



Proteomic Analysis of Food Allergens by MALDI TOF/TOF Mass Spectrometry

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Abstract

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is largely recognized as an important tool in the analysis of many biomolecules such as proteins and peptides. The MS analysis of digested peptides to identify a protein or some of its modifications is a key step in proteomics. MALDI-MS is well suited for the peptide mass fingerprinting (PMF) technique, as well as selected fragmentation of various precursors using collisional-induced dissociation (CID) or post-source decay (PSD).

In the last few years, MALDI-MS has played a significant role in food chemistry, especially in the detection of food adulterations, characterization of food allergens, and investigation of protein structural modifications induced by various industrial processes that could be an issue in terms of food quality and safety.

Here, we present simple extraction protocols of allergenic proteins in food commodities such as milk, egg, hazelnut, and lupin seeds. Classic bottom-up approaches based on Sodium Dodecyl Sulphate (SDS) gel electrophoresis separation followed by in-gel digestion or direct in-solution digestion of whole samples are described. MALDI-MS and MS/MS analyses are discussed along with a comparison of data obtained by using the most widespread matrices for proteomic studies, namely, α -cyano-4-hydroxy-cinnamic acid (CHCA) and α -cyano-4-chloro-cinnamic acid (CCICA). The choice of the most suitable MALDI matrix is fundamental for high-throughput screening of putative food allergens.

Key words MALDI TOF/TOF, Mass spectrometry, Proteomics, Food, Allergens, Egg, Milk

1 Introduction

The introduction of “soft” ionization techniques as matrix-assisted laser desorption/ionization (MALDI) [1] and electrospray ionization (ESI) [2] has revolutionized mass spectrometry as a molecular characterization tool in many fields from proteomics [3, 4] to lipidomics [5, 6], food science and safety [7, 8]. Indeed, MALDI-MS has become a fundamental technique even in the analysis of low-molecular-weight compounds, including chlorophylls [9, 10],

sugars [11–13], diols [14], vitamins [15, 16], and so on, by the introduction of novel rationally designed matrices [17, 18].

However, proteomic strategies still represent the workhorse of this analytical technique for clinical chemistry [4], microbiology [19], and food science [20]. In this area, MALDI represents a valuable analytical tool for food authentication [21], identification of food spoilers and food-borne pathogens [22], food allergen identification [23], control of the technological processes [3], impact on the chemical properties of food proteins and safety assessment of genetically modified foods [24].

Food allergy is nowadays regarded as a significant health problem in our society, which counts around 6–8% of children and up to 3% of adults affected. Allergic reactions are triggered by the immune system against food components and they can manifest by various symptoms from mild to fatal effects as anaphylaxis [25]. Until now, the only effective choice to avoid allergic reactions remains the strict avoidance of hurting foods. Conceivably, this requires clear classification and accurate labeling of the potential allergenic food ingredients. Ninety percent of food allergies are caused mainly by eight food groups referred to as “The Big 8,” including egg, fish, milk, peanut, shellfish, soy, tree nuts, and wheat. It is recognized that food allergies are caused by proteins, also termed allergens, which are not generally considered damaging to the human body except for sensitized people [26]. About plant food allergens, the most relevant protein families are represented by 2S albumins, seed storage proteins, nonspecific lipid transfer protein, while for animal foods the most common protein families remain tropomyosin and EF-hand proteins [27]. Recently, it has been reported that lipids as well might represent an adjuvant for peanut allergens in sensitization via the skin [28] even if very few studies have been retrieved on this topic. Since it is recognized that even allergen amounts at ppm level can activate a negative reaction, it stays of paramount importance for the protection of allergic consumers to develop robust and sensitive analytical techniques, able to detect eventual residues of allergens contaminating foods, also referred to as “hidden” allergens. Current strategies use an antibody-based recognition as enzyme-linked immunosorbent assay (ELISA) or lateral flow devices [29, 30]. Some of these methods are commercialized and routinely used by industry and administration agencies because of their ease of use and limited technical requirements. However, they encounter several drawbacks, such as lack of accuracy and robustness due to crossreactivity phenomena, presence of interfering compounds in complex food matrices (false positives), allergen modification occurring during food processing or sample preparation that could mask the allergen itself (false negatives) [31–34]. Due to the high sensitivity, accuracy, and performance of last-generation mass analyzers, MS techniques really represent alternative powerful methods for the

sensitive and selective detection of allergens in food products [35]. MS may potentially turn into a high-throughput screening tool, able, within a single run, to quantify multiplex allergenic ingredients or traces in several food commodities at high molecular level of selectivity. The first step for their identification is the extraction of proteins from complex food samples by applying conventional protocols based on solid/liquid or liquid/liquid extraction with buffers as Tris-HCl, TRIZMA, or chaotropic agents, i.e., ammonium sulfate and sodium chloride [36]. Even if intact proteins can be analyzed, the choice to investigate peptides could be crucial since it allows to identify and localize minor modifications in the protein sequence, in order to obtain useful information for food authenticity [37, 38] or to discover unique diagnostic peptides related to hidden food allergens. So detection is usually achieved by a “bottom-up” approach analyzing peptides obtained after proteolytic digestion of the extracted proteins [39]. Proteins are usually first treated with reducing agents as dithiothreitol (DTT) to reduce any disulfide bridges and subsequently with iodoacetamide to block the SH groups before adding specific proteolytic enzymes such as trypsin, LysC, AspN, or Glu-C. Trypsin is the most used enzyme since it generates peptides within the mass range of 500–4000 Da that are easily suitable for successive investigation by liquid chromatography (LC)-MS or MALDI-MS. Masses of the generated peptides originate a characteristic dataset called peptide mass fingerprinting (PMF). According to this approach, experimental data are compared with *in silico* protein digest to find the best match that should identify the unknown protein. Moreover, it is possible also to recognize the amino acid sequence through MS/MS acquisitions and search on databases. If the digestion is performed on a single protein, as for example on a spot arising from a 2D gel electrophoresis separation, the output score will be higher compared to the same protein digested in a complex mixture [40]. However, consistent information can also be obtained by digesting a complex protein mixture and exploiting MS/MS analyses, with the great advantage of a considerable time saving. In the case of food allergens, it is critical to identify marker peptides since the robustness and sensitivity of the whole analytical method is strictly dependent on the reliability of the signature peptides (uniqueness, specificity, stability) reflecting the target offending proteins. The approach is usually developed on standard proteins or reference materials and then applied to processed food since it is known that food processing might affect the reliability of target peptides. The global scheme of the described approach is depicted in Fig. 1.

Here, we describe protocols for the treatment of some food commodities belonging to the “Big 8” group, such as milk, egg, hazelnut, and lupin seed flour, which are often employed as raw ingredients in several baked foods or can be present as hidden

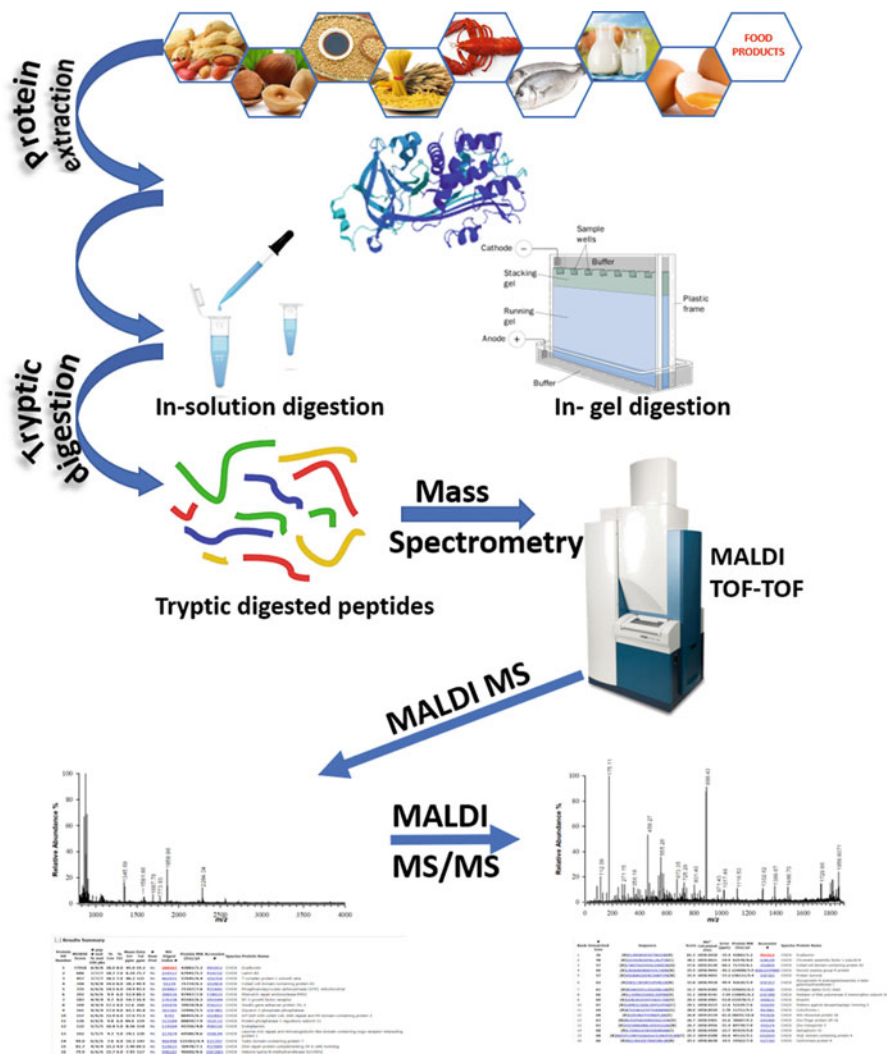


Fig. 1 Scheme of the whole bottom-up approach proposed for detection and characterization of allergen proteins from “Big 8” foods

allergens or contaminants due to their ubiquitous presence in many food industries. The proteins of milk can be grouped into three major classes, namely, caseins, milk fat globule membrane (MFGM), and whey proteins. Cow milk contains several proteins that are considered antigenic and responsible for inducing immune responses: lactoglobulins (β -LG), caseins (CN), and α -lactalbumin (ALA) are the major allergens, followed by minor proteins such as bovine serum albumin, lactoferrin, and immunoglobulins [41]. After cow’s milk, hen’s egg allergy is the second most common food allergy, with about 2% of prevalence in infants and children. The major allergens in egg white, containing more than 20 different proteins and glycoproteins, are ovomucoid (Gal d 1),

ovalbumin (Gal d 2), conalbumin (ovotransferrin) (Gal d 3), and lysozyme (Gal d 4), while in egg yolk the allergen responsible for syndrome has been identified in α -livetin (Gal d 5) [42].

As for the hazelnut proteome, at least five protein types of hazelnut appear to be involved in allergic reactions. The first major allergen, Cor a 1 (18 kDa, Bet v 1 family), is a typical pollen allergen. The second one, Cor a 2 (14 kDa), belongs to the profilin family and could be found in pollens and seeds. The other three ones belong to the seed storage protein family. Cor a 8 (9 kDa) is a nonspecific lipid transfer protein associated with severe allergic reactions to hazelnuts. The other major food allergens present in hazelnut are Cor a 9 (Corylin), a 59-kDa protein, which belongs to the legumin (11S globulin protein family) and Cor a 11 (48 kDa), which belongs to the 7S seed storage globulin family [43, 44]. Regarding the seed storage proteins of *Lupinus* species, conglutins are the major allergens. The two major fractions are β -conglutin (vicilin-like protein or acid 7S globulin) and α -conglutin (legumin-like protein or 11S globulin), whereas the two minor components are δ -conglutin (2S sulfur-rich albumin) and γ -conglutin (basic 7S globulin) [45].

Simple, rapid, and sensitive procedures for proteomic MALDI-MS/MS analysis of these foods are described in the following. A comparison between data obtained using two different matrices, namely, α -cyano-4-hydroxy-cinnamic acid (CHCA) and α -cyano-4-chloro-cinnamic acid (CClCA), is reported to underline their chief role in this field. The approach here described was able to identify the signature allergenic peptides actually available [36] for the selected foods.

2 Materials

Prepare all solutions using ultrapure water and analytical-grade reagents. Prepare and store all aqueous solutions at 4 °C and the organic solutions at room temperature. Store the reagents following the manufacturer's instructions. Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Protein Extraction

2.1.1 Protein Extraction from Milk [38]

1. Pyrex[®] glass centrifuge tube, tube capacity: 10 mL.
2. Acetic acid.
3. Centrifuge and centrifugal vacuum concentrator.
4. Pasteur pipettes.
5. Diethyl ether.
6. PVDF filters (0.45- μ m porosity).

2.1.2 *Protein Extraction
from Hazelnut* [23]

1. Porcelain pestle and mortar.
2. Eppendorf® test tube, tube capacity: 1.5 mL.
3. Aqueous isopropanol: 20% (v/v).
4. Centrifuge and centrifugal vacuum concentrator.
5. Pasteur pipettes.
6. Thermo shaker.
7. Cold acetone: 100%.

2.1.3 *Protein Extraction
from Egg*

1. Pyrex® glass suitable container/beaker, volume capacity: 100 mL.
2. Falcon centrifuge tube, tube capacity: 25 mL.
3. Pure water.
4. Aqueous ethanol: 85% (v/v).
5. Centrifuge.
6. Pasteur pipettes.
7. Thermo shaker.
8. NaCl or (NH₄)₂SO₄: 10% (w/v).
9. HCl: 6 M.

2.1.4 *Protein Extraction
from Lupin Seed Flour* [46]

1. Pyrex® glass centrifuge tube, tube capacity: 15 mL.
2. Eppendorf® test tube, tube capacity: 1.5 mL.
3. 0.2 M Tris-HCl buffer (pH: 8.2).
4. Centrifuge.
5. Pasteur pipettes.
6. Thermo shaker.
7. Filters made of 0.2-µm regenerated cellulose.

**2.2 Protein
Separation
and Digestion**

2.2.1 *SDS Gel
Electrophoresis* [47]

1. Protein electrophoresis buffer: Dilute ten times with Millipore water the 10× Tris/Glycine/SDS buffer (Bio-Rad Laboratories, Hertfordshire, UK) (*see Note 1*).
2. Sample loading buffer: Laemmli sample buffer (Bio-Rad Laboratories) (*see Note 2*).
3. DTT solution: DL-Dithiothreitol 50 mM in ultrapure water (freshly prepared).
4. Thermo shaker.
5. Hamilton syringe.
6. 4–15% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories, Hertfordshire, UK).
7. Molecular-weight marker “Precision Plus Protein™ Standards Dual Color” (Bio-Rad Laboratories).

8. Mini-PROTEAN[®] II Electrophoresis cell (Bio-Rad Laboratories).
9. Fixing solution: 50% (v/v) Millipore water, 40% (v/v) ethanol, 10% (v/v) acetic acid.
10. Staining solution: 0.116 g Blue Coomassie R250 in 100 mL of 67% (v/v) Millipore water, 25% (v/v) ethanol, 8% (v/v) acetic acid.
11. Destaining solution: 67% (v/v) Millipore water, 25% (v/v) ethanol, 8% (v/v) acetic acid.

2.2.2 In-Gel Digestion [48]

1. Sterile cutter.
2. 1.5-mL microfuge tubes.
3. Organic buffer A: NH_4HCO_3 200 mM in 40% (v/v) of ACN.
4. Organic buffer B: NH_4HCO_3 50 mM in 9% (v/v) of ACN.
5. Thermo shaker.
6. Centrifugal vacuum concentrator.
7. Trypsin solution in organic buffer B (Trypsin Proteomics Grade, Dimethylated, Sigma-Aldrich).
8. Aqueous 10% (v/v) trifluoroacetic acid (TFA).

2.2.3 In-Solution Digestion [49]

1. 0.5-mL microfuge tubes.
2. Aqueous buffer: 400 mM NH_4HCO_3 in 8 M urea (*see Note 3*).
3. DTT solution: DL-Dithiothreitol 50 mM in NH_4HCO_3 200 mM (freshly prepared).
4. IAA solution: iodoacetamide 150 mM in NH_4HCO_3 200 mM (freshly prepared).
5. Ultrapure water.
6. Trypsin solution in organic buffer B.
7. Aqueous 10% (v/v) TFA.

2.3 MALDI MS/MS Analysis

1. Matrix solution (*see Notes 4 and 5*).
2. Calibration peptide mixture: Mass standards kit for calibration was purchased from AB Sciex (Ontario, Canada) and contains Neurotensin, des-Arg¹-Bradykinin, angiotensin I, [Glu]¹-fibrinopeptide, ACTH fragments 18–39 (concentration ranging from 0.2 to 9.2 µg/vial) (*see Note 6*).
3. Acetonitrile, water, aqueous TFA 0.1% or formic acid 1% (v/v).
4. MALDI sample plate.
5. MALDI TOF/TOF mass spectrometer.

All experiments were performed using a 5800 MALDI ToF/-ToF analyzer (AB SCIEX, Darmstadt, Germany) equipped with a

neodymium-doped yttrium lithium fluoride (Nd:YLF) laser (345 nm) and operated in reflectron positive mode, with a typical mass accuracy of 5 ppm. In MS and MS/MS mode, 2000 laser shots were typically accumulated by a random rastering pattern, at a laser pulse rate of 400 and 1000 Hz, respectively. MS/MS experiments were performed setting a potential difference of 1 kV between the source and the collision cell; ambient air was used as the collision gas, with a medium pressure of 10^{-6} Torr. The delayed extraction (DE) time was set at 280 ns.

DataExplorer software 4.0 (AB Sciex) was used to control the acquisitions and to perform the initial elaboration of data, while SigmaPlot 11.0 was used to graph final mass spectra. ChemDraw Pro 8.0.3 (CambridgeSoft Corporation, Cambridge, MA, USA) was employed to draw chemical structures. Isotopic pattern contribution was achieved by Xcalibur 4.0 with Foundation 3.1 (Thermo Fisher Scientific). Any commercial MALDI mass spectrometer should be capable of obtaining data as discussed here.

3 Methods

3.1 Protein Extraction from Milk (See Notes 7 and 8)

1. Centrifuge 5 mL of milk samples at $5000 \times g$ for 30 min at 4 °C.
2. Remove the film of fat formed at the top of the solution.
3. Add 250 μ L of acetic acid in order to precipitate caseins.
4. Centrifuge at $5000 \times g$ for 30 min.
5. Store the casein pellet.
6. Add an equal volume of diethyl ether to the remaining solution (containing whey proteins).
7. Remove lipid and filter whey proteins through PVDF filters (0.45- μ m porosity).
8. Dry both casein and whey fractions in a centrifugal vacuum concentrator.
9. Redissolve in water (1 mg/mL) and subject to in-solution digestion or SDS-PAGE run.

3.2 Protein Extraction from Hazelnuts (See Note 8)

1. Ground into a fine powder 30 mg of roasted hazelnuts.
2. Add 1 mL of isopropanol 20% (v/v).
3. Shake for 60 min at $500 \times g$.
4. Centrifuge at $12,000 \times g$ for 10 min at 4 °C.
5. Collect the supernatant and add 6 mL of cold acetone.
6. Vortex the mixture and incubate overnight at -20 °C.
7. Centrifuge at $3000 \times g$ for 30 min.
8. Dry the pellet at room temperature (around 30 min).

3.3 Protein Extraction from Egg

1. Separate the yolk from the white by cracking the egg and collect them in two suitable container/beakers.
2. Prepare a 20% (w/v) solution of egg white in pure water (distilled or deionized).
3. Centrifuge at $3000 \times g$ for 30 min.
4. Collect the supernatant (albumin fraction).
5. To 1 g of egg yolk, add about 2 mL of pure water (distilled or deionized).
6. Centrifuge at $4000 \times g$ for 10 min.
7. To the precipitate, add 4 volumes of ethanol 85%, vortex and centrifuge at $4000 \times g$ for 10 min.
8. Discard the supernatant if not necessary (it contains lipids).
9. Extract protein using 9 volumes of NaCl or $(\text{NH}_4)_2\text{SO}_4$ 10%.
10. Centrifuge at $4000 \times g$ for 10 min and collect supernatant for successive purification (*see* **Note 9**) and enzymatic digestion.

3.4 Protein Extraction from Lupin Seed Flour (*See Note 8*)

1. Suspend 0.5 g of lupin seed flour in 12 mL of 0.2 M Tris-HCl buffer (pH: 8.2) (*see* **Note 10**).
2. Keep under magnetic stirring for 6 h at 60 °C.
3. Centrifuge at $10,000 \times g$ for 20 min at 4 °C.
4. Filter on 0.2- μm regenerated cellulose filters.

3.5 SDS-Page

1. Mix a volume of 10 μL of each sample with 10 μL of Laemmli sample buffer (1:1, v/v).
2. Add 1 μL of DTT 50 mM and keep at 90 °C for 5 min in a thermo shaker.
3. Load a volume of 15 μL onto the gel using Hamilton syringe (*see* **Note 11**).
4. Run the gel with a constant voltage (200 V) and a variable current (run time ca. 25 min).
5. After the run, put the gel in the fixing solution for 30 min.
6. Stain the gel in the staining solution for 30 min.
7. Destain for 2 h in the destaining solution and then overnight in water.

3.6 In-Gel Digestion

1. Cut the bands in small pieces and put in 0.5-mL microfuge tubes.
2. Cover the pieces with 200 μL of organic buffer A.
3. Incubate samples at 37 °C for 30 s in a thermo shaker.
4. Repeat washing until the gel pieces are decolorized.

5. Dry the gel fragments in a centrifugal vacuum concentrator for 15 s.
6. Add porcine trypsin (0.05 µg/µL) and 50 µL of organic buffer B.
7. Incubate at 37 °C overnight.
8. Collect the liquid and acidify with aqueous TFA 10% (v/v).

3.7 In-Solution Digestion

1. Mix 10 µL of aqueous sample with 40 µL of aqueous buffer.
2. Add 5 µL of DTT solution and incubate for 40 min at 60 °C.
3. Chill at room temperature.
4. Add 5 µL of IAA solution and incubate in the dark at room temperature for 45 min.
5. Add 120 µL of water and 20 pmol of porcine trypsin (*see Note 12*).
6. Incubate overnight at 37 °C.
7. Stop the digestion with aqueous TFA 10% (v/v) (reach pH ca 2).

3.8 MALDI MS/MS Analysis

1. Mix the tryptic digests 1:1 (v/v) with the matrix.
2. Spot 1 µL of the sample/matrix solution and let drying in air.
3. Wash the spot twice with ultrapure water (*see Note 13*) and then analyze by MALDI-TOF-MS/MS.
4. Acquire spectra in reflectron positive ion mode.
5. For peptide analysis, the explored mass range is usually 700–4000 *m/z*; perform mass calibration using the appropriate peptide mixtures (*see Note 6*).

3.9 Protein Identification

For PMF analysis, the raw files relevant to tryptic digests were searched against the nonredundant database Swiss-Prot utilizing Mascot (Matrix Science Ltd., London, UK) or MS-Fit (Protein Prospector, California) database search engines (*see Note 14*). The search was performed against the taxonomies *Other Mammalia* (or *Bos Taurus*) for milk proteins, *Gallus gallus* for egg proteins, and *Green plants* for hazelnut and lupin seeds, setting an error of 20 ppm for mass accuracy. The following modifications were set: carbamidomethylation of cysteines as a fixed modification (for in-solution digestion), oxidation of methionines, and phosphorylation of serines, tyrosines and threonines as variable modifications. Up to three missed cleavages were accepted (*see Note 14*). For MS/MS analyses, the search was performed utilizing MS-Tag (Protein Prospector, California) and the same parameters except for error that was set to 0.1 Da for MS and 0.3 Da for MS/MS.

3.10 MALDI-MS and Tandem MS Analysis

Representative examples of mass spectra data obtained on some allergenic proteins extracted from food as described in the experimental section are discussed. In MALDI analyses, a key role to have a robust and sensitive detection of the unique, selective, and reliable signature peptides is played by the chosen matrix. Figure 2 reports a comparison of MALDI TOF spectra relevant to a tryptic digest arising from one SDS-PAGE spot of a commercial milk sample by using CHCA (Plot a) and CCICA (Plot b) as matrices. The PMF results from database search clearly indicate that β -casein was properly digested and positively identified irrespective of the matrix used, but the coverage was 21% (MOWSE score: 7066) for CHCA and 30% (MOWSE score: 54132) when CCICA was employed. As previously reported [16], CCICA shows a proton affinity lower than CHCA (198 vs. 201 kcal/mol), leading to an increased proton transfer to the analyte; the result is higher ionization efficiency for less basic peptides compared to CHCA. Then, it is likely that the positive effects detected in peptide mass fingerprint of β -casein (Fig. 2), i.e., enhancement in signal intensities and uniform instrumental response to peptides, no matter their amino acid composition, could be explained by the increased ion yield generated by the CCICA matrix, confirming its importance in detecting likely marker peptides. Notably, three phosphorylated peptides (*see label P* in Fig. 2b), which got undetected using CHCA, could also be identified. Indeed, phosphopeptides are challenging analytes (i.e., difficult to perceive without specific sample pretreatments) since they occur at low concentration, are suppressed during the ionization by nonphosphorylated peptides, and do not bind to C18 resin usually used for sample desalting. This observation suggests that the use of CCICA provides an enough enrichment of phosphopeptides directly on spot. To confirm the assignment of these peptides as potentially phosphorylated, MS/MS acquisitions were run on the native peptide at m/z 1981.7 (Fig. 3a) and the relevant phosphorylated form at m/z 2061.8 (Fig. 3b). Searching on the MS/MS database, the peptide at m/z 1981.7 was identified as FQSEEQQQTEDELQDK of β -casein by identifying the predominant b - and y -type ions [50] arising from the cleavage of the thermally labile C–N amide bonds of the polypeptide backbone. The peptide and precursor ion at m/z 2061.8 were instead identified as phosphorylated because the intense product ion at m/z 1963.9 is most likely due to the neutral loss of H_3PO_4 (=97.9 Da) used as a marker of fragmentation. Moreover, product ions b - and y -type explained by the database (labeled in plot 3b) correspond also to phosphorylated ion fragments, which allowed to assign the sequence as FQpSEEQQQTEDELQDK with phosphorylation on serine 35 [51].

While the aforementioned example was referred to an in-gel tryptic digest obtained from SDS PAGE purified sample, Fig. 4 shows the MALDI-TOF MS spectrum of an in-solution tryptic

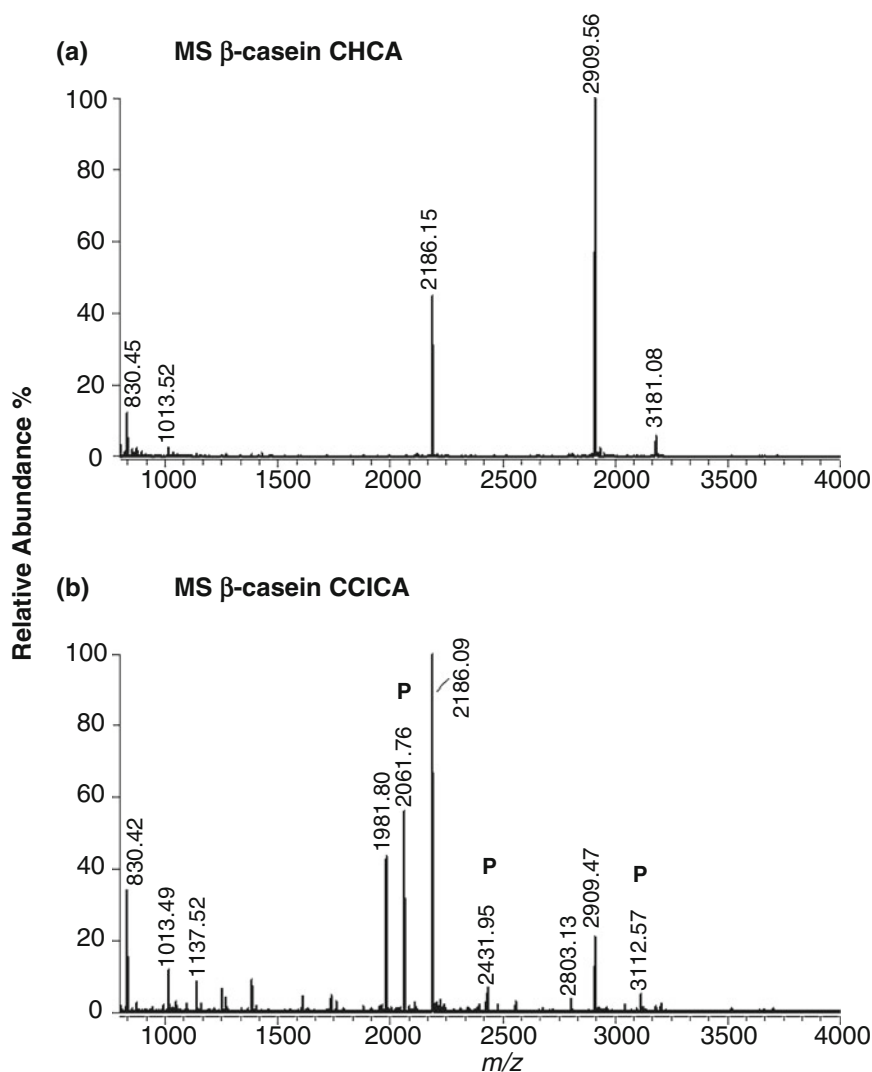


Fig. 2 MALDI TOF spectra of a tryptic digest arising from an SDS-PAGE spot of β -casein when a commercial milk sample was analyzed by CHCA (a) and CCICA (b) as matrices. Phosphopeptide species are labeled (P)

digest of lupin seed flour extract obtained following the method reported before by using CCICA as a matrix. The database search allowed to recognize the most abundant lupin seed proteins attributable to globulin family as β -conglutinin 7 and β -conglutinin 2. In this case, the MS spectrum exhibited many peaks, including minor peptides not predictable by PMF. A 2D gel separation could help in the characterization of lupin seed proteins. Alternatively, the MS/MS spectra carried out on selected peptides can also be useful to distinguish between different conglutinin families. For instance, in Fig. 5 is reported the MS/MS spectrum of the precursor ion at m/z 1446.7 that was chosen since it is known to be a signature allergenic peptide to reveal the occurrence of lupin seed proteins. The b - and

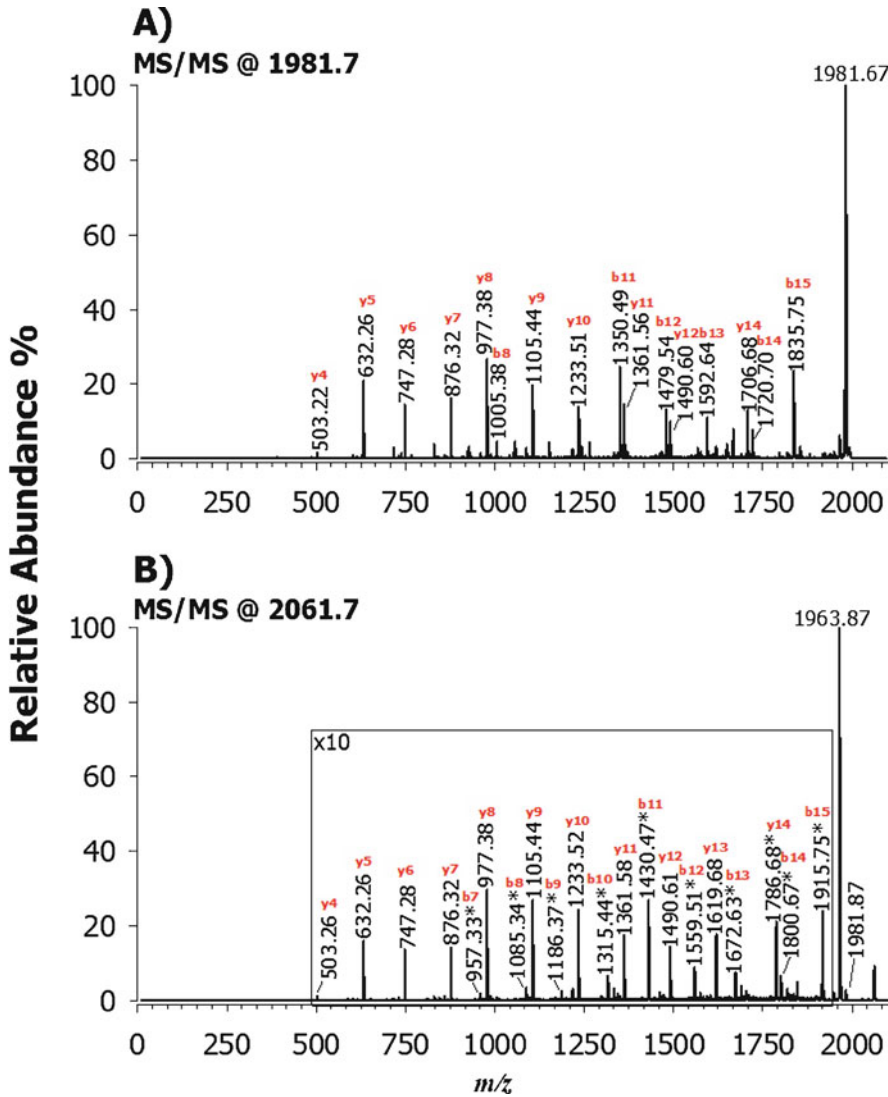


Fig. 3 MALDI TOF/TOF spectra relevant to the peptide FQSEEQQTDELQDK at m/z 1981.7 (**a**) and to the corresponding phosphorylated form at m/z 2061.76 (**b**) from β -casein digest. CCICA was used as a matrix. Phosphorylated fragments are labeled (*)

y -type ions allowed to assign the peptide as LLGFGINADENQR, as previously described [36, 46]. Unfortunately, the high homology among various isoforms of β -conglutinin did not allow to unambiguously identify the subspecies between β -conglutinin 2, 3, 4, 5, and 6, but the peptide remains informative and stable to monitor the presence of lupin seeds.

Finally, also for another complex matrix such as egg white extract, the fragmentation of selected precursor ions enables the identification of the common allergens associated with this foodstuff. Figure 6 reports the tandem MS spectrum of the precursor

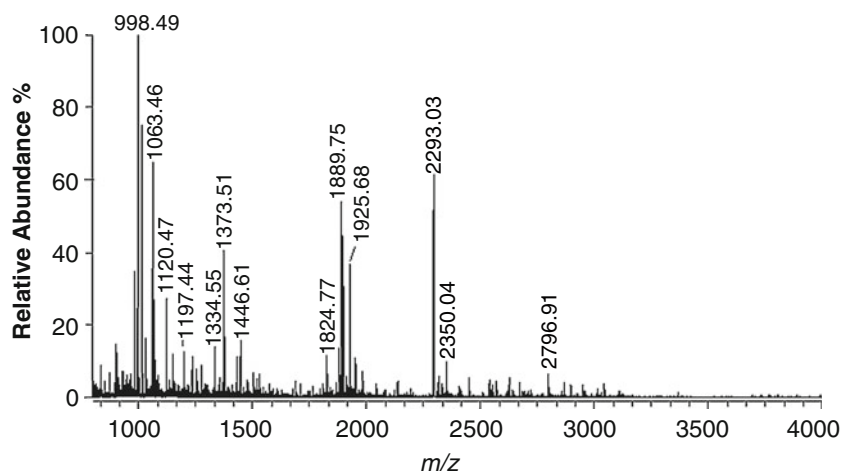


Fig. 4 MALDI TOF spectrum of a tryptic digest of proteins extracted from lupin seed flour, as described in the experimental section. MALDI matrix: CCICA

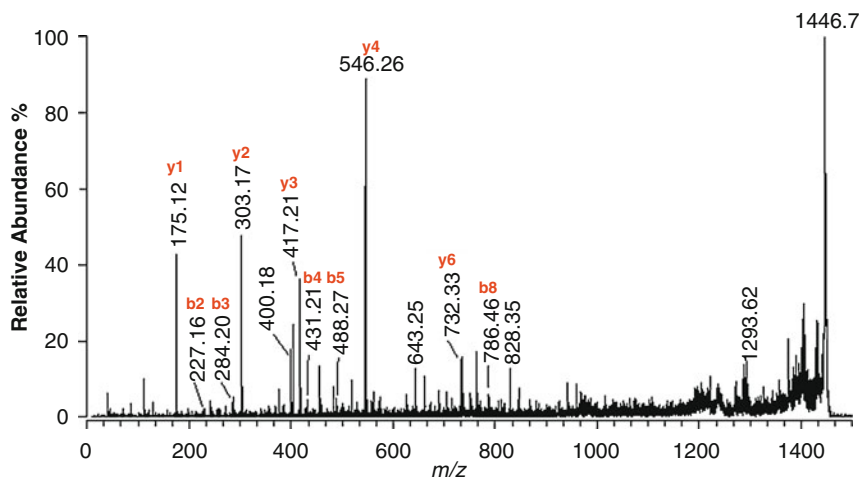


Fig. 5 MALDI TOF/TOF spectrum of the protonated peptide LLGFGINADENQR at m/z 1446.7 assigned to β -conglutinin from lupin seed flour extract. The main y and b ions are reported. MALDI matrix: CCICA

ion at m/z 1858.8 identified as ELINSWVESQTNGIIR and well known to be an allergenic peptide of ovalbumin [36]. Therefore, if gel electrophoresis is not available, the tandem MS acquisition of MALDI-MS along with the right matrix choice is very important to search for the signature peptides of allergens introduced as hidden ingredients of foods.

4 Notes

1. The formulation of the commercial protein electrophoresis buffer 1 $\times$ consists of 25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH = 8.3. A simple method of preparing

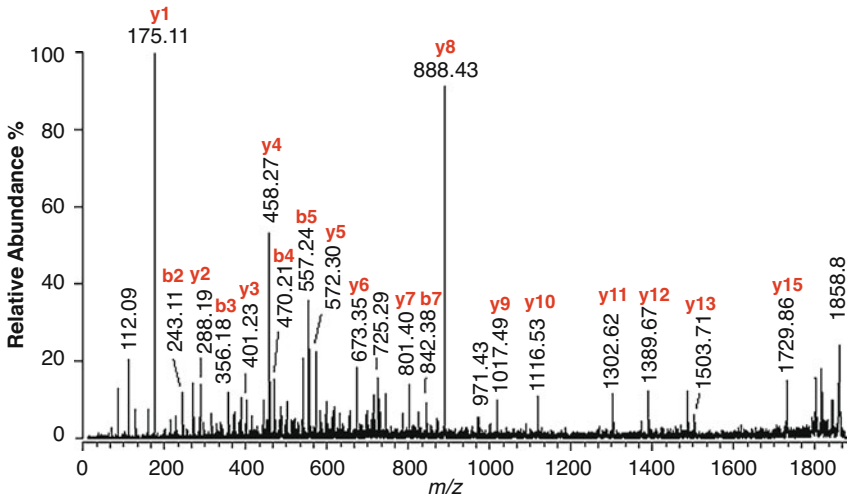


Fig. 6 MALDI TOF/TOF spectrum of the protonated peptide ELINSWVESQTNGIIR at m/z 1858.8 of ovalbumin from egg white. The main y and b ions are reported. MALDI matrix: CCICA

running buffer: Weigh 15.1 g of Tris, 71 g of glycine, and 0.5 g of SDS, mix, and make it to 500 mL with water.

2. The formulation of the commercial loading buffer consists of 62.5 mM Tris, pH = 6.8, 2% (w/v) SDS, 25% (v/v) glycerol, 0.01% (w/v) bromophenol blue. A simple method of preparing running buffer: Weigh 0.6 g of Tris and dissolve in 10 mL of water (pH 8.5). Take 2.5 mL of Tris buffer and add 0.31 g DTT, 0.4 g of SDS, 5.5 mL of water, 2 mL of glycerol, and a few crystals of bromophenol blue.
3. Alternatively, for complex samples, the denaturation can be performed by adding 40 μ L of RapiGest™ (0.1% in 50 mM ammonium bicarbonate) reagent (Waters) instead of the aqueous buffer described. RapiGest™ SF Surfactant is a reagent used to enhance enzymatic digestion of proteins since it helps in solubilizing proteins, making them more susceptible to enzymatic cleavage without inhibiting enzyme activity. It is compatible with enzymes such as Trypsin, Lys-C, Asp-N, and Glu-C and other enzymes and it improves efficiency of digestion.
4. The matrix was usually prepared in ACN:H₂O 2:1 with 0.1% (v/v) TFA at a concentration of 10 mg/mL. The most common matrices for peptide analysis are α -cyano-4-hydroxycinnamic acid (CHCA) and α -cyano-4-chloro-cinnamic acid. For matrix recrystallization, *see* **Note 5**.
5. Recrystallization was carried out in 70% acetonitrile/30% (v/v) water. A saturated solution of the matrix in 70% acetonitrile/30% (v/v) water was heated until boiling. When the solid dissolved completely, the solution was cooled to room

temperature, then on ice until a precipitate was observed. Finally, the precipitate was filtered, and the procedure repeated in triplicate.

6. Instead of buying a specific kit, the mass calibration can also be carried out by manually preparing a mixture of standard peptides such as angiotensin I, [Glu]¹-fibrinopeptide, ACTH fragments 18–39, with decreasing concentration from the lightest to the heaviest peptide.
7. The in-solution tryptic digestion on whole milk samples originated MALDI mass spectra dominated by casein-related peptides, while whey proteins were almost absent. Thus, a preliminary procedure (see experimental) to separate caseins from whey fractions, which were then subjected to independent processing and analysis, was adopted.
8. All the described samples can be usually defatted using a 1:10 (w/v) sample/hexane mixture by end-over-end rotation at ambient temperature for 15 min. The hexane layer can be decanted, and the defatting process repeated two additional times. The defatted sample can be vacuum-dried and resuspended in the appropriate extraction buffer.
9. The purification step is a key phase in developing sensitive methods for the detection of allergens since it is necessary to concentrate proteins and eliminate interferences from the supernatant. Purification techniques usually involve solid-phase extraction (SPE), protein precipitation, ultrafiltration, and size exclusion chromatography (SEC) [52].
10. The same protocol can be applied to lupin seeds after grinding samples under liquid nitrogen to obtain a fine and homogeneous powder.
11. Centrifuging the samples prior to the run helps to remove insoluble debris, which could produce streaks in the protein lanes (revealed when stained with Coomassie Blue).
12. When using the common denaturants, such as SDS or urea, it is very important to dilute the sample before the addition of enzyme to avoid the denaturation of trypsin (or other proteolytic enzymes). This problem does not occur using *RapiGest* SF since it does not modify peptides or suppress protease activity.
13. If the concentration level of impurities is high, it could be nearly impossible to obtain useful signals. If you suspect that extraneous salts are interfering with data collection, a simple spot-washing step after cocrystallization of the sample and matrix may be useful. Simply apply a microliter of Milli-Q water to the spot and allow it to stand for several seconds. The water can then be removed with a pipette tip. Sometimes,

the wash on target is not enough to completely remove salts. In this case, a Zip Tip purification can be performed following the indications of the supplier Millipore. In the first step, the resin is washed and activated with acetonitrile ($2 \times 10 \mu\text{L}$) and with TFA 0.1% (v/v) ($2 \times 10 \mu\text{L}$). Then, the sample is loaded by taking up (ten times) the liquid containing the peptide mixture. Washing is performed with TFA 0.1% (v/v) ($3 \times 10 \mu\text{L}$) in order to eliminate contaminants and finally the sample is eluted with 1–3 μL of matrix (*see Note 4*) directly on the target plate.

14. Often, modified peptides are not attributed to any theoretical protein and no database matching is obtained. For these reasons, a different approach can be followed. A number of well-known proteins normally present in food samples (for instance, in our case, globulin-like proteins, whey proteins, caseins) is created and the corresponding database codes are retrieved. Comparison of the experimental data with those related to peptides arising from these proteins is carried out by means of the FindPept software. In this search mode, one protein at a time was compared with the experimental m/z data in order to recognize unmodified as well as modified peptides. In this way, almost all signals of the mass spectra could be attributed to peptides and thus to proteins. This approach is very useful when investigations are carried out searching the modifications likely occurring during the heating processes, as deamidation, lactosylation, glycation.

Acknowledgments

This work was supported by 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.”

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