [20] Marchisotto MJ, Harada L, Blumenstock JA, Bilaver LA, Wasserman S, Sicherer S, et al. Global perceptions of food allergy thresholds in 16 countries. *Allergy* [Internet]. 2016;**71**(8):1081-1085. Available from: <http://doi.wiley.com/10.1111/all.12933> [Accessed: 16 March 2017]
- [21] BUCCHINI, Luca, GUZZON, Antonella, POMS, Roland, et al. Analysis and critical comparison of food allergen recalls from the European Union, USA, Canada, Hong Kong, Australia and New Zealand. *Food Additives & Contaminants: Part A*, 2016;**33**(5):760-771
- [22] Gendel Sm, Zhu J. Analysis of U.S. food and drug administration food allergen recalls after Implementation of the food allergen labeling and consumer protection act. *Journal of Food Protection* [Internet]. 2013;**76**(11):1933-1938. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24215698> [Accessed: 13 April 2017]
- [23] U S Food and Drug Administration Home Page [Internet].. Available from: <https://www.fda.gov/> [Accessed: 13 April 2017]
- [24] The Rapid Alert System for Food and Feed https://ec.europa.eu/food/safety/rasff_en
- [25] Bienvenue à l'Agence canadienne d'inspection des aliments - Agence canadienne d'inspection des aliments [Internet].. Available from: <http://www.inspection.gc.ca/fra/1297964599443/1297965645317> [Accessed: 13 April 2017]
- [26] Recalls | Product Safety Australia [Internet]. Available from: <https://www.productsafety.gov.au/recalls?source=recalls> [Accessed: 13 April 2017]
- [27] VITAL ® Voluntary Incidental Trace Allergen Labelling | Allergen Bureau [Internet].. Available from: <http://allergenbureau.net/vital/> [Accessed: 13 April 2017]
- [28] Home | NVWA [Internet].. Available from: <https://www.nvwa.nl/> [Accessed: 13 April 2017]
- [29] Tolin S, Pasini G, Curioni A, Arrigoni G, Masi A, Mainente F, et al. Mass spectrometry detection of egg proteins in red wines treated with egg white. *Food Control*. 2012;**23**:87-94
- [30] Weber D, Raymond P, Ben-Rejeb S, Lau B. Development of a liquid chromatography-tandem mass spectrometry method using capillary liquid chromatography and nano-electrospray ionization-quadrupole time-of-flight hybrid mass spectrometer for the

- detection of milk allergens. *Journal of Agricultural and Food Chemistry* [Internet]. 2006;**54**(5):1604-1610. Available from: <https://vpn.gw.ulg.ac.be/doi/abs/10.1021/DanaInfo=pubs.acs.org+jf052464s> [Accessed: 28 April 2016]
- [31] Korte R, Lepski S, Brockmeyer J. Comprehensive peptide marker identification for the detection of multiple nut allergens using a non-targeted LC-HRMS multi-method. *Analytical and Bioanalytical Chemistry* [Internet]. 2016;**408**(12):3059-3069. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26894760> [Accessed: 23 September 2016]
- [32] Houston NL, Lee D-G, Stevenson SE, Ladics GS, Bannon GA, McClain S, et al. Quantitation of soybean allergens using tandem mass spectrometry. *Journal of Proteome Research* [Internet]. 2011;**10**(2):763-773. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21141899> [Accessed: 27 March 2017]
- [33] Planque M, Wallace A, Gillard N. [Application Note] Targeted and Sensitive Detection of Food Allergens in Complex and Processed Foodstuffs Using UPLC-MS/MS [APPLICATION NOTE]. 2016;1-6
- [34] Taylor SL, Baumert JL, Kruizinga AG, Remington BC, Crevel RWR, Brooke-Taylor S, et al. Establishment of Reference Doses for residues of allergenic foods: Report of the vital expert panel. *Food and Chemical Toxicology*. 2014;**63**:9-17
- [35] Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, et al. Food processing and allergenicity. *Food and Chemical Toxicology: An International Journal published for the British Industrial Biological Research Association* [Internet]. 2015;**80**:223-240. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25778347> [Accessed: 1 April 2016]
- [36] SAIFI, Maryam, SWAMY, Nithya, CRAIN, Maria, et al. Tolerance of a high-protein baked-egg product in egg-allergic children. *Annals of Allergy, Asthma & Immunology*, 2016;**116**(5):415-419
- [37] Allen KJ, Remington BC, Baumert JL, Crevel RWR, Houben GF, Brooke-Taylor S, et al. Allergen reference doses for precautionary labeling (VITAL 2.0): Clinical implications. *The Journal of Allergy and Clinical Immunology* [Internet]. 2014;**133**(1):156-164. Available from: <http://www.sciencedirect.com/science/article/pii/S0091674913010592> [Accessed: 26 April 2016]
- [38] Muraro A, Hoffmann-Sommergruber K, Holzhauser T, Poulsen LK, Gowland MH, Akdis CA, et al. EAACI food allergy and anaphylaxis guidelines. Protecting consumers with food allergies: Understanding food consumption, meeting regulations and identifying unmet needs. *Allergy* [Internet]. 2014;**69**(11):1464-1472. Available from: <http://doi.wiley.com/10.1111/all.12453> [Accessed: 15 March 2017]
- [39] Downs ML, Taylor SL. Effects of thermal processing on the enzyme-linked immunosorbent assay (ELISA) detection of milk residues in a model food matrix. *Journal of Agricultural and Food Chemistry* [Internet]. 2010;**58**(18):10085-10091. Available from: <http://pubs.acs.org/doi/abs/10.1021/jf101718f> [Accessed: 22 November 2016]

- [40] Sanchiz Á, Ballesteros I, Martin A, Rueda J, Pedrosa MM, Dieguez M del C, et al. Detection of pistachio allergen coding sequences in food products: A comparison of two real time PCR approaches. *Food Control* [Internet]. 2017;**75**:262-270. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0956713516306880> [Accessed: 23 March 2017]
- [41] Khuda S, Slate A, Pereira M, Al-Taher F, Jackson L, Diaz-Amigo C, et al. Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: Sugar cookies. *Journal of Agricultural and Food Chemistry* [Internet]. 2012;**60**(17):4195-4203. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22486175> [Accessed: 26 April 2016]
- [42] Gomaa A, Boye JI. Impact of thermal processing time and cookie size on the detection of casein, egg, gluten and soy allergens in food. *FRIN*. 2013;**52**:483-489
- [43] Iqbal A, Shah F, Hamayun M, Ahmad A, Hussain A, Waqas M, et al. Allergens of *Arachis hypogaea* and the effect of processing on their detection by ELISA. *Food & Nutrition Research* [Internet]. 2016;**60**:28945. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4773821&tool=pmcentrez&rendertype=abstract> [Accessed: 26 April 2016]
- [44] Benedé S, López-Expósito I, Molina E, López-Fandiño R, Boyce JA, Assa'ad A, et al. Egg proteins as allergens and the effects of the food matrix and processing. *Food & Function* [Internet]. 2015;**6**(3):694-713. Available from: <http://xlink.rsc.org/?DOI=C4FO01104J> [Accessed: 10 June 2016]
- [45] Nesvizhskii AI. Protein Identification by Tandem Mass Spectrometry and Sequence Database Searching. In: *Mass Spectrometry Data Analysis in Proteomics* [Internet]. New Jersey: Humana Press; 2007. pp. 87-120. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17185772> [Accessed: 24 March 2017]
- [46] Sealey-Voyksner J, Zweigenbaum J, Voyksner R. Discovery of highly conserved unique peanut and tree nut peptides by LC-MS/MS for multi-allergen detection. *Food Chemistry* [Internet]. 2016;**194**:201-211. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26471545> [Accessed: 8 March 2017]
- [47] Shefcheck KJ, Musser SM. Confirmation of the allergenic peanut protein, Ara h 1, in a model food matrix using liquid chromatography/tandem mass spectrometry (LC/MS/MS). *Journal of Agricultural and Food Chemistry* [Internet]. 2004;**52**(10):2785-2790. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15137814> [Accessed: 8 March 2017]
- [48] Cucu T, De Meulenaer B, Devreese B. MALDI based identification of soybean protein markers – Possible analytical targets for allergen detection in processed foods. *Peptides* [Internet]. 2012;**33**(2):187-196. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0196978111005183> [Accessed: 8 March 2017]
- [49] MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: An open source document editor for creating and analyzing targeted proteomics experi-

- ments. *Bioinformatics* [Internet]. 2010;**26**(7):966-968. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20147306> [Accessed: 15 March 2017]
- [50] Mead JA, Bianco L, Ottone V, Barton C, Kay RG, Lilley KS, et al. MRMAid, the web-based tool for designing multiple reaction monitoring (MRM) transitions. *Molecular & Cellular Proteomics* [Internet]. 2009;**8**(4):696-705. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19011259> [Accessed: 15 March 2017]
- [51] Rogniaux H, Pavlovic M, Lupi R, Lollier V, Joint M, Mameri H, et al. Allergen relative abundance in several wheat varieties as revealed via a targeted quantitative approach using MS. *Proteomics* [Internet]. 2015;**15**(10):1736-1745. Available from: <http://doi.wiley.com/10.1002/pmic.201400416> [Accessed: 20 December 2016]
- [52] Hoofnagle AN, Whiteaker JR, Carr SA, Kuhn E, Liu T, Massoni SA, et al. Recommendations for the generation, quantification, storage, and handling of peptides used for mass spectrometry-based assays. *Clinical Chemistry* [Internet]. 2015;**62**(1):48-69. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26719571> [Accessed: 10 March 2017]
- [53] Heick J, Fischer M, Kerbach S, Tamm U, Popping B. Application of a liquid chromatography tandem mass spectrometry method for the simultaneous detection of seven allergenic foods in flour and bread and comparison of the method with commercially available ELISA test kits. *Journal of AOAC International* [Internet]. 2011;**94**(4):1060-1068. Available from: <http://europemc.org/abstract/med/21919338> [Accessed: 28 April 2016]
- [54] Wang H, Tu Z-C, Liu G-X, Liu C-M, Huang X-Q, Xiao H. Comparison of glycation in conventionally and microwave-heated ovalbumin by high resolution mass spectrometry. *Food chemistry* [Internet]. 2013;**141**(2):985-991. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/23790877> [Accessed: 26 April 2016]
- [55] Heick J, Fischer M, Pöpping B. First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *Journal of Chromatography A* [Internet]. 2011;**1218**(7):938-943. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21227428> [Accessed: 28 April 2016]
- [56] PILOLLI, Rosa, DE ANGELIS, Elisabetta, et MONACI, Linda. Streamlining the analytical workflow for multiplex MS/MS allergen detection in processed foods. *Food Chemistry*, 2017;**221**:1747-1753
- [57] Lamberti C, Cristina L, Acquadro E, Elena A, Corpillo D, Davide C, et al. Validation of a mass spectrometry-based method for milk traces detection in baked food. *Food chemistry* [Internet]. 2016;**199**:119-127. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0308814615302776> [Accessed: 20 December 2016]
- [58] Pedreschi R, Nørgaard J, Maquet A. Current challenges in detecting food allergens by shotgun and targeted proteomic approaches: A case study on traces of peanut allergens in baked cookies. *Nutrients* [Internet]. 2012;**4**(12):132-150. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22413066> [Accessed: 10 March 2017]

- [59] Huschek G, Bönick J, Löwenstein Y, Sievers S, Rawel H. Quantification of allergenic plant traces in baked products by targeted proteomics using isotope marked peptides. *LWT - Food Science and Technology* [Internet]. 2016;**74**:286-293. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0023643816304674> [Accessed: 3 April 2017]
- [60] Planque M, Arnould T, Dieu M, Delahaut P, Renard P, Gillard N. Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs. *Journal of Chromatography A*. 2016;**1464**:115-123
- [61] Parker CH, Khuda SE, Pereira M, Ross MM, Fu T-J, Fan X, et al. Multi-allergen quantitation and the Impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry* [Internet]. 2015;**63**(49):10669-10680. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26595064> [Accessed: 23 September 2016]
- [62] Gomaa A, Boye J. Simultaneous detection of multi-allergens in an incurred food matrix using ELISA, multiplex flow cytometry and liquid chromatography mass spectrometry (LC-MS). *Food Chemistry* [Internet]. 2015;**175**:585-592. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25577123> [Accessed: 28 April 2016]
- [63] Sayers RL, Johnson PE, Marsh JT, Barran P, Brown H, Mills ENC. The effect of thermal processing on the behaviour of peanut allergen peptide targets used in multiple reaction monitoring mass spectrometry experiments. *The Analyst* [Internet]. 2016;**141**(13):4130-4141. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27113917> [Accessed: 8 March 2017]
- [64] Campbell L, Raikos V, Euston SR. Modification of functional properties of egg-white proteins. *Nahrung/Food* [Internet]. 2003;**47**(6):369-376. Available from: <http://doi.wiley.com/10.1002/food.200390084> [Accessed: 10 June 2016]
- [65] Davis PJ, Williams SC. Protein modification by thermal processing. *Allergy* [Internet]. 1998;**53**(46 Suppl):102-105. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9826012> [Accessed: 8 March 2017]
- [66] Mueller GA, Maleki SJ, Johnson K, Hurlburt BK, Cheng H, Ruan S, et al. Identification of Maillard reaction products on peanut allergens that influence binding to the receptor for advanced glycation end products. *Allergy* [Internet]. 2013;**68**(12):1546-1554. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24266677> [Accessed: 8 March 2017]
- [67] Monaci L, Pilolli R, De Angelis E, Godula M, Visconti A. Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *Journal of Chromatography A* [Internet]. 2014;**1358**:136-144. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25042441> [Accessed: 26 April 2016]
- [68] Bignardi C, Mattarozzi M, Penna A, Sidoli S, Elviri L, Careri M, et al. A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by liquid chromatography–tandem mass spectrometry.

- Food Analytical Methods [Internet]. New York: Springer. 2013;**6**(4):1144-1152. Available from: <http://link.springer.com/10.1007/s12161-012-9521-4> [Accessed: 10 March 2017]
- [69] Dumont V, Kerbach S, Poms R, Johnson P, Mills C, Popping B, et al. Development of milk and egg incurred reference materials for the validation of food allergen detection methods. *Quality Assurance and Safety of Crops & Foods* [Internet]. 2010;**2**(4):208-215. Available from: <http://doi.wiley.com/10.1111/j.1757-837X.2010.00081.x> [Accessed: 23 September 2016]
- [70] Kirsch S, Fourdrilis S, Dobson R, Scippo M-L, Maghuin-Rogister G, De Pauw E. Quantitative methods for food allergens: A review. *Analytical and Bioanalytical Chemistry* [Internet]. 2009;**395**(1):57-67. Available from: <http://link.springer.com/10.1007/s00216-009-2869-7> [Accessed: 20 December 2016]
- [71] Lutter P, Parisod V, Weymuth H. Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. *Journal of AOAC International* [Internet]. 2010;**94**(4):1043-1059. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21919337> [Accessed: 20 December 2016]
- [72] Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: A critical review. *Analytical and Bioanalytical Chemistry* [Internet]. 2007;**389**(4):1017-31. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17668192> [Accessed: 10 March 2017]
- [73] Ong S-E, Mann M. Mass spectrometry-based proteomics turns quantitative. *Nature Chemical Biology* [Internet]. 2005;**1**(5):252-262. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16408053> [Accessed: 10 March 2017]
- [74] Paez V, Barrett WB, Deng X, Diaz-Amigo C, Fiedler K, Fuerer C, et al. AOAC SMPR(®) 2016.002. *Journal of AOAC International* [Internet]. 2016;**99**(4):1122-1124. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27455945> [Accessed: 22 November 2016]
- [75] Monaci L, van Hengel AJ. Development of a method for the quantification of whey allergen traces in mixed-fruit juices based on liquid chromatography with mass spectrometric detection. *Journal of Chromatography A* [Internet]. 2008;**1192**(1):113-120. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/18394633> [Accessed: 10 March 2017]
- [76] Careri M, Costa A, Elviri L, Lagos J-B, Mangia A, Terenghi M, et al. Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara h 3/4 for food control by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* [Internet]. 2007;**389**(6):1901-1907. Available from: <http://link.springer.com/10.1007/s00216-007-1595-2> [Accessed: 20 December 2016]
- [77] Mattarozzi M, Bignardi C, Elviri L, Careri M. Rapid shotgun proteomic liquid chromatography-electrospray ionization-tandem mass spectrometry-based method for the lupin (*Lupinus albus* L.) multi-allergen determination in foods. *Journal of Agricultural*

- and Food Chemistry [Internet]. 2012;**60**(23):5841-5846. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22612429> [Accessed: 10 March 2017]
- [78] Zhang J, Lai S, Zhang Y, Huang B, Li D, Ren Y. Multiple reaction monitoring-based determination of bovine α -lactalbumin in infant formulas and whey protein concentrates by ultra-high performance liquid chromatography-tandem mass spectrometry using tryptic signature peptides and synthetic peptide stand. *Analytica Chimica Acta* [Internet]. 2012;**727**:47-53. Available from: <http://dx.doi.org/10.1016/j.aca.2012.03.034>
- [79] Posada-Ayala M, Alvarez-Llamas G, Maroto AS, Maes X, Muñoz-Garcia E, Villalba M, et al. Novel liquid chromatography–mass spectrometry method for sensitive determination of the mustard allergen Sin a 1 in food. *Food Chemistry* [Internet]. 2015;**183**:58-63. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S0308814615003428> [Accessed: 23 March 2017]
- [80] Ahsan N, Rao RSP, Gruppuso PA, Ramratnam B, Salomon AR. Targeted proteomics: Current status and future perspectives for quantification of food allergens. *Journal of Proteomics* [Internet]. 2016;**143**:15-23. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27113134> [Accessed: 10 March 2017]
- [81] Brun V, Masselon C, Garin J, Dupuis A. Isotope dilution strategies for absolute quantitative proteomics. *Journal of Proteomics* [Internet]. 2009;**72**(5):740-749. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S1874391909001079> [Accessed: 23 March 2017]
- [82] Newsome GA, Scholl PF. Quantification of allergenic bovine milk α_{s1} -casein in baked goods using an intact 15 N-Labeled protein internal standard. *Journal of Agricultural and Food Chemistry* [Internet]. 2013;**61**(24):5659-5668. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22670623> [Accessed: 10 March 2017]
- [83] Chen Q, Zhang J, Ke X, Lai S, Tao B, Yang J, et al. Quantification of bovine β -casein allergen in baked foodstuffs based on ultra-performance liquid chromatography with tandem mass spectrometry. *Food Additives & Contaminants Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment* [Internet]. 2015;**32**(1):25-34. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25413212> [Accessed: 09 March 2017]
- [84] Liao Y-S, Kuo J-H, Chen B-L, Tsuei H-W, Lin C-Y, Lin H-Y, et al. Development and validation of the detection method for wheat and barley glutens using mass spectrometry in processed foods. *Food Analytical Methods* [Internet]. Springer US. 2017;1-9. Available from: <http://link.springer.com/10.1007/s12161-017-0827-0> [Accessed: 23 March 2017]
- [85] Planque M, Arnould T, Renard P, Delahaut P, Dieu M, Gillard N. Highlight on bottlenecks in food allergen analysis: Detection and quantification by mass spectrometry. *Journal of AOAC International* [Internet]. 2017; Available from: <http://www.ingentaconnect.com/content/10.5740/jaoacint.17-0005> [Accessed: 15 March 2017]
- [86] Chen J, Wang M, Turko IV. Quantification of amyloid precursor protein isoforms using quantification concatamer internal standard. *Analytical Chemistry* [Internet]. 2013;**85**(1):303-307. Available from: <http://pubs.acs.org/doi/abs/10.1021/ac3033239> [Accessed: 05 April 2017]

- [87] Chen Q, Zhang J, Ke X, Lai S, Li D, Yang J, et al. Simultaneous quantification of α -lactalbumin and β -casein in human milk using ultra-performance liquid chromatography with tandem mass spectrometry based on their signature peptides and winged isotope internal standards. *Biochimica et Biophysica Acta (BBA): Proteins and Proteomics* [Internet]. 2016;**1864**(9):1122-1127. Available from: <http://linkinghub.elsevier.com/retrieve/pii/S1570963916301157> [Accessed: 10 March 2017]
- [88] Korte R, Brockmeyer J. MRM3-based LC-MS multi-method for the detection and quantification of nut allergens. *Analytical and Bioanalytical Chemistry* [Internet]. 2016;**408**(27):7845-7855. Available from: <http://link.springer.com/10.1007/s00216-016-9888-y> [Accessed: 20 December 2016]
- [89] Ippoushi K, Sasanuma M, Oike H, Kobori M, Maeda-Yamamoto M. Absolute quantification of Pru av 2 in sweet cherry fruit by liquid chromatography/tandem mass spectrometry with the use of a stable isotope-labelled peptide. *Food Chemistry* [Internet]. 2016;**204**:129-134. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26988485> [Accessed: 28 March 2017]
- [90] Abdel Rahman AM, Kamath SD, Gagné S, Lopata AL, Helleur R. Comprehensive proteomics approach in characterizing and quantifying allergenic proteins from northern shrimp: Toward better occupational asthma prevention. *Journal of Proteome Research* [Internet]. American Chemical Society. 2013;**12**(2):647-656. Available from: <http://pubs.acs.org/doi/abs/10.1021/pr300755p> [Accessed: 28 March 2017]

