Received: 30 May 2023 | Revised: 4 August 2023 | Accepted: 10 August 2023

DOI: 10.1002/pmic.202200427

Proteomics
Proteomics and Systems Biology

REVIEW

Food allergen detection by mass spectrometry: From common to novel protein ingredients

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Abstract

Food allergens are molecules, mainly proteins, that trigger immune responses in susceptible individuals upon consumption even when they would otherwise be harmless. Symptoms of a food allergy can range from mild to acute; this last effect is a severe and potentially life-threatening reaction. The European Union (EU) has identified 14 common food allergens, but new allergens are likely to emerge with constantly changing food habits. Mass spectrometry (MS) is a promising alternative to traditional antibody-based assays for quantifying multiple allergenic proteins in complex matrices with high sensitivity and selectivity. Here, the main allergenic proteins and the advantages and drawbacks of some MS acquisition protocols, such as multiple reaction monitoring (MRM) and data-dependent analysis (DDA) for identifying and quantifying common allergenic proteins in processed foodstuffs are summarized. Sections dedicated to novel foods like microalgae and insects as new sources of allergenic proteins are included, emphasizing the significance of establishing stable marker peptides and validated methods using database searches. The discussion involves the *in-silico* digestion of allergenic proteins, providing insights into their potential impact on immunogenicity. Finally, case studies focussing on microalgae highlight the value of MS as an effective analytical tool for ensuring regulatory compliance throughout the food control chain.

KEYWORDS
allergens, marker peptides, novel foods, processed foods, proteomics, tandem mass spectrometry

1 | INTRODUCTION

1.1 | Food allergy

Food allergy is an adverse immune response that occurs in susceptible individuals upon repeated exposure to certain foodstuffs. Proteins are often responsible for causing and eliciting these unwanted reactions. The primary mechanism of food allergy involves the interaction between allergenic proteins and immunoglobulin E (IgE) antibodies. Specifically, the allergenicity of an antigen protein is linked to its amino acid (aa) sequences called "*epitopes*" (Figure 1). These epitopes can be classified as (A) linear or *sequential*, characterized by a specific aa sequence, and (B) conformational or *discontinuous*, which are recognized as three-dimensional complexes by the immune system. The interaction between antigens and antibodies is non-covalent but rather relies on weak interactions such as

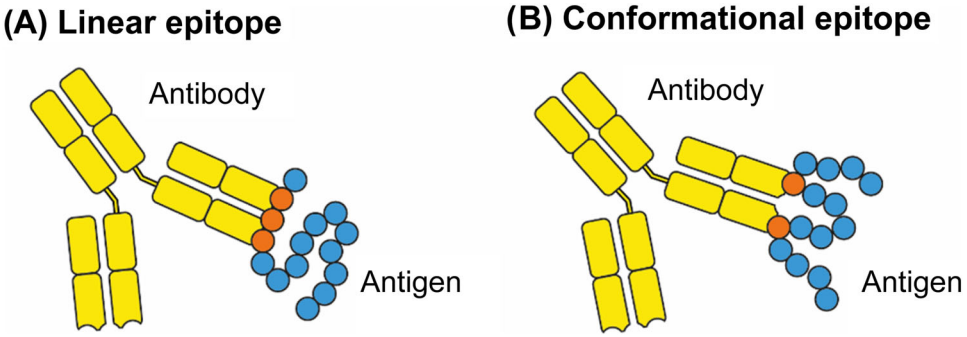

FIGURE 1 Antibody binding of linear and conformational epitopes.

hydrogen bonds, and electrostatic, van der Waals, and hydrophobic forces [1].

Food allergy follows a common disease mechanism and develops in two phases [2]. First, during *sensitization*, the antigen repeatedly interacts with the intestinal mucosa without causing any immediate consequence. Even when sensitized individuals eat low levels of an allergen, an allergic reaction may occur shortly after. Sensitization can result from dietary intake, as well as other exposure pathways such as inhalation and skin contact [3–5]. IgE-mediated food allergies cause a range of symptoms, including cutaneous ones such as urticaria and angioedema, gastrointestinal such as nausea and abdominal cramps, and cardiovascular such as hypotension and arrhythmia. In severe cases, anaphylaxis can occur as an acute reaction [6, 7].

There are various tests used to diagnose IgE-mediated food allergies, including skin prick tests (SPTs), food-specific IgE determination, and oral food challenges (OFCs). SPTs are the most commonly used diagnostic tools due to their low cost, safety, rapidity, and convenience [8]. During an SPT, a drop of each allergen extract is placed on the patient's cleaned skin, and a prick device is passed through the drop, allowing small amounts of allergen to penetrate just below the epidermis. If the patient is sensitized to the allergen, a wheal will be formed [8]. In a food-specific IgE test, the patient's serum is incubated on a device containing immobilized allergens. Specific serum IgE binds to the antigens, which are then identified by a fluorescently labelled anti-IgE. Both tests have a selectivity above 85% but there is a possibility of some cross-reaction with related proteins [9].

The gold standard for diagnosing food allergies is the OFC test [10], which follows a careful route where the amount of the suspected food allergen is gradually increased. It is administered in an open or blind placebo-controlled manner until the patient exhibits symptoms, or the test produces a negative result. Due to the potential for severe reactions, the OFC test must be conducted in a controlled environment with access to appropriate medical equipment and trained personnel [11, 12]. Currently, there is no specific cure for food allergies, and avoiding allergenic ingredients is the only viable solution for those affected [13]. Therefore, it is crucial to identify common and novel food allergenic proteins in complex and processed foodstuffs to protect the health of sensitized persons.

1.2 | Analytical methods for food allergen detection

Analytical methods employed for identifying and quantifying proteins are classified as direct or indirect ones. Direct methods detect proteins, while indirect methods detect the DNA sequence that encodes the protein. Direct immunochemical methods include radioallergosorbent test (RAST), enzyme-linked allergosorbent test (EAST), enzyme-linked immunosorbent assay (ELISA), and a combination of gel electrophoresis and immunoblotting. All these methods have some limitations, such as generating false-positive results due to complex food matrices or interferences during food processing [14–16]. Indirect methods based on polymerase-chain-reaction (PCR) involve several steps, such as DNA extraction and purification, amplification, and detection. Real-time PCR, which employs a probe for the spectrofluorometric quantification of DNA, is usually used for quantitative determinations [17, 18]. This method is selective and sensitive, less prone to cross-reactivity phenomena, and offers rapid performance. Moreover, it comes at a moderate cost per analysis and demonstrates resistance to high-temperature treatment of foods [19] and conversion factors are needed to define the allergenic protein content since PCR quantifies DNA fragments [20]. Additionally, the food matrix composition plays a key role, and some metabolites, such as fats and sugar, can hinder the correct classification [21, 22].

Recently, highly selective analytical methods based on mass spectrometry (MS) have been developed to overcome these limitations in food allergen analysis. MS enables clear-cut identification of allergens by detecting the aa sequence, thus avoiding false positive results and complications encountered in secondary/tertiary structure variations due to heat treatment. The direct detection of allergens eliminates the need for conversion factors in quantification, streamlining the process. However, the initial step of extracting proteins from complex food matrices remains a critical challenge. The efficiency, reproducibility, and quality of allergen extracts depend on various factors, such as foodstuff, composition, and consistency [23, 24]. It is necessary therefore to account for matrix effects and optimize the extraction process on a case-by-case methodology. Different protocols may be required for solid versus liquid matrices or matrices with high-fat or protein contents; this topic has been thoroughly addressed elsewhere [25, 26]. Moreover, MS coupled with liquid chromatography (LC) enables

the analysis of complex matrices, facilitating the identification and quantification of allergens even in trace amounts [27] within a single chromatographic run, even though MS analysis necessitates expensive instrumentation and specialized personnel. More detailed information regarding MS strategies, specific methodologies and approaches employed for the identification and quantification of conventional and novel allergens in a wide series of matrices, are reported in section 3.

2 | FOOD ALLERGENS

Ninety per cent of food allergies stem from eight main common foodstuffs, including milk, eggs, peanuts, crustaceans, soybean, nuts, fish, and wheat [21, 28]. In 2011, the allergenic list was expanded to fourteen by the European Union (EU), with the addition of molluscs, celery, mustard, sesame, lupin, and sulfur dioxide [29]. Table 1 lists the main allergy-associated proteins and additional allergenic ingredients. Each protein is identified by an allergen name that consists of three letters from the genus, one letter from the species epithet, and an Arabic numeral revealing the chronological order of protein isolation from the same source. For example, Ara h1 is the first isolated seed storage protein from *Arachis hypogaea* (peanuts). The allergen naming system was adopted by the World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) [30], which revise names when new information or further details become available.

Cow's milk allergy is caused by several proteins found in both milk fractions, caseins, and whey, mainly consisting of lactoglobulins and albumins [31]. The main egg allergens have been found in egg white, such as ovomucoid, ovalbumin, and ovotransferrin [32], with fewer allergens present in egg yolk [33]. Peanuts, as well as tree nuts such as almonds, hazelnuts, cashews, Brazil nuts, and pistachios, contain numerous allergenic proteins like cupins, 2S albumins, profilin, oleosins, and defensins [34–36]. Soybean allergenic proteins include glycines, β-conglycines, 2S albumin, and profilin [37], while tropomyosin, which has highly conservative primary structures [38], is the most allergenic protein in crustaceans, followed by troponin C, arginine kinase, and myosin.

Fish allergies are mainly caused by parvalbumins, which are calcium-binding proteins present in two isoforms: α-parvalbumins, which are non-allergenic, and β-parvalbumins, which are IgE-reactive [39]. Different fish species, such as Atlantic cod, yellowfin tuna, and Atlantic salmon have various forms of parvalbumins [40]. Despite minimal primary structure similarity, the secondary and tertiary structures of these proteins are highly conservative and contain antigenic regions that can cause cross-reactions in individuals allergic to other fish species [41]. Parvalbumins are also highly stable, making processed foods risky for susceptible individuals. Enolase and aldolase, two enzymes involved in metabolic glycolysis, are other proteins found in fish [40].

Wheat allergies can either be non-IgE-mediated, as in coeliac disease, or IgE-mediated and involve various proteins, such as α-purothionin, lipid transfer proteins, and gliadins [42, 43]. Like crustaceans, molluscs' major allergenic proteins are tropomyosins. In addition, arginine kinase has been identified as an important allergen for molluscs such as octopus [44]. Celery allergenic proteins can pose a severe health risk to people who are cross-reactive to birch pollen and lipid transfer proteins [45]. Proteins of the cupin family, including 11S globulin, 7S vicilin, and 2S albumin and globulins, are the most abundant allergens in mustard [46], sesame [47], and lupin seeds [48, 49].

3 | IDENTIFICATION OF FOOD ALLERGENS BY MASS SPECTROMETRY

A widely used technique in proteomics studies for characterizing food allergens is MS, which can be used to identify proteins and other compounds not only in conventional food products but also in novel food sources. In the latter case, MS-related data can be helpful to determine whether proteins have the potential to cause allergic reactions in humans. By analysing the aa sequences of proteins in novel foods, like insect and microalgae flours, the similarity to known allergens can be established, thus predicting their potential for cross-reactivity.

MS techniques are advantageous in identifying and quantifying allergens in complex and processed foodstuffs through two main proteomics approaches: top-down and bottom-up. The top-down strategy involves the analysis of intact proteins by MS and tandem MS, providing information on isoforms and post-translational modifications (PTMs), and enabling the reconstruction of the primary structure by database searching. In general, PTMs can be obtained through biological processes mediated by enzymes (e.g., glycosylation, phosphorylation, acetylation, etc.) or by food processing (e.g., deamidation or glycation). The definition of PTMs related to allergenic proteins is carried out using both top-down and bottom-up approaches [50, 51], as proteins with specific PTMs have been associated with increased allergenic possibility. However, the true impact on allergenicity is still unknown [52].

Commonly used for intact protein characterization and identification of PTM modifications is matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF)/TOF MS [53, 54]. When LC is exploited for intact protein separation and is then coupled to MS via electrospray ionization (ESI), a more complex data interpretation is demanded. Indeed, multi-charged spectrum are typically obtained from a single protein when subjected to ESI making identification difficult. Moreover, the relative abundance of peak signals related to specific charge states is usually influenced by solution pH and may thus change when passing from the standard solution to a real sample. This can also complicate label-free quantification if a specific peak signal in the multi-charge spectrum is adopted to evaluate the MS response. The likely lack of chromatographic resolution between two or more proteins may also lead to the overlap of the respective multi-charge spectra, thus interpreting the peak signals related to a specific protein is rather challenging. The complexity becomes even greater if high-resolution/accuracy (HR) MS, usually facilitating the recognition of a specific charge state in a multi-charge spectrum, is not available. Although constant improvements in terms of intact protein chromatographic separation and HRMS have extended the application

TABLE 1 Common allergenic proteins declared by the EU.

Allergenic ingredients and examples of sources	Allergenic proteins	Allergen name[a]
Milk		
-*Bos domesticus*	αS1-casein	Bos d 9
	αS2-casein	Bos d 10
	β-casein	Bos d 11
	κ-casein	Bos d 12
	α-lactalbumin	Bos d 4
	β-lactoglobulin	Bos d 5
	serum albumin	Bos d 6
Eggs		
-*Gallus domesticus*	Ovomucoid	Gal d 1
	Ovalbumin	Gal d 2
	Ovotransferrin	Gal d 3
	Lysozyme	Gal d 4
	Serum Albumin	Gal d 5
	Vitellogenin	Gal d 6
Crustaceans		
-*Crangon*	Tropomyosin	Cra c 1, Pen a 1, Pen m 1, …[b]
-*Homarus americanus*	Arginine kinase	Cra c 2, Lit v 2, Pen m 2, …[b]
-*Litopenaeus vannamei*	Troponin C	Cra c 6, Hom a 6, Pen m 6, …[b]
-*Penaeus aztecus, Penaeus monodon*	Myosin light chain	Cra c 5, Lit v 3, Pen m 3, …[b]
Fish		
-*Gadus morhua*	Parvalbumin beta	Gad m 1, Pan h 1, Sal s 1, Thu a 1, …[b]
-*Oreochromis mossambicus*	Beta enolase	Gad m 2, Pan h 2, Thu a 2, …[b]
-*Pangasianodon hypophthalmus*	Aldolase	Gad m 3, Pan h 3, Sal s 3, Thu a 3, …[b]
-*Salmo Salar*	Tropomyosin	Ore m 4, Pan h 4, Sal s 4, …[b]
-*Thunnus albacares*	Triosephosphate Isomerase	Pan h 8, Sal s 8, …[b] [b]
Mollusks		
Crassostrea gigas, Octopus	Tropomyosin	Cra g 1, Sac g 1, Tod p 1, …[b]
-*Saccostrea glomerata, Todarodes pacificus*	Arginine kinase	Cra a 2, Oct f 2, Oct v 2
Peanuts		
- *Arachis hypogaea*	7S Vicilin	Ara h 1
	2S Albumin	Ara h 2, Ara h 6, Ara h 7
	11S Globulin	Ara h 3
	Profilin	Ara h 5
	Lipid Transfer Proteins	Ara h 9, Ara h 16, Ara h 17
	Oleosin	Ara h 10, Ara h 11, Ara h 14, Ara h 15
	Defensin	Ara h 12, Ara h 13
Soybean		
-*Glycine max*	Profilin	Gly m 3
	Beta-conglycinin	Gly m 5
	Glycinin	Gly m 6
	Seed biotinylated protein	Gly m 7
	2S albumin	Gly m 8

(Continues)

TABLE 1 (Continued)

Allergenic ingredients and examples of sources	Allergenic proteins	Allergen name[a]
Nuts		
-*Anacardium occidentale*	Profilin	Cor a 2, Jug r 7, …[b]
-*Corylus avellana*	11S Globulin	Ana o 2, Cor a 9, Jug r 4, Pis v 2, …[b]
-*Juglans regia*	7S Vicilin	Ana o 1, Cor a 11, Jug r 2, Pis v 3, …[b]
-*Pistacia vera*	2S Albumin	Ana o 3, Cor a 14, Jug r 1, Pis v 1, …[b]
Wheat		
-*Triticum aestivum*	Alpha purothionin	Tri a 37
	Lipid transfer protein	Tri a 14
	Thioredoxin	Tri a 25
	Gliadins	Tri a 19, Tri a 20
Celery		
-*Apium graveolens*	Bet v 1-like	Api g 1
	Lipid Transfer Protein	Api g 2, Api g 6
	Profilin	Api g 4
	Flavoprotein	Api g 5
Mustard		
-*Brassica juncea*	2S Albumin	Bra j 1, Bra r 1, Sin a 1
-*Brassica rapa*	11S Globulin	Sin a 2
-*Sinapis alba*	Lipid Transfer Protein	Sin a 3
	Profilin	Sin a 4
Sesame		
-*Sesamum indicum*	2S Albumin	Ses i 1, Ses i 2
	7S Vicilin	Ses i 3
	Oleosin	Ses i 4, Ses i 5
	11S Globulin	Ses i 6, Ses i 7
Lupin		
-*Lupinus albus*	Conglutin alpha	Lup a alpha_Conglutin[c], Lup an alpha_Conglutin[c]
-*Lupinus angustifolius*	Conglutin beta	Lup a 1, Lup an 1
	Conglutin gamma	Lup a gamma_Conglutin[c], Lup an gamma_Conglutin[c]
	Conglutin delta	Lup a delta_Conglutin[c], Lup an delta_Conglutin[c]

[a]The allergen name contains three letters from the genus, one from the species epithet, and a number indicating the chronological order of its isolation [30].
[b]This allergenic protein has also been detected in other sources.
[c]Allergen names that do not follow the nomenclature system established by the WHO/IUIS Allergen Nomenclature Sub-Committee.

of top-down proteins in the last 10 years, especially in clinical research [55], the discussed constraints, along with the limited availability of commercial protein standards, still make the bottom-up approach the preferred one in the identification and quantification of food allergenic proteins [23].

3.1 | The bottom-up approach and marker peptides for allergenic proteins

The bottom-up approach involves the enzymatic digestion of the protein extract to produce identifiable peptides for each protein. Trypsin is commonly used due to its high purity, efficient digestion, and ability to generate peptides within the mass range of most MS analysers; it has a high cleavage frequency on lysine (K) and arginine (R). However, other enzymes such as pepsin [56], which cleaves at the carboxyl side of phenylalanine (F), tryptophan (W), and tyrosine (Y), or chymotrypsin 57–59], which cleaves at the carboxyl side of Y, W, F, leucine (L), and methionine (M), are also used. To generate a large number of shorter peptides, thus increasing the protein sequence coverage, a combination of enzymes can be employed as well [60, 61]. Double enzymes can be useful for analysing proteins that are resistant to digestion, such as hydrophobic or membrane proteins, or when identifying low-abundance peptides [62]. However, in the field

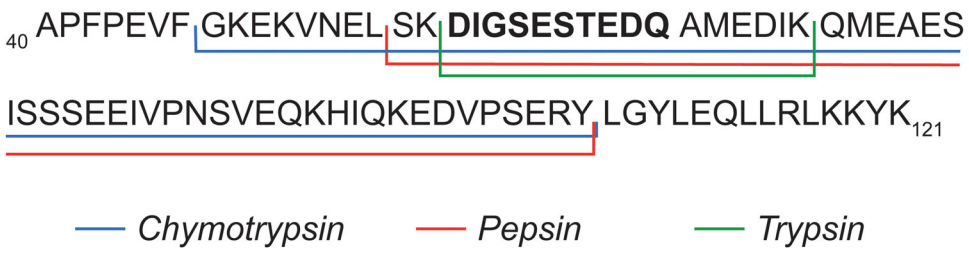

FIGURE 2 *In-silico* digestion of the αS1-casein, which is a milk-allergenic protein, using chymotrypsin, pepsin, and trypsin shows that the proteolytic enzymes are not able to remove the linear DIGSESTEDQ epitope.

of allergens, this strategy can increase the complexity of the resulting peptide mixture and make data analysis more challenging.

From an allergenic standpoint, numerous human proteolytic enzymes participate in the digestion of food, and certain allergenic epitopes can remain unaltered during this process. For example, in the case of αS1-casein, an allergenic milk protein, over three hundred linear epitopes have been identified using the freely accessible Immune Epitope Database and Analysis Resource (https://www.iedb.org/result_v3.php?cookie_id=efa362). Through *in-silico* digestion of this protein with key enzymes like trypsin, chymotrypsin, and pepsin, it becomes evident that certain linear epitopes are not affected. As an example, Figure 2 illustrates a portion of the primary structure of αS1-casein, highlighting the presence of the survey linear epitope DIGSESTEDQ within peptides *in-silico* generated by chymotrypsin (blue), pepsin (red), and trypsin (green) action. Therefore, the employment of multiple enzymes may pose analytical challenges in the discovery and characterization of allergenic proteins in food, while it could potentially serve as a strategy to modulate food allergenicity in the future [63]. However, further research is necessary to assess the actual in vivo reactivity of conserved epitopes.

The complexity of protein artificial digests can be remarkably simplified by performing a preliminary separation of intact proteins using mono- or bi-dimensional sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then proceeding to in-gel digestion. Indeed, bands referring to certain proteins can be cut and digested separately and the resulting, simpler digests, can be more easily characterized by MS and MS/MS, coupled or not with LC. Depending on the objective of the study, "targeted" or "untargeted" approaches are implemented in bottom-up proteomics for food allergens. The targeted approach is widely used because the sequences of several allergenic proteins are known and likely marker peptides can be predicted *in-silico*, thus MS and MS/MS acquisitions can be planned accordingly. The untargeted approach, often implying a data-dependent acquisition (DDA) for MS/MS data collection, is employed when limited information on the samples is available. This allows for the collection of many tandem mass spectra and the following elucidation of aa sequences by available software tools. The detection of allergenic proteins and the selection of candidate marker peptides can be thus achieved in a single chromatographic run. According to guidelines established in the literature [64–66], marker peptides (see Figure 3) should meet the following criteria:

- They must be specific and unique to the allergenic protein, evaluating potential interferences from isomeric aa such as I (iso-leucine)/L, E (glutamic acid)/Q (glutamine), E/K, Q/K, I/N (asparagine), L/N, and N/D (aspartic acid);
- They should be between 7 and 20 aa in length, as shorter peptides may not be unique and longer peptides may exhibit low ionization efficiency [64];
- They should have a reproducible enzymatic digestion pattern, without missed cleavages or post-translation modifications [65];
- They should not contain aa susceptible to modification, such as methionine (M) for oxidation, cysteine (C) for disulfide bond formation, or the N-G (glycine) motif for deamidation [66].

To identify and quantify allergens in a food sample, two types of marker peptides are needed: "qualifier" and "quantifier". Qualifier marker peptides confirm the presence of allergens, while quantifier marker peptides are employed to establish their content [67] and the protein sub-sequences including them should remain stable during processing or heat treatment of the food matrix. During LC-ESI-MS analysis, typically two qualifier and one quantifier marker peptides are monitored to detect allergenic proteins in a food matrix sample. Table 2 lists the main marker peptides reported in the literature for some major allergenic ingredients, including their exact (monoisotopic) mass values. Non-compliant marker peptides (i.e., commonly used marker peptides that do not follow the rules mentioned above) are also reported. As an example, Figure 3 shows tryptic peptides arising from cow's milk allergen Bos d 9 that might not be used as marker peptides, along with five peptides that are compliant with the previously described criteria.

3.2 | Untargeted bottom-up approach for expected/hidden allergens in foods

When an untargeted approach is adopted, the most reliable method for detecting and quantifying marker peptides of allergenic proteins is based on HRMS. HR tandem mass data, obtained through either DDA or data-independent acquisition (DIA), can be utilized as input for bioinformatics-based data processing. This approach offers the advantage of employing more stringent criteria for identifying precursor and product ions. Consequently, it enables the identification of multiple allergenic proteins through their marker peptides in extracts

TABLE 2 Proteins marker peptides for some of the major allergenic foods, along with sequences and monoisotopic masses of neutral peptides.

		Marker Peptides		Non-compliant marker peptides		
Food	**Allergenic Protein**	**Sequence**	**Exact mass**	**Sequence**	**Exact mass**	**Refs**
Milk	αS1-casein (Bos d 9)	HQGLPQEVLNENLLR	1758.9377	VNELSK[a]	688.3756	[27], [66],
		FFVAPFPEVFGK	1383.7227	EKVNELSK[b]	945.5131	[86], [87],
		YLGYLEQLLR	1266.6972	DIGSESTEDQAMEDIK[c]	1766.7516	[88], [89],
		EDVPSER	830.377	HIQKEDVPSER[b]	1336.6735	[92], [93],
		EGIHAQQK	909.4668			[172]
	αS2-casein (Bos d 10)	NAVPITPTLNR	1194.6721			[173],
		ALNEINQFYQK	1366.6881			[174],
		FALPQYLK	978.5539			[175],
	β-casein (Bos d 11)	GPFPIIV	741.4425	EMPFPK[a,c]	747.3625	[176],
		VLPVPQK	779.4905	VMPVLK[a,c]	685.4197	[177],
		AVPYPQR	829.4446	DMPIQAFLLYQEPVLGPVR[c]	2185.1605	[178],
	β-lactoglobulin (Bos d 5)	VLVLDTDYK	1064.5754	VYVEELKPTPEGDLEILLQK[b]	2312.2515	[179],
		TPEVDDEALEK	1244.5772	IPAVFK[a]	673.4163	
				LIVTQTMK[c]	932.5365	
				LSFNPTQLEEQCHI[c]	1657.777	
Egg	Ovalbumin (Gal d 2)	GGLEPINFQTAADQAR	1686.8325	LTEWTSSNVMEER[c]	1580.7141	[27], [66],
		ISQAVHAAHAEINEAGR	1772.8918	ELINSWVESQTNGIIR[c]	1857.9585	[86],[88],
		HIATNAVLFFGR	1344.7303	YPILPEYLQCVK[c]	1464.7687	[89], [92],
				DILNQITKPNDVYSFSLASR[b]	2280.175	[93],
				ELYRGGLEPINFQTAADQAR[b]	2248.1236	[173],
	Lysozyme (Gal d 4)	FESNFNTQATNR	1427.643			[174],
		NTDGSTDYGILQINSR	1752.8279			[176],
		GTDVQAWIR	1044.5352			[177]
	Vitellogenin (Gal d 6)	EALQPIHDLADEAISR	1776.9006			
		NIPFAEYPTYK	1341.6605			
		NIGELGVEK	957.5131			
		LPLSLPVGPR	1047.6441			

(Continues)

TABLE 2 (Continued)

		Marker Peptides		Non-compliant marker peptides		
Food	**Allergenic Protein**	**Sequence**	**Exact mass**	**Sequence**	**Exact mass**	**Refs**
Peanut	7S Vicilin (Ara h 1)	DLAFPGSGEQVEK	1375.662	AMVIVVVNK[c]	971.5838	[27], [66],
		VLLEENAGGEQEER	1571.7427			[86], [87],
		GTGNLELVAVR	1127.6299			[88], [89]
		SFNLDEGHALR	1257.6102			[92], [93]
		NNPFYFPSR	1140.5352			[174],
		NTLEAAFNAEFNEIR	1737.8322			[179],
	2S Albumin (Ara h 2)	GAGSSQHQER	1055.4744	CCNELNEFENNQR[c]	1611.6406	[180],
		DEDSYGR	840.325	NLPQQCGLR[c]	1027.5233	[181],
		DPYSPSPYDR	1195.5146	CDLEVESGGR[c]	1063.4604	[182],
		QQEQQFK	934.4508	CMCEALQQIMENQSDR[c]	1897.7791	
	11S Globulin (Ara h 3)	SPDIYNPQAGSLK	1388.6936	AHVQVVDSNGNR[c]	1294.6378	
		FNLAGNHEQEFLR	1573.7637	QQPEENACQFQR[c]	1476.6416	
		WLGLSAEYGNLYR	1540.7674	RPFYSNAPQEIFIQQGR[b]	2050.0385	
		TANDLNLLILR	1254.7296			
Hazelnut	11S Globulin (Cor a 9)	LNALEPTNR	1026.5458	VQVVDDNGNTVFDDELR[c]	1933.9017	[27], [66],
		INTVNSNTLPVLR	1439.8096			[87, 88]
		ADIYTEQVGR	1150.5619			[89, 93]
		ALPDDVLANAFQISR	1628.8522			
		TNDNAQISPLAGR	1355.6793			
		QGQVLTIPQNFAVAK	1612.8937			
		WLQLSAER	1001.5294			
	7S Vicilin (Cor a 11)	LLSGIENFR	1047.5713			
		AFSWEVLEAALK	1362.7184			
		ELAFNLPSR	1045.5556			

(Continues)

TABLE 2 (Continued)

Food	Allergenic Protein	Marker Peptides		Non-compliant marker peptides		Refs
		Sequence	Exact mass	Sequence	Exact mass	
Soybean	Beta-conglycinin (Gly m 5)	QQQEEQPLEVR	1382.679	TISSEDKPFNLR[b]	1405.7201	[27], [66],
		DSYNLQSGDALR	1337.6212	AIVILVINEGDANIELVGLKDSYNLHPGDAQR[a,b]	3445.8205	[87],
		ESYFVDAQPK	1182.5557	LITLAIPVNKPGR[b]	1390.866	[89], [92]
	Glycinin (Gly m 6)	VFDGELQEGR	1148.5462	LNALKPDNR[b]	1039.5774	[93],
		VLIVPQNFVVAAR	1424.8504	EAFGVNMQIVR[c]	1262.6441	[173],
		LSAEFGSLR	978.5134	NLQGENEGEDKGAIVTVK[b]	1899.9538	[174],
		SQSDNFEYVSFK	1449.6412			[176],
		ISTLNSLTLPALR	1397.8242			
		FYLAGNQEQEFLK	1585.7777			
Crustaceans	Tropomyosin	ADTLEQQNK	1045.504	SQLVENELDHAQEQLSAATHK[a]	2347.1404	[38],
		IQLLEEDLER	1256.6612			[96],
		LAEASQAADESER	1375.6216			[183],
		SLSDEER	834.3719			[184]
		FLAEEADR	949.4505			
		IVELEEELR	1128.6027			
		SLEVSEEK	919.4498			
		ANIQLVEK	913.5233			
		SEEEVFGLQK	1164.5663			
	Arginine kinase	VSSTLSSLEGELK	1348.7086	AVFDQLKEK[b]	1076.5866	
		TFLVWVNEEDHLR	1656.826	LEEVAGKYNLQVR[b]	1517.8202	
		FINVDPEGTFVISTR	1693.8675			
		VSSTLSSLEGELK	1348.7086			
		SFLVWVNEEDQLR	1633.81			
		LVSAVNEIEK	1100.6077			
	Troponin C	AAEFNFR	853.4083	KGFMTPER[b,c]	964.48	
		DYEINELNIQVNDLR	1846.9061	FLIEEDEEALKTELR[b]	1833.936	
				DEEALKTELR[b]	1202.6143	
	Myosin light chain	SSGESDDDDVVAASIR	1621.7067	KGG**X**NVFDMFTQK[b,c]	—[d]	

(Continues)

TABLE 2 (Continued)

Food	Allergenic Protein	Marker Peptides		Non-compliant marker peptides		Refs
		Sequence	Exact mass	Sequence	Exact mass	
				EGFQLMDR[c]	994.4542	
	Myosin heavy chain	LTQEAVADLER	1243.6408	DEAGGATSAQIELNKKR[b]	1786.9173	
		LDEAGGATSAQIELNK	1615.8053	KANAVNGELEESR	1415.7005	
		QIEEAEEIAALNLAK	1640.8621			
		VVSQAPAER	955.5087			
		LEELEEDVEHER	1525.6896			
		NELLAAVDSTK	1159.6084			
Lupin	Conglutin alpha	ISSVNSLTLPILR	1411.8399	VIIPPTMRPR[b,c]	1178.6958	[83],
		TLTSLDFPILR	1274.7234			[94],
	Conglutin beta	NTLEATFNTR	1165.5728	ATITIVNPDRR[b]	1254.7044	[110]
		LLGFGINADENQR	1445.7263	IVEFQSKPNTLILPK[b]	1726.0029	[185]
		NPYHFSSQR	1134.5207	DKPSQSGPFNLR[b]	1344.6786	
		AVNELTFPGSAEDIER	1746.8424			
	Conglutin delta	QQEQQLEGELEK	1457.6998	ALQQIYENQSEQCQGR[c]	1893.8639	
		QEEQLLEQELENLPR	1866.9323			
	Conglutin gamma	VGFNTNSLK	978.5134	ISGGVPSVDLIMDK[c]	1429.7487	

Note. If available, the nomenclature system proposed by the WHO/IUIS Allergen Nomenclature Sub-Committee is utilized. Non-compliant marker peptides are also reported.

[a]Too short/too long

[b]Contains missed cleavage

[c]Contains susceptible aa

[d]Due to the presence of X in the reported sequence, the exact mass cannot be computed.

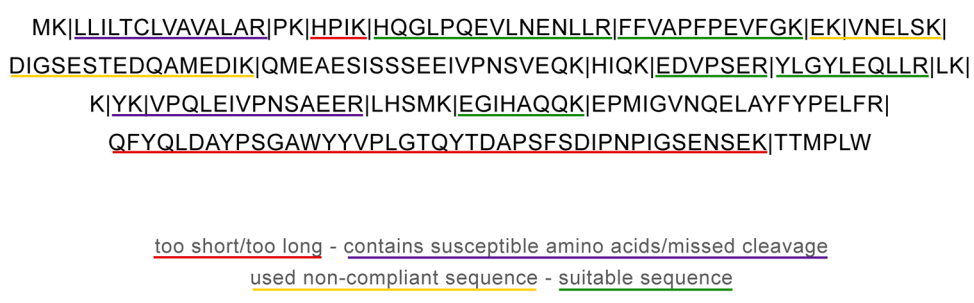

FIGURE 3 Structural criteria limiting the choice of marker peptides exemplified for the cow's milk allergen Bos d 9.

derived from complex and processed food products. Moreover, it saves time, samples, and solvents, while also providing valuable information about charge states and isotopic patterns that aid in the identification of peptides. The untargeted approach constantly needs innovative software tools and methodologies to analyse, process, and store data, creating a persistent demand in the field [68, 69]. For example, the progressive spread of DIA methods, which generate complex tandem MS spectra resulting from the convolution of various MS/MS spectra with different precursor ions poses a significant challenge for data processing [70]. To meet these requirements, there has been an ongoing development of new tools and software. However, compiling an all-encompassing list of software used in proteomics remains intriguing [71]. Among the most frequently employed ones are those developed by instrument vendors, such as Proteome Discoverer™ (ThermoFisher Scientific, https://www.thermofisher.com/order/catalog/product/OPTON-31014), ProteinPilot (Sciex, https://sciex.com/products/software/proteinpilot-software), ProteinLynx (Waters, https://www.waters.com/waters/en_US/ProteinLynx-Global-SERVER-(PLGS)/nav.htm?cid=513821), ProteoScape (Bruker, https://www.bruker.com/content/bruker/int/en/products-and-solutions/mass-spectrometry/ms-software/proteoscape.html), and MassHunter (Agilent, https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software/data-analysis). These software solutions are widely utilized due to their compatibility and integration with specific instruments, facilitating continuous data processing and analysis in proteomics research. As an example, Proteome Discoverer allows the processing of raw files (e.g., in DDA acquisition mode), facilitating the identification of aa sequences of peptides through tandem mass spectra and subsequently linking these peptides to proteins present in the input database. Indeed, it is focal to acknowledge that the landscape of software is continuously evolving, and researchers constantly explore and adopt new tools and methods to remain at the forefront of scientific advancements. Freeware software alternatives are particularly popular among proteomics studies due to their accessibility and affordability. Among these options, some notable ones include MaxQuant (https://www.maxquant.org/), OpenMS (https://openms.de/), and Skyline (https://skyline.ms/project/home/software/Skyline/begin.view). However, conducting an in-depth comparison of the capabilities and advantages of each software is beyond the scope of this review.

3.2.1 | Untargeted HR-MS by All Ion Fragmentation (AIF)

The development of highly sensitive untargeted analytical methods for detecting trace amounts of allergenic ingredients in complex food samples is an important achievement in ensuring the safety of allergic consumers. Cross-contamination that can occur in production plants poses risks of including unpredictable allergens in processed foods, often referred to as hidden allergens, which can be potentially life-threatening to individuals with allergies. Furthermore, unexpected allergenic proteins may be present in food products or beverages due to practices involving other food-related compounds. These practices aim to enhance properties such as texture, colour, and flavour. The untargeted all-ion fragmentation (AIF)-MS approach allows for the fragmentation on all ions within the scanned MS range, avoiding pre-selection of the precursor ion [72]. AIF-MS can be used for identifying allergenic proteins in complex samples, such as foreign proteins added during the clarification processes of white wines. As an example, Monaci et al. [73] addressed the issue of foreign allergenic proteins by validating a method for quantifying milk and egg proteins in white wines by combining full scan and AIF MS acquisitions. The method allowed for the simultaneous quantification of αs1-casein (Bos d 9) and β-casein (Bos d 11), and ovalbumin (Gal d 2) and lysozyme (Gal d 4) of milk and egg ingredients, respectively, with detection limits (LOD) below 1.1 mg/Kg [73]. The *m/z* values of the peptides obtained from the full MS spectra were compared with the theoretical *m/z* values of the milk and egg peptides generated in vitro using Expasy software. Three *y*-type product ions from each of those peptides were selected and searched for AIF MS spectra. The retention times of the precursor peptide and related product ions in the AIF acquisitions were compared to confirm the assignment. As an example, plot **A** of Figure 4 shows the extracted ion current (EIC) chromatogram referred to the *m/z* value (791.3640) of the peptide doubly charged ion, while the lower panel (plot **B**) shows the EIC of three of its product ions (y_8, y_9, and y_{10}). After matching the retention times, the assignment to the tryptic peptide LTEWTSSNVMEER from ovalbumin was confirmed. The full mass spectrum reported in plot **C** of the figure reveals that the ion at *m/z* 791.3640 is predominant with respect to those of co-eluting peptides. A mono-charged target peptide ion at *m/z* 1581.7190 is also present; the resulting tandem mass spectrum is shown in plot **D** of Figure 4.

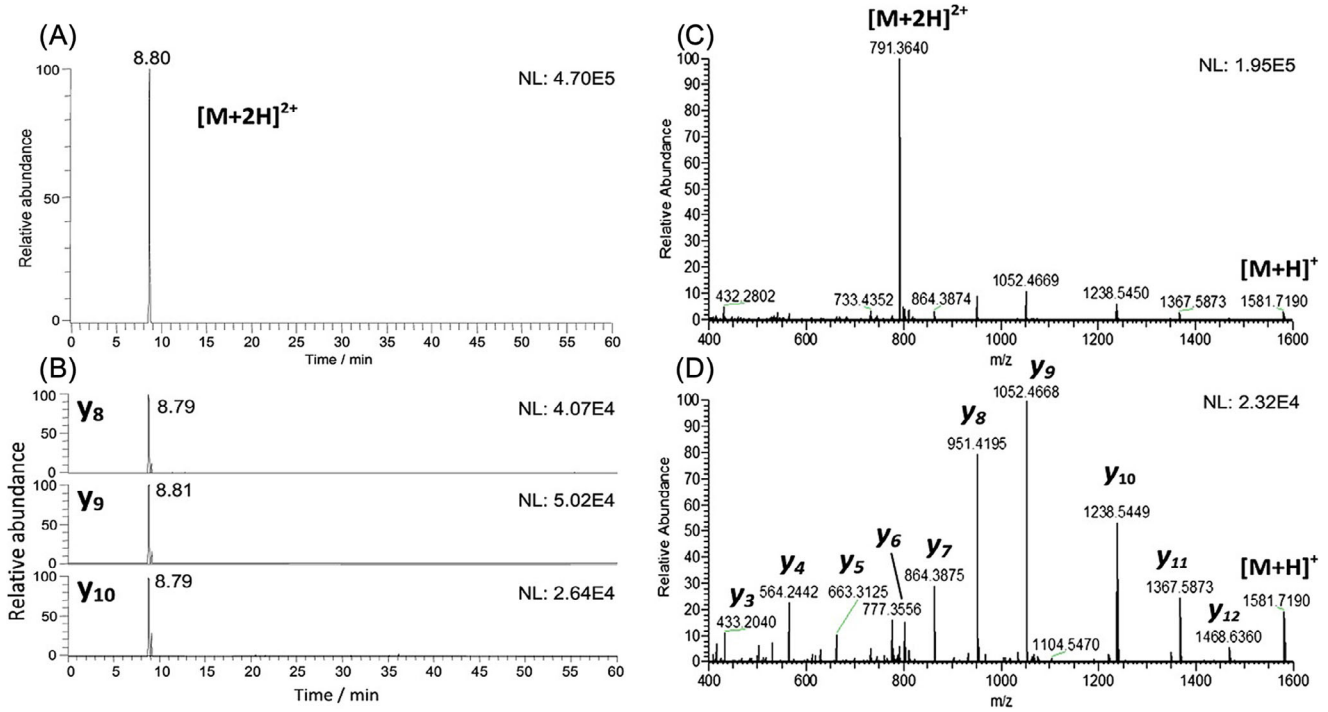

FIGURE 4 (A) Extracted ion current (EIC) chromatogram of the peptide precursor ion LTEWTSSNVMEER at a retention time of 8.8 min obtained as a tryptic digest of a standard solution of ovalbumin (10 μg/mL). (B) Tandem HCD-MS spectra of three of its generated product ions: y_8, y_9, and y_{10}. (C) Full scan MS and (D) HCD-MS/MS spectra of the doubly protonated peptide at the retention time of 8.8 min. Reproduced from [73] with permission from John Wiley & Sons Ltd.

This approach was also used to identify allergenic milk proteins in cookies [74].

3.2.2 | HRMS DIA acquisitions

The DIA approach enables the fragmentation of very low-abundance ions in a specific *m/z* interval; in particular, the range under investigation is divided into regions of variable width and MS/MS spectra are collected in each one by isolating the entire *m/z* range. This approach ensures that tandem MS spectra contain fragment ions generated from all precursors within the specific *m/z* range [75]. Unlike the DDA approach, the DIA method does not rely on precursor-fragment ion correlation. Bioinformatic approaches allow for the fragment's assignment so that by combining the MS/MS information, even low-abundance species, such as allergenic proteins, can be accurately identified in complex matrices. In a study reported by Uvackova et al. [76], DIA was employed to identify and quantify 15 isoforms of allergenic proteins belonging to amylase/trypsin inhibitors, γ-gliadins, and glutenins in nine different milled wheat grain samples. Aiming to identify and quantify allergenic proteins in wheat samples, the quantified reported content by using internal standards ranged between 1.5–6.8 mg/Kg of sample for amylase/trypsin inhibitors, 4.9-60.9 mg/Kg for gliadins, and 1.6–90.4 mg/Kg for glutenins. Moreover, the DIA approach was successfully applied to identifying and quantifying wheat amylase/trypsin inhibitor proteins in 60 different European bread wheat varieties [77].

3.2.3 | HRMS DDA acquisitions

In complex matrix analysis, where many interfering ions co-elute with the target ones, making it difficult to recognize peptide fragments, DDA mode may be a better choice. DDA provides specific information between precursor and product ions that cannot be obtained directly from the AIF or DIA scan. For example, LC-HRMS-DDA was successfully employed [78] to identify and quantify several almond, cashew, hazelnut, peanut, pistachio, and walnut allergenic proteins in various foodstuffs such as milk chocolate, vanilla ice cream, commercial bread, and breakfast cereals. The limits of detection (LODs), reported as $mg_{Tot.protein}/Kg_{matrix}$, were 0.34–1.92 for almonds, 0.78–2.02 for cashews, 0.49–1.01 for hazelnuts, 0.26–2.08 for peanuts, 0.91–1.90 for pistachios, and 0.80–5 for walnuts [78]. Once peptides and proteins were identified using an ad hoc nuts/peanuts database, marker peptides were selected based on the rules described earlier. It should be noted that some chosen peptides did not meet all the described criteria, such as the inclusion of M or C, or missed cleavages. Although these peptides were recovered with highly reproducible responses in real samples, the Authors recommend their use with caution [78].

Marker peptides of white and yolk eggs in processed foodstuffs like mayonnaise and chocolate have been validated using an HRMS untargeted approach [79]. Likewise, peanut marker peptides in fermented peanut milk, caramelized peanuts, and chocolate have been successfully investigated [80]. The identification and quantification of target peptides were achieved through two digestion processes based on either trypsin alone or in synergy with Lys-C, which cleaves at the carboxyl side of K.

In determining marker peptides and developing methods for identifying and quantifying allergenic proteins in complex foodstuffs, the concurrent use of targeted and nontargeted MS-based methods is highly effective. Monaci et al. [81] used this procedure to define specific marker peptides for peanut proteins and develop a method for their quantification in food matrices containing hazelnuts, almonds, walnuts, and pistachios. Despite the high similarity between the different nut species, the Authors were able to prove the occurrence of allergenic proteins with a limit of detection (LOD) of 4 $mg_{peanut\ proteins}/Kg_{matrix}$.

Targeted HRMS methods can quantify allergens even in the presence of matrix interference by selecting well-defined marker peptides. This is particularly important for individuals with severe food allergies, where trace amounts of allergenic proteins can trigger a potentially life-threatening reaction. Our recent work [82] provides a representative example of searching for allergenic milk proteins in meat food products that were marketed without any label indication of their possible presence or even declaring their absence. The identification and quantification of milk proteins with LODs in the range 3.8–7.1 mg_{ing}/Kg_{matrix}, depending on the examined meat matrices, were achieved using LC-HRMS and MS/MS methods. Given the significant problem of hidden allergens in food, efforts are underway to develop more selective and sensitive analytical methods to tackle it.

3.2.4 | Low resolution (LR)MS for allergens determination

While HRMS has the potential to overcome the limitations of classical methods and provide detailed structural elucidation, LR instruments like triple quadrupoles (QqQ) remain popular due to their lower cost. Indeed, multiple reaction monitoring (MRM) using LR mass analysers is the most common approach for identifying allergens with MS methods. MRM involves selecting two to three intense product ions related to a marker peptide, which improves selectivity when analysing complex matrices. Several studies have used MRM to identify multiple allergens in processed foods [64, 83–94]. Planque et al. [92, 93] detected several allergenic proteins by LC-ESI-MS/MS and identified ten allergenic ingredients in biscuits, tomato sauce, chocolate, and ice cream with different LODs. Hoffmann et al. [94] proposed a similar strategy to quantify foreign proteins from soybeans, peas, and lupins in meat products, which are commonly added to improve meat properties. After HRMS-DDA analysis, tandem mass spectra were processed to obtain a list of peptides that were then reduced according to defined selection rules for marker peptides. Three or four markers were preferred for each allergenic ingredient, and three transitions for MRM acquisition were defined for each one. The validated procedure achieved LODs of 2 mg/kg for lupin, 5 mg/kg for pea, and 4 mg/kg for soy proteins. Meat products without pre-existing soy, lupin, and pea proteins were also analysed to rule out the presence of matrix interferents. As demonstrated in Figure 5, the MRM chromatogram referred to meat products with the addition of lupin flour and pea/soy protein isolate allowed for the adoption of this method for detecting multiple allergens. These studies are very useful for detecting food fraud, where undeclared allergenic proteins can pose a serious health risk to sensitive consumers.

LC-MS methods in which marker peptides are further fragmented after MRM acquisitions (i.e., MRM^3) have been described in other studies [95–98], resulting in a better signal-to-noise ratio for the selected marker peptides. Typically, two transitions were identified for MRM^3 acquisition, excluding the most common ones, such as y_1 ions or loss of water and ammonia. This operation mode also decreases the matrix interferences on marker peptides that could occur in MRM [95].

3.2.5 | Validation of LC-MS methods for allergens quantification

Validation of LC-MS methods, for quali- and quanti-tative purposes, embraces the evaluation of several parameters such as linear range, accuracy, recovery, LOD, limit of quantification (LOQ), matrix effects, and processing effects. During food processing, changes in protein conformation or the formation of aggregates with matrix components may take place, and the matrix effect must be considered since other ingredients could mask marker peptide signals during MS analysis [99, 100–104]. To address these issues, both matrix and processing effects need to be estimated during method validation, usually by treating spiked and incurred samples, respectively. By considering these effects, it is possible to accurately quantify allergens in food products.

For quantification purposes, isotopically labelled internal standards and label-free methods can be applied [105, 106]. Isotopically labelled standard proteins/peptides are commonly used in most published works [107–109] because they can be added before the extraction and/or digestion process. However, labelled standards are expensive, and a feasible alternative is represented by label-free proteins/peptides. This last approach allows for the quantification of allergenic proteins by using external calibration curves or standard addition methods [110–112]. Accordingly, a comprehensive overview that resumes the above-discussed classical steps for the quantification of allergenic proteins in real samples is depicted in Figure 6.

Improving the sensitivity of these strategies is a key factor when dealing with cross-contamination. As mentioned earlier, complete avoidance of allergenic ingredients is the only cure for food allergies. For this reason, the EU strictly regulates food labelling and requires that allergenic ingredients must be declared on the label in a font that is distinguishable from the ingredients [29]. However, in recent years food industries have abused precautionary allergen labelling (PAL), which is often added on food labels to list potential allergenic

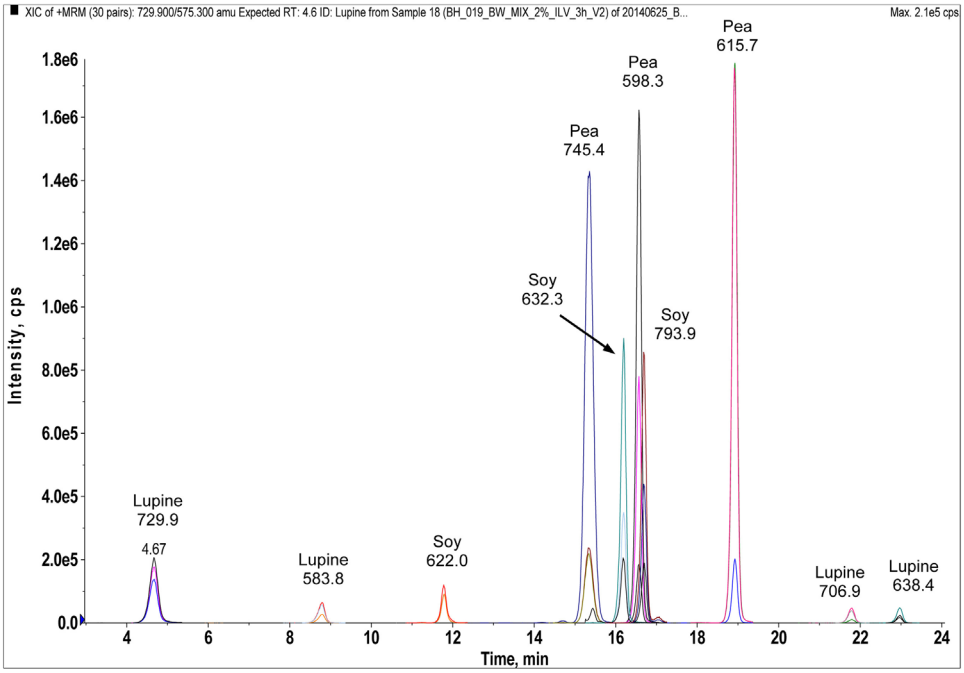

FIGURE 5 Chromatogram of the marker peptides for lupin, pea, and soy in meat products. Reproduced from [94] with permission from Elsevier B.V.

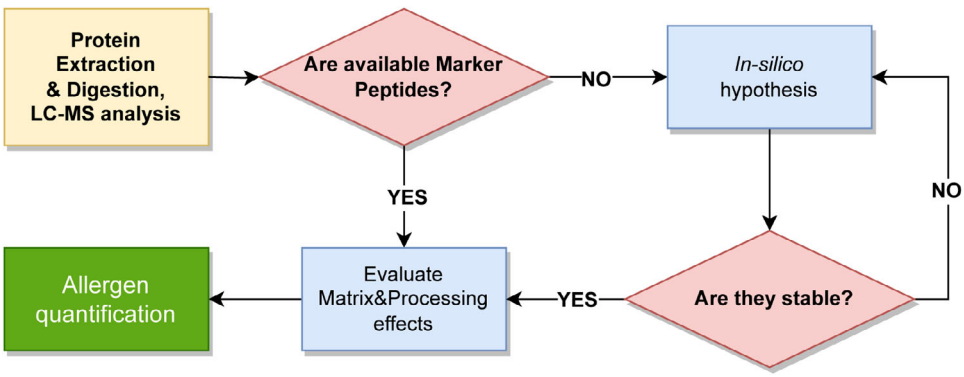

FIGURE 6 Steps commonly followed to quantify allergenic proteins in foodstuffs.

ingredients that may be present in marketed products. This is typically preceded by expressions such as "*may contain*," "*produced with shared equipment*," or "*not suitable for people with a specific allergy*" [113]. Unfortunately, this approach may confuse allergy sufferers, and in many cases, the label is ignored, posing a serious health risk to sensitised consumers [114]. Presently, there is no regulation governing the use of PAL, which is left to the discretion of food manufacturers. To address this issue, some national agencies have developed the Voluntary Incidental Trace Allergen Labelling (VITAL) program [19, 115], which establishes reference doses and action levels for each allergenic ingredient. Action levels are set as thresholds indicating the amount of an allergenic ingredient in a food product that could be dangerous to consumers. If this threshold is surpassed, the allergenic ingredient must be listed on the label. Reliable analytical methods capable of quantifying allergenic proteins from cross-contamination are highly valuable, as they help restrict the use of precautionary allergen labelling (PAL) and ease the burden for sensitive consumers when choosing food products.

3.3 | Allergenicity of novel foods

Considering the increasing global population and the resulting surge in demand for animal-based food proteins, it becomes imperative to prioritize sustainable consumption practices and mitigate the environmental impact effectively [116–118]. One way to do this is by reducing portion sizes or incorporating alternative protein sources into our diets. Novel foods such as algae, microorganisms, and insects

provide a comparable protein content to common sources like meat and have a minimal environmental impact during the production process [119, 120]. These *novel foods*, that is, *any food that was not commonly consumed in the EU before May 1997* [121], are now becoming more widespread in the market, either as stand-alone products or as ingredients in processed foods [122]. Despite the presence of numerous nutritional benefits [123–130] including high protein content, essential aa, polyunsaturated fatty acids (omega-3 and omega-6), carbohydrates, lipids, vitamins, and other bioactive compounds such as antihypertensive agents [131], it is imperative to take into account also potential side effects, such as allergenicity [132]. Food allergies can vary based on factors such as location, dietary practices, methods of food preparation, and the age at which the food was first introduced [133]. Several adverse reactions were reported from consuming novel foods, such as microalgae like spirulina [134, 135] and chlorella [136–139], as well as insects [140, 141]. In certain cases, it remains unclear how an adverse reaction may emerge following the first ingestion of a novel food. Most likely, this occurs when novel proteins share epitopes with known allergenic proteins, allowing IgE produced for known allergens to bind to new food proteins and trigger an allergic reaction. This was demonstrated by Palmer et al. [142], who found that various insect species exhibited varying degrees of cross-reactivity when exposed to the IgE of individuals sensitized to shrimp tropomyosin. The likelihood of shared epitopes and allergic reactions rises with the level of structural similarity between novel and known allergenic proteins. Identifying potential allergenic proteins in novel foods, including those that may cross-react with known allergens, is crucial. However, confirming their allergenic potential involves specific tests like SPTs, IgE tests, and the gold standard OFC, which come with challenges. To address some of these difficulties associated with traditional methods, *in-silico* evaluations have been suggested as a means to identify potentially allergenic proteins in novel foods. As outlined in Figure 7, combining MS analysis and bioinformatic processing can facilitate the explanation of *in-silico* allergenicity. A comparison of novel food proteins' aa sequences with known allergens provides a list of novel proteins with high similarity, requiring further investigation.

3.4 | *In-silico* evaluation of the potential allergenic risk

Two general rules for defining putative allergens were established by WHO and Food of Agriculture Organization (FAO) [143]. It has been agreed by several studies [144, 145] that putative allergen identification requires either (i) an identity percentage higher than 35% in an 80 aa sequence or (ii) at least 6 identical contiguous aa between a novel food protein and a known allergen. Even though these rules do not apply to conformational allergens, several software programs and dedicated databases are available for similarity comparisons, such as Allergen.org [30], AllFam [146], Allergome [147], Allermatch [148], COMPARE [149], AllergenOnline (AO) [150], Structural Database of Allergenic Proteins (SDAP) [151], Allergen Database for Food Safety [152], and AllerCatPro 2.0 [153]. Unfortunately, the effectiveness of identifying potential cross-reactive allergenic proteins in novel foods is heavily dependent on the reference database used. A brief review and commentary on the most employed database and software is provided in Table 3, with a focus on emphasizing their technical details, features and capabilities.

3.5 | Potential allergenic risk of microalgae

As previously mentioned, microalgae *Arthrospira platensis* (known as spirulina) and *Chlorella vulgaris* are recognized as valuable nutrient sources. To identify potential allergenic proteins in these microalgae, a bottom-up approach with RPLC-FTMS in DDA mode was applied [154]. A dedicated microalgae database for protein assignment was employed and a set of Matlab instructions to query the Allergome database and retrieve known allergenic proteins was developed. The phycocyanin beta subunit as a known allergen related to *A. platensis* microalga was identified and AO was used to examine all identified proteins for both microalgae. Another custom algorithm was engaged to filter the results and to obtain a list of microalgae proteins similar to known allergens with E-score values lower than 1×10^{-7}. Accordingly, five and two putative allergenic proteins of *A. platensis* and *C. vulgaris*, respectively, that match with known pistachio, fish, and crustacean allergens, were identified. The study highlighted the importance of using multiple databases for predicting putative allergenic proteins. For example, LC-MS data processed with the Allergome database identified the C-phycocyanin beta subunit, which was not identified with AO, as AO does not contain this protein.

As discussed before, marker peptides are necessary for the identification and quantification of potential allergens, especially for novel foods. Thus, two qualifiers (ETYLALGTPGSSVAVGVGK and YVTYAVFAGDASVLEDR) and one quantifier (ITSNASTIVSNAAR) marker peptides were chosen for the allergenic C-phycocyanin beta subunit (P72508) after *in-silico* digestion of allergenic proteins [155]. These peptides were used for method validation, that is, to assess linearity, recovery, reproducibility, matrix effects, processing effects, LOD, and LOQ. The main goal was to establish the presence of spirulina in commercial foodstuffs, such as pasta, crackers, and homemade bread, that were incurred with the microalga. The potential inclusion of the designated peptides was also demonstrated by MRM using a linear ion trap MS instrument.

With the same aims, Polikovsky et al. [156] developed an *in-silico* food allergenic risk assessment of proteins extracted from the macroalga *Ulva sp*. The study evaluated various protein extraction methods, and the resulting digests were analysed by LC-MS using DDA for proteome characterization. AO and SDAP with different E-values were applied to perform *in-silico* prediction of allergens for all extracted proteins. The results recommended that retrieved putative allergenic proteins were dependent on the employed extraction method. In total, four allergenic proteins of Ulva macroalga were predicted [156]. Some studies suggest that increasing the match criteria above 35% identity or decreasing the E-score below 1×10^{-7} could be a good choice to avoid over-predicting the potential risk of allergy [157].

TABLE 3 A summary and commentary on the most employed databases and software with a focus on emphasizing their definite features and capabilities.

Name	Site	DB dimension	Last update (YYYY-MM-DD)	Description	Example of application
Allergen.org	www.allergen.org	*1009 + isoforms*	—[a,b]	*The official site for systematic allergen nomenclature, approved by WHO and IUIS. It contains numerous entries with comprehensive information and undergoes stringent controls before new entries are included.*	Fish collagen [186]
AllFam	www.meduniwien.ac.at/allfam/	*1042*	*2017-03-07*	*First uploaded in 2008. Classify allergens into protein families.*	*Prunus dulci* [187]
Allergome	www.allergome.org	*4981*	*2023-02-28*	*First uploaded in 2003. It provides information on molecules that cause IgE-mediated diseases. Its large database consists of many proteins with comprehensive information about immune triggers.*	Spirulina and chlorella microalgae [154]
Allermatch	www.allermatch.org	*2569*	*2022-09-05*	*User-friendly interface to enter the input sequence and retrieve the outcomes of interest in an accurate, concise, and understandable format. The tool can be used to analyze one protein at a time.*	Skipjack tuna and Nile tilapia [188]
COMPARE	https://comparedatabase.org	*2631*	*2023-01-26*	*A recent database allows similarity searches through the COMPASS utility, but only one protein at a time can be inspected. Its database includes most of the proteins reported in Allergen.org but is not as extensive as that of Allergome.*	Oyster mushroom [189]
AllergenOnline (AO)	www.allergenonline.org	*2233*	*2021-02-14*	*Probably the most widely used software. It allows for searching for potentially allergenic proteins by simultaneously loading hundreds of FASTA sequences and evaluates the alignment, identity, and statistical expectation score (E-score) on the whole protein. The E-score represents the probability of a chance alignment, with very low E-scores and high identities often revealing a high likelihood of cross-reactivity.*	*Chlorella variabilis, Galdieria sulphuraria, and Fusarium strain Flavolapis* [157]
Structural Database of Allergenic Proteins (SDAP)	https://fermi.utmb.edu/	*1883*	*2023-01-26*	*A web server that provides database information and computational tools. It contains information on allergen names, sources, sequences, structures, IgE epitopes, and literature references. SDAP uses an original algorithm to identify regions of known allergens similar to user-supplied peptides or selected from the SDAP database of IgE epitopes. SDAP offers a flexible interface that allows users to check the FAO/WHO allergenicity rules against each allergen in its database individually.*	*Tarom GM rice* [190]

(Continues)

TABLE 3 (Continued)

Name	Site	DB dimension	Last update (YYYY-MM-DD)	Description	Example of application
Allergen Database for Food Safety (ADFS)	https://allergen.nihs.go.jp/ADFS/	*2403*	*2023-04-13*	*Maintained by the Japan National Institute of Health Sciences (NIHS), provides up-to-date allergen information to help evaluate food safety. ADFS offers tools to assess the potential for allergenic cross-reactivities using four different types of sequence identity search. The homology search tool uses the FASTA program to determine whether query proteins are homologous to known IgE epitopes or relatively identical to allergens. The FAO/WHO tool uses criteria proposed by a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology to analyze the potential allergenicity of query proteins. The AllerSTAT tool uses a data-driven approach and machine learning to detect aa subsequences that contribute to allergenicity and assess the allergen potential of query proteins.*	*Transgenic proteins expressed in genetically modified crops* [191]
AllerCatPro 2.0	https://allercatpro.bii.a-star.edu.sg/	*4979 + 162 low allergenic proteins, and 165 autoimmune allergens*	–[a,b]	*A web server that provides comprehensive analysis and prediction of allergenicity potential from protein or nucleotide sequences. It also offers visualization of 3D models for input proteins based on the similarity of 3D surface epitopes. The user-friendly interface allows users to identify protein allergenicity potential with detailed results for cross-reactivity, protein information, functionality, and clinical relevance of IgE prevalence. AllerCatPro 2.0 predicts allergenic proteins using the similarity of both their aa sequences and 3D structures compared with a comprehensive dataset of known allergens from various sources. The new version has improved prediction accuracy by extending the dataset of known allergens and enabling nucleotides alongside protein sequences as input. These datasets currently include 4979 protein allergens, 162 low-allergenic proteins, and 165 autoimmune allergens.*	*Moringa oleifera leaves* [192]

[a]The isoforms are not computed

[b]Not available information on the online site.

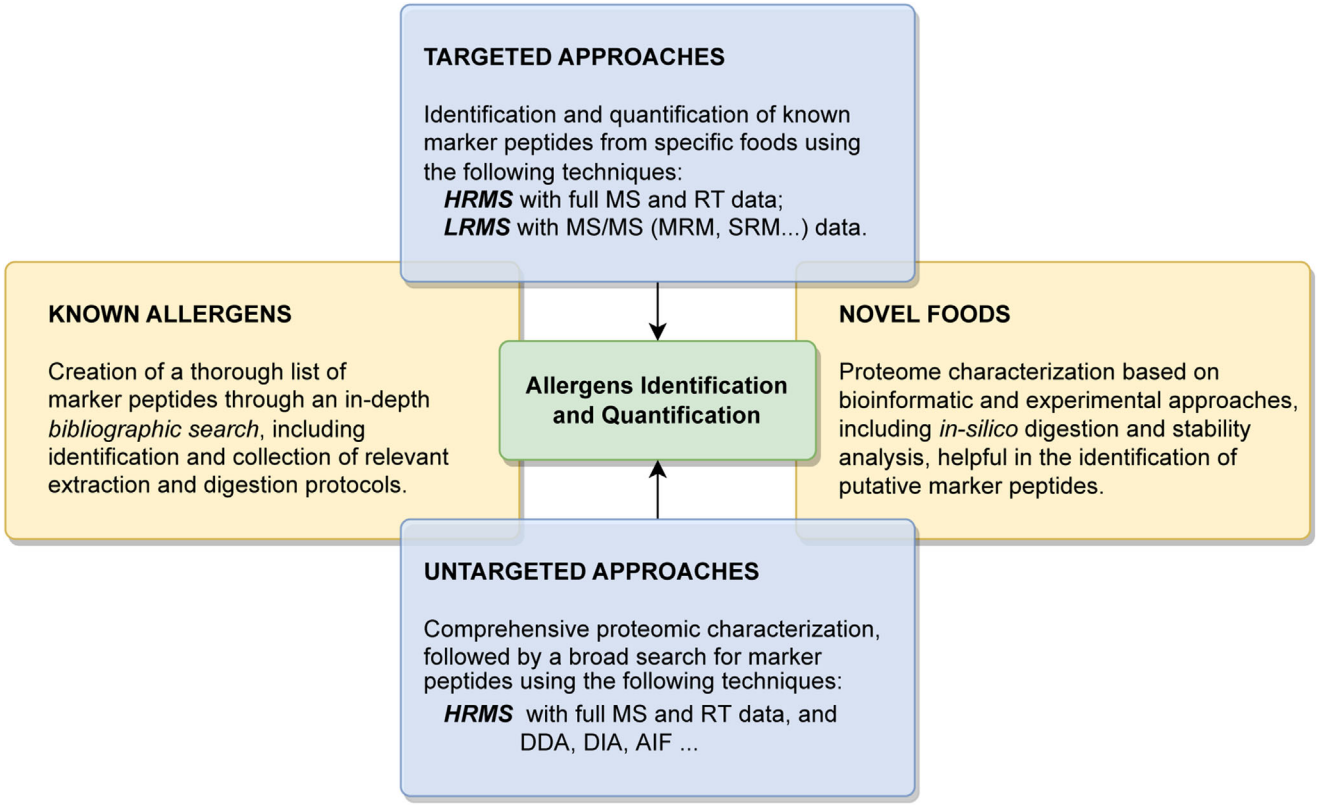

FIGURE 7 Workflow for allergen identification and quantification in foodstuffs by mass spectrometry.

In their comprehensive review of current analytical methods used in allergenic risk assessment, Mazzucchelli et al. [158] highlighted the strengths and limitations of these methods. Ensuring the harmonization of protocols and calibration assays with appropriate reference proteins is crucial in the current assessment process. The Authors also emphatized the importance of uderstanding the correlation between food processing and how the matrix interacts with protein allergenicity. To enhance the reliability of single techniques and methods, collaborative ring trials could be implemented as a potential policy. Furthermore, it is essential to develop and validate other cellular assays and animal models for the allergenic risk assessment of novel foods or food proteins lacking any homology to known allergenic structures. The Authors established a partnership called COST action ImpARAS (Improved Allergenicity Risk Assessment Strategy) [159], dedicated to enhancing the current risk assessment system. This collaborative effort aims to develop new strategies for predicting the safe consumption of novel foods through joint work.

3.6 | Exploring the safety concerns of edible insects as novel foods

Recently, there has been significant discussion about introducing insects as a food source in Europe. Historical records show that insects have been used for both medicinal and nutritional purposes within and outside Europe for centuries, but they have not been a primary food source. Instead, insects have been sporadically added to the diet, even during times of food scarcity, suggesting a certain level of reluctance [160]. However, attitudes may be changing as evidenced by the recent approval of several insect species as novel foods by the EU [161], and their safety issue is becoming more interesting. For example, the safety of eating frozen and dried formulations from whole-house crickets (*Acheta domesticus*) was studied by the EFSA Panel on *Nutrition, Novel Foods, and Food Allergens* [162]. The report concluded that the frozen and dried formulations of whole-house crickets are safe for human consumption when produced in compliance with relevant food safety legislation and processes. Nevertheless, the Panel identified allergenicity as a prospective safety concern due to the potential for allergic reactions in individuals with allergies to crustaceans, mites, and molluscs [162]. This is attributable to the fact that both insects and these types of arthropods belong to the phylum *Arthropoda* and share highly conserved allergenic proteins such as tropomyosin and arginine kinase (vide supra). Barre et al. [163], using a proteomic- and bioinformatic-based approach, analysed the diversity of proteins found in commonly consumed edible insects (i.e., silkworms, crickets, African migratory locusts, yellow mealworms, red palm weevils, and giant mealworm beetles) and discovered that the majority of these proteins are phylogenetically-related allergens widely distributed among various groups of arthropods and molluscs. Besides, several proteins belonging to discrete protein families, including chemosensory proteins, hexamerins, and odorant-binding proteins, were also discovered, which are highly specific to edible insects. The Authors suggested that

these proteins could serve as probes for the specific detection of insect proteins in food products to ensure their safety for consumption, particularly for individuals with allergies to edible insects. Food allergy to various insects has been described, allergens have been identified, and cross-reactivity (i.e., IgE binds to structurally homologous proteins that share common epitopes) and/or co-sensitization (i.e., different IgE produced against multiple allergens) with other allergenic species have been demonstrated [142, 164]. For example, cross-allergenicity (i.e., allergic symptoms triggered by cross-reactive IgE binding to multiple sources) between shrimp and two types of crickets (*Gryllus bimaculatus* and *Acheta domesticus*) has been reported [165, 166]. Despite shrimp-sensitized patients' IgE recognizing several cricket proteins, tropomyosin has been determined to be the major allergen in both shrimp and gryllus. De Marchi et al. [166] found that tropomyosin of *A. domesticus* is more stable for gastric digestion than its shrimp counterpart, and baking enhances the stability of IgE-binding proteins. Thus, the stability of cricket tropomyosin represents a risk for primary sensitization. Broekman et al. [167] established that 14 out of 15 shrimp-allergic patients were sensitized to either mealworm tropomyosin and/or arginine kinase, and 13 out of 15 reacted positively in a double-blind placebo-controlled food challenge (DBPCFC) with mealworm. In another work, Broekman et al. [168] tested several other edible insects suggested for human consumption, including yellow (*Tenebrio molitor*), giant (*Zophobas morio*) and lesser mealworms (*Alphitobius diaperinus*), large wax moths (*Galleria mellonella*), black soldier flies (*Hermetia illucens*) all in their final larval stage, as well as crickets (*A. domesticus*) and grasshoppers (*Locusta migratoria migratorioides*). Results showed that all shrimp-allergic patients were sensitized to multiple insects with similar response profiles for all the examined insects. Likewise, IgE from patients with allergies to house dust mites and crustaceans cross-reacted with yellow mealworm proteins, causing basophil activation [169]. The main cross-reactive proteins were identified as tropomyosin and arginine kinase, which are known allergens in arthropods, with moderate stability in a pepsin test [170].

Overall, MS can be used as a detection method for food allergens, which is important for ensuring food safety and compliance with labelling regulations. Since the extraction methods play an important role in allergens determination, eight protein extraction protocols were compared by Bose et al. [171]. By employing *in-silico* prediction through the AllerCatPro database, 20 putative allergen families were identified, including three arginine kinase proteoforms. To analyse the arginine kinaseproteoforms, a targeted LC-MRM-MS assay was developed and applied to a subset of extracts. The findings indicated that the abundance and detectability of allergens differ depending on the extraction and food processing methods used.

4 | CONCLUSIONS

Food allergies are frequently diagnosed in children but can develop at any age. The only way to prevent an allergic reaction is to avoid allergenic foods, which is neither simple nor obvious. While food labels are required to list major allergens, cross-contamination during food production or preparation can still occur. Mass spectrometry is a valuable tool for investigating the entire proteome of food, which helps identifying potential new allergens and allergenic protein families. By using carefully selected marker peptides, MS can also quantify the amount of allergenic protein in food samples, enabling the assessment of allergen contamination severity. Additionally, MS aids in food authentication by detecting food fraud, adulteration, and the presence of undeclared allergens. Overall, MS is a powerful tool for contributing to the safety of food products for individuals with food allergies. To fully leverage MS measurements and bioinformatics tools in allergen detection, understanding, and possibly predicting the effects of certain epitopes in novel foods will be an important challenge.

ACKNOWLEDGMENTS

This work was supported by projects: (i) PONa3_00395/1 "BIOSCIENZE & SALUTE (B&H)" and (ii) Progetto di Ricerca di Interesse Nazionale—PRIN 2017YER72K—"Development of novel DNA-based analytical platforms for the rapid, point-of-use quantification of multiple hidden allergens in food samples", financed by the Italian Ministero per l'Istruzione, l'Università e la Ricerca (MIUR). This work was also supported by a Project funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3 - Call for tender No. 341 of 15 March 2022 of the Italian Ministry of University and Research funded by the European Union—Next Generation EU. Project code PE00000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001, Project title "ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods".

CONFLICT OF INTEREST STATEMENT

The authors have declared that no competing interest exists.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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How to cite this article: Bianco, M., Ventura, G., Calvano, C. D., Losito, I., & Cataldi, T. R. I. (2023). Food allergen detection by mass spectrometry: From common to novel protein ingredients. *Proteomics*, e2200427.
https://doi.org/10.1002/pmic.202200427