

Next Generation of Food Allergen Quantification Using Mass Spectrometric Systems

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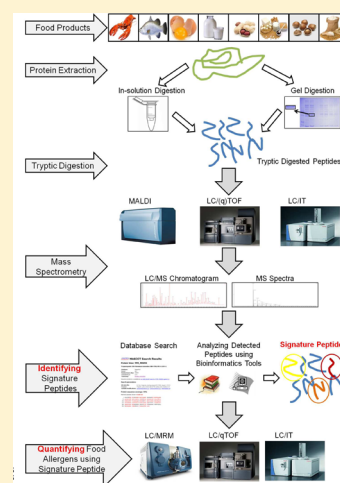
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S Supporting Information

ABSTRACT: Food allergies are increasing worldwide and becoming a public health concern. Food legislation requires detailed declarations of potential allergens in food products and therefore an increased capability to analyze for the presence of food allergens. Currently, antibody-based methods are mainly utilized to quantify allergens; however, these methods have several disadvantages. Recently, mass spectrometry (MS) techniques have been developed and applied to food allergen analysis. At present, 46 allergens from 11 different food sources have been characterized using different MS approaches and some specific signature peptides have been published. However, quantification of allergens using MS is not routinely employed. This review compares the different aspects of food allergen quantification using advanced MS techniques including multiple reaction monitoring. The latter provides low limits of quantification for multiple allergens in simple or complex food matrices, while being robust and reproducible. This review provides an overview of current approaches to analyze food allergens, with specific focus on MS systems and applications.

KEYWORDS: food allergy, mass spectrometry, MRM, quantification, signature peptide, soy, wheat, peanut, crustacean



1. INTRODUCTION

Food allergy is a hypersensitive reaction of the human immune system. Currently, sensitization rates to one or more allergens among children are globally 40–50%.¹ Worldwide an estimated 220–250 million people suffer from food allergy.¹ Food allergies are caused by proteins, also termed allergens, which are not generally considered harmful to the human body. Typical allergic symptoms include urticaria, vomiting, asthma, and life-threatening anaphylaxis. More details of different types of allergy and symptoms can be found elsewhere.^{2–7}

Ninety percent of all food allergies are caused by eight food groups. These eight groups, often referred to as “The Big 8” food allergies, include: egg, fish, milk, peanut, shellfish, soy, tree nuts, and wheat. Currently, more than 600 food allergens are known on the molecular level. Of these, 206 are officially registered by the allergen nomenclature subcommittee established by the International Union of Immunological Societies (IUIS) <http://www.allergen.org>. To complicate the analysis, different food sources are known to have more than one allergenic protein. The Allergome database (<http://www.allergome.org>) reports 4248 allergens derived from 2294 species. In this database, for example, celery has 6 known allergens, mollusks 2, peanut 13, lupine only 1, and mustard 5. In soybean, at least 212 proteins are reported to be possibly

allergenic, of which ~20 proteins are confirmed to be allergenic. Figure 1 summarizes the most common allergens grouped by “The Big 8” food allergies.

The prevalence of food allergies is increasing worldwide, as is the reported number of identified food allergens. Children have a higher prevalence of food allergies with about 4–8% and adults about 1–5% based on large population studies.^{2,5,6,8} The overall prevalence of allergenic sensitization to “The Big 8” food allergies for adults and children is shown in Figure 2. Over 80% of allergic children are sensitized to milk, peanut, egg, and tree nuts. In contrast, the vast majority of allergic adults are sensitized to shellfish, peanut, tree nuts, and fish.

Legislation requires food labeling to protect increasing numbers of food allergic individuals from serious health problems. The basis for labeling in all countries is provided by the International Codex Alimentarius Commission (a joint committee with delegates from both the Food and Agriculture Organization of the United Nations and the World Health Organization).¹ However, as shown in Table 1, different countries mandate a different selection of allergens for food labeling. Already 14 different food groups are required for

Received: March 12, 2014

Published: May 13, 2014

	Crustacean/ Shellfish	Egg	Fish	Milk	Peanut	Soy	Tree nuts	Wheat
								
	Myosin light chain	Lyozyme C	β -pavalbumin (and isoforms)	S100 calcium binding protein A7	Lipid transfer protein	Defensin	2S albumin	Lipid transfer protein
	Troponin C	Ovomucoid (α , β)	Aldolase A	α -lactalbumin	Defensin	Profilin	Lipid transfer protein	α -purothionin
	Sarcoplasmic calcium binding protein	Ovalbumin	β -enolase	β -lactoglobulin	Oleosin	PR-10, Bet v 1 family member	Ribosomal protein P2	Thioredoxin
	Triphosphosphate isomerase	Serum albumin		Caseins (α , β , γ , κ)	Profilin	Hydrophobic protein	S albumin	Profilin
	Tropomyosin	Ovotransferrin		Lipocalin	α -amylase	Glycinin (legumin, 11S globulin)	Profilin	Trypsin inhibitor
	α -actine	Vitellogenin II		Serum albumin	Conglutin (2S albumin)	β -conglycinin (vicilin, 7S globulin)	2S albumin	Amylase Inhibitor (α , β)
	Arginine kinase	Ovomucin			PR-10, Bet v 1 family member	Biotinylated protein	Oleosin	Agglutinin Isolectin
	SERCA/ Ca ²⁺ ATPase				Agglutinin		PR-10, Bet v 1 family member	Gliadin (α , γ , ω)
	Myosin heavy chain				Cupin (Vicilin-type, 7S globulin)		Manganese superoxide dismutase	Peroxidase 1
					Cupin (11S globulin, Glycinin)		11S globulin	LMW Glutenin
					Phospholipase D		Vicilin-like protein	Serpin
					Conarachin I		Legumin-like protein	β -D galactan exohydrolase
					α -arachin		Vicilin	HMW Glutenin
							Prunin	
							Amandin	

Figure 1. Common food allergens from “The Big 8” food allergen groups. Allergens are ordered from top to bottom in increasing molecular weight. Yellow highlights indicate allergens investigated with MS systems.

allergen labeling in the European Union compared with Japan mandating just five allergens.^{10–12}

In summary, food allergies are a serious health concern with increasing worldwide prevalence and increasing numbers of food allergens. However, detecting and quantifying food allergens remains problematic. Analysis is complicated by complex food matrices, multiple allergens, and different food sources. Food labeling is required by law to protect allergic individuals. However, a lack of standardized analytical methods means legislation cannot be properly policed. Therefore, sensitive, reliable, robust, fast, reproducible, and standardized methods are necessary for improved allergen analysis and interpretation. This can be achieved using advanced MS approaches.

2. CURRENT METHODS FOR FOOD ALLERGEN QUANTIFICATION

Current methods for food allergen analysis are mainly antibody-based using enzyme-linked immunosorbent assay (ELISA) or lateral flow devices. ELISA kits are available for most of “The Big 8” food allergens. Most ELISA kits, however, target only a single allergen from a single food product. For example, for the analysis of milk allergens, ELISA kits are commercially available to detect casein, β -lactoglobulin, and total allergen content (casein and β -lactoglobulin). None of the other known milk allergens include α -lactoglobulin, which is also considered to be a major allergen in milk, is targeted.¹³

Antibody-based methods require the availability of either monoclonal or polyclonal antibodies, preferably both.¹⁴ Many

Prevalence to “The Big 8” food allergens

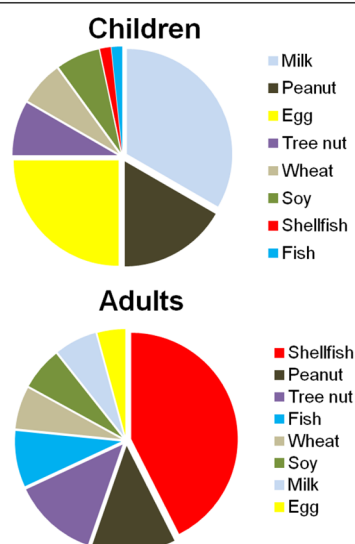


Figure 2. Prevalence profile of allergic sensitization to “The Big 8” food allergens among children and adults. The highest prevalence data are listed in decreasing order. Data are derived from U.S. and Australian studies.^{6,8,9}

antibodies are commercially available; however, often antibodies are poorly characterized.¹⁵ Cross-reactivity can occur when using antibody-based methods, leading to potential false-positive results.^{15–18} Complex food matrices can comprise interfering components, for example, polyphenols or tannins, which interact with or bind to proteins and antibodies.^{19,20} In addition, food processing or sample preparation can modify allergens, which subsequently are not recognized by the target antibody, leading to potential false-negative results.^{21,22}

A comparative study by Heick et al.²³ of two commercially available ELISA kits for soy noted that the detection for spiked flour samples varied by a factor of 10. When they examined hazelnut in spiked processed bread they observed that results between ELISA kits varied by a factor of three. Quantifying

hazelnut in both unprocessed and processed samples using two different ELISA kits resulted in significant differences of up to 40%.²³ A similar comparative study of commercial ELISA kits for hazelnut detection by Cucu et al.²⁴ demonstrated that all kits evaluated produced false-positive and false-negative results. In some kits the actual hazelnut protein concentration was 17–49% underestimated, and another kit overestimated the concentration by 27%.

Johnson et al.¹⁵ performed a multilaboratory evaluation of egg and milk allergens and demonstrated that all kits underestimated the concentration of egg. Only one kit quantified the milk protein content with acceptable accuracy at 6 and 15 mg/kg. All milk and egg kits were able to detect the lowest spiked concentration (3 mg/kg, however, the limit of quantification (LOQ) was for egg ~10 mg/kg and for milk 30 mg/kg). This highlights that the current methods would have difficulties to detect allergens in certain types of food consumed in larger quantities. Furthermore, some ELISA kits have a very low dynamic range, and the generated results are difficult to compare.^{15,25}

In summary, for many food allergens ELISA kits are commercially available. However, these methods are restricted to single well-known allergens, and less well-characterized allergens are excluded. The lack of general consistent standards and reference material for allergen analysis makes a comparison of results between different ELISA kits very difficult. These insufficiencies clearly highlight that alternative methods for the quantification of food allergens are urgently needed.

3. ADVANTAGES OF MASS SPECTROMETRY SYSTEMS FOR ALLERGEN DETECTION

The disadvantages of current established methods for allergen analyses are numerous; hence alternatives have been investigated in recent years. In particular, nonimmunological methods, such as mass spectrometry (MS) systems, have been investigated and developed to overcome the drawbacks of ELISA. Different MS systems are commonly used in proteomics. A mass spectrometer is composed of three different parts: ion source, mass analyzer, and detector. As ion source, matrix-assisted laser desorption/ionization (MALDI) or

Table 1. Food Products Requiring Allergen Labeling^a

source/allergen	Codex Alimentarius	United States	European Union	Australia/ New Zealand	Canada	China	Hong Kong	Japan	Korea	Mexico
crustacean	☆	☆	☆	☆	☆	☆	☆	☆	☆	☆
egg	☆	☆	☆	☆	☆	☆	☆	☆	☆	☆
fish	☆	☆	☆	☆	☆	☆	☆		☆	☆
milk	☆	☆	☆	☆	☆	☆	☆	☆	☆	☆
soy	☆	☆	☆	☆	☆	☆	☆		☆	☆
peanut	☆	☆	☆	☆	☆	☆	☆			☆
tree nuts	☆	☆	☆	☆	☆	☆	☆			☆
wheat/cereals	☆	☆	☆	☆	☆	☆	☆	☆	☆	☆
sesame			☆	☆	☆					
shellfish/mollusks			☆		☆					
mustard			☆		☆					
celery			☆							
lupine			☆							
sulfur oxide and sulphites			☆							

^a“The Big 8” food allergens are ordered alphabetically, and additional allergens are below them as currently required. “☆” indicates that the allergen needs to be labeled on every food product.

electrospray ionization (ESI) are used most commonly. For mass analyzer, time-of-flight (TOF) and ion trap (IT) are used in proteomics. Combining different ion sources and mass analyzers leads to hybrid MS systems, such as ESI-qTOF (referred as qTOF in this review), ESI-IT (referred as IT in this review), or MALDI-TOF (referred as MALDI in this review). These hybrid MS systems can be used to identify proteins and peptides.^{26,27} Quantitative qTOF and IT systems have the advantage of identification and quantification through fragmentation settings in the MS collision cell.¹² Figure 3 compares and

Comparison between immunological and chemical methods for allergen analysis						
Food matrix containing allergen						
Antibody based			MS based			
	ELISA	Immunoblot	MALDI	qTOF/IT	MRM	
Extraction Buffer	Strong detergents	Strong detergents	Weak buffers	Weak buffers	Weak buffers	
Treatment	No	No	Yes/No	Digestion	Digestion	
Detection Method	Spectrometry	Visual	Mass spectrometry	Mass spectrometry	Mass spectrometry	
Allergens can be analyzed	One	One	Multiple	Multiple	Multiple	
Standard required	Yes (internal kit standard)	No	No	Yes/No	Yes	
Cross Reactivity	Yes	Yes	No	No	No	
Species Specificity	No	No	Yes	Yes	Yes	
Can be standardized	No	No	Yes	Yes	Yes	
Results comparable	No	No	Yes	Yes	Yes	
Time	Time consuming	Time consuming	Fast analysis	Fast analysis	Fast analysis	
Cost	Medium	Low	Medium	Medium	Medium	
Quantification	LOD (lowest 0.2ppm) LOQ (lowest 0.3ppm)	Semi quantitative	Semi quantitative	LOD (lowest 0.06ppm) LOQ (lowest 3.7ppm)	LOD (lowest 0.001ppm) LOQ (lowest 0.01ppm)	

Figure 3. Comparison between antibody-based immunological methods and MS systems for the quantification of food allergens.

summarizes the major differences in antibody-based immunological methods allergen analysis and the chemical procedures using MS-based methods. The application of MS for allergen identification is, however, not commonly applied for routine analysis, as discussed in detail later.^{12,25,28,29}

The advantages of MS are ease of sample preparation, fast analysis, and analysis of more than one allergen at a time. Moreover, MS is robust and stable and can easily be automated and standardized compared with other methods with potential low LOD and LOQ.^{22,23,30} Another advantage is to have better defined standards, which makes the comparison of results between methods and laboratories much easier. Nevertheless, the MS analysis of proteins requires a set of methodological steps that includes proteins digestion to generate peptides and their introduction into the MS system for analysis (Figure 4). The MS spectra of digested peptides, via MALDI, LC/TOF, or LC/IT, are then analyzed using bioinformatics tools and protein databases. Databases most commonly used for this purpose are SEQUEST and Mascot.^{12,22} Peptides are identified according to their mass-to-charge ratio and the allergenic protein identified by comparing the derived amino acid sequences with known proteins.^{12,22,31} The peptide sequences that are uniquely specific for a particular allergen are termed "signature peptides".

Post-translational modifications (PTMs), for example, occurring during heat treatment, can result in a possible

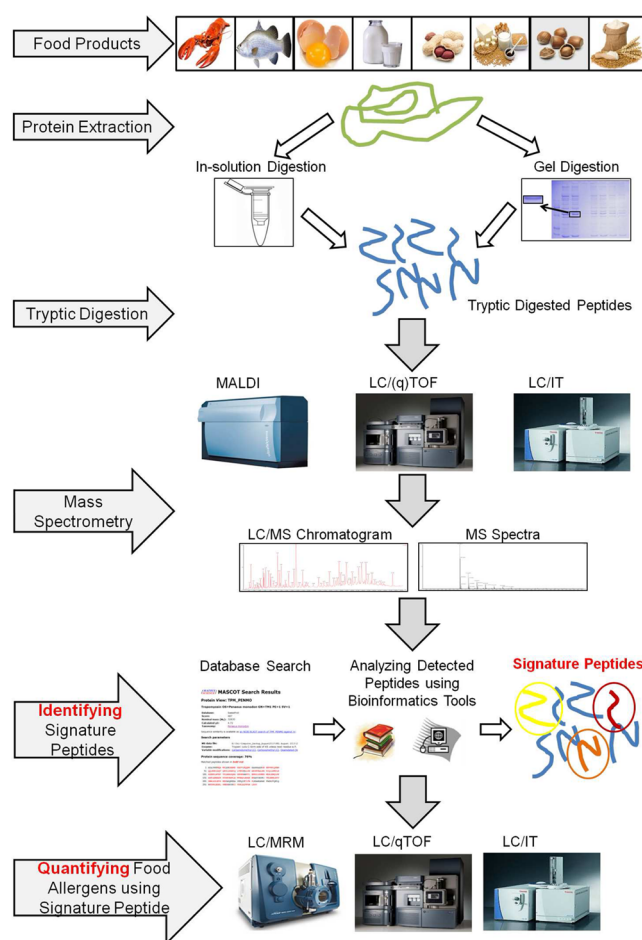


Figure 4. Signature peptide identity and characterization workflow for detection and quantification of food allergens.

change to the secondary and tertiary structure of an allergen. The sensitivity and specificity of an ELISA can depend on the 3-D structure of allergens, whereas MS is based on the structurally independent amino acid sequence. PTMs can therefore have a huge impact on allergenicity of the protein, which can lead to false-positive or false-negative results in ELISA techniques.^{12,27} High resolving power and sensitivity coupled to this independence from structural changes allows MS to detect allergens in trace amounts.²⁰ With LC-MS, it is possible to detect PTM, providing additional information on primary and secondary protein structure.^{25,32}

In summary, MS systems have been developed in the past few years for food allergen analysis. MS analysis overcomes the major drawbacks of established methods, such as nonspecific antibody-allergen reaction and unknown modifications. Moreover, MS systems make it possible to generate information about amino acid sequences of allergens as well as identifying PTMs and isoforms.

3.1. Sample Digestion for Allergen Analysis with MS Systems

Digestion of the allergenic protein is commonly performed prior to MS analysis following the bottom-up strategy, explained in detail by Monaci and Visconti.¹² Digestion cleaves large proteins into smaller peptides, and thus potentially matrix interferences and associated interactions with other proteins are reduced. These reductions remove complicating factors and make the analysis with LC-MS more reproducible.³³ Ideally, a

complete digestion is achieved in a very short time with a maximum of peptides generated and without missed cleavage sites.^{22,30} The peptides generated should be stable over time and easily detected by MS. In some food samples, enzymes are naturally present and unwanted degradation of proteins occurs. Thus, the digestion process depends on the individual allergen structure with careful consideration of disulfide bridges, structural folding, solubility, and glycosylation. Comparing methods adequately requires a total, robust, and reproducible digestion method.

Various enzymes are available with specific cleavage sites. However, the most commonly used enzyme is trypsin due to the well-known cleavage sites between the amino acid arginine (R) and lysine (K). R and K occur in proteins common enough to derive sufficient peptide fragments from most proteins. As more information about trypsin peptides is available, protein identification is more likely using database searches. Trypsin is also preferred because it occurs naturally in the stomach and therefore is representative in *in vivo* cleavage of allergen proteins.¹² Abdel Rahman et al.³² compared different enzymes and demonstrated that V8 enzymes have poor efficiency for *in-gel* digestion due to their enzyme size. Many peptides digested with V8 had missed cleavage sites (maximum five missed cleavage sites). Carrera et al.³⁴ showed that more peptides could be identified when fish species were digested with trypsin compared with Glu-C. In contrast, Sealey-Voyksner et al.³⁵ showed that pepsin showed the highest yield of peptides compared with trypsin and chymotrypsin when analyzing wheat gluten. Surprisingly, this study found that a higher sample concentration, with the optimal enzyme to protein ratio, did not lead to increased yield of generated peptides due to incomplete digestion.

In summary, when identifying and detecting allergens using MS systems, sample preparation is a critical step. Allergen proteins are extracted from food matrices and digested with enzymes generating peptides. We recommend for most applications trypsin digestion for allergen detection using MS-based systems. The tryptic digestion approach is most commonly referenced in the literature and has the most data publicly available. Trypsin digestion also occurs naturally in the stomach when allergens are ingested.

3.2. Identifying Signature Peptides by MS

Specific criteria must be fulfilled for the designation of a signature peptide (Figure 4). It is critical that signature peptides are unique to the target protein and detectable by the MS systems of choice.^{22,36} The selected signature peptides do not need to be the most intense signals found in MS spectra, but they do need to be sufficiently intense to allow clear separation from other peptides or MS background, even if they are only present in low quantities.²⁵ The most important steps on how to select an appropriate signature peptide are summarized in Figure 5. Peptides with amino acids prone to PTMs should not be selected.^{22,25,28,36} Peptides with missed cleavage sites after digestion should be avoided in signature peptide selection;^{22,25,26} preferably, the peptide should be between 10 and 20 amino acids long.^{25,26,28,37}

The selection of a signature peptide for allergen analysis depends on the investigative approach. One option is to choose a signature peptide that is unique for the allergen selected. A more advanced approach, which is not always possible due to allergen amino acid sequence similarity, is to select a signature peptide that is unique for the allergen as well as for the species.

Critical Steps for selecting signature peptide and transition	
Signature peptide selection	• Unique • Reproducible (digestion) • Easy detected with MS systems • Abundant in food matrix • Stable • No modifications • Easily extracted • 6-12 amino acid long
Transition selection	• Easy detected with MS systems • Abundant in food matrix • No modifications • Reproducible transition time • Reproducible retention time • Precursor ion: double or triple charged • Product ion: reproducible transitions specific to peptide

Figure 5. Selection criteria for signature peptides and transitions for the quantitation of food allergens.

As seen in Supplementary Table 1 in the Supporting Information, Carrera et al.³⁸ tried to identify different signature peptides for the fish allergen β -parvalbumin in different species. Although different peptides were combined, only 5 species out of 19 were unique. The remaining 14 species have identical or similar peptides identified. For β -parvalbumin, the sequence identity for different species is between 60 and 80%.^{2,5} Houston et al.³⁹ identified 10 allergens in soy and were aiming to report two signature peptides per allergen (total of 20), but because of signature peptides selection criteria could find only 15 ideal synthetic peptides. For other allergens, such as the major allergen in crustacea, tropomyosin, the homology is as high as 98%.³ Ortea et al.⁴⁰ were able to distinguish six different prawn species by MALDI analyzing the minor allergen arginine kinase (Supplementary Table 1 in the Supporting Information). Therefore, MS systems have potential for species differentiation.

In summary, signature peptides should be carefully selected. The more information available, the higher the certainty that the peptide represents the allergen and species of interest. Unfortunately, because of the sequence homology of allergens in different species, it is not always possible that signature peptides are species specific.

3.2.1. Signature Peptides Identified with MS Systems.

Signature peptides have been identified for several of the most common food allergens in "The Big 8". Allergens analyzed using different MS systems are highlighted in yellow (Figure 1). Currently, 39 different allergens have been identified from "The Big 8" food allergen group. Table 1 demonstrates the major allergens in crustacean and crustacean species that have been investigated by MS. A complete summary of all allergen, species, and food sources can be found in Supplementary Table 1 in the Supporting Information. Currently, allergenic food proteins are identified by MS for crustacean (8 allergens), egg (5 allergens), fish (1 allergen), milk (7 allergens), peanut (3 allergens), soy (7 allergens), tree nuts (5 allergens), and wheat (10 allergens).

Crustacean. Eight different crustacean allergens have been investigated by three different research groups (Table 2 and Supplementary Table 1 in the Supporting Information).^{37,40–47} Arginine kinase is the most investigated allergen in this allergen source. Ortea et al.⁴² attempted to identify different peptides for different species with MALDI and IT. However, in five crustacean species, identical peptides were identified, and unique peptides were identified for only two species. Abdel Rahman et al.^{32,44} were successful in identifying different peptides for tropomyosin from black tiger prawn and snow crab.

Table 2. Alphabetical Summary of the Major Allergens in Crustacean Analyzed with Different MS Systems Peptides Published in the Literature^a

allergen (registered allergen)	peptides identified (*recommended signature peptides)	species/allergen source	LOD (LOQ)	MS system used	ref
Crustacean					
arginine kinase (Pen m 2)	FLQAANAC#R GTRGEHTEAEGGIYDISNK	(<i>Penaeus monodon</i>)	N.D.	MALDI and IT	40
arginine kinase (Lit v 2)	FLQAANAC#R GTRGEHTEAEGGIYDISNK	(<i>Litopenaeus vannamei</i>)	N.D.	MALDI and IT	
arginine kinase (Pen m 2)	*AVFDQLKEK *VSSTLSSLEGELK *TFLVWVNEEDHLR *LEEVAGKYNLQVR	(<i>Penaeus monodon</i>)	N.D.	LC/IT	42
arginine kinase (Lit v 2)	*VSSTLSSLEGELK *TFLVWVNEEDHLR *LEEVAGKYNLQVR	(<i>Litopenaeus vannamei</i>)	N.D.	LC/IT	
arginine kinase	*LVSANVEIEK	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF, and LC/MRM	37
arginine kinase	AVFDQLKEK VSSTLSSLEGELK GTYPLTGMSK LIDHFLFK	(<i>Penaeus monodon</i>)	N.D.	MALDI and LC/qToF	44
myosin light chain (Lit v 3)	KGXNVFDMFTQK SSGESDDDDVVAASIR	(<i>Litopenaeus vannamei</i>)	N.D.	LC/MALDI	46
myosin light chain	EGFQLMDR	(<i>Pandalus borealis</i>)	N.D.	MALDI, LC/qToF, and LC/MRM	41
sarcoplasmic Ca-binding (Lit v 4)	YMYDIDDDGFLK NDFECLAVR GEFSAADYANNQK NLWNEIAELADFNKDG	(<i>Litopenaeus vannamei</i>)	N.D.	LC/IT	47
sarcoplasmic calcium binding protein	VATVSLPR	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF, and LC/MRM	32
tropomyosin (Pen m 1)	*ANQLVEK	(<i>Penaeus monodon</i>)	N.D.	LC/qToF	44
tropomyosin	*SQLVENELDHAQEQLSAATHK	(<i>Chionoecetes opilio</i>)	3 nM	LC/MRM	45
tropomyosin	*SEEEVFLQK	(<i>Pandalus borealis</i>)	0.25 nM	MALDI, LC/qToF, and LC/MRM	41

^a“*” indicates recommendation use as a signature peptide. Allergen name in brackets confirms the registration with the international union of immunological societies (IUIS). LOD and LOQ given are provided in ppm as published. {C# = carbamidomethylated cysteine; N.D. = not determined}.

Egg. Five different allergens are identified in egg (Supplementary Table 1 in the Supporting Information) by five different research groups.^{20,23,30,48–50} Interestingly, identical peptides are reported for the same allergens investigated, with the exception of Azarnia et al.,⁴⁸ who identified a different signature peptide for the Gal d 2 allergen.

Fish. The major allergen in fish is parvalbumin and was investigated by Carrera et al.^{34,38} (Supplementary Table 1 in the Supporting Information). In 2010, Carrera et al.³⁴ could fully de novo sequence 25 new parvalbumin isoforms. An additional 16 new isoforms were partially sequenced, investigating 13 species of the Merlucciidae family, which includes cod-like fish, including most hakes. Outcomes of the study in 2012 are described in Section 3.2.

Milk. Seven allergens are identified in milk (Supplementary Table 1 in the Supporting Information).^{13,20,30,51–54,69} The identified signature peptides for α -S1 casein and β -casein are identical between all five research groups.^{13,20,30,52} One peptide is consistent with two of the four research groups for β -S2 casein, which may be due to different MS systems applied. κ -Casein was reported with two different peptides due to the fact that Molle and Leonil⁵¹ were trying to identify and quantify different glycosylated and nonglycosylated forms of the macro peptide κ -casein.

Peanut. The three major peanut allergens (Supplementary Table 1 in the Supporting Information)^{19,30,33,55–70} include

various isoforms of cupin and conglutin. Chassaigne et al.⁵⁸ detected unique peptides for all three peanut allergens from two different isoforms. However, only the Ara h 1 signature peptide from the 2007 Chassaigne et al.^{57,58} study were found again in the 2009 study. Four publications investigating Ara h 1 reported similar peptides, in contrast with Shefcheck and Musser¹⁹ and Helbing et al.⁷⁰ who chose the most abundant peptides and opposed the identified criteria set out in Section 3.2. Ara h 2 was analyzed by three groups, with some corresponding peptides reported. Heick et al.³⁰ identified different peptides for Ara h 3/4 than Chassaigne et al.,^{57,58} which may again be explained by the different MS systems used.

Soy. Four research groups investigated seven different allergens in soy (Supplementary Table 1 in the Supporting Information).^{30,39,59,60} Cucu et al.⁶⁰ reported one signature peptide each for Gly m 5 and Gly m 6. Both peptides are not modified during food processing, such as the Maillard Reaction. Heick et al.³⁰ reported one peptide for glycinin consistent with Cucu et al.⁶⁰ and a different peptide consistent with Houston et al.³⁹ None of the reported peptides are identical between Houston et al.³⁹ and Cucu et al.⁶⁰

Tree Nut. Tree nuts, such as Brazil nut, walnut, hazelnut, and almond (Supplementary Table 1 in the Supporting Information) were investigated by Moreno et al.⁶¹ and Heick et al.,³⁰ with up to six isoforms for the 2S albumin.

Wheat. Ten different allergens have been reported for wheat allergens by five research groups (Supplementary Table 1 in the Supporting Information).^{35,62–65} Wheat allergens are therefore the most investigated food allergens. α -Amylase inhibitor with similar peptides was identified by two groups. The investigated β -amylase resulted in only one common peptide using MALDI and LC/qTOF.⁶⁴

Overall, the current literature demonstrates that the analysis of allergens with different MS systems is successful. Many different peptides for different allergens have been reported. Similar or identical peptides are reported for the specific allergens investigated, despite the different MS systems used. LOD and LOQ derived with qTOF and IT are comparable to reported ELISA values. These findings highlight that MS is an outstanding approach in detecting and quantifying specific food allergens. However, consistent signature peptides, which can be used as standards and reference materials, need to be further evaluated.

4. MRM SYSTEMS FOR ABSOLUTE ALLERGEN QUANTIFICATION

The use of multiple reaction monitoring (MRM) for peptide analysis has been a more recent approach for clinical applications and for allergen analysis. New MRM systems are now available like Triple Quadruples (QQQ) with extended mass ranges.²⁶ However, MRM does not provide accurate mass determinations when compared with other MS systems, such as MALDI, qTOF, and (q)IT, used for peptide/protein/allergen analysis. The great advantage of MRM is that it allows the precise quantitative determination of target proteins in complex samples.^{22,25,50} MRM also offers a broad dynamic range (up to five magnitudes), which is essential for quantification of allergens with highly variable concentrations.⁵⁰

The high sensitivity of MRM allows precise quantification of individual proteins as well as different isoforms.²³ This is an advantage because allergens, for example, parvalbumin, have different isoforms and cannot be easily detected using antibody-based methods. Because the quantification of proteins by MRM is a relatively new technique, the development of methods has to be carefully investigated, designed, and validated,^{22,25} as illustrated in Figure 4. Selecting an appropriate tryptic signature peptide for MRM is the most critical step.^{22,25,29} Ionization of selected peptides must be complete and distinguishable from the matrix.^{25,29} The quantification can be based on a single peptide, if specifically unique to the target protein; however, two to three peptides per proteins are preferred to achieve better specificity.^{22,25,29}

In MRM approaches, the time window of a selected signature peptide is scanned for a defined time period according to its elution or retention time in the chromatographic run.^{22,23} More recent MRM systems are able to narrow the scanning window of precursor ions to 0.2 m/z, which aids selectivity and accuracy.⁶⁶ These narrow windows make it possible to analyze many different signature peptides in one single LC–MRM run,²⁶ as demonstrated by Picotti et al.⁵⁰

4.1. Standards Used for Absolute Allergen Quantification Using MRM

Well-characterized standards are required to ensure that the correct peptide, matching the target allergen, is quantified. To analyze allergens, we should base these standards on well-characterized signature peptides. These signature peptides can be generated from a recombinant allergen, synthetic peptides,

or identical isotopic-labeled peptides.^{26,28,29,67} With an appropriate standard, LC/MRM parameters can be optimized to achieve sensitivity of the target peptide.^{26,50} MRM approaches quantifying small molecules have most commonly used isotope-labeled standards.^{22,26,28,29}

Different standards are used for MRM quantification in literature and will be described in the following paragraphs. Johnson et al.³¹ suggest that isotopically labeled peptides are a realistic alternative for the development of reference methods,³¹ although the best standards are isotopically labeled proteins. However, these proteins are prohibitively expensive. Isotopically labeled peptides have been used successfully for protein quantification over the last two decades.^{27,44} For example, Kitteringham et al.²⁶ and Meng and Veenstra²⁸ suggest using isotopic-labeled standards, as they match characteristics of the original peptide.^{27,45} These characteristics include the physicochemical and chromatographic performance as well as ionization efficiency.^{25,28,45} Isotopic-labeled peptides have a slightly different mass-to-charge ratio, generated from the elemental isotope label used, and can therefore be distinguished from the native peptide.^{36,45} The mass difference between label and native peptide should be between 5 and 6 Da. At least three to four transitions should be selected per labeled peptide.^{25,28} The major disadvantage of isotopic-labeled standards remains their cost.

Alternatives to isotopic-labeled standards have been investigated to reduce the expense of method development. Synthetic peptides without an isotopic label are one option. Rauh²⁵ suggests optimizing MRM parameters with synthetic peptides or crude peptides, as all LC and MS parameters will match the native protein, as previously explained. Because the synthetic peptide is not distinguishable from the native peptide, absolute quantification is still successful.^{25,36,50,68} Sealey-Voyksner et al.³⁵ considered the use of isotopic label standards in their study. The recovery, accuracy, and injection to injection precision of various control samples demonstrated that isotopic-labeled standards were not necessary. Pak et al.⁶⁸ also found accuracy and reproducibility via spiking samples with synthetic standards was suitable. This study also found an acceptable dynamic range for qTOF and IT. However, LC/MRM methods using unlabeled spikes need to be more consistent and reliable to be able to achieve absolute quantification.²³

Another alternative is the use of recombinant proteins because they are generally well-defined and better characterized molecularly and chemically. Recombinant proteins can be produced in large amounts and are easier to purify compared with traditional native protein extract purifications.^{22,67} Recombinant allergen proteins are also useful to investigate digestion efficiency, losses during sample preparation, peptides generation, LC retention time, and transitions times.^{22,28} Parameters optimized with recombinant proteins are equivalent to native allergens.^{25,28} Recombinant proteins can be generated from different species, which may differ by only few amino acids and therefore may assist in characterizing species specific allergens.²⁸ Because recombinant proteins are usually expressed in *E. coli*, they are, however, not suitable for the determination of PTM.²⁸ While recombinant proteins are ideal for the development of precise and accurate methods using MRM,^{25,27,29} only a few highly purified recombinant proteins are currently available.²²

In summary, MRM quantification requires standards to confirm reproducibility and accuracy of the target molecules.

Standards used most commonly are isotopic-labeled proteins or peptides. When establishing MRM methods for absolute quantification of allergens, we suggest the use of both recombinant proteins and isotopic labeled standards. Once the method is established, optimized and validated synthetic peptides without isotopic labels can replace the labeled standards.

4.2. Using MRM for Absolute Allergen Quantification

Many biomarkers are published in the allergy field, but only one per year is actually well-characterized and methods fully validated.²⁸ To establish, evaluate, and validate new MRM methods, we should consider several critical steps. A brief workflow is shown in Figure 4. First, signature peptides best fitting the selection criteria should be used. Optimization of LC/MRM parameters should be performed as previously described. Second, samples should be spiked with synthetic or isotopic-labeled peptides to demonstrate the inter- and intralaboratory method reproducibility and precision. All sample preparation steps and precursor/product ion generation must be proven to be stable and reproducible. Synthetic or isotopic-labeled standards should be spiked into the matrix before and after sample preparation to identify losses in the extraction process and obtain recovery information. The absence of peptides in blank samples should also be demonstrated. Third, using recombinant protein, repeated digestions should be carried out to demonstrate that the same signature peptides can be identified consistently and losses during sample preparation evaluated. Finally, the method reproducibility, precision, linearity, accuracy, and recoveries should be calculated.^{29,35,45}

Signature peptides used with MRM for absolute allergen quantification should be selected according to the criteria that are required for identifying signature peptides. The most important steps are summarized in Figure 5. Each MRM transition should be optimized with MS to achieve maximum sensitivity.^{23,25,29} In MRM mode, peptides will be fragmented with mainly y-ions and b-ions generated. Sealey-Voyksner et al.³⁵ also demonstrated that cleavage sites between amino acid F (phenylalanine) and P (proline) exhibited different product ion fragment patterns. The fragment spectra generated provides essential information about the amino acid sequence of the peptide and the protein source.²⁵ Because fragmentation can vary from instrument to instrument, it is important to optimize each peptide individually to determine the best balance of signal-to-noise ratio, transition time, and collision energy.^{28,29}

At least one, preferably three, signature peptides, should be selected per protein. These peptides should not interfere with other peptides or matrix compounds.^{25,28,29} For each of these three peptides, the precursor ions need to be selected. For each precursor ion, at least two, preferably three, product ions should be selected.^{25,28} In total, per protein, at least six, preferably nine, MRM transition will be chosen. The more transitions are chosen, the more specific the MRM quantification will be for the target protein. Too many transitions, however, will lead to a loss of sensitivity.^{25,28}

If the amino acid sequence of the detected peptide is specific to the target protein, then the MRM transition is also specific. The precursor peptides chosen should not have too many charge states and should be double or at the most triple charge.^{30,31} The higher the charges are on peptides, the more transitions should be chosen to quantify precursor ion accurately.²⁶ The product ions chosen for MRM transition

time should also have a higher m/z than the precursor ion to guarantee peptide specificity.³⁰ Sealey-Voyksner et al.³⁵ reported that a longer dwell time improved the signal-to-noise ratio by two- to three-fold. In general, the larger the investigated allergen, the easier false-positive transition time and fragments can occur.²⁶ Peptides used as standards must therefore be reproducible after digestion and have very small LC retention time windows.^{26,35}

In summary, many factors have to be considered when establishing and validating a new MRM method for the absolute allergen quantification. These results can be a reliable, robust, accurate, and sensitive method for allergen quantification. Standards used in the MRM method must be well-characterized. In contrast with antibody-based methods, MRM methods for absolute allergen quantification are reproducible for different food samples and comparable between laboratories.

4.3. LOD/LOQ Identified with MRM Systems

The first study to use synthetic peptide for allergen analysis using MRM system was in 2005 by Molle and Leonil.⁵¹ However, LOD and LOQ data were not published. Using the same LC system, Monaci and van Hengel⁵³ demonstrated MRM to be almost 10 times more sensitive compared with a UV detector in an LC method for milk allergen detection. MRM can detect peptides in a low femtomol and attomolar range, equivalent to low ppm and ppb range calculated by ELISA.^{22,28,50} Heick et al.³⁰ proved that one method, using LC/qIT, was able to detect more than one allergen. Monaci et al.⁵² used milk allergen standards and demonstrated an LOD of 1 ppm using LC/qTOF. The LOD for spiked wine samples was 5 ppm, and thus Monaci et al.⁵² recommends that MRM sensitivity can be increased and warrants further investigation.

When comparing identical preparation and digestion with LC/qTOF and LC/MRM, Shefcheck et al.³³ showed that sensitivity of the latter was 10 times higher compared with LC/qTOF. Molle and Leonil⁵¹ identified and quantified by MRM total casein macropeptide variant A, variant B, and a glycosylated macropeptide in different dairy samples with good sensitivity and accuracy. These peptides originated from κ -casein with a LOQ of 10 pmol. Subsequently, in 2008, Monaci and van Hengel⁵³ analyzed milk-allergen-spiked samples, achieving an LOD and LOQ of 1 and 4 ppm, respectively. Careri et al.⁵⁵ calculated LOD and LOQ for Ara h 2 to be 5 and 13 ppm, respectively. In the same study, LOD and LOQ for Ara h 3/4 was about 1 and 3.7 ppm, respectively. Abdel Rahman et al.^{41,45} reported a crustacean tropomyosin and arginine kinase LOQ of 3 and 0.25 nM (nM), respectively. Sealey-Voyksner et al.³⁵ were able to detect trace levels of cereal allergens by LC/MRM with an LOD and LOQ of 0.01 to 0.03 ppm and 0.01 to 0.1 ppm, respectively. These studies demonstrate that MRM is comparable and can be more sensitive than allergen detection by ELISA.

Studies quantifying allergens in the past five years using MRM systems have confirmed that low LOD and LOQ can be easily achieved with reported values lower than antibody-based systems. MRM methods are easy to compare and standardize; therefore, validated MRM methods should be used for absolute allergen quantification.

5. CONCLUDING REMARKS

The prevalence of food allergies is increasing worldwide and therefore represents a growing public health concern. Govern-

ments protect allergic consumers by regulating the labeling of food products containing potential allergens. Currently more than 600 different food allergens are known, which demonstrates the variety of existing allergens and the difficulty in allergen analysis and subsequent food labeling. To date, the most common quantitative method for allergen analysis is the ELISA. However, ELISA methods have several drawbacks. Therefore, the development of new methods for the quantification of food allergens is suggested, which are robust, reliable, comparable, stable, fast, and easy to standardize. MRM fulfills all of these factors and in addition demonstrates better LOD and LOQ compared with ELISA, which makes MRM the method of choice for absolute allergen quantification. Well-characterized standards are required for all MS-based methods, which can be used as reference materials for inter- and intra-laboratory comparison. Further research is needed to define signature peptides for most of the major food allergens.

■ ASSOCIATED CONTENT

■ Supporting Information

Supplementary Table 1. Alphabetical summary of all food allergens currently analyzed with different MS systems and peptides published in the literature. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge The National Measurement Institute (NMI) for financial support and scholarship for M.K. A.L.L. is the holder of an Australian Research Council (ARC) Future Fellowship.

■ ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; MS, mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MALDI, matrix-assisted laser desorption/ionization; TOF, time of flight; m/z , mass-to-charge ratio; LC, liquid chromatography; PTMs, post-translational modifications; IUIS, International Union of Immunological Societies; MRM, multiple reaction monitoring; IT, ion trap

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Allergen (registered Allergen)	Peptides Identified (*Recommended Signature peptides)	Species/ Allergen source	LOD (LOQ)	MS system used	Ref.
Crustacean					
α -Actin	• AGFAGDDAP • AVFPSIVGRPR • DAYVGDEAQSKR • RGILTLK • IAPESPVLLTEAPLNPK • TTGIVLDTGDGVTHTVPIYEGYCLPHAILR • LDLAGRDLTAYLTK • GYSFTTTAEREIVR • SYELPDGQVITIGNER • CDIDIRK • KDLFANNVLSGGTTMYPGIADR • EITALAPPTIK • IKIIAPPER • IIAPPERK • EEYDESGPGIVHR	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF and LC/MRM	³⁸
Actin (isoforms)	• VAPEEHPVLLWEAPLNP	(<i>Pandalus</i>)		MALDI,	⁴²

	K • DITNYLGK • SYELPDGQVITISNER • GYSFTTTAER • EEYDESGPGIVHRK • SYELPDGQVITISNER • AVFPSIVGR • EGYSFTTTAER • SYELPDGQVITIGNER • EITGLAPSSIK • EITALAPSSIK	<i>borealis</i>) Crude extract		LC/qToF and LC/MRM	
Arginine Kinase (Pen m 2)	• FLQAANAC#R • GTRGEHTEAEGGIYDIS NK	(<i>Penaeus</i> <i>monodon</i>)	N.D.	MALDI and IT	41
Arginine Kinase (Lit v 2)	• FLQAANAC#R • GTRGEHTEAEGGIYDIS NK	(<i>Litopenaeus</i> <i>vannamei</i>)	N.D.	MALDI and IT	
Arginine Kinase	• FLQAANAC#R • GTRGEHTEAEGGIYDIS NK	(<i>Fenneropenaeus</i> <i>indicus</i>)	N.D.	MALDI and IT	
	• FLQAANAC#R • GTRGEHTEAEGGIYDIS	(<i>Farfantepenaeus</i> <i>notialis</i>)	N.D.	MALDI and IT	

	NK • FLQAANAC#R • GTRGEHTEAEGGIYDIS NK • FLQAANAC#R • LVDDHFLFVSGDR	<i>(Pleoticus muelleri)</i> <i>(Pandalus borealis)</i>	N.D. N.D.	MALDI and IT MALDI and IT	
Arginine Kinase (Pen m 2)	• *AVFDQLKEK • *VSSTLSSLEGELK • *TFLVWVNEEDHLR • *LEE VAGKYNLQVR	<i>(Penaeus monodon)</i>	N.D.	LC/IT	43
Arginine Kinase (Lit v 2)	• *VSSTLSSLEGELK • *TFLVWVNEEDHLR • *LEE VAGKYNLQVR	<i>(Litopenaeus vannamei)</i>	N.D.	LC/IT	
Arginine Kinase	• *LTNAVNEIEKR	<i>(Pleoticus muelleri)</i>	N.D.	LC/IT	
	• *ALFDQLKDKK	<i>(Fenneropenaeus merguensis)</i>	N.D.	LC/IT	
	• *TFLVWVNEEDHLR				
	• *LEE VAGKYNLQVR				
	• *SFLVWVNEEDQLR	<i>(Pandalus borealis)</i>	N.D.	LC/IT	
	• *TFLVWVNEEDHLR	<i>(Fenneropenaeus indicus)</i>	N.D.	LC/IT	
	• *LEE VAGKYNLQVR				

	• *VSSTLSSLEGELK • *TFLVWVNEEDHLR	(<i>Farfantepenaeus notialis</i>)	N.D.	LC/IT	
Arginine Kinase	• *LVSAVNEIEK	(<i>Chionoecetes opilio</i>) This signature peptide can be used for arginine kinase from other crustacean species	N.D.	MALDI, LC/qToF and LC/MRM	³⁸
Arginine Kinase	• AVFDQLKEK • VSSTLSSLEGELK • GTYYPLTGMSK • LIDDHFLFK • IISMQMGGDLGQVFRR • LTSAVNEIEKR • IPFSHHDR • GTRGEHTEAEGGIYDIS NK	(<i>Penaeus monodon</i>)	N.D.	MALDI and LC/qToF	⁴⁵
Arginine Kinase (isoforms)	• *QQLVDDHFLFVSGDR • SIDGFGLSPGITK • VGVENLMK • KQLVDDHFLFMSGDR	(<i>Pandalus borealis</i>) Synthetic and isotopically labeled synthetic	LOD 0.25nM	MALDI, LC/qToF and LC/MRM	⁴²

	• NLQVAGMERDWPEGR • GIFHNAEK • TFLVWVVEEDQLR • TFLVWLVEEDQLR • TFLVWTVEEDHLR • AVGDSVK • YGYVHSCPTNLGTGMR - ASVHVDLPGTWK • CEELKVQPR • KHRLGYSEVELVQCMID - GVNTLYAEDVALQK - K	signature peptide			
Glyceraldehyde-3-phosphate dehydrogenase	• GIDGFGR • HVYNEMKPENIPWSK • GAGQNIIPSSTGAAK • AGAHMKGGAK • AGAEYIVESTGVFTTIEK • AGAHMKGGAK • LTGMAFR • VPTPDVSVVDLTVR • AGIQLSK	(<i>Pandalus borealis</i>) Crude extract		MALDI, LC/qToF and LC/MRM	⁴²
Myosin light	• KGGXNVFDMFTQK	(<i>Litopenaeus</i>)	N.D.	LC/MAL	⁴⁷

chain (Lit v 3)	• SSGESDDDDVVAASIR	<i>vannamei)</i>		DI	
Myosin light chain	• EGFQLMDR	<i>(Pandalus borealis)</i>	N.D	MALDI, LC/qToF and LC/MRM	⁴²
Myosin heavy chain	• LTQEAVADLER • ELQARIEEL • LDEAGGATSAQIELNK • DEAGGATSAQIELNKKR • DLKLTQEAV • DLLRQLEEA • ELQARIEEL • ELSQVRQEI • LTQEAVADLER • QIEEAEEIAALNLAK	<i>(Pandalus borealis)</i> Crude extract	N.D	MALDI, LC/qToF and LC/MRM	⁴²
Sarcoplasmic Ca-binding (Lit v 4)	• YMYDIDDDGFLDK • NDFECLAVR • GEFSAADYANNQK • NLWNEIAELADFNKDG • DGEVTVDEFK • VFIANQFKAIDVNGDGK	<i>(Litopenaeus vannamei)</i>	N.D.	LC/IT	⁴⁸

	• AIDVNGDGK • VGLDEYR • SAFAEVKEIDDAYNK • EIDDAYDK • LTTEDDRK • KAGGLTLER • AGGLTLER • YQELYAQFISNEDEK				
Sarcoplasmic calcium binding protein	• VATVSLPR	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF and LC/MRM	³⁸
Sarcoplasmic calcium binding protein (isoforms)	• DNRVKYVVRMYDIDN • NGFLDKNDFECLAVKN TLI • DGFLDKNDFECLALRNT LI • ECRGEWSAEKYAANQK • IMSNLWNEIAELADFNK • TFUANQFK • TIDVNGDGL • TVDVNGDGL	(<i>Pandalus borealis</i>) Crude extract	N.D	MALDI, LC/qToF and LC/MRM	⁴²

	• VGVDEYRLDCITR • AGGINIARYQELYAQFIS NPDEK • CNAVYLFGPLKEVV				
SERCA/ smooth endoplasmic reticulum Ca ²⁺ ATPase	• YGPNELPAEEGK • NAESAIEALKEYEPEMG K • EIVPGDLVEISVGDKIPA DLR • IDQSILTGESVSVIK • NILFSGTNVAAGK • TQMAETEEIKTPLQKQ • VGEATETALIVLGEK • EFTLEFSR • VIVITGDNK • KAEIGIAMGSGTAVAK	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF and LC/MRM	³⁸
Tropomyosin (Pen m 1)	• *ANIQLVEK	(<i>Penaeus monodon</i>)	N.D.	LC/qToF	⁴⁵
(Pen m 1)	• AIKKKMAMKLE • LAEASQAADER	(<i>Penaeus monodon</i>)	N.D.	MALDI	

	• *SQLVENELDHAQEQLS AATHK	(<i>Chionoecetes opilio</i>)	N.D.	MALDI and LC/qToF	³³
	• *SQLVENELDHAQEQLS AATHK	(<i>Chionoecetes opilio</i>)	3nM	LC/MRM	⁴⁶
Tropomyosin (full sequence coverage)	• *SEEEVFGQLK	(<i>Pandalus borealis</i>) Synthetic and isotopically labeled synthetic peptide	LOD 0.25nM	MALDI, LC/qToF and LC/MRM	⁴²
Troponin C	• KGFMTPER • AAEFNFR	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF and LC/MRM	³⁸
Troponin C	• DYEINELNIQVNDLR • DKKKLFEGGW • FLIEEDEEALKTEL • DEEALKTEL • GLDPEALTGKHPPK	(<i>Pandalus borealis</i>) Crude extract	N.D	MALDI, LC/qToF and LC/MRM	⁴²
SERCA/smooth endoplasmic	• YGPNELPAEEGK • NAESAIEALKEYEPEMG	(<i>Chionoecetes opilio</i>)	N.D.	MALDI, LC/qToF	³⁸

reticulum Ca ²⁺ ATPase	K - EIVPGDLVEISVGDKIPA - DLR - IDQSILTGESVSVIK - NILFSGTNVAAGK - TQMAETEEIKTPLQQK - VGEATETALIVLGEK - EFTLEFSR; VIVITGDNK - KAEIGIAMGSGTAVAK			and LC/MRM	
Egg					
Lysozyme C (Gal d 4)	- NTDGSTDYGILQINSR - FESNFNTQATNR	Detected in wine from species <i>Gallus gallus</i>	N.D.	LC/qTOF	²⁰
Ovalbumin	- HIATNAVLFFGR - YPILPEYLQCVK - DILNQITKPNDVYSFSLA - SR - ELINSWVESQTNGIIR	Synthetic standards	0.58ppm in standar d; 42ppm in spiked bread	LC/IT	^{23, 31}
Ovalbumin	ISQAVHAAHAEINEAGR	Detected in whole	N.D.	LC/qTOF	⁵⁰

(Gal d 2)		egg, egg biscuit and mayonnaise from species <i>Gallus gallus</i>			
Ovalbumin (Gal d 2)	• HIATNAVLFGR • LTEWTSSNVMEER • GGLEPINFQTAADQAR • VASMASEK • AFKDEDTQAMPFR • ISQAVHAAHAEINEAGR • ELYRGGLEPINFQTAAD QAR • VTEQESKPVQMMYQIG LFR • NVLQPSSVDSQTAMVL VNAIVFK • LYAEER • ILELPFASGTMSMLVLLP DEVSGLEQLESIINFE K • DILNQITKPNDVYSFSLA SR	Detected In wine from species <i>Gallus gallus</i>	0.1ppm	LC/qTOF	²⁰

Ovalbumin (Gal d 2)	• *EDTQAMPFRV	Ovalbumin, egg white and whole egg, Raw pasta incurred with 1000 ppm whole egg	N.D.	LC/qTOF	⁴⁹
Ovotransferrin (Gal d 3)	• GAIEWEGIESGSVEQAV AK	Detected in whole egg and mayonnaise from species <i>Gallus gallus</i>	N.D.	LC/qTOF	⁵¹
	• GTEFTVNDLQGK • TDERPASYFAVAVAR • GAIEWEGIESGSVEQAV AK • KGTEFTVNDLQGK • DQLTPSPR • AQSDFGVDTK	Detected In wine from species <i>Gallus gallus</i>	N.D.	LC/qTOF	²⁰
Serum albumin precursor (Gal d 5)	• MPQVPTDLLLETGKK	Detected in mayonnaise <i>Gallus gallus</i>	N.D.	LC/qTOF	⁵¹
Vitellogenin II	• SAVSASGTTETL	Detected in whole egg, egg biscuit	N.D.	LC/qTOF	⁵¹

		<i>carolinensis)</i>			
• *SGFIEEDELK		<i>(Xenopus laevis)</i>	N.D.	LC/IT	
• *SGFIEEEEELK					
• *LFLQNFSAGAR					
• *LFLQNFSASAR					
• *IGVEEFQALVK					
• *SGFIEEDELK		<i>(Xenopus</i>	N.D.	LC/IT	
• *SGFIEEEEELK		<i>tropicalis)</i>			
• *LFLQNFSAGAR					
• *LFLQNFSASAR					
• *SGFIEEEEELK		<i>(Rana</i>	N.D.	LC/IT	
• *IGVEEFQALVK		<i>catesbeiana)</i>			
• *SGFIEEEEELK		<i>(Cavia porcellus)</i>	N.D.	LC/IT	
• *SGFIEEEEELK		<i>(Triakis</i>	N.D.	LC/IT	
		<i>semifasciata)</i>			
• *LFLQNFSAGAR		<i>(Latimeria</i>	N.D.	LC/IT	
• *SGYIEEEEELK		<i>chalumnae)</i>			
• *IGVEEFQALVK		<i>(Rana</i>	N.D.	LC/IT	
		<i>temporaria)</i>			
• *IGVEEFQALVK		<i>(Rana esculenta)</i>	N.D.	LC/IT	
• *IGVEEFQALVK		<i>(Limnonectes</i>	N.D.	LC/IT	
		<i>macrodon)</i>			

Milk					
α -S1 casein	• YLGYLEQLLR • FFVAPFPEVFGK	Synthetic standards	0.11ppm standard; 5ppm in spiked bread	LC/IT	³¹
	• *YLGYLEQLLR • *FFVAPFPEVFGK	Detected in white wine	1ppm	LC/qTOF	⁵³
	• YLGYLEQLLR • HQGLPQEVLENLLR • FFVAPFPEVFGKEK	Detected in wine from species <i>Bos taurus</i>	0.06ppm	LC-qTOF	²⁰
	• *YLGYLEQLLR • *FFVAPFPEVFGK	Detected in chocolates, cookies, baby foods, frozen desserts	1.25ppm	LC/qTOF	¹³
	• HQGLPQEVLENLLR (¹³C¹⁵N-labeled), • YLGYLEQLLR (¹³C¹⁵N-	CFSAN cookies FAPAS Biscuits;	CFSAN cookies 0.4 and	LC/qIT	⁷⁰

	labeled) FFVAPFPEVFGK (¹³C¹⁵N-labeled)		06fmol (LOQ: 1.3 fmol) FAPAS Biscuits ; LOD 0.5 and 0.7fmol (LOQ: 6fmol)		
α -S2 casein	- NAVPITPTLNR - FALPQYLK	Synthetic standards	0.11ppm standard; 5ppm in spiked bread	LC/IT	³¹
	*YIPIQYVLSR *NAVPITPTLNR	Detected in white wine	1ppm	LC/qTOF	⁵³
β -casein	*DMPIQAFLLYQEPVLG PVR	Detected in white wine	1ppm	LC/qTOF	⁵³

	• *GPFPIIV				
	• DMPIQAFLLYQEPVLGP VR • VLPVPQK • AVYPYQR	Detected in wine from species <i>Bos taurus</i>	0.06ppm	LC-qTOF	²⁰
κ -casein	• *SPAQLQWQVLSNTVP AK	Detected in white wine	1ppm	LC/qTOF	⁵³
	• VQVTSTAV	Synthetic peptide	N.D.	LC/UV and LC/MRM	⁵²
α -lactalbumin	• m/z = 1419.7 • m/z = 1577.3	Spiked and detected in mixed-fruit juices	1ppm (LOQ: 4ppm)	LC/MRM	⁵⁴
α -lactalbumin	• *VGINYWLAHK • KILDKVGINNYWLAHK ALCSE	Synthetic standard spiked in infant formula	(LOQ: 100ppm)	LC/MRM	⁵⁵
α -lactoglobulin	• m/z = 1082.2 • m/z = 1149.7 • m/z = 1226.2 • m/z = 1313.6	Spiked and detected in mixed-fruit juices	1ppm (LOQ: 4ppm)	LC/MRM	⁵⁴
β -lactoglobulin	• m/z = 1077.1 • m/z = 1144.4	Spiked and detected in	1ppm (LOQ:	LC/MRM	⁵⁴

	• m/z = 1220.5 • m/z = 1307.5	mixed-fruit juices	4ppm)		
Peanut					
Cupin: Vicillin-type, 7S globulin (Ara h 1)	• *VLLEENAGGEGEER • *DLAFPGSEQVEK	Raw, mild roasted and strong roasted peanut (<i>Arachis hypogaea</i>)	7-40ng/protein	LC/qTOF	⁵⁸
	• DLAFPGSGEQVEK • GTGNLELVAVR	Synthetic standards	0.2ppm standard; 11ppm in spiked bread	LC/qIT	³¹
	• *SFNLDEGHALR • *NNPFYFPSR • *IFLAGDKDNVIDQIEK • *NTLEAAFNAEFNEIR	Spiked in ice cream	10ppm	LC/qTOF	¹⁹
	• *VLLEENAGGEGEER • *DLAFPGSEQVEK	Spiked in dark chocolate	2ppm	LC/MRM	³⁴
	• NNPFYFPSR • IFLAGDKDNVIDQIEK	Raw and roasted peanut flour	N.D.	LC/IT	⁷¹

Cupin: Vicillin-type, 7S globulin isoform 1 (Ara h 1)	• VASISATHAKSSPYQKK TENPC • ASVSATQKSPYRKT • WGPAEPE • EGEQEWGTPGSEVR • IVQIEAR • SSEMMEGVIVK • KGSEEGDOTNPINLR • EEEEEDEEEEEGSNR	Raw peanut extract (<i>Arachis hypogaea</i>)	N.D.	LC/qTOF	⁵⁹
Cupin: Vicillin-type, 7S globulin isoform 2 (Ara h 1)	• ASVSATQKSPYRKT • WGPAEPE • EGEQEWGTPGSEVR • IVQIEAR;EHVQELTK • GSEEDITNPINLR		N.D.	LC/qTOF	⁵⁹
Conglutin (2S albumin) (Ara h 2)	• *CCNELNEFENNQR • *CMCEALQQIMENQSDR	In rice crispy and chocolate based snacks	5ppm (LOQ: 14ppm)	LC/qTOF and LC/MRM	⁵⁶
	• *RQQWELQGDR	Raw, mild roasted and strong roasted peanut (<i>Arachis hypogaea</i>)	7-40ng/protein	LC/qTOF	⁵⁸
	• CCNELNEFENNQR	Raw and roasted	N.D.	LC/IT	⁷¹

	• APQRCDELVESGGR • CMCEALQQIMENQSDR • CDLELVESGGR	peanut flour			
Conglutin (2S albumin) isoform 1 (Ara h 2)	• DLELVESGGRD	Raw peanut extract (<i>Arachis hypogaea</i>)	N.D.	LC/qTOF	⁵⁹
Conglutin (2S albumin) isoform 2 (Ara h 2)	• DEDSYERDPY • HPSQDPD	Raw peanut extract (<i>Arachis hypogaea</i>)	N.D.	LC/qTOF	
Cupin: Legumin-type, 11S globulin, Glycinin (Ara h 3/4)	• *AHYQVVDSNGDT • *SPDIYNPQAGSLK	In rice crispy and chocolate based snacks	1ppm (LOQ: 3.7ppm)	LC/qTOF and LC/MRM	⁵⁶
	• AHYQVVDSNGDR • SPDIYNPQAGSLK		3ppm (LOQ: 10ppm)	LC/IT	⁵⁷
	• *AHYQVVDSNGDT • *SPDIYNPQAGSLK • *AQSENYEYLAFK	Raw, mild roasted and strong roasted peanut (<i>Arachis hypogaea</i>)	7-40ng/protein	LC/qTOF	⁵⁸

	• RPFYSNAPQEIFIQQGR • WLGLSAEYGNLYR	Synthetic standards	0.2ppm standard; 11ppm in spiked bread	LC/IT	³¹
Cupin: Legumin-type, 11S globulin, Glycinin Isoform 1 (Ara h 3/4)	• GETESEEGAIIVTVR	Raw peanut extract (<i>Arachis hypogaea</i>)	N.D.	LC/qTOF	⁵⁹
Cupin: Legumin-type, 11S globulin, Glycinin Isoform 4 (Ara h 3/4)	• FQGQDQSQQQDQSHQK • AHVQVVDSDNGDR	Raw peanut extract (<i>Arachis hypogaea</i>)	N.D.	LC/qTOF	⁵⁹
	• SQSENFYVAFK • SPDIYNPQAGSLKTAND LNLLILR	Raw and roasted peanut flour	N.D.	LC/IT	⁷¹

Soy					
α -chain of the conglycinin (Gly m 5)	• *(R)QQQEEQPLEVRK(Y)	(<i>Glycine max</i>)	N.D.	MALDI/q TOF	⁶¹
β -conglcinin α - subunit (Gly m 5)	• *LITLAIPVKNPGR	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
G1 glycinin (Gly m 6)	• *(R)VFDGELQEGR(V)	(<i>Glycine max</i>)	N.D.	MALDI/q TOF	⁶¹
G1 glycinin (Gly m 6)	• *VLIVPQN FVVAAR • LSAEFGSLR	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
G2 glycinin (Gly m 6)	• *NLQGENEEEDSGAIVT VK • LSAQYGSLR	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
G3 glycinin (Gly m 6)	• *FYLAGNQEQEFLQYQP QK • LSAQFGSLR	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
G4 glycinin (Gly m 6)	• *VESEGGLIQTWNSQHP ELK	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
Glycinin	• *NGLHLPSYSPYPR	20 varieties of	N.D.	LC/IT and	⁴⁰

precursor (Gly m 6)		soybean (<i>Glycine max</i>)		LC/MRM	
Glycinin	- NLQGENEGEDKGAIQTVK - VFDGELQEGR - SQSDNFEYVSFK - EAFGVNMQIVR	Synthetic standards	0.24ppm in spiked bread	LC/IT	³¹
Gly m Bd 28K	DGPLEFFGFSTSR	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
Gly m Bd 30K	EESETLVSR	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
Kunitz trypsin inhibitor 1	*DTVDGWFNIER	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
Kunitz trypsin inhibitor 2	*FIAEGHPLSLK *VSDDEFNNYK	20 varieties of soybean (<i>Glycine max</i>)	N.D.	LC/IT and LC/MRM	⁴⁰
Profilin	YMVIQGEPGAVIR	In soymilk and	N.D.	LC/qTOF	⁶⁰

(Gly m 3)	• KPGGGVTVK • GPGGGVTVKK	soybean meal			
Tree Nut					
2S albumin (six different isoforms)	• QDCPEQMQR • EQMQRQQMLSHCR • QQMLSHCR • MYMRQMMK • CEGLRMMMR • MMRMMQKQ • MQQEEMQPR • LAENIPSR • CNLSPQR • MMMRMAENLPSR	Brazil Nut <i>(Bertholletia excels)</i> purified extract	N.D.	MALDI	⁶²
2S sulfur-rich seed storage albumin (Ber e 1)	• QEECREQMQR • EQMQRQQMLSHCR • GRSEQQCR • QQMLSHCR • QQQLNHCR • GEEPHLDECCEQLER • MQQEEMQPR • QREEMELQGEQMQR • KAENLLSR	Brazil Nut <i>(Bertholletia excels)</i> purified extract	N.D.	MALDI	⁶²

	• LAENIPSR • MAENLPSR • LAENIPSRCNLSPMR • MAENLPSRCNLSPMR • CNLSPMR				
2S albumin (Jug r 1)	• DLPNECGISSQR • QCCQQLSQMDEQCQCE GLR • GEEMEEMVQSAR	Walnut Synthetic standards	10ppm standar d; 70ppm in spiked bread	LC/IT	³¹
11S globulin (Cor a 9)	• ADIYTEQVGR • INTVNSNTLPVLR • QGQVLTIPQNFAVAK • ALPDDVLANAFQISR	Hazelnut Synthetic standards	0.32pp m standar d; 5ppm in spiked bread	LC/IT	³¹
Prunin	• GNLDVFQPPR • GVLGAFSGCPETFEESSQ QSSQQGR	Almond Synthetic standards	0.13pp m standar	LC/IT	³¹

	• ALPDEVLANAYQISR • NGLHLPSYSNAPQLIYIV QGR		d; 3ppm in spiked bread		
Wheat					
α -amylase inhibitor	• LQCNGSQVPEAVVRDC CQQLANISEWCR • DCCQQLANISEWCRC ALYNMLDSMYK • CDALYNMLDSMYKEHG AQEGQAGTGAFPR • EHGAQEGQAGTGAFPR CR	In wheat flour (<i>Triticum aestivum</i> L.)	N.D.	MALDI- TOF/TOF	⁶³
	• SGPWMCYPGQAFQVPA LPACR • EHGAQEGQAGTGAFPR • LATIHNVR • DCCQQLAHISEWCR • QEGQAGTGAFPR	In wheat flour (<i>Triticum aestivum</i> and <i>Triticum spelta</i>)	N.D.	MALDI/T OF and LC/qTOF	⁶⁵
β -amylase inhibitor	• FFVDNGTYLTEQGR • ASLNFTCAEMR	In wheat flour (<i>Triticum</i>)	N.D.	MALDI/T OF and	⁶⁵

	• YDPTAYNTILR • SAPEELVQQVLSAGWR • DAGQYNDAPQR • SFPGIGEFICYDK • AAAAMVGHPEWEFPR	<i>aestivum</i> and <i>Triticum spelta</i>)		LC/qTOF	
CM3 α - amylase/trypsin inhibitor (Tri a 30)	• IALPVPSQPVDPR • FIALPVPSQPVDPR	18 samples analyzed belonged to <i>Triticum turgidum</i> spp durum species; two different varieties (D240, Levante and Svevo) and cultivation areas	N.D.	LC/IT	⁶⁴
β -D- Glucanexohydro lase	• TAGTTILSAIK	In wheat flour (<i>Triticum</i> <i>aestivum</i> and <i>Triticum spelta</i>)	N.D.	MALDI/T OF and LC/qTOF	⁶⁵
γ -gliadin	• NFLLQQCNHVSLSLV SIILPR	In wheat flour (<i>Triticum</i>	N.D.	MALDI- TOF/TOF	⁶³

	• TLPTMCNVYVPPDCSTI NIPYANIDAGIGGQ	<i>aestivum</i> L.)			
γ -gliadin (isoforms)	• QPQQPFP • QTQQPQQPFP • LALQTLPAMC • YIPPHCSTTI	In wheat grain	N.D	LC/qTOF	⁶⁶
LMW glutenin	• VFLQQQCSPVAMPQSLA R • SQMLQQSSCHVMQQQC CQQLPQIPQQSR • TLPTMCNVNVSLYR • VPFGVGTGVGGY	In wheat flour (<i>Triticum aestivum</i> L.)	N.D.	MALDI- TOF/TOF	⁶³
Peroxidase 1	• GAVVSCADILALAAR • DSVVVS GGPDYR	In wheat flour (<i>Triticum aestivum</i> and <i>Triticum spelta</i>)	N.D.	MALDI/T OF and LC/qTOF	⁶⁵
Profilin (Tri a12)	• YMVIQGE PGVVIR • YMVIQGE PGVVIR • DFEEPGHLAPTGLFLGG TK	In wheat flour (<i>Triticum aestivum</i> and <i>Triticum spelta</i>)	N.D.	MALDI/T OD and LC/qTOF	⁶⁵
Serpin (Tri a 33)	• LSIAHQTR • VAFANGVFVDASLQLK	In wheat flour (<i>Triticum</i>	N.D.	MALDI- TOF/TOF	⁶³

	PSFQELAVCK - QFSMYILLPEAPGGLSN LAEK - VTTGLIK - LSAEPEFLEQHIPR - LASTISSNPK - SAASNAAFSPVSLHSAL SLLAAGAGSATR - EDTSGVVLFIGHVVNPL LSS - AAEVTTQVNSWVEKVT SGR - NILPSGSVDNTTKLVLA NALYFK - LVLANALYFK - VLKLPHYK - LSAEPDFLER - QGGDNRQFSMYILLPEA PGGLSSLAEK - CLGLQLPFSDEADFSEM VDSPMPQGLR	<i>aestivum</i> L.)			
wheat gluten	LQPQNPSQQQPQEQVPL	Synthetic	0.001–	LC/MRM	³⁶

	• TQQPQQPFPQQPQQPFP Q • VPVPQLQPQNPSQQQPQ EQVPL • RPQQPYQPQPQY • QPQQPFPQTQQPQQPFP Q • PQQSPF	standards and various different flours and different food products	0.03 ppm (LOQ: 0.01- 0.1ppm)		
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2 “**Supplementary table 1.** Alphabetical summary of all food allergens currently analyzed with
3 different MS systems and peptides published in the literature. “*” indicates recommendation use
4 as a signature peptide. Allergen name in brackets confirm the registration with the international
5 union of immunological societies (IUIS). LOD and LOQ given are provided in ppm as
6 published. {C# carbamidomethylated cysteine; N.D = Not Determined}.”

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