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Optimization of a sample preparation workflow based on UHPLC-MS/MS method for multi-allergen detection in chocolate: An outcome of the ThRAll project

Jean Henrottin [a], Rosa Pilolli [b], Anne-Catherine Huet [a], Christof van Poucke [c], Chiara Nitride [d], Marc De Loose [c], Olivier Tranquet [e,f], Colette Larré [e], Karine Adel-Patient [g], Hervé Bernard [g], E.N. Clare Mills [d], Nathalie Gillard [a,**], Linda Monaci [b,*]

[a] CER Groupe, Rue du point du Jour, 8, 6900, Marloie, Belgium
[b] Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR), Via Giovanni Amendola 122/O, 70126, Bari, Italy
[c] Flanders Research Institute for Agriculture, Fisheries and Food, Brusselsesteenweg 370, 9090, Melle, Belgium
[d] School of Biological Sciences, Division of Infection, Immunity and Respiratory Medicine, Manchester Academic Health Science Centre, Manchester Institute of Biotechnology, University of Manchester, UK
[e] UR1268 BIA, Rue de la Géraudière, BP 71627, 44316, Nantes, France
[f] INRAE, Aix Marseille University, Biodiversité Et Biotechnologie Fongiques (BBF), UMR1163, 13009, Marseille, France
[g] Université Paris-Saclay, CEA, INRAE, UMR MTS, Service de Pharmacologie et d'Immunoanalyse, Laboratoire d'Immuno-Allergie Alimentaire, F-91191, Gif-sur-Yvette, France

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ABSTRACT

Developing reliable methodologies for detecting and quantifying allergens in processed food commodities is crucial to support food business operators in allergen risk assessment and properly implementing precautionary allergen labels whenever required to safeguard the health of allergic consumers. Multiple Mass Spectrometry (MS) methods have been developed so far and applied for single and multi-allergen detection in foods, generating a heterogeneous literature on this topic, with little attention paid to the extraction and the digestion steps, crucial in delivering accurate allergen measurements.

This investigation carried out within an international consortium specifically built up to convey a prototype MS based reference method, reports on the first part of the method development, namely the optimization of the sample preparation protocol for six allergens detection (cow's milk, hen's egg, soy, peanut, hazelnut, and almond) in chocolate. The latter was chosen as model complex food matrix, having a high lipid and polyphenol content.

Different steps of the sample preparation protocol have been taken into consideration: (i) sampling, (ii) composition of the extraction buffer, (iii) protein purification, (iv) protein enzymatic digestion, (v) peptide purification and pre-concentration, and some experiments were carried out by two independent laboratories and two different MS platforms to provide a first assessment of the robustness of the method under development. Fifty target peptides were monitored in multiple reaction monitoring mode and validated in different laboratories to trace the six allergenic ingredients in the incurred chocolate and the best performing protocol for sample preparation was identified. This work paves the way of the forthcoming full analytical validation of a prototype reference method for MS-based allergen quantification.

1. Introduction

Food allergens are responsible for food product recalls and incidents of fatal or severe allergic reactions globally representing a management issue for food business operators [World Health Organization—International Food Safety Authorities Network]. Since the only

* Corresponding author.
** Corresponding author.
E-mail addresses: n.gillard@cergroupe.be (N. Gillard), linda.monaci@ispa.cnr.it (L. Monaci).

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effective treatment for food-allergic individuals is a strict long-life allergen-free diet, worldwide regulations have been implemented for foodstuff labeling [Remington et al., 2020]. A list of 'priority' allergenic foods, which prevalently responsible for allergic reaction in the population and can be responsible for severe symptoms, to be declared on food labels, has been published by the Codex Alimentarius Commission [FAO/WHO Food Standards, 2018] and it is current being reviewed by a panel of *ad hoc* Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens. In the European Union (EU), the original Codex list has been expanded and now includes 14 different allergenic foods [European Commission, 2011].

While mandatory allergen labeling supports allergic consumers in making informed decisions about the foods they can eat, confusion remains about the meaning of voluntary precautionary allergen labels (PAL; *e.g.*, 'may contain … ' or 'may contain traces of … '). PAL warns of the potential presence of unintended allergens, resulting from cross-contamination of raw materials and/or finished foods [DunnGalvin et al., 2019]. Analytical methods (including namely ELISA-, PCR-, and MS-based methods) support food operators in implementing the allergen risk assessment evaluations and confirm whether a contamination has happened. However, PAL has often been applied in response to potential unintended allergen presence, with no risk assessment performed as a protective measure by food operators. This inconsistent application of PAL has led to a loss of trust in allergic consumers, which do not fully understand their purpose [DunnGalvin et al., 2019, DunnGalvin et al., 2015]. Irrespective of whether the allergen risk assessment is performed appropriately or comprehensively, the lack of a mandatory threshold reflecting clinical reactivity makes the decision for PAL difficult. However, the accumulation of clinical data would make the establishment of mandatory thresholds possible. Such thresholds have already been set at 10 mg of total ingredient protein/kg in Japan, and the VITAL initiative takes into account clinical data to extrapolate thresholds currently implemented in Australia and New-Zealand to support the PAL [Taylor et al., 2014].

Accurate and reliable methodologies enabling the detection and quantitation of allergen traces in foodstuffs are urgently needed to support the risk assessment. Mass Spectrometry (MS) is one of the most promising techniques that proved to be successfully applied to allergen detection, identification, quantification, and characterization for over a decade now, and has much promise as a reference method for food allergen analysis [Monaci & van Hengel, 2008; Monaci, Losito, De Angelis, Pilolli, & Visconti, 2013; Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014; Heick, Fischer, & Pöpping, 2011; Parker et al., 2015; De Angelis, Pilolli, & Monaci, 2017; Boo et al., 2018; Pilolli et al., 2017a; Pilolli, De Angelis, & Monaci, 2018; ; Nitride et al., 2019; Sayers et al., 2016; Sayers et al., 2018; Planque et al., 2016; Planque et al., 2017a; ; Planque et al., 2017bPlanque et al., 2019; Henrottin et al., 2019; Gavage et al., 2020; Hands, Sayers, Nitride, Gethings, & Mills, 2020]. A recent review of the methods published in this area compared different aspects of food allergen quantification using advanced MS techniques, highlighting the main gaps that need to be addressed in terms of harmonization and results comparability across independent laboratories [Monaci et al., 2018].

In this context, the European Food Safety Agency (EFSA) has funded the ThRAll (Thresholds and Reference method for Allergen detection method) project aiming at the '*Detection and quantification of allergens in foods and minimum eliciting doses in food allergic individuals*'. The project focused on the development of a harmonized and quantitative MS-based reference method for the simultaneous detection and quantification of six food allergens in standardized incurred food matrices by multiple reaction monitoring (MRM) acquisition mode [Mills et al., 2019]. The target allergens within the project include two animal-derived food allergens (cow's milk and hen's eggs) and four plant food allergens (soy, peanut, hazelnut, and almond), all of which are included in Annex II of EU Regulation No 1169/2011 [European Commission, 2011]. A multi-analyte method is being developed to determine all these allergens in two model and standardized incurred food matrices, namely chocolate and broth powder [Huet et al., 2022], which are very challenging matrices for analysis. Previous studies aiming at developing multiplex methods for allergen analysis used milk chocolate and dark chocolate as model matrices [Shefcheck et al., 2006, New, Schreiber, Stahl-Zeng, & Liu, 2018, Planque et al., 2016], but recoveries of allergenic marker peptides were found to be low and not satisfactory, highlighting the need for optimization of extraction and digestion approaches for challenging matrices where proteins may be bound to polyphenols and tannins [New et al., 2020]. Both matrices were produced within the ThRAll project in a food pilot plant after careful characterization of the allergenic ingredients [Huet et al., 2022].

Under this frame, the present work addresses the development of a prototype reference method as commissioned by EFSA and focus on the optimization of a reliable, straightforward, reproducible, and harmonized sample preparation protocol for multi-allergen detection in processed foodstuffs by MS analysis. This report describes the optimization of a multiplex MS method including 50 marker peptides (from 5 to 13 specific peptides [and from 1 to 3 proteins] for each allergenic ingredient). The UHPLC-MS/MS analyses were carried out in two independent laboratories using different triple quadrupole LC-MS platforms to confirm the robustness and reliability of the protocol developed herein. Since the detection of the peptides depends of the MS platform used, the MS parameters of each peptide transition have to be duly optimized on the MS platform to be used to maximize the detection of these peptides. Typical method transfer procedures were also described herein, allowing to easily and universally transposing this harmonized multi-allergen MS-based detection method to various MS platforms. Besides MS parameters, sample preparation workflow was also duly optimized, including: composition of the extraction buffer (also including denaturation agents), inclusion of technical aids for protein and peptide purification (*i.e.* several stationary phases), and optimization of the tryptic digestion (incubation time, enzyme to protein ratio, addition of chemical aids to improve proteolytic activity (*e.g.*, RapiGest SF)). The impact of each parameter/modification on the signature peptide detection was investigated in incurred chocolate (as an example of complex food matrix) according to a systematic approach to achieve the best response for the selected markers tracing for the six allergens under analysis. This will provide a solid foundation to base a viable reference MS method for food allergens detection.

2. Materials and methods

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris), urea, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate (ABB) were from Sigma-Aldrich (Bornem, Belgium). Trypsin Gold (mass spectrometry grade) was from Promega (V5280; Leiden, The Netherlands). Acetic acid was from Acros Organics (Geel, Belgium), hydrochloric acid was from Fisher Chemical (Loughborough, UK), and RapiGest SF was from Waters (Milford, Massachusetts, USA). Acetonitrile, water, methanol (ULC-MS grade), and formic acid were obtained from Biosolve (Valkenswaard, The Netherlands). Acrodisc® syringe filter with Versapor® membrane (PALL laboratory; 5 μm, 25 mm) was obtained from VWR (Leuven, Belgium; #28143-963). PD-10 desalting columns pre-packed with Sephadex G-25 M resin used for protein extract pre-purification were purchased from Cytiva (GE Healthcare, Hoegaarden, Belgium; #17085101). Sep-Pak C18 solid phase extraction (SPE) columns (1 cc, 50 mg; WAT054955) and Strata-X polymeric reversed phase (33 μm; 30 mg; 1 mL; 8B–S100-TAK) were purchased from Waters (Milford, Massachusetts, USA) and Phenomenex (Torrance, California, USA), respectively, and used for peptide purification and enrichment.

2.2. Model food matrix

The model food matrix used for the optimization of the parameters was an incurred chocolate bar prepared within the ThRAll project, in a food pilot plant in order to mimic real production process [Huet et al., 2022]. Briefly, chocolate refiner flakes were weighed and an appropriate amount of each targeted allergenic ingredient (cow's milk, hen's egg, peanut flour (lightly roasted and partially defatted powder), full fat soy flour (non-toasted), hazelnut flour (not roasted), and almond flour (blanched)) was added and carefully mixed by vigorously shaking. The mixture was applied several times to a three-roll mill (Exakt 80E) to obtain a mixture with equal particle (final particle size of 20 μm). The obtained pre-mix of chocolate refiner flakes was further diluted with blank chocolate refiner flakes. These chocolate refiner flakes were melted in a dry heat chocolate melter (Mol D'Art) at 40 °C–45 °C. Once the refiner flakes were liquid, 200 g of cocoa butter were added and gently mixed. Ammonium phosphatide (20 g), used as emulsifier, was added and mixed until a glazy mass was obtained. The liquid chocolate was then transferred to a pastry bag and was dripped into pellets of about 5 g. Pellets were kept overnight at 4 °C, packed in sealed aluminum laminate and finally stored at 4 °C. The chosen contamination level for method development was 40 mg total protein of each allergenic ingredient/kg of chocolate bar (40 ppm).

2.3. Multi-allergen UHPLC-MS/MS analysis parameters

2.3.1. UHPLC-MS/MS parameters and MRM selection

The UHPLC-MS/MS analyses were carried out in two independent laboratories using different triple quadrupole mass spectrometry instruments. Specifically, the following instrumental platforms and conditions were used for method development.

Most of the optimization experiments were carried out on an Acquity liquid chromatography (UHPLC) system coupled to a Waters Xevo TQ-S triple quadrupole system. Peptide separation was performed on a Waters Acquity UPLC peptide BEH C18 column (130 A, 1.7 μm, 2.1 × 150 mm) at 50 °C and with a flow rate of 0.2 mL/min. Elution was carried out for 26 min as follows: 0–3 min: 92% A; 3–18 min: 92%–58% A, 18.0–18.1 min: 58%–15% A; 18.1–22.5 min: 15% A; 22.5–22.6 min: 15%–92% A, 22.6–26 min: 92% A (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile) [Henrottin et al., 2019]. MRM detection in positive electrospray mode was performed with a Waters Xevo TQ-S triple quadrupole system and set up at unit resolution in both Q1 and Q3. The cone nitrogen flow was set at 150 L/h, the collision gas flow at 0.13 mL/min, the capillary voltage at 2.5 kV, and the source temperature at 150 °C. The desolvation temperature was set at 500 °C and the nitrogen flow at 650 L/h.

Additional experiments were carried out on a UHPLC LX-50 system coupled with a QSight® 220 triple quadrupole mass analyzer (PerkinElmer). Chromatographic separation was performed on a Brownlee SPP Peptide ES-C18 column (2.1 × 150 mm; 2.7 μm; 160 Å) at 30 °C with the following elution gradient (flow 0.25 mL/min): 0–33 min, from 10% to 35% B; 33.0–33.2 from 35% to 90% B, constant at 90% B for 10 min, from 43.2 to 43.4 min from 90% to 10% of solvent B, column equilibration for 16 min. Timed-MRM acquisition in positive ion mode was set up at unit resolution in both Q1 and Q3, and with 2 min wide acquisition windows. Electrospray source parameters were set as follows: drying gas (nitrogen): 120 (arbitrary units), hot-surface induced desolvation (HSID™) Temp: 250 °C; nebulizer gas: 300 (arbitrary units), Electrospray V1: 4500, ion source Temp: 400 °C. All instrument control, analysis, and data processing were performed using the Simplicity™ 3Q software platform v. 1.6.

Marker peptide selection for the six allergenic ingredients has been described by Pilolli et al.[Pilolli et al., 2020; Pilolli et al., 2021]. Since the MS settings (including detected MRM transitions, collision energies, etc.) may vary across MS platforms, the experimental optimization of the mass spectrometry parameters was carried out as first optimization step. Optimal cone voltage and collision energies were determined for all peptide markers under evaluation. Proteins were extracted from the six ingredients with the same protocol described below (see section 2.4.1.2) digested with trypsin (enzyme/protein ratio 1:50) and injected without any further purification to identify the optimal MRM transitions and collision energies (CE, with a step size of 1 eV) to apply at each targeted peptide. The open source *Skyline* software (version #: 20.1.0.76; https://skyline.ms/project/home/software/Skyline/begin.view) was used to this purpose. The following options were selected: b or y fragments generated (with a minimum length of 3 amino acids, in order to guarantee the maximum specificity), fixed carbamidomethylation of cysteines, precursor ion charge +2 or +3, and product ion charge +1. In addition, due to the multi-target MS/MS method under development, the number of MRM transitions were limited to a maximum of five for each selected peptide. The optimal parameters determined for each MRM on the two different mass spectrometers are summarized in Table S1. Chocolate samples incurred at 40 ppm were then analyzed by monitoring the selected transitions under these optimized conditions.

2.4. Sample preparation protocol – optimization

2.4.1. Sample preparation

2.4.1.1. Chocolate pre-treatment. Different sample pre-treatment procedures were investigated before extraction: grinding, melting, melting followed by defatting.

a) Grinding

To avoid chocolate melting, samples, as well as the blade and stainless-steel container of the grinder, were kept at −20 °C for a minimum of 2 h before use. 15 g of chocolate sample were placed in a Waring laboratory blender. Two pulse cycles of 3 s at maximum speed were applied. The stainless-steel container was removed, and the contents were manually shaken. The container and its content were stored at −20 °C for 5 min. These pulse cycles were repeated twice. Optionally, the sample can be manually sieved (1 mm sieve). The sample was weighed (2.00 ± 0.02 g of sample in 50 mL Falcon tube) and stored at 4 °C up to its use.

b) Melting

Chocolate was weighed (*ca.* 15 g) in a 50 mL Falcon tube. The chocolate was melted at 37 °C in a water bath. Melted chocolate was weighed (2.00 ± 0.02 g) in a 50 mL Falcon tube. Once weighed, the melted chocolate was used immediately.

c) Melting and defatting

As a third sample preparation procedure, hexane (20 mL) was added to the 2.00 ± 0.02 g of melted chocolate. The sample was mixed (head-over-head shaking) at room temperature (RT) for 15 min and centrifuged (4660×*g*; 5 min; 10 °C). The supernatant was discarded, and the defatting procedure was repeated. The crude defatted chocolate was dried at 30 °C under nitrogen flow. This defatting resulted in approximately 10% weight loss. Once dried, the sample was either used for the extraction or stored at 4 °C until use.

2.4.1.2. Sample extraction. Extraction buffer (20 mL of Tris HCl 200 mM, pH 9.2 with urea 2 M or 5 M), was added to the ground (2.4.1.1 *a*), melted (2.4.1.1. *b*), or melted and defatted (2.4.1.1. *c*) chocolate sample kept beforehand at room temperature (RT, 25 °C) for at least 15 min. The solution was then mixed (head-over-head shaking; 30 min, RT), sonicated in a water bath (15 min), and centrifuged (4660×*g*; 10 min; 10 °C).

2.4.1.3. Protein purification. The supernatant recovered at the end of the extraction step was filtrated on an Acrodisc® syringe filter with a 5 μm Versapor® membrane. The resulting filtrated solution (2.5 mL) was purified by size exclusion chromatography (SEC; 5 kDa cut-off) on disposable cartridge (PD-10 desalting columns pre-packed with Sephadex G-25 M resin, from Cytiva). The columns were conditioned beforehand with three aliquots of water (4 mL each) followed by four aliquots of 50 mM ammonium bicarbonate buffer (ABB 50, 4 mL each). Both the "Spin elution" and "Gravity elution" protocols, which involved respectively the centrifugation of the cartridge and the gravitational elution of the sample, were carried out according to the producer instructions. These elution protocols provide different dilution factors for the purified samples recovered after elution: no dilution for spin elution and 1.4 times dilution for gravity elution. The option of sample purification via SEC procedures was also compared with the extract direct dilution in ABB 200 mM to decrease the urea concentration down to 1 M, limit compatible with proper trypsin activity.

2.4.1.4. Enzymatic digestion. The protein concentration of the extracts was determined using bicinchoninic acid (BCA) assay (Sigma Aldrich) according to the manufacturer's instructions. The resulting assayed values were used to adjust the amount of trypsin to the sample according to the enzyme to substrate ratios of 1:50, 1:100, and 1:200. Prior to protease digestion, proteins were denatured, reduced, and alkylated. First, the protein extracts (0.5 mL) recovered after either SEC protein purification or dilution were transferred into a LoBind Eppendorf tube (1.5 mL) and heated at 95 °C for 15 min (600 rpm) for denaturation. Second, once cooled down on ice, DTT (50 μL, 500 mM dissolved in ABB 200 mM) was added, and the solution was incubated at 60 °C for 30 min (600 rpm). Third, the solution was cooled down on ice, and IAA (100 μL, 100 mM dissolved in ABB 200 mM) was added; the resulting solution was incubated in the dark, at 37 °C, for 30 min (600 rpm). The reduced and alkylated protein solution was then digested with trypsin. Trypsin Gold solution (1 μg/μL in acetic acid 50 mM) was added (10 μL; theoretical trypsin to protein ratio: 1/100) and the digestion was performed at 37 °C for 16 h (900 rpm). The digestion was quenched by the addition of hydrochloric acid (70 μL, 1 M) and centrifuged (14 800 rpm; 10 min).

2.4.1.5. Peptide purification and sample extract concentration. The resulting centrifuged digest was purified either on Strata-X SPE column (1 cc, 30 mg) or on Sep-Pak C18 SPE column (1 cc, 50 mg). The Strata-X SPE column was activated with methanol (3 mL) and conditioned with water (3 mL). The sample (0.5 mL) was loaded onto the SPE and washed with water (2 mL) followed by water/methanol solution (95/5 (v/v); 1 mL). The peptides were eluted with ACN/MeOH (1/1 (v/v)) and 2% formic acid (1 mL). The SPE column was then dried for 2 min. The Sep-Pak C18 SPE column was activated with ACN (3 mL) and conditioned using 0.1% formic acid in water (3 mL). The sample was loaded onto the SPE and washed with 0.1% formic acid in water (3 mL). The peptides were eluted with acetonitrile/0.1% formic acid in water mixture 80/20 (v/v) (1 mL). The SPE column was then dried for 2 min. The eluted solution was concentrated by evaporation under nitrogen flow (N_2; 40 °C) up to dryness. The dried extract was solubilized in 5% acetonitrile in 0.1% formic acid in water solution (100 μL), vortexed, and

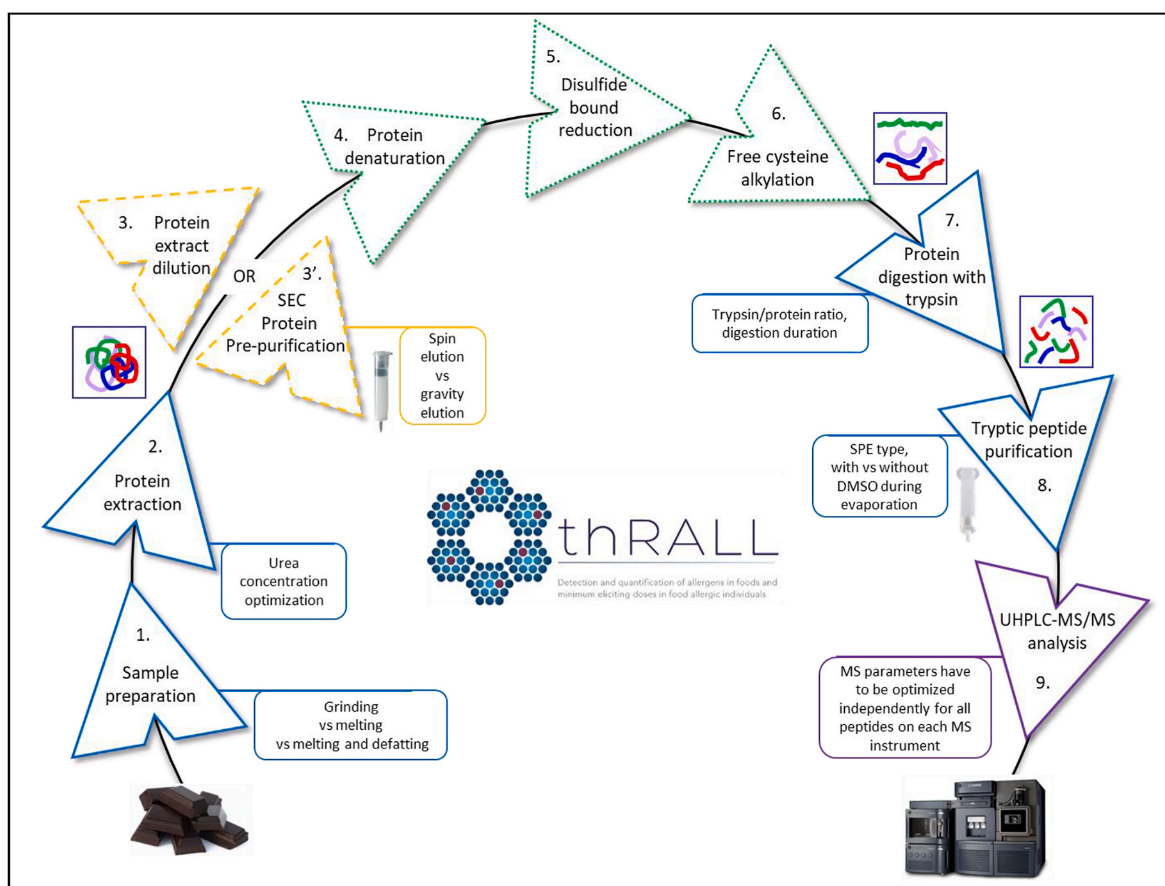

Fig. 1. Optimization of the various steps of the sample protocol—overview from sample preparation to its analysis.
In blue: the mandatory steps in which at least one parameter has been studied and optimized; in dashed orange: optional steps necessary to reach a theoretical urea concentration not exceeding 1 M as required for the subsequent digestion step; in dotted green: usual steps not optimized in this study (denaturation was performed by sample heating, while DTT and IAA were added to the sample for reduction and alkylation steps, respectively); and in purple: the UHPLC-MS/MS analysis step for which the MS parameters have to be optimized independently for all peptides on each MS instrument (courtesy of Waters).

centrifuged (4660 g; 5 min; 10 °C). The supernatant was transferred into an injection vial and analyzed by UHPLC-MS/MS.

2.4.1.6. Statistical treatment. In terms of method development, as shown in the scheme reported in Fig. 1, five steps of the sample preparation protocol were considered. For all selected procedures, three independent replicates were carried out for statistical relevance of the comparisons ($n = 3$). Mean and standard deviation of the peak areas for each protocol were calculated and compared by an unpaired Student's t-test (two-tailed distribution, equal variances) at a 5% significance level to evaluate the influence of each parameter on detection sensitivity. The equality of variances of the two independent groups was assessed by an F-test (at a 5% significance level). For the digestion kinetics experiment, a Tukey's *post hoc* ANOVA test was performed for multiple comparisons of mean values. For each digestion time ($t = 1, 4, 16, 24$ h), mean values that are not significantly different will be marked with equal labels (a, b, c, d) in relevant plots.

3. Results and discussion

In this investigation, the optimization of a sample preparation workflow for multi-allergen detection in chocolate has been carried out. The accomplishment of this task is very important to understand and compensate for the effects that the specific matrix composition may have on the reliability and sensitivity of the LC-MS based detection [Croote, Braslavsky, & Quake, 2019; Korte, Oberleitner, & Brockmeyer, 2019], especially when complex foodstuffs are going to be analyzed [Mattarozzi et al., 2014]. Indeed, matrix components might promote the establishment of covalent or non covalent interactions with the target proteins, thus affecting their detection, with potential enhancement or impairment depending on the specific case [Alves et al., 2017]. Chocolate, in particular, is a very challenging matrix due to its high content of sugars, tannins and polyphenolic compounds which might account for a masking effect of the target allergenic protein [Bignardi et al., 2013; Khuda, Jackson, Fu, & Williams, 2015; Korte et al., 2019; New et al., 2020]. Therefore a great focus have been placed on this investigation on the proper purification of the analytical sample both before trypsin digestion and LC-MS analysis.

3.1. Optimization of MS parameters

Ancillary to the actual optimization of the sample preparation workflow, a preliminary tuning of the MS platforms involved in this work has been carried out. A list of fifty target peptides previously selected as markers (Supplementary data – Table S1) [Pilolli et al., 2020; Pilolli et al., 2021] has been set up on two instruments (both based on triple quadrupoles analyzers) with four transitions/peptide monitored in MRM mode. Several parameters have been optimized including: cone voltage (Supplementary data – Figure S1A), desolvation temperature (Supplementary data – Figure S1.B), and collision energy (Supplementary data – Table S1). To perform this task, protein extracts from each allergenic ingredient have been prepared and digested according to the protocols described in sections 2.4.2 and 2.4.4, respectively, without further purification.

The optimization of the MS parameters was described herein by applying two independent techniques. First, collision energy was optimized on the Xevo TQ-S mass spectrometer (Waters) by using the *Skyline* software: the energy was changed automatically (with a step size set to 1 eV) for each MRM of each selected peptide marker. Given that the method developed here is a multi-target UHPLC-MS/MS, the number MRM was limited to a maximum of three to five MRM transitions for each selected peptide (Table S1). This maximum of three to five MRM transitions per peptide marker was chosen according to the three following main factors: the number of peptide markers to analyze, their respective acquisition windows, and the dwell time, which have an impact on the signal stability, and on the number of points per peak. As depicted in Figure S1A, the higher the desolvation temperature, the higher the observed peak area. The observed peak area also increases rapidly in line with the cone voltage, before reaching a plateau from 10 to 35 V; for some peptides, this plateau can be observed up to 45 V (Figure S1.B). For higher cone voltages, the observed peak area decreases. Therefore, a desolvation temperature of 500 °C and a cone voltage of 35 V were selected as the most appropriate compromises for the detection of all allergen peptides on the Xevo TQ-S triple quadrupole spectrometer instrument. A second method was applied for MS parameters optimization on the second alternative triple quadrupole (Q-Sight®, PerkinElmer; see section 2.3 and in Table S1) used in this investigation. For this latter, the MS method was optimized by the direct infusion of the allergenic ingredient digests with a t-line configuration. The source parameters (drying gas, hot-surface induced desolvation (HSID™) temperature, nebulizer gas, electrospray voltage, and source temperature) were tuned by maximizing the total ion current of the Full-MS mass spectra acquired. In addition, the experimental m/z values both for precursors and transitions were checked and the three main parameters related to the MRM acquisition (entrance voltage, voltage on flat lens, and collision energy) were automatically optimized by running individual ramps.

3.2. Sample preparation optimization

Each step of the sample preparation workflow displayed in Fig. 1 has been investigated and optimized by varying individual parameters highlighted in this figure. Chocolate bar incurred at 40 ppm concentration level with milk, egg, soy, peanut, almond, and hazelnut, was chosen as the model sample for this optimization. Three independent samples were tested ($n = 3$), and the resulting MRM peak areas (quantitative transition only) were compared by unpaired statistical *t*-test to highlight statistically significant differences of recorded mean values.

3.2.1. Chocolate pre-treatment

For a proper sampling of representative and homogeneous portions of the chocolate bar, two physical approaches have been compared: grinding [Gu et al., 2018; Korte & Brockmeyer, 2016; Korte et al., 2019] and melting [Huet et al., 2022]. As for the grinding protocol special attention was required to avoid unintended melting caused by overheating. Most of the detected peptides (38 peptides out of the 47 detected) did not show any significant impact of the sample preparation (melting vs grinding) on their detection (Table 1 and in Figures S2 and S3 (Supplementary Data)). Some differences were disclosed only for soybean, since four specific peptides (out of the 13 peptides selected) showed a higher sensitivity when grinding was used (Fig. 2). All four peptides originated from the 11S globulin named *glycinin* (known as the allergen *Gly m 6*), one of the major soy allergens associated with severe allergic reactions to soybean in children [Holzhauser et al., 2009; Ito et al., 2011].

In addition, a dual step preparation including melting and defatting of chocolate sample, was also compared with the grinding procedure. The defatting step might contribute to reduce the matrix complexity, by removal of the lipophilic components potentially interfering with the enzymatic proteolysis and/or with the peptide detection [New et al., 2018; New et al., 2020; Xiong 2021]. However, also in this case, most of the peptides signals were not influenced by the two procedure (39 out of 47), whereas only six peptides, from egg (two peptides), peanut (two peptides), hazelnut (one peptide) and soy (one peptide), appeared to be promoted by the melting-defatting procedure and two promoted, on the contrary, by the grinding procedure (see Figure S3 for details). However, the melting-defatting procedure suffers from several drawbacks, being time-consuming and neither user nor environmentally friendly. Therefore, as the impact of an additional hexane defatting step is limited to some peptides, the grinding procedure was preferred and chosen for the preparation and homogenization of the sample, being simple and

Table 1
Number of allergen peptides for which the MS detection is significantly influenced by a modification in the sample preparation workflow/parameters (based on t-test).

Sample preparation step modified/optimized	Milk			Egg			Peanut	Hazelnut	Soy	Almond	Number of SD and not SD peptides over total number of detected peptides
	Casein	β-lactoglobulin	*Total*	White	Yolk	*Total*					
Grinding	0	0	*0*	0	1	*1*	0	1	4	0	**6/47**
Melting	0	1	*1*	0	0	*0*	1	1	0	0	**3/47**
Not SD	5	3	*8*	3	3	*6*	4	7	9	4	**38/47**
Grinding	0	0	*0*	0	0	*0*	1	0	0	1	**2/47**
Melting & Defatting	0	0	*0*	2	0	*2*	2	1	1	0	**6/47**
Not SD	5	4	*9*	1	4	*5*	2	8	12	3	**39/47**
2 M Urea*	1	0	*1*	2	2	*4*	0	2	1	1	**9/47**
5 M Urea*	0	0	*0*	0	0	*0*	3	1	0	0	**4/47**
Not SD*	4	4	*8*	1	2	*3*	2	6	12	3	**34/47**
2 M Urea**	0	1	*1*	2	1	*3*	1	1	0	1	**7/38**
5 M Urea**	0	0	*0*	0	1	*1*	4	1	0	0	**6/38**
Not SD**	5	3	*8*	1	2	*3*	0	5	6	3	**25/38**
with SEC (gravity)	5	1	*6*	1	3	*4*	1	7	6	3	**27/47**
without SEC (dilution)	0	1	*1*	1	0	*1*	0	0	2	0	**4/47**
Not SD	0	2	*2*	1	1	*2*	4	2	5	1	**16/47**
Gravity SEC elution	0	0	*0*	0	0	*0*	0	0	1	0	**1/49**
Spin SEC elution	4	4	*8*	2	4	*6*	6	9	11	4	**44/49**
Not SD	1	0	*1*	1	0	*1*	0	0	1	1	**4/49**
with RapiGest SF**	0	1	*1*	0	0	*0*	1	0	0	0	**2/38**
without RapiGest SF**	5	1	*6*	2	0	*2*	2	4	0	2	**16/38**
Not SD**	0	2	*2*	1	4	*5*	2	3	6	2	**20/38**
Trypsin/protein ratio 1/50	0	2	*2*	2	2	*4*	0	2	4	0	**12/49**
Trypsin/protein ratio 1/100	0	0	*0*	0	0	*0*	0	0	1	0	**1/49**
Not SD	5	2	*7*	1	2	*3*	6	7	8	5	**36/49**
Trypsin/protein ratio 1/50	3	4	*7*	2	2	*4*	1	4	5	0	**21/49**
Trypsin/protein ratio 1/200	0	0	*0*	0	1	*1*	0	2	2	0	**5/49**
Not SD	2	0	*2*	1	1	*2*	5	3	6	5	**23/49**
Trypsin/protein ratio 1/100	3	3	*6*	2	2	*4*	1	4	4	1	**20/49**
Trypsin/protein ratio 1/200	0	0	*0*	0	1	*1*	0	0	2	0	**3/49**
Not SD	2	1	*3*	1	1	*2*	5	5	7	4	**26/49**
StrataX SPE	2	0	*2*	1	0	*1*	0	0	1	0	**4/47**
C18 SPE	0	0	*0*	0	0	*0*	0	1	1	0	**2/47**
Not SD	3	4	*7*	2	4	*6*	5	8	11	4	**41/47**
with DMSO	0	0	*0*	0	0	*0*	0	0	0	0	**0/47**
without DMSO	0	0	*0*	0	1	*1*	1	1	1	0	**4/47**
Not SD	5	4	*9*	3	3	*6*	4	8	12	4	**43/47**

SD: Significantly different; * Xevo TQ-S (Waters); ** QSight® 220 TQ (Perkin Elmer).

environmental/user friendly, also confirming the protocols previously applied by independent research groups on similar matrixes [Bignardi et al., 2013; Sayers et al., 2018; Gu et al., 2018; Korte & Brockmeyer, 2016; Korte et al., 2019].

3.2.2. *Protein extraction – extraction buffer*

The next step of the sample protocol (Fig. 1) is protein extraction, which usually requires the use of Tris-buffered saline (TBS), NH_4HCO_3, or Tris.HCl, at a pH value of 8.0–9.2 [Gu et al., 2018; Martinez-Esteso et al., 2020, Shefcheck et al., 2006, Planque et al., 2016, Monaci et al., 2014]. These buffers can be used in combination with denaturing, reducing agents, and/or surfactants such as urea, thiourea, dithiotreitol, sodium dodecyl sulphate (SDS), tween, octyl β-D-glucopyranosid and RapiGest SF, in order to improve the protein extraction rate [Martinez-Esteso et al., 2020, Monaci et al., 2014, New et al., 2018, New et al., 2020, Sayers et al., 2018, Sagu, Huschek, Homann, & Rawel, 2021, Xiong 2021]. However, some of these additives can interfere with the enzymatic digestion step (*e.g.*, proteases like trypsin are inhibited by urea concentrations higher than 1 M) or may adversely affect the MS analysis (*e.g.*, SDS is not MS compatible); therefore whenever added to improve the protein extraction yield, such additives require proper removal steps in the preparation workflow such as solid phase extraction, cut-off filtration or dilution down to compatible concentrations, to avoid any interference with the final detection [Boo et al., 2018, Croote et al., 2019, Monaci et al., 2020, Planque et al., 2016, Planque et al., 2019, Xiong 2021]. In addition, to further improve the extraction yield, a sonication step may also be included as physical aid [Monaci et al., 2014; Pilolli et al., 2017b; Pilolli et al., 2018; Planque et al., 2016], particularly efficient in promoting the recovery of specific allergenic proteins such as caseinate proteins from milk [Monaci et al., 2014].

A basic extraction protocol for multi-allergen detection was chosen from previous investigations [Planque et al., 2019] based on Tris.HCl buffer (200 mM, pH 9.2) added with 2 M urea: the sample was mixed with this buffer and sonicated to improve the extraction. The possibility to include a higher concentration of urea (5M) was assessed for potential application in highly processed samples. The results of such comparison (2M vs 5M urea) obtained in two independent laboratories with two different triple quadrupole spectrometers (lab 1: Xevo TQ-S [Waters]; lab 2: QSight® 220 TQ [PerkinElmer]) were generally consistent (Supplementary data – Table S1). Interestingly, the urea concentration had a limited impact on the extraction/detection of peptide targets from soy, almond, hazelnut, and milk (Supplementary Data – Figure S4 and S5),

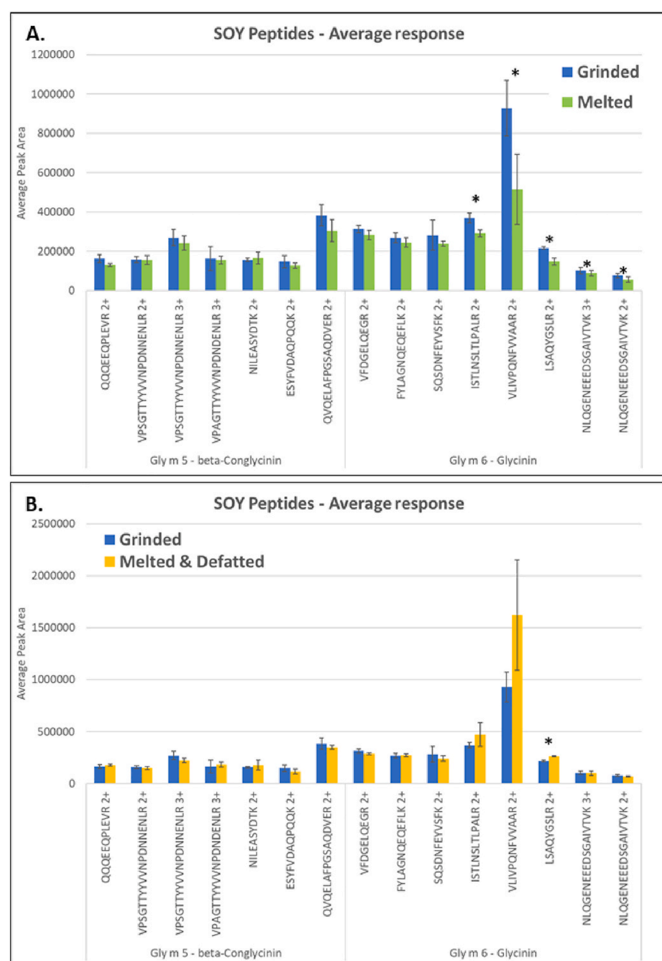

Fig. 2. Impact of the sample preparation and homogenization on the soy peptide detection.
Comparison of the average peak area ($n = 3$) between sample grinding (blue bars) and (A.) sample melting (green bars) or (B.) sample melting and defatting (orange bars) procedures. * Significantly different based on *t*-test calculation ($\alpha = 5\%$).

whereas had a considerable impact on protein extraction from egg and peanut (Fig. 3) with opposite trends for the two ingredients. Indeed, depending on the MS platform, the detection of three to four egg peptides (out of the seven targeted for white and yolk proteins) was significantly improved by extracting with 2 M urea instead of 5 M urea (Table 1, Fig. 3A and C). In contrast, 5 M urea significantly favors peanut protein extraction (Table 1, Fig. 3B and D). Indeed, the higher urea concentration (5 M) improves the extraction and detection of lightly roasted peanuts, which is the only extensively processed ingredient in this incurred chocolate matrix, confirming previous investigations on the proteomic profiling of this ingredient [Johnson et al., 2016]. This observation is especially important in the perspective to extend the current method to other extensively processed samples [Mattarozzi & Careri, 2019]. Based on these findings, which are similar on both MS platforms, the higher urea concentration (5 M) was chosen for the final extraction protocol due to the improved allergens extraction yield from incurred processed food commodities.

3.2.3. SEC protein purification

When used as a chaotropic agent in protein extraction, a major drawback of urea is that concentrations higher than 1 M can denature trypsin, reducing the effectiveness of the digestion step. This can be overcome by diluting the extract with ammonium bicarbonate buffer before the digestion step to give a theoretical urea concentration below 1 M [Croote et al., 2019, Planque et al., 2016, Planque et al., 2017b, Planque et al., 2019]. Alternatively, technical aids for buffer exchange using disposable cartridge-based size exclusion chromatography (SEC) can be implemented to remove urea. This option also has the additional advantage of simplifying the sample composition by removing low molecular weight interfering compounds (5 kDa cut-off of the stationary phase), such as polyphenols, that might be co-extracted from the matrix, thus reducing the background signal from the chocolate matrix [Pilolli et al., 2017b, Pilolli et al., 2018, Pilolli et al., 2021]. Therefore, direct dilution and SEC protein purification procedures (using either a spin or gravity elution protocol) were compared on protein extracts prepared with Tris.HCl buffer containing 2 M urea (Table 1). The SEC based proteins purification significantly improved the detection of peptides from milk, egg, hazelnut, soy, and almond proteins compared to dilution. For peanut, only one peptide (SPDIYNPQAGSLK^{2+}) showed a significant improvement in its MS detection after SEC protein purification (Supplementary Data – Figure S6). Of the SEC elution procedures (gravity *versus* spin protocols), the spin elution significantly improved the detection of 90% of the peptides (44 out of 49 peptides), irrespective of the allergen or protein fraction (Table 1; Supplementary Data – Figure S7), compared to gravity elution protocol. Based on these results, the use of an additional purification step at protein level is highly recommended, and the SEC purification using centrifugation was chosen to be included in the final optimized protocol.

3.2.4. Protein digestion with trypsin

The next step involves the proteolytic digestion of the extracted proteins into peptides using a protease with specific cleavage sites such as trypsin (which cleaves proteins after arginine and lysine residues) after the proper reduction and alkylation of cysteines residues.

Further tests were performed to evaluate the potential effect of the acid labile surfactant RapiGest SF, on the efficiency of in-solution enzymatic digestion [Johnson et al., 2016, Sayers et al., 2018]. Unlike other commonly used denaturants, RapiGest SF does not modify peptides or protease (trypsin) activity and is hydrolysable at acid pH (half-life 8 min at pH 2), forming water-immiscible by-products which can be removed, allowing LC-MS analyses. RapiGest SF was added to the protein extract at 0.1% (final concentration in the digest sample) just before proteins thermal denaturation (at 95 °C), and hydrolyzed during protease quenching by HCl addition (section 2.4.4.). However, according to the results, detection of several peptides belonging to five out of six targeted allergens (except for soy peptides) was negatively affected by the RapiGest SF (Table 1 and Supplementary Data – Figure S8), which was thus not included in further optimization steps and the final protocol.

Additional digestion assays were performed with a focus on optimizing the trypsin to protein ratio (protein concentration in the extract being estimated by BCA assays) corresponding to either 1/50, 1/100, or 1/200. The detection of most peptide markers was significantly improved when using a 1/50 or 1/100 compared to a 1/200 trypsin to protein ratio, indicating the latter ratio was too low to provide the complete release of the peptides (Table 1) [Nitride et al., 2019]. This observation did not apply to the peptide markers for almond and peanut, where peptide generation was independent of the trypsin to protein ratio (Table 1). The trypsin to protein ratio had a relatively limited impact on detection of peptides for milk and hazelnut, especially when 1/50 and 1/100 ratios are compared (only four peptides being significantly affected for these two allergens; Table 1). However, a more significant effect was observed for soy and egg peptides. It is noteworthy that this influence is quite different depending on the target allergen (egg or soy). Most of the egg peptides displayed a significant improved detection when using a 1/50 trypsin to protein ratio, while for soy a lower trypsin to protein ratio significantly favored detection of the most intense peptide VLIVPQNFVVAAR^{2+} (Supplementary Data – Figure S9). Based on these results, the 1/100 trypsin to protein ratio was found to be the best compromise, allowing effective protein digestion at a lower cost. To

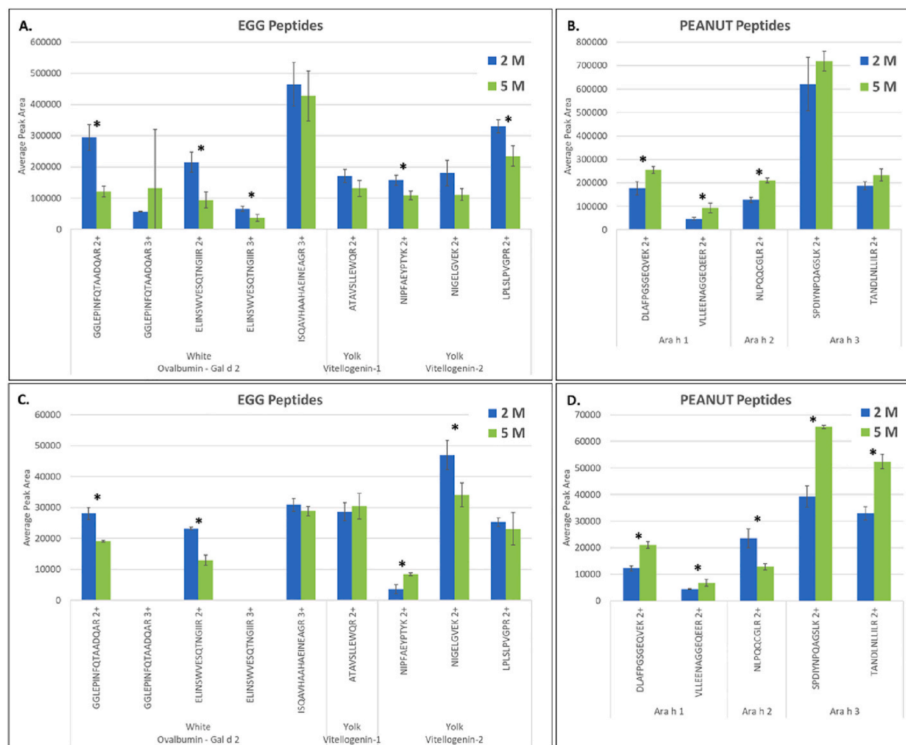

Fig. 3. Comparison of egg and peanut target peptide detection regarding the urea concentration in the extraction buffer (2 M urea blue bars; 5 M urea green bars).
Average peak area ($n = 3$) for egg (A.) and peanut (B.) peptides detected on a Xevo TQ-S (Waters). Average peak area for egg (C.) and peanut (D.) peptides detected on a QSight® 220 TQ (PerkinElmer). * Significantly different based on t-test calculation ($\alpha = 5\%$). Depending on the MS instrument used, some peptides, such as GGLEPINFQTAADQAR and ELINSWVESQTNGIIR, are detected in only one charge state (2+) on QSight® 220 TQ (PerkinElmer), or in two charge states (2+ and 3+) on a Xevo TQ-S (Waters) instrument.

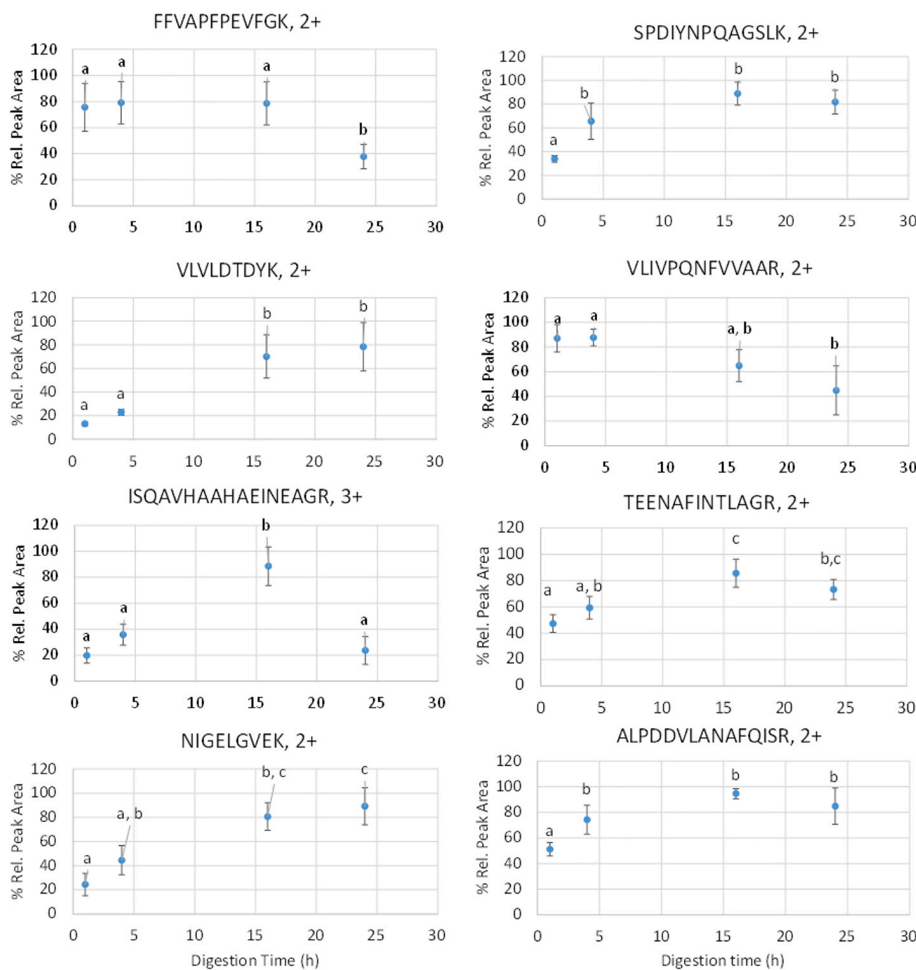

Fig. 4. Kinetics release of the peptide markers.
The experiment was performed on chocolate bar incurred at 40 ppm level. Peak areas were normalized by the highest value recorded in each series. The labels reported (a, b, c, d) represent the results of the Tukey statistical test for multiple comparisons ($n = 3$); equal labels highlight mean values that are not significantly different.

reduce furtherly the analysis costs, the digestion of a lower absolute amount of extract (0.5 mL instead of 1.0 mL) with this 1/100 trypsin to protein ratio was considered and found to still be representative of the sample and sufficient to guarantee the reproducibility of the analysis. These digestion conditions were thus preferred for the final optimized protocol and applied in the following optimization steps, confirming similar protocols already described in the literature [Xiong et al., 2021].

A peptide acting as a reliable quantifier must be fully released. Therefore, the rate of protein digestion was investigated by monitoring peptide release from its constituent protein using a time course experiment [Nitride et al., 2019, Korte & Brockmeyer, 2016]. A single batch of the 40 ppm allergen incurred chocolate sample was extracted with Tris-HCl containing 5 M urea, subjected to SEC protein purification, and aliquoted to carry out independent digestions at fixed trypsin to protein ratio (1/100) and different time course: 1, 4, 16, and 24 h (Fig. 4 and Supplementary Data Figure S10). The effect of digestion time on the measured peak intensity is markedly different depending not only on the specific protein but also on the peptide sequence. For example, the peptides monitored for the soybean allergens *Gly m 5* and *Gly m 6*, or almond allergen *Pru du 6* displayed peculiar kinetics depending on the specific sequence (Fig. 4 and Supplementary Data – Figure S10). This experimental evidence can likely be ascribed to the accessibility of the peptide bonds specifically cleaved by trypsin. For some of the selected markers, the resulting digestion rate was very high, and the peptide appeared to be fully released after only 1 h digestion (see, for example, FFVAPFPEVFGK^{2+} and YLGYLEQLLR^{2+} from α-S1 casein (*Bos d 9*); VLIVPQNFVVAAR^{2+} and SQSDNFEYVSFK^{2+} from soy *Gly m 6*; GNLDFNVQPPR^{2+} and ALPDEVQNAFR^{2+} from almond *Pru du 6*; and ADIYTEQVGR^{2+} and INTVNSNTLPVLR^{2+} from hazelnut *Cor a 9*). However, significantly different trends were observed for several other markers, which required much longer incubations (*e.g.*, 16 h) to achieve maximum release from the intact protein. For example, the peptides NAVPITPTLNR^{2+} and FALPQYLK^{2+} from α-S2 casein, *Bos d 10*; VYVEELKPTEGDLEILLQK^{3+} and VLVLDTDYK^{2+} from β-lactoglobulin *Bos d 5*, GGLEPINFQTAADQAR^{2+} and ISQAVHAAHAEINEAGR^{3+} from ovalbumin *Gal d 2*, VLLEENAGGEQEER^{2+} from peanut *Ara h 1*, NILEASYDTK^{2+} from soybean *Gly m 5*, TEENAFINTLAGR$^{2+/3+}$ and ADIFSPR^{2+} from almond *Pru du 6*, and ALPDDVLANAFQISR^{2+} from hazelnut *Cor a 9* accomplished full release after 16 h of incubation. These results confirm previous data from similar investigations carried out from independent research groups on milk, egg [Nitride et al., 2019], peanut, and tree nuts [Korte et al., 2019]. It is also worth noting that while most of the peptides fully released after 1 h remained stable over an incubation of 16 h, a very limited number of peptides were not stable over this longer digestion duration (16 h) and slightly degraded. This can be observed for VLIVPQNFVVAAR^{2+} from soy *Gly m 6* (Fig. 4), QVQELAFPGSAQDVER^{2+} from soy *Gly m 5*, NLPQQCGLR^{2+} from peanut *Ara h 2*, GNLDFVQPPR$^{2+/3+}$ and ALPDEVLQNAFR^{2+} almond *Pru du 6*, and (Supplementary Data – Figure S10). This must be taken into account in the final peptide selection in the validation, as technically these peptides would lead to underestimation whenever long incubation time are applied. Accordingly, digestion for 16 h was chosen for the final optimized method to ensure that the molar amount of all the peptide markers can be representative of the moles of protein present in the extract (Supplementary Data – Figure S10).

3.2.5. Tryptic peptides purification

Due to the high complexity of the chocolate matrix, a further step of purification has been considered, applied to the peptide pool generating from the trypsin digestion. Solid phase extraction (SPE) with disposable cartridges was tested, with the dual aim of (i) removing polar interfering compounds from the matrix, potentially co-extracted with the target proteins and (ii) concentrating the peptide pool in a smaller volume of solvents suitable for the LC-MS analysis. According to the information available in the literature, two types of SPE columns were compared, namely the polymeric phase Strata-X® [Korte & Brockmeyer, 2016, Korte et al., 2019, Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017] and a classical reverse phase C18 [Huschek et al., 2016, Planque et al., 2016, Monaci et al., 2020]. In general, both formats performed equally well (Table 1 and Supplementary Data – Figure S11). Some exceptions were two peptides from milk casein (HQGLPQEVLNENLLR$^{2+/3+}$ and NAVPITPTLNR^{2+}), one from egg white (ISQAVHAAHAEINEAGR^{3+}), and one from soy *Gly m 6* (ISTLNSLTLPALR^{2+}) which showed a significant improvement with the Strata-X SPE. In contrast, the signal intensity was significantly improved for only one peptide from hazelnut (ALPDDVLANAFQISR^{2+}) and another from soy *Gly m 5* (VPSGTTYYVVNPDNNENLR^{2+}) when using the C18 SPE column. Although both SPE formats could be used (since they lead to very similar results), it was decided to use Strata-X SPE cartridges for the final optimized protocol.

After tryptic peptide purification on the SPE column, the solvent was evaporated to allow peptide concentration. Noteworthy, solvent evaporation to dryness might result in a partial loss of peptides due to their adsorption on the vial walls, therefore, it is recommended to use low-binding vials. An option to prevent this effect, might be to add the SPE eluate with a small volume of DMSO, which does not evaporate under these conditions and so avoids complete dryness of the peptides pool. This option was tested and compared with the complete solvent evaporation, and as a fact most of the detected peptide markers (43/47) did not show any significant impact on their detection when DMSO is present (Supplementary Data – Figure S12), while four peptides (LPLSLPVGPR^{2+} from egg yolk protein, TANDLNLLILR^{2+} from peanut, ALPDDVLANAFQISR^{2+} from hazelnut, and ISTLNSLTLPALR^{2+} from soy *Gly m 6*) (Supplementary Data – Figure S13) were even significantly better detected when evaporation was performed in the absence of DMSO. Given this results, the use of DMSO was not included during the evaporation step in the final optimized method even though this solvent may facilitate crude extract solubilization.

3.3. Summary of the sample preparation workflow and final considerations

The optimization of the sample preparation workflow for the detection of six allergenic ingredients incurred in a hard-to-analyze food matrix, such as chocolate bar, by tandem mass spectrometry has been herein described. This investigation took advantage of previous knowledge on the analysis of such a complex matrix testing and comparing solutions proposed by several independent groups in analogous case studies. Each individual step of this protocol has been thoroughly optimized and tuned in order to deliver a final method with high sensitivity and reliability, with minimized interference from the matrix itself. As described and summarized in Fig. 5 (main optimized parameters being highlighted in bold), the developed protocol employs an *ad hoc* step for the chocolate pre-treatment and homogenization, by grinding with short repeated cycles, with temperature under control. Subsequently, proteins are extracted with tris buffer, added with a 5M urea to maximize the extraction efficiency from the incurred chocolate and potentially provide the same efficiency also in other processed food commodities. Then, the protein extract is purified on SEC disposable cartridge, and subjected to specific proteolytic digestion with trypsin added at a 1/100 (w/w) enzyme to protein ratio for 16 h. A final clean-up step, including the purification of tryptic peptides on SPE cartridge and their concentration, is applied to improve the sensitivity of the final method under development.

4. Conclusions

The paper describes the development of an analytical protocol for the extraction and quantification of six allergens in a complex food like chocolate by using low resolution mass spectrometry. In the present study, different parameters influencing protein and peptide recoveries were investigated and duly optimized in order to maximize the detection

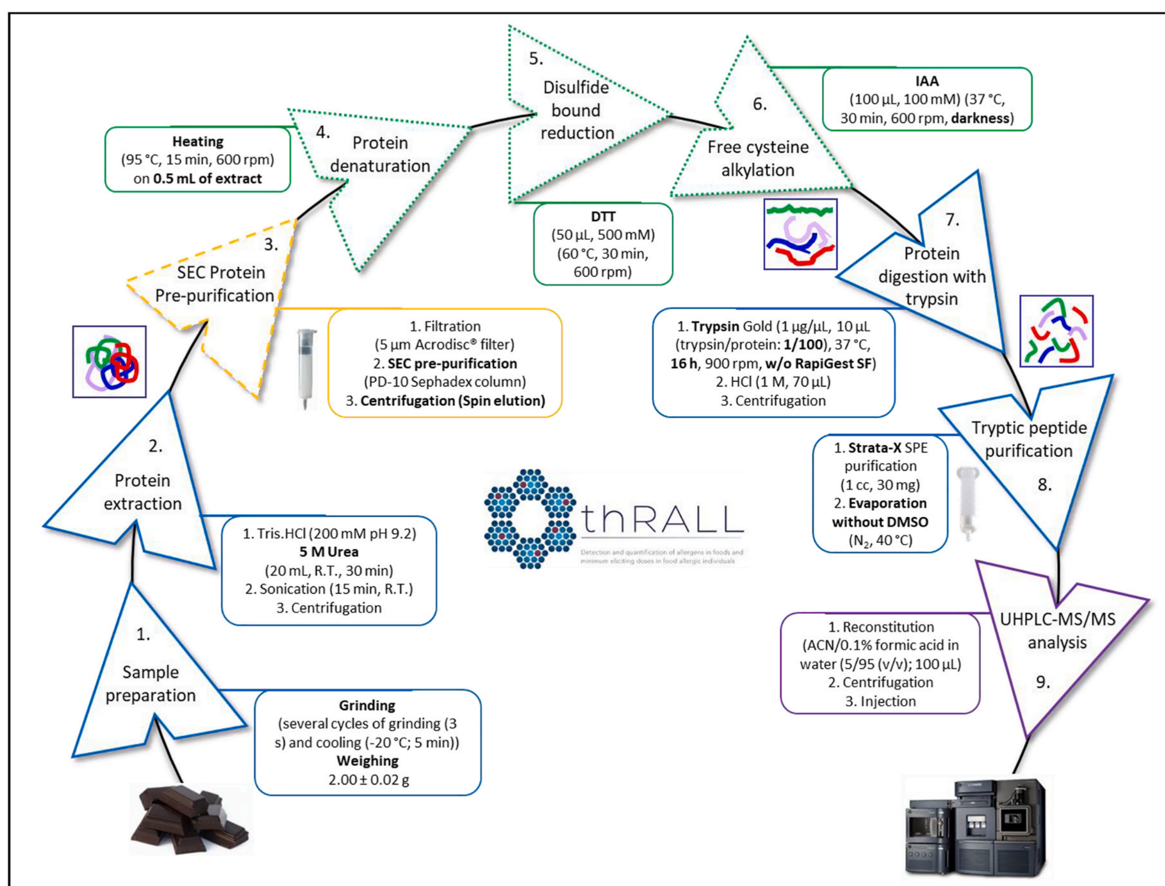

Fig. 5. Optimized harmonized reference protocol for use in sample preparation for multi-allergen detection by UHPLC-MS/MS analysis.
This protocol was applied and optimized with chocolate bar incurred at 40 ppm concentration level in milk, egg, soy, peanut, almond, and hazelnut, and chosen as the reference matrix (courtesy of Waters).

sensitivity. Fifty peptide markers tracing for the six allergenic ingredients have been validated in two independent laboratories, irrespective of the instrumental set-up (MS-platforms and chromatographic conditions) and operators involved, thus assessing the robustness of the method under development.

Work is currently in progress to validate the method not only intra-laboratory but also at inter-laboratory scale to have more insights on the analytical performance as prototype reference method for quantitative analysis.

CRediT authorship contribution statement

Jean Henrottin: Conceptualization, Formal analysis, Writing – original draft. **Rosa Pilolli:** Conceptualization, Formal analysis, Data curation, Writing – original draft. **Anne-Catherine Huet:** Writing – review & editing. **Christof van Poucke:** Writing – review & editing. **Chiara Nitride:** Writing – review & editing. **Marc De Loose:** Writing – review & editing. **Olivier Tranquet:** Writing – review & editing. **Colette Larré:** Writing – review & editing. **Karine Adel-Patient:** Writing – review & editing. **Hervé Bernard:** Writing – review & editing. **E.N. Clare Mills:** Writing – review & editing, Project administration. **Nathalie Gillard:** Conceptualization, Writing – review & editing. **Linda Monaci:** Conceptualization, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2022.109256.

References

Alves, R. C., Pimentel, F. B., Nouws, H. P. A., Silva, T. H. B., Oliveira, M. B. P. P., & Delerue-Matos, C. (2017). Improving the extraction of Ara h 6 (a peanut allergen) from a chocolate-based matrix for immunosensing detection: Influence of time, temperature and additives. *Food Chemistry, 218*, 242–248. https://doi.org/10.1016/j.foodchem.2016.09.085

Bignardi, C., Mattarozzi, M., Penna, A., Sidoli, S., Elviri, L., Careri, M., & Mangia, A. (2013). A rapid size-exclusion solid-phase extraction step for enhanced sensitivity in multi-allergen determination in dark chocolate and biscuits by liquid

chromatography–tandem mass spectrometry. *Food Analytical Methods, 6*, 1144–1152. https://doi.org/10.1007/s12161-012-9521-4

Boo, C. C., Parker, C. H., & Jackson, L. S. (2018). A targeted LC-MS/MS method for the simultaneous detection and quantitation of egg, milk, and peanut allergens in sugar cookies. *Journal of AOAC International, 101*, 108–117. https://doi.org/10.5740/jaoacint.17-0400

Croote, D., Braslavsky, I., & Quake, S. R. (2019). Addressing complex matrix interference improves multiplex food allergen detection by targeted LC−MS/MS. *Analytical Chemistry, 91*, 9760–9769. https://doi.org/10.1021/acs.analchem.9b01388

De Angelis, E., Pilolli, R., & Monaci, L. (2017). Coupling SPE on-line pre-enrichment with HPLC separation and MS/MS detection for the sensitive detection of allergens in wine. *Food Control, 73*, 814–820. https://doi.org/10.1016/j.foodcont.2016.09.031

DunnGalvin, A., Chan, C. H., Crevel, R., Grimshaw, K., Poms, R., Schnadt, S., Taylor, S. L., Turner, P., Allen, K. J., Austin, M., Baka, A., Baumert, J. L., Baumgartner, S., Beyer, K., Bucchini, L., Fernandez-Rivas, M., Grinter, K., Houben, G. F., Hourihane, J., … Roberts, G. (2015). Precautionary allergen labelling: Perspectives from key stakeholder groups. *Allergy, 70*. https://doi.org/10.1111/all.12614, 1369-1051.

DunnGalvin, A., Roberts, G., Regent, L., Austin, M., Kenna, F., Schnadt, S., Sanchez-Sanz, A., Hernandez, P., Hjorth, B., Fernandez-Rivas, M., Taylor, S., Baumert, J., Sheikh, A., Astley, S., Crevel, R., & Mills, C. (2019). Understanding how consumers with food allergies make decisions based on precautionary labelling. *Clinical and Experimental Allergy, 49*, 1446–1454. https://doi.org/10.1111/cea.134793

European Commission. (2011). Regulation (EU) No. 1169/2011 of the European parliament and of the council of 25 October 2011, on the provision of food information to consumers. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32011R1169.

FAO/WHO Food Standards. (2018). Codex Alimentarius. General standard for the labelling of prepackaged foods. *Codex Stan*, 1–1985. Rev. 2018.

Gavage, M., Van Vlierberghe, K., Van Poucke, C., De Loose, M., Gevaert, K., Dieu, M., Renard, P., Arnould, T., Filée, P., & Gillard, N. (2020). Comparative study of concatemer efficiency as an isotope-labelled internal standard for allergen quantification. *Food Chemistry, 332*, Article 127413. https://doi.org/10.1016/j.foodchem.2020.127413

Gu, S., Chen, N., Zhou, Y., Zhao, C., Zhan, L., Qu, L., Cao, C., Han, L., Deng, X., Ding, T., Song, C., & Ding, Y. (2018). A rapid solid-phase extraction combined with liquid chromatography-tandem mass spectrometry for simultaneous screening of multiple allergens in chocolates. *Food Control, 84*, 89–96. https://doi.org/10.1016/j.foodcont.2017.07.033

Hands, C. M., Sayers, R. L., Nitride, C., Gethings, L. A., & Mills, E. N. C. (2020). A multiple reaction monitoring method for determining peanut (Arachis hypogea) allergens in serum using quadrupole and time-of-flight mass spectrometry. *Analytical and Bioanalytical Chemistry, 412*(12), 2815–2827. https://doi.org/10.1007/s00216-020-02508-9

Heick, J., Fischer, M., & Pöpping, B. (2011). First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *Journal of Chromatography A, 1218*, 938–943. https://doi:10.1016/j.chroma.2010.12.067.

Henrottin, J., Planque, M., Huet, A. C., Marega, R., Lamote, A., & Gillard, N. (2019). Gluten analysis in processed foodstuffs by a multi-allergens and grain-specific UHPLC-MS/MS method: One method to detect them all. *Journal of AOAC International, 102*(5), 1286–1302. https://doi.org/10.5740/jaoacint.19-0057

Hoffmann, B., Münch, S., Schwägele, F., Neusüß, C., & Jira, W. (2017). A sensitive HPLCMS/MS screening method for the simultaneous detection of lupine, pea, and soy proteins in meat products. *Food Control, 71*, 200–209. https://doi.org/10.1016/j.foodcont.2016.06.021

Holzhauser, T., Wackermann, O., Ballmer-Weber, B. K., Bindslev-Jensen, C., Scibilia, J., Perono-Garoffo, L., Utsumi, S., Poulsen, L. K., & Vieths, S. (2009). Soybean (Glycine max) allergy in Europe: Gly m 5 (b-conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *The Journal of Allergy and Clinical Immunology, 123*(2), 452–458. https://doi.org/10.1016/j.jaci.2008.09.034

Huet, A. C., Paulus, M., Henrottin, J., Brossard, C., Tranquet, O., Bernard, H., Pilolli, R., Nitride, C., Larré, C., Adel-Patient, K., Monaci, L., Mills, E. N. C., De Loose, M., Gillard, N., & Van Poucke, C. (2022). Development of incurred chocolate bars and broth powder with 6 fully characterised food allergens as test materials for food allergen analysis. *Analytical and Bioanalytical Chemistry, 414*, 2553–2570. https://doi.org/10.1007/s00216-022-03912-z

Huschek, G., Bönick, J., Löwenstein, Y., Sievers, S., & Rawel, H. (2016). Quantification of allergenic plant traces in baked products by targeted proteomics using isotope marked peptides. *LWT–Food Science and Technology, 74*, 286–293. https://doi.org/10.1016/j.lwt.2016.07.057

Ito, K., Sjölander, S., Sato, S., Moverare, R., Tanaka, A., Söderström, L., Borres, M., Poorafshar, M., & Ebisawa, M. (2011). IgE to Gly m 5 and Gly m 6 is associated with severe allergic reactions to soybean in Japanese children. *The Journal of Allergy and Clinical Immunology, 128*(3), 673–675. https://doi.org/10.1016/j.jaci.2011.04.025

Johnson, P. E., Sayers, R. L., Gethings, L. A., Balasundaram, A., Marsh, J. T., Langridge, J. I., & Mills, E. N. C. (2016). Quantitative proteomic profiling of peanut allergens in food ingredients used for oral food challenges. *Analytical Chemistry, 88*, 5689–5695. https://doi.org/10.1021/acs.analchem.5b04466

Khuda, S. E., Jackson, L. S., Fu, T. J., & Williams, K. M. (2015). Effects of processing on the recovery of food allergens from a model dark chocolate matrix. *Food Chemistry, 168*, 580–587. https://doi.org/10.1016/j.foodchem.2014.07.084

Korte, R., & Brockmeyer, J. (2016). MRM3-based LC-MS multi-method for the detection and quantification of nut allergens. *Analytical and Bioanalytical Chemistry, 408*, 7845–7855. https://doi.org/10.1007/s00216-016-9888-y

Korte, R., Oberleitner, D., & Brockmeyer, J. (2019). Determination of food allergens by LC-MS: Impacts of sample preparation, food matrix, and thermal processing on peptide detectability and quantification. *Journal of Proteomics, 196*, 131–140. https://doi.org/10.1016/j.jprot.2018.11.002

Martinez-Esteso, M. J., O'Connor, G., Nørgaard, J., Breidbach, A., Brohée, M., Cubero-Leon, E., Nitride, C., Robouch, P., & Emons, H. (2020). A reference method for determining the total allergenic protein content in a processed food: The case of milk in cookies as proof of concept. *Analytical and Bioanalytical Chemistry, 412*, 8249–8267. https://doi.org/10.1007/s00216-020-02959-0

Mattarozzi, M., & Careri, M. (2019). The role of incurred materials in method development and validation to account for food processing effects in food allergen analysis. *Analytical and Bioanalytical Chemistry, 411*, 4465–4480. https://doi.org/10.1007/s00216-019-01642-3

Mattarozzi, M., Milioli, M., Bignardi, C., Elviri, L., Corradini, C., & Careri, M. (2014). Investigation of different sample pre-treatment routes for liquid chromatography-tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine. *Food Control, 38*, 82–87. https://doi.org/10.1016/j.foodcont.2013.10.015

Mills, E. N. C., Adel-Patient, K., Bernard, H., De Loose, M., Gillard, N., Huet, A. C., Larré, C., Nitride, C., Pilolli, R., Van Poucke, C., & Monaci, L. (2019). Detection and quantification of allergens in foods and minimum eliciting doses in food-allergic individuals (ThRAll). *Journal of AOAC International, 102*(5), 1346–1353. https://doi.org/10.5740/jaoacint.19-0063

Monaci, L., De Angelis, E., Guagnano, R., Ganci, A. P., Garaguso, I., Fiocchi, A., & Pilolli, R. (2020). Validation of a MS Based Proteomics Method for Milk and Egg Quantification in Cookies at the Lowest VITAL Levels: An Alternative to the Use of Precautionary Labeling. *Foods, 9*, 1489. https://doi.org/10.3390/foods9101489.

Monaci, L., De Angelis, E., Montemurro, N., & Pilolli, R. (2018). Comprehensive overview and recent advances in proteomics MS based methods for food allergens analysis. *Trends in Analytical Chemistry, 106*, 21–36. https://doi.org/10.1016/j.trac.2018.06.016

Monaci, L., Losito, I., De Angelis, E., Pilolli, R., & Visconti, A. (2013). Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry. *Rapid Communications in Mass Spectrometry, 27*. https://doi.org/10.1002/rcm.6662, 2009–2018.

Monaci, L., Pilolli, R., De Angelis, E., Godula, M., & Visconti, A. (2014). Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. *Journal of Chromatography A, 1358*, 136–144. https://doi.org/10.1016/j.chroma.2014.06.092

Monaci, L., & van Hengel, A. J. (2008). Development of a method for the quantification of whey allergen traces in mixed-fruit juices based on liquid chromatography with mass spectrometric detection. *Journal of Chromatography A, 1192*, 113–120. https://doi.org/10.1016/j.chroma.2008.03.041

New, L. S., Schreiber, A., Stahl-Zeng, J., & Liu, H.-F. (2018). Simultaneous analysis of multiple allergens in food products by LC-MS/MS. *Journal of AOAC International, 101*, 132–145. https://doi.org/10.5740/jaoacint.17-0403

New, L. S., Stahl-Zeng, J., Schreiber, A., Cafazzo, M., Liu, A., Brunelle, S., & Liu, H.-F. (2020). Detection and quantitation of selected food allergens by liquid chromatography with tandem mass spectrometry: First action 2017.17. *Journal of AOAC International, 103*, 570–583. https://doi.org/10.5740/jaoacint.19-0112

Nitride, C., Nørgaard, J., Omar, J., Emons, H., Esteso, M. J. M., & O'Connor, G. (2019). An assessment of the impact of extraction and digestion protocols on multiplexed targeted protein quantification by mass spectrometry for egg and milk allergens. *Analytical and Bioanalytical Chemistry, 411*, 3463–3475. https://doi.org/10.1007/s00216-019-01816-z

Parker, C. H., Khuda, S. E., Pereira, M., Ross, M. M., Fu, T. J., Fan, X., Wu, Y., Williams, K. M., De Vries, J., Pulvermacher, B., Bedford, B., Zhang, X., & Jackson, L. S. (2015). Multi-allergen quantitation and the impact of thermal treatment in industry processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 63*, 10669–10680. https://doi.org/10.1021/acs.jafc.5b04287

Pilolli, R., Chaudhari, R., Palmisano, F., & Monaci, L. (2017). Development of a mass spectrometry immunoassay for unambiguous detection of egg allergen traces in wines. *Analytical and Bioanalytical Chemistry, 409*, 1581–1589. https://doi.org/10.1007/s00216-016-0099-3

Pilolli, R., De Angelis, E., & Monaci, L. (2017). Streamlining the analytical workflow for multiplex MS/MS allergen detection in processed foods. *Food Chemistry, 221*, 1747–1753. https://doi.org/10.1016/j.foodchem.2016.10.110

Pilolli, R., De Angelis, E., & Monaci, L. (2018). In-house validation of a high resolution mass spectrometry Orbitrap-based method for multiple allergen detection in a processed model food. *Analytical and Bioanalytical Chemistry, 410*, 5653–5662. https://doi.org/10.1007/s00216-018-0927-8

Pilolli, R., Nitride, C., Gillard, N., Huet, A. C., Van Poucke, C., De Loose, M., Tranquet, O., Larré, C., Adel-Patient, K., Bernard, H., Mills, E. N. C., & Monaci, L. (2020). Critical review on proteotypic peptide marker tracing for six allergenic ingredients in incurred foods by mass spectrometry. *Food Research International, 128*. https://doi.org/10.1016/j.foodres.2019.108747. Article 108747.

Pilolli, R., Van Poucke, C., De Angelis, E., Nitride, C., De Loose, M., Gillard, N., Huet, A. C., Tranquet, O., Larré, C., Adel-Patient, K., Bernard, H., Mills, E. N. C., & Monaci, L. (2021). Discovery based high resolution MS/MS analysis for selection of allergen markers in chocolate and broth powder matrices. *Food Chemistry, 343*. https://doi.org/10.1016/j.foodchem.2020.128533. Article 128533.

Planque, M., Arnould, T., Delahaut, P., Renard, P., Dieu, M., & Gillard, N. (2019). Development of a strategy for the quantification of food allergens in several food products by mass spectrometry in a routine laboratory. *Food Chemistry, 274*, 35–45. https://doi.org/10.1016/j.foodchem.2018.08.095

Planque, M., Arnould, T., Dieu, M., Delahaut, P., Renard, P., & Gillard, N. (2016). Advances in ultra high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and

processed foodstuffs. *Journal of Chromatography A, 1464*, 115–123. https://doi.org/10.1016/j.chroma.2016.08.033

Planque, M., Arnould, T., Dieu, M., Delahaut, P., Renard, P., & Gillard, N. (2017). Liquid chromatography coupled to tandem mass spectrometry for detecting ten allergens in complex and incurred foodstuffs. *Journal of Chromatography A, 1530*, 138–151. https://doi:10.1016/j.chroma.2017.11.039.

Planque, M., Arnould, T., & Gillard, N. (2017). In S. S. Athari (Ed.), *Food allergen analysis: Detection, quantification and validation by mass spectrometry*. London: IntechOpen. https://doi.org/10.5772/intechopen.69361. *Allergen* (pp. 7–41.

Remington, B. C., Westerhout, J., Meima, M. Y., Blom, W. M., Kruizinga, A. G., Wheeler, M. W., Taylor, S. L., Houben, G. F., & Baumert, J. L. (2020). Updated population minimal eliciting dose distributions for use in risk assessment of 14 priority food allergens. *Food and Chemical Toxicology, 139*, Article 111259. https://doi.org/10.1016/j.fct.2020.111259

Sagu, S. T., Huschek, G., Homann, T., & Rawel, H. M. (2021). Effect of sample preparation on the detection and quantification of selected nuts allergenic proteins by LC-MS/MS. *Molecules, 26*, 4698. https://doi.org/10.3390/molecules26154698

Sayers, R. L., Gethings, L. A., Lee, V., Balasundaram, A., Johnson, P. E., Marsh, J. A., Wallace, A., Brown, H., Rogers, A., Langridge, J. I., & Mills, E. N. C. (2018). Microfluidic separation coupled to mass spectrometry for quantification of peanut allergens in a complex food matrix. *Journal of Proteome Research, 17*, 647–655. https://doi.org/10.1021/acs.jproteome.7b00714

Sayers, R. L., Johnson, P. E., Marsh, J. T., Barran, P., Brown, H., & Mills, E. N. C. (2016). The effect of thermal processing on the behavior of peanut allergen peptide targets used in multiple reaction monitoring mass spectrometry experiments. *The Analyst, 141*(13), 4130–4141. https://doi.org/10.1039/C6AN00359A

Shefcheck, K. J., Callahan, J. H., & Musser, S. M. (2006). Confirmation of peanut protein using peptide markers in dark chocolate using liquid Chromatography−Tandem mass spectrometry (LC-MS/MS). *Journal of Agricultural and Food Chemistry, 54*, 7953–7959. https://doi.org/10.1021/jf060714e

Taylor, S. L., Baumert, J. L., Kruizinga, A. G., Remington, B. C., Crevel, R. W. R., Brooke-Taylor, S., & Allen, K. J. (2014). The allergen bureau of Australia & New Zealand, & houben, Establishment of reference doses for residues of allergenic foods: Report of the VITAL Expert panel. *Food and Chemical Toxicology, 63*, 9–17. https://doi.org/10.1016/j.fct.2013.10.032

World Health Organization—International Food Safety Authorities Network (INFOSAN), INFOSAN information note No. 3/2006 – food allergies.

Xiong, W., Parker, C. H., Boo, C. C., & Fiedler, K. L. (2021). Comparison of allergen quantification strategies for egg, milk, and peanut in food using targeted LC-MS/MS. *Analytical and Bioanalytical Chemistry, 413*, 5755–5766. https://doi.org/10.1007/s00216-021-03550-x