



**HAL**  
open science

## 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, Rosa Pilolli, Anne-Catherine Huet, Christof van Poucke, Chiara Nitride, Marc de Loose, Olivier Tranquet, Colette Larré, Karine Adel-Patient, Hervé Bernard, et al.

### ► To cite this version:

Jean Henrottin, Rosa Pilolli, Anne-Catherine Huet, Christof van Poucke, Chiara Nitride, et al.. Optimization of a sample preparation workflow based on UHPLC-MS/MS method for multi-allergen detection in chocolate: An outcome of the ThRAll project. *Food Control*, 2023, 143, pp.109256. 10.1016/j.foodcont.2022.109256 . hal-03761018

**HAL Id: hal-03761018**

**<https://hal.inrae.fr/hal-03761018v1>**

Submitted on 15 Jan 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Optimization of a sample preparation workflow based on UHPLC-MS/MS method for**  
2 **multi-allergen detection in chocolate: an outcome of the ThRAI project**

3

4

5 Jean Henrottin<sup>a</sup>, Rosa Pilolli<sup>b</sup>, Anne-Catherine Huet<sup>a</sup>, Christof van Poucke<sup>c</sup>, Chiara Nitride<sup>d</sup>,  
6 Marc De Loose<sup>c</sup>, Olivier Tranquet<sup>e,f</sup>, Colette Larré<sup>e</sup>, Karine Adel-Patient<sup>g</sup>, Hervé Bernard<sup>g</sup>,  
7 E.N. Clare Mills<sup>d</sup>, Nathalie Gillard<sup>a,\*</sup>, and Linda Monaci<sup>b,\*</sup>.

8

9 <sup>a</sup> CER Groupe, Rue du point du Jour, 8, 6900 Marloie, Belgium.

10 <sup>b</sup> Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR),  
11 Via Giovanni Amendola 122/O, 70126 Bari, Italy.

12 <sup>c</sup> Flanders Research Institute for Agriculture, Fisheries and Food, Brusselsesteenweg 370,  
13 9090 Melle, Belgium.

14 <sup>d</sup> School of Biological Sciences, Division of Infection, Immunity and Respiratory Medicine,  
15 Manchester Academic Health Science Centre, Manchester Institute of Biotechnology,  
16 University of Manchester, UK.

17 <sup>e</sup> UR1268 BIA, Rue de la Géraudière, BP 71627, 44316 Nantes, France.

18 <sup>f</sup> INRAE, Aix Marseille University, Biodiversité Et Biotechnologie Fongiques  
19 (BBF), UMR1163, 13009 Marseille, France.

20 <sup>g</sup> Université Paris-Saclay, CEA, INRAE ; UMR MTS, Service de Pharmacologie et  
21 d'Immunoanalyse, Laboratoire d'Immuno-Allergie Alimentaire, F-91191 Gif-sur-Yvette,  
22 France.

23

24 \* Corresponding authors: [n.gillard@cergroupe.be](mailto:n.gillard@cergroupe.be), and [linda.monaci@ispa.cnr.it](mailto:linda.monaci@ispa.cnr.it).

**25 Abstract**

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

33 This investigation carried out within an international consortium specifically built up to  
34 convey a prototype MS based reference method, reports on the first part of the method  
35 development, namely the optimization of the sample preparation protocol for six allergens  
36 detection (cow's milk, hen's egg, soy, peanut, hazelnut, and almond) in chocolate. The latter  
37 was chosen as model complex food matrix, having a high lipid and polyphenol content.  
38 Different steps of the sample preparation protocol have been taken into consideration: (i)  
39 sampling, (ii) composition of the extraction buffer, (iii) protein purification, (iv) protein  
40 enzymatic digestion, (v) peptide purification and pre-concentration, and some experiments  
41 were carried out by two independent laboratories and two different MS platforms to provide a  
42 first assessment of the robustness of the method under development. Fifty target peptides  
43 were monitored in multiple reaction monitoring mode and validated in different laboratories  
44 to trace the six allergenic ingredients in the incurred chocolate and the best performing  
45 protocol for sample preparation was identified. This work paves the way of the forthcoming  
46 full analytical validation of a prototype reference method for MS-based allergen  
47 quantification.

48

49 **Keywords:** food allergen detection, sample preparation, reference method, mass  
50 spectrometry, ThRAI.

51

## 52 **1. Introduction**

53 Food allergens are responsible for food product recalls and incidents of fatal or  
54 severe allergic reactions globally representing a management issue for food business  
55 operators [WHO 2006 INFOSAN; Remington 2020]. Since the only effective treatment for  
56 food-allergic individuals is a strict long-life allergen-free diet, worldwide regulations have  
57 been implemented for foodstuff labeling [Henrottin 2019, Remington 2020]. A list of  
58 ‘priority’ allergenic foods, which prevalently responsible for allergic reaction in the  
59 population and can be responsible for severe symptoms, to be declared on food labels, has  
60 been published by the Codex Alimentarius Commission [Codex STAN] and it is current  
61 being reviewed by a panel of *ad hoc* Joint FAO/WHO Expert Consultation on Risk  
62 Assessment of Food Allergens. In the European Union (EU), the original Codex list has been  
63 expanded and now includes 14 different allergenic foods (Annex II of the European  
64 Regulation (EU) No 1169/2011).

65 While mandatory allergen labeling supports allergic consumers in making  
66 informed decisions about the foods they can eat, confusion remains about the meaning of  
67 voluntary precautionary allergen labels (PAL; e.g., ‘may contain...’ or ‘may contain traces  
68 of...’). PAL warns of the potential presence of unintended allergens, resulting from cross-  
69 contamination of raw materials and/or finished foods [DunnGalvin 2019]. Analytical  
70 methods (including namely ELISA-, PCR-, and MS-based methods) support food operators  
71 in implementing the allergen risk assessment evaluations and confirm whether a  
72 contamination has happened. However, PAL has often been applied in response to potential  
73 unintended allergen presence, with no risk assessment performed as a protective measure by

74 food operators. This inconsistent application of PAL has led to a loss of trust in allergic  
75 consumers, which do not fully understand their purpose [DunnGalvin 2019, DunnGalvin  
76 2015]. Irrespective of whether the allergen risk assessment is performed appropriately or  
77 comprehensively, the lack of a mandatory threshold reflecting clinical reactivity makes the  
78 decision for PAL difficult. However, the accumulation of clinical data would make the  
79 establishment of mandatory thresholds possible. Such thresholds have already been set at 10  
80 mg of total ingredient protein /kg in Japan, and the VITAL initiative takes into account  
81 clinical data to extrapolate thresholds currently implemented in Australia and New-Zealand  
82 to support the PAL [Taylor 2014].

83           Accurate and reliable methodologies enabling the detection and quantitation of  
84 allergen traces in foodstuffs are urgently needed to support the risk assessment. Mass  
85 Spectrometry (MS) is one of the most promising techniques that proved to be successfully  
86 applied to allergen detection, identification, quantification, and characterization for over a  
87 decade now, and has much promise as a reference method for food allergen analysis [Monaci  
88 2008, 2013, 2014; Heick 2011; Parker 2015; De Angelis 2017; Boo 2018; Pilolli 2017a,  
89 2017b, 2018; Nitride 2019; Sayers 2016, 2018; Planque 2016-2017-2017bis-2019; Henrottin  
90 2019; Gavage 2020; Hands 2020]. A recent review of the methods published in this area  
91 compared different aspects of food allergen quantification using advanced MS techniques,  
92 highlighting the main gaps that need to be addressed in terms of harmonization and results  
93 comparability across independent laboratories [Monaci 2018].

94           In this context, the European Food Safety Agency (EFSA) has funded the  
95 ThRAIl (Thresholds and Reference method for Allergen detection method) project aiming at  
96 the '*Detection and quantification of allergens in foods and minimum eliciting doses in food*  
97 *allergic individuals*'. The project focused on the development of a harmonized and  
98 quantitative MS-based reference method for the simultaneous detection and quantification of

99 six food allergens in standardized incurred food matrices by multiple reaction monitoring  
100 (MRM) acquisition mode [Mills 2019]. The target allergens within the project include two  
101 animal-derived food allergens (cow's milk and hen's eggs) and four plant food allergens (soy,  
102 peanut, hazelnut, and almond), all of which are included in Annex II of EU Regulation No  
103 1169/2011 [EU Regulation – 1169-2011]. A multi-analyte method is being developed to  
104 determine all these allergens in two model and standardized incurred food matrices, namely  
105 chocolate and broth powder [Huet 2022], which are very challenging matrices for analysis.  
106 Previous studies aiming at developing multiplex methods for allergen analysis used milk  
107 chocolate and dark chocolate as model matrices [Shefcheck 2006, New 2018, Planque 2016],  
108 but recoveries of allergenic marker peptides were found to be low and not satisfactory,  
109 highlighting the need for optimization of extraction and digestion approaches for challenging  
110 matrices where proteins may be bound to polyphenols and tannins [New 2020]. Both matrices  
111 were produced within the ThRAAll project in a food pilot plant after careful characterization of  
112 the allergenic ingredients [Huet 2022].

113           Under this frame, the present work addresses the development of a prototype  
114 reference method as commissioned by EFSA and focus on the optimization of a reliable,  
115 straightforward, reproducible, and harmonized sample preparation protocol for multi-allergen  
116 detection in processed foodstuffs by MS analysis. This report describes the optimization of a  
117 multiplex MS method including 50 marker peptides (from 5 to 13 specific peptides [and from  
118 1 to 3 proteins] for each allergenic ingredient). The UHPLC-MS/MS analyses were carried  
119 out in two independent laboratories using different triple quadrupole LC-MS platforms to  
120 confirm the robustness and reliability of the protocol developed herein. Since the detection  
121 of the peptides depends of the MS platform used, the MS parameters of each peptide  
122 transition have to be duly optimized on the MS platform to be used to maximize the detection  
123 of these peptides. Typical method transfer procedures were also described herein, allowing to

124 easily and universally transpose this harmonized multi-allergen MS-based detection method  
125 to various MS platforms. Besides MS parameters, sample preparation workflow was also  
126 duly optimized, including: composition of the extraction buffer (also including denaturation  
127 agents), inclusion of technical aids for protein and peptide purification (*i.e.* several stationary  
128 phases), and optimization of the tryptic digestion (incubation time, enzyme to protein ratio,  
129 addition of chemical aids to improve proteolytic activity (*e.g.*, RapiGest SF)). The impact of  
130 each parameter/modification on the signature peptide detection was investigated in incurred  
131 chocolate (as an example of complex food matrix) according to a systematic approach to  
132 achieve the best response for the selected markers tracing for the six allergens under analysis.  
133 This will provide a solid foundation to base a viable reference MS method for food allergens  
134 detection.

135

## 136 **2. Materials and Methods**

### 137 **2.1. Materials**

138 Tris(hydroxymethyl)aminomethane (Tris), urea, dimethyl sulfoxide (DMSO),  
139 DL-dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate (ABB) were from  
140 Sigma-Aldrich (Bornem, Belgium). Trypsin Gold (mass spectrometry grade) was from  
141 Promega (V5280; Leiden, The Netherlands). Acetic acid was from Acros Organics (Geel,  
142 Belgium), hydrochloric acid was from Fisher Chemical (Loughborough, UK), and RapiGest  
143 SF was from Waters (Milford, Massachusetts, USA). Acetonitrile, water, methanol (ULC-MS  
144 grade), and formic acid were obtained from Biosolve (Valkenswaard, The Netherlands).  
145 Acrodisc® syringe filter with Versapor® membrane (PALL laboratory; 5 µm, 25 mm) was  
146 obtained from VWR (Leuven, Belgium; #28143-963). PD-10 desalting columns pre-packed  
147 with Sephadex G-25 M resin used for protein extract pre-purification were purchased from  
148 Cytiva (GE Healthcare, Hoegaarden, Belgium; #17085101). Sep-Pak C18 solid phase

149 extraction (SPE) columns (1 cc, 50 mg; WAT054955) and Strata-X polymeric reversed phase  
150 (33  $\mu\text{m}$ ; 30 mg; 1 mL; 8B-S100-TAK) were purchased from Waters (Milford, Massachusetts,  
151 USA) and Phenomenex (Torrance, California, USA), respectively, and used for peptide  
152 purification and enrichment.

153

## 154 **2.2. Model food matrix**

155 The model food matrix used for the optimization of the parameters was an  
156 incurred chocolate bar prepared within the ThRAll project, in a food pilot plant in order to  
157 mimic real production process [Huet 2022]. Briefly, chocolate refiner flakes were weighed  
158 and an appropriate amount of each targeted allergenic ingredient (cow's milk, hen's egg,  
159 peanut flour (lightly roasted and partially defatted powder), full fat soy flour (non-toasted),  
160 hazelnut flour (not roasted), and almond flour (blanched)) was added and carefully mixed by  
161 vigorously shaking. The mixture was applied several times to a three-roll mill (Exakt 80E) to  
162 obtain a mixture with equal particle (final particle size of 20  $\mu\text{m}$ ). The obtained pre-mix of  
163 chocolate refiner flakes was further diluted with blank chocolate refiner flakes. These  
164 chocolate refiner flakes were melted in a dry heat chocolate melter (Mol D'Art) at 40°C to  
165 45°C. Once the refiner flakes were liquid, 200 g of cocoa butter were added and gently  
166 mixed. Ammonium phosphatide (20 g), used as emulsifier, was added and mixed until a  
167 glazy mass was obtained. The liquid chocolate was then transferred to a pastry bag and was  
168 dripped into pellets of about five grams. Pellets were kept overnight at 4°C, packed in sealed  
169 aluminum laminate and finally stored at 4°C. The chosen contamination level for method  
170 development was 40 mg total protein of each allergenic ingredient/ kg of chocolate bar (40  
171 ppm).

172

## 173 **2.3. Multi-allergen UHPLC-MS/MS analysis parameters**



## 174 2.3.1. UHPLC-MS/MS parameters and MRM selection

175 The UHPLC-MS/MS analyses were carried out in two independent  
176 laboratories using different triple quadrupole mass spectrometry instruments. Specifically, the  
177 following instrumental platforms and conditions were used for method development.  
178 Most of the optimization experiments were carried out on an Acquity liquid chromatography  
179 (UHPLC) system coupled to a Waters Xevo TQ-S triple quadrupole system. Peptide  
180 separation was performed on a Waters Acquity UPLC peptide BEH C18 column (130 Å, 1.7  
181 µm, 2.1 x 150 mm) at 50 °C and with a flow rate of 0.2 mL/min. Elution was carried out for  
182 26 min as follows: 0–3 min: 92% A; 3–18 min: 92% to 58% A, 18.0–18.1 min: 58% to 15%  
183 A; 18.1–22.5 min: 15% A; 22.5–22.6 min: 15% to 92% A, 22.6–26 min: 92% A (solvent A:  
184 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile) [Henrottin 2019].  
185 MRM detection in positive electrospray mode was performed with a Waters Xevo TQ-S  
186 triple quadrupole system and set up at unit resolution in both Q1 and Q3. The cone nitrogen  
187 flow was set at 150 L/h, the collision gas flow at 0.13 mL/min, the capillary voltage at 2.5  
188 kV, and the source temperature at 150 °C. The desolvation temperature was set at 500 °C and  
189 the nitrogen flow at 650 L/h.

190 Additional experiments were carried out on a UHPLC LX-50 system coupled  
191 with a QSight® 220 triple quadrupole mass analyzer (Perkin Elmer). Chromatographic  
192 separation was performed on a Brownlee SPP Peptide ES-C18 column (2.1 x 150 mm; 2.7  
193 µm; 160 Å) at 30 °C with the following elution gradient (flow 0.25 mL/min): 0–33 min, from  
194 10% to 35% B; 33.0–33.2 from 35% to 90% B, constant at 90% B for 10 min, from 43.2 to  
195 43.4 min from 90% to 10% of solvent B, column equilibration for 16 min. Timed-MRM  
196 acquisition in positive ion mode was set up at unit resolution in both Q1 and Q3, and with 2  
197 min wide acquisition windows. Electrospray source parameters were set as follows: drying  
198 gas (nitrogen): 120 (arbitrary units), hot-surface induced desolvation (HSID™) Temp: 250

199 °C; nebulizer gas: 300 (arbitrary units), Electrospray V1: 4500, ion source Temp: 400°C. All  
200 instrument control, analysis, and data processing were performed using the Simplicity™ 3Q  
201 software platform v. 1.6.

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

221

## 222           **2.4. Sample preparation protocol – optimization**

### 223           2.4.1. Sample preparation

## 224 2.4.1.1 Chocolate pre-treatment

225 Different sample pre-treatment procedures were investigated before extraction: grinding,  
226 melting, melting followed by defatting

227 *a) Grinding*

228 To avoid chocolate melting, samples, as well as the blade and stainless-steel container of the  
229 grinder, were kept at -20°C for a minimum of 2 hours before use. 15 g of chocolate sample  
230 were placed in a Waring laboratory blender. Two pulse cycles of 3 s at maximum speed were  
231 applied. The stainless-steel container was removed, and the contents were manually shaken.  
232 The container and its content were stored at -20°C for 5 min. These pulse cycles were repeated  
233 twice. Optionally, the sample can be manually sieved (1 mm sieve). The sample was weighed  
234 ( $2.00 \pm 0.02$  g of sample in 50 mL Falcon tube) and stored at 4 °C up to its use.

235

236 *b) Melting*

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

240

241 *c) Melting and defatting*

242 As a third sample preparation procedure, hexane (20 mL) was added to the  
243  $2.00 \pm 0.02$  g of melted chocolate. The sample was mixed (head-over-head shaking) at room  
244 temperature (RT) for 15 min and centrifuged (4660 x g; 5 min; 10 °C). The supernatant was  
245 discarded, and the defatting procedure was repeated. The crude defatted chocolate was dried  
246 at 30 °C under nitrogen flow. This defatting resulted in approximately 10% weight loss. Once  
247 dried, the sample was either used for the extraction or stored at 4 °C until use.

248

249                   2.4.1.2 Sample extraction

250                   Extraction buffer (20 mL of Tris HCl 200 mM, pH 9.2 with urea 2 M or 5 M),  
251 was added to the ground (2.4.1.1 *a*), melted (2.4.1.1. *b*), or melted and defatted (2.4.1.3. *c*)  
252 chocolate sample kept beforehand at room temperature (RT, 25°C) for at least 15 min. The  
253 solution was then mixed (head-over-head shaking; 30 min, RT), sonicated in a water bath (15  
254 min), and centrifuged (4660 x g; 10 min; 10 °C).

255

256                   2.4.1.3 Protein purification

257                   The supernatant recovered at the end of the extraction step was filtrated on an  
258 Acrodisc<sup>®</sup> syringe filter with a 5 µm Versapor<sup>®</sup> membrane. The resulting filtrated solution  
259 (2.5 mL) was purified by size exclusion chromatography (SEC; 5 kDa cut-off) on disposable  
260 cartridge (PD-10 desalting columns pre-packed with Sephadex G-25 M resin, from Cytiva).  
261 The columns were conditioned beforehand with three aliquots of water (4 mL each) followed  
262 by four aliquots of 50 mM ammonium bicarbonate buffer (ABB 50, 4 mL each). Both the  
263 “Spin elution” and “Gravity elution” protocols, which involved respectively the  
264 centrifugation of the cartridge and the gravitational elution of the sample, were carried out  
265 according to the producer instructions. These elution protocols provide different dilution  
266 factors for the purified samples recovered after elution: no dilution for spin elution and 1.4  
267 times dilution for gravity elution. The option of sample purification via SEC procedures was  
268 also compared with the extract direct dilution in ABB 200 mM to decrease the urea  
269 concentration down to 1 M, limit compatible with proper trypsin activity.

270

271                   2.4.1.4 Enzymatic digestion

272                   The protein concentration of the extracts was determined using bicinchoninic  
273 acid (BCA) assay (Sigma Aldrich) according to the manufacturer’s instructions. The resulting

274 assayed values were used to adjust the amount of trypsin to the sample according to the  
275 enzyme to substrate ratios of 1:50, 1:100, and 1:200. Prior to protease digestion, proteins  
276 were denatured, reduced, and alkylated. First, the protein extracts (0.5 mL) recovered after  
277 either SEC protein purification or dilution were transferred into a LoBind Eppendorf tube  
278 (1.5 mL) and heated at 95 °C for 15 min (600 rpm) for denaturation. Second, once cooled  
279 down on ice, DTT (50 µL, 500 mM dissolved in ABB 200 mM) was added, and the solution  
280 was incubated at 60 °C for 30 min (600 rpm). Third, the solution was cooled down on ice,  
281 and IAA (100 µL, 100 mM dissolved in ABB 200 mM) was added; the resulting solution was  
282 incubated in the dark, at 37 °C, for 30 min (600 rpm). The reduced and alkylated protein  
283 solution was then digested with trypsin. Trypsin Gold solution (1 µg/µL in acetic acid 50  
284 mM) was added (10 µL; theoretical trypsin to protein ratio: 1/100) and the digestion was  
285 performed at 37 °C for 16 hours (900 rpm). The digestion was quenched by the addition of  
286 hydrochloric acid (70 µL, 1 M) and centrifuged (14 800 rpm; 10 min).

287

#### 288 2.4.1.5 Peptide purification and sample extract concentration

289 The resulting centrifuged digest was purified either on Strata-X SPE column  
290 (1 cc, 30 mg) or on Sep-Pak C18 SPE column (1 cc, 50 mg). The Strata-X SPE column was  
291 activated with methanol (3 mL) and conditioned with water (3 mL). The sample (0.5 mL)  
292 was loaded onto the SPE and washed with water (2 mL) followed by water/methanol solution  
293 (95/5 (v/v); 1 mL). The peptides were eluted with ACN/MeOH (1/1 (v/v)) and 2% formic  
294 acid (1 mL). The SPE column was then dried for 2 min. The Sep-Pak C18 SPE column was  
295 activated with ACN (3 mL) and conditioned using 0.1% formic acid in water (3 mL). The  
296 sample was loaded onto the SPE and washed with 0.1% formic acid in water (3 mL). The  
297 peptides were eluted with acetonitrile/0.1% formic acid in water mixture 80/20 (v/v) (1 mL).  
298 The SPE column was then dried for 2 min. The eluted solution was concentrated by

299 evaporation under nitrogen flow (N<sub>2</sub>; 40 °C) up to dryness. The dried extract was solubilized  
300 in 5% acetonitrile in 0.1% formic acid in water solution (100 µL), vortexed, and centrifuged  
301 (4660 g; 5 min; 10 °C). The supernatant was transferred into an injection vial and analyzed  
302 by UHPLC-MS/MS.

#### 303 2.4.1.6 Statistical treatment

304 In terms of method development, as shown in the scheme reported in Figure 1,  
305 five steps of the sample preparation protocol were considered. For all selected procedures,  
306 three independent replicates were carried out for statistical relevance of the comparisons ( $n =$   
307 3). Mean and standard deviation of the peak areas for each protocol were calculated and  
308 compared by an unpaired Student's t-test (two-tailed distribution, equal variances) at a 5%  
309 significance level to evaluate the influence of each parameter on detection sensitivity. The  
310 equality of variances of the two independent groups was assessed by an F-test (at a 5%  
311 significance level). For the digestion kinetics experiment, a Tukey's *post hoc* ANOVA test  
312 was performed for multiple comparisons of mean values. For each digestion time ( $t = 1, 4,$   
313 16, 24 h), mean values that are not significantly different will be marked with equal labels (a,  
314 b, c, d) in relevant plots.

315

### 316 3. Results and Discussion

317 In this investigation, the optimization of a sample preparation workflow for  
318 multi-allergen detection in chocolate has been carried out. The accomplishment of this task is  
319 very important to understand and compensate for the effects that the specific matrix  
320 composition may have on the reliability and sensitivity of the LC-MS based detection [Croote  
321 2019, Korte 2019], especially when complex foodstuffs are going to be analyzed [Mattarozzi  
322 2014]. Indeed, matrix components might promote the establishment of covalent or non  
323 covalent interactions with the target proteins, thus affecting their detection, with potential

324 enhancement or impairment depending on the specific case [Alves 2017, 2015]. Chocolate, in  
325 particular, is a very challenging matrix due to its high content of sugars, tannins and  
326 polyphenolic compounds which might account for a masking effect of the target allergenic  
327 protein [Bignardi 2013, Khuda 2015, Korte 2019, New 2020]. Therefore a great focus have  
328 been placed on this investigation on the proper purification of the analytical sample both  
329 before trypsin digestion and LC-MS analysis.

### 330 **3.1. Optimization of MS parameters**

331 Ancillary to the actual optimization of the sample preparation workflow, a  
332 preliminary tuning of the MS platforms involved in this work has been carried out. A list of  
333 fifty target peptides previously selected as markers (Supplementary data – Table S1) [Pilolli  
334 2020 and 2021] has been set up on two instruments (both based on triple quadrupoles  
335 analyzers) with four transitions/peptide monitored in MRM mode. Several parameters have  
336 been optimized including: cone voltage (Supplementary data – Figure S1.A), desolvation  
337 temperature (Supplementary data – Figure S1.B), and collision energy (Supplementary data –  
338 Table S1). To perform this task, protein extracts from each allergenic ingredient have been  
339 prepared and digested according to the protocols described in sections 2.4.2 and 2.4.4,  
340 respectively, without further purification.

341 The optimization of the MS parameters was described herein by applying two  
342 independent techniques. First, collision energy was optimized on the Xevo TQ-S mass  
343 spectrometer (Waters) by using the *Skyline* software: the energy was changed automatically  
344 (with a step size set to 1 eV) for each MRM of each selected peptide marker. Given that the  
345 method developed here is a multi-target UHPLC-MS/MS, the number MRM was limited to a  
346 maximum of three to five MRM transitions for each selected peptide (Table S1). This  
347 maximum of three to five MRM transitions per peptide marker was chosen according to the  
348 three following main factors: the number of peptide markers to analyze, their respective

349 acquisition windows, and the dwell time, which have an impact on the signal stability, and on  
350 the number of points per peak. As depicted in Figure S1.A, the higher the desolvation  
351 temperature, the higher the observed peak area. The observed peak area also increases rapidly  
352 in line with the cone voltage, before reaching a plateau from 10 to 35 V; for some peptides,  
353 this plateau can be observed up to 45 V (Figure S1.B). For higher cone voltages, the observed  
354 peak area decreases. Therefore, a desolvation temperature of 500 °C and a cone voltage of 35  
355 V were selected as the most appropriate compromises for the detection of all allergen  
356 peptides on the Xevo TQ-S triple quadrupole spectrometer instrument. A second method was  
357 applied for MS parameters optimization on the second alternative triple quadrupole (Q-  
358 Sight<sup>®</sup>, Perkin Elmer; see section 2.3 and in Table S1) used in this investigation. For this  
359 latter, the MS method was optimized by the direct infusion of the allergenic ingredient  
360 digests with a t-line configuration. The source parameters (drying gas, hot-surface induced  
361 desolvation (HSID<sup>™</sup>) temperature, nebulizer gas, electrospray voltage, and source  
362 temperature) were tuned by maximizing the total ion current of the Full-MS mass spectra  
363 acquired. In addition, the experimental m/z values both for precursors and transitions were  
364 checked and the three main parameters related to the MRM acquisition (entrance voltage,  
365 voltage on flat lens, and collision energy) were automatically optimized by running  
366 individual ramps.

367

### 368 **3.2. Sample preparation optimization**

369 Each step of the sample preparation workflow displayed in Figure 1 has been  
370 investigated and optimized by varying individual parameters highlighted in this figure.  
371 Chocolate bar incurred at 40 ppm concentration level with milk, egg, soy, peanut, almond,  
372 and hazelnut, was chosen as the model sample for this optimization. Three independent  
373 samples were tested ( $n = 3$ ), and the resulting MRM peak areas (quantitative transition only)



374 were compared by unpaired statistical t-test to highlight statistically significant differences of  
375 recorded mean values.

376

### 377 3.2.1. Chocolate pre-treatment

378 For a proper sampling of representative and homogeneous portions of the  
379 chocolate bar, two physical approaches have been compared: grinding [Gu et al. 2018, Korte  
380 et al. 2016, 2019] and melting [Huet 2022]. As for the grinding protocol special attention was  
381 required to avoid unintended melting caused by overheating. Most of the detected peptides  
382 (38 peptides out of the 47 detected) did not show any significant impact of the sample  
383 preparation (melting vs grinding) on their detection (Table 1 and in Figures S2 and S3  
384 (Supplementary Data)). Some differences were disclosed only for soybean, since four  
385 specific peptides (out of the 13 peptides selected) showed a higher sensitivity when grinding  
386 was used (Figure 2). All four peptides originated from the 11S globulin named *glycinin*  
387 (known as the allergen *Gly m 6*), one of the major soy allergens associated with severe  
388 allergic reactions to soybean in children [Holzhauser 2009, Ito 2011].

389 In addition, a dual step preparation including melting and defatting of  
390 chocolate sample, was also compared with the grinding procedure. The defatting step might  
391 contribute to reduce the matrix complexity, by removal of the lipophilic components  
392 potentially interfering with the enzymatic proteolysis and/or with the peptide detection [New  
393 2018, 2020, Xiong 2021]. However, also in this case, most of the peptides signals were not  
394 influenced by the two procedure (39 out of 47), whereas only six peptides, from egg (two  
395 peptides), peanut (two peptides), hazelnut (one peptide) and soy (one peptide), appeared to be  
396 promoted by the melting-defatting procedure and two promoted, on the contrary, by the  
397 grinding procedure (see Figure S3 for details). However, the melting-defatting procedure  
398 suffers from several drawbacks, being time-consuming and neither user nor environmentally

399 friendly. Therefore, as the impact of an additional hexane defatting step is limited to some  
400 peptides, the grinding procedure was preferred and chosen for the preparation and  
401 homogenization of the sample, being simple and environmental/user friendly, also confirming  
402 the protocols previously applied by independent research groups on similar matrixes  
403 [Bignardi 2013, Sayers 2018, Gu 2018, Korte 2016, 2019].

404

### 405 3.2.2. Protein extraction – Extraction buffer

406 The next step of the sample protocol (Figure 1) is protein extraction, which  
407 usually requires the use of Tris-buffered saline (TBS),  $\text{NH}_4\text{HCO}_3$ , or Tris.HCl, at a pH value  
408 of 8.0 to 9.2 [Gu 2018, Martinez-Esteso 2020, Shefcheck 2006, Planque 2016, Monaci 2014].  
409 These buffers can be used in combination with denaturing, reducing agents, and/or  
410 surfactants such as urea, thiourea, dithiotreitol, sodium dodecyl sulphate (SDS), tween, octyl  
411  $\beta$ -D-glucopyranosid and RapiGest SF, in order to improve the protein extraction rate  
412 [Martinez-esteso 2020, Monaci 2014, New 2018, 2020, Sayers 2018, Sagu 2021, Xiong  
413 2021]. However, some of these additives can interfere with the enzymatic digestion step (*e.g.*,  
414 proteases like trypsin are inhibited by urea concentrations higher than 1 M) or may adversely  
415 affect the MS analysis (*e.g.*, SDS is not MS compatible); therefore whenever added to  
416 improve the protein extraction yield, such additives require proper removal steps in the  
417 preparation workflow such as solid phase extraction, cut-off filtration or dilution down to  
418 compatible concentrations, to avoid any interference with the final detection [Boo 2018,  
419 Croote 2019, Monaci 2020, Planque 2016-2019, Xiong 2021]. In addition, to further improve  
420 the extraction yield, a sonication step may also be included as physical aid [Monaci 2014,  
421 Pilolli 2017a, Planque 2016], particularly efficient in promoting the recovery of specific  
422 allergenic proteins such as caseinate proteins from milk [Monaci 2014].

423                   A basic extraction protocol for multi-allergen detection was chosen from  
424 previous investigations [Planque 2019] based on Tris.HCl buffer (200 mM, pH 9.2) added  
425 with 2 M urea: the sample was mixed with this buffer and sonicated to improve the  
426 extraction. The possibility to include a higher concentration of urea (5M) was assessed for  
427 potential application in highly processed samples. The results of such comparison (2M vs 5M  
428 urea) obtained in two independent laboratories with two different triple quadrupole  
429 spectrometers (lab 1: Xevo TQ-S [Waters]; lab 2: QSight<sup>®</sup> 220 TQ [Perkin Elmer]) were  
430 generally consistent (Supplementary data – Table S1). Interestingly, the urea concentration  
431 had a limited impact on the extraction/detection of peptide targets from soy, almond,  
432 hazelnut, and milk (Supplementary Data – Figure S4 and S5), whereas had a considerable  
433 impact on protein extraction from egg and peanut (Figure 3) with opposite trends for the two  
434 ingredients. Indeed, depending on the MS platform, the detection of three to four egg  
435 peptides (out of the seven targeted for white and yolk proteins) was significantly improved by  
436 extracting with 2 M urea instead of 5 M urea (Table 1, Figures 3A and 3C). In contrast, 5 M  
437 urea significantly favors peanut protein extraction (Table 1, Figures 3B and 3D). Indeed, the  
438 higher urea concentration (5 M) improves the extraction and detection of lightly roasted  
439 peanuts, which is the only extensively processed ingredient in this incurred chocolate matrix,  
440 confirming previous investigations on the proteomic profiling of this ingredient [Johnson  
441 2016]. This observation is especially important in the perspective to extend the current  
442 method to other extensively processed samples [Mattarozzi 2019]. Based on these findings,  
443 which are similar on both MS platforms, the higher urea concentration (5 M) was chosen for  
444 the final extraction protocol due to the improved allergens extraction yield from incurred  
445 processed food commodities.

446

447                   3.2.3. SEC protein purification

448                   When used as a chaotropic agent in protein extraction, a major drawback of  
449 urea is that concentrations higher than 1 M can denature trypsin, reducing the effectiveness of  
450 the digestion step. This can be overcome by diluting the extract with ammonium bicarbonate  
451 buffer before the digestion step to give a theoretical urea concentration below 1 M [Croote  
452 2019, Planque 2016-2017a-2017b-2019]. Alternatively, technical aids for buffer exchange  
453 using disposable cartridge-based size exclusion chromatography (SEC) can be implemented  
454 to remove urea. This option also has the additional advantage of simplifying the sample  
455 composition by removing low molecular weight interfering compounds (5kDa cut-off of the  
456 stationary phase), such as polyphenols, that might be co-extracted from the matrix, thus  
457 reducing the background signal from the chocolate matrix [Pilolli 2017b, 2018, 2021].  
458 Therefore, direct dilution and SEC protein purification procedures (using either a spin or  
459 gravity elution protocol) were compared on protein extracts prepared with Tris.HCl buffer  
460 containing 2 M urea (Table 1). The SEC based proteins purification significantly improved  
461 the detection of peptides from milk, egg, hazelnut, soy, and almond proteins compared to  
462 dilution. For peanut, only one peptide (SPDIYNPQAGSLK<sup>2+</sup>) showed a significant  
463 improvement in its MS detection after SEC protein purification (Supplementary Data –  
464 Figure S6). Of the SEC elution procedures (gravity *versus* spin protocols), the spin elution  
465 significantly improved the detection of 90% of the peptides (44 out of 49 peptides),  
466 irrespective of the allergen or protein fraction (Table 1; Supplementary Data – Figure S7),  
467 compared to gravity elution protocol. Based on these results, the use of an additional  
468 purification step at protein level is highly recommended, and the SEC purification using  
469 centrifugation was chosen to be included in the final optimized protocol.

470

471                   3.2.4. Protein digestion with trypsin

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

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

487                   Additional digestion assays were performed with a focus on optimizing the  
488 trypsin to protein ratio (protein concentration in the extract being estimated by BCA assays)  
489 corresponding to either 1/50, 1/100, or 1/200. The detection of most peptide markers was  
490 significantly improved when using a 1/50 or 1/100 compared to a 1/200 trypsin to protein  
491 ratio, indicating the latter ratio was too low to provide the complete release of the peptides  
492 (Table 1) [Nitride 2019]. This observation did not apply to the peptide markers for almond  
493 and peanut, where peptide generation was independent of the trypsin to protein ratio (Table  
494 1). The trypsin to protein ratio had a relatively limited impact on detection of peptides for  
495 milk and hazelnut, especially when 1/50 and 1/100 ratios are compared (only four peptides  
496 being significantly affected for these two allergens; Table 1). However, a more significant

497 effect was observed for soy and egg peptides. It is noteworthy that this influence is quite  
498 different depending on the target allergen (egg or soy). Most of the egg peptides displayed a  
499 significant improved detection when using a 1/50 trypsin to protein ratio, while for soy a  
500 lower trypsin to protein ratio significantly favored detection of the most intense peptide  
501 VLIVPQNFVVAAR<sup>2+</sup> (Supplementary Data – Figure S9). Based on these results, the 1/100  
502 trypsin to protein ratio was found to be the best compromise, allowing effective protein  
503 digestion at a lower cost. To reduce furtherly the analysis costs, the digestion of a lower  
504 absolute amount of extract (0.5 mL instead of 1.0 mL) with this 1/100 trypsin to protein ratio  
505 was considered and found to still be representative of the sample and sufficient to guarantee  
506 the reproducibility of the analysis. These digestion conditions were thus preferred for the  
507 final optimized protocol and applied in the following optimization steps, confirming similar  
508 protocols already described in the literature [Xiong 2021].

509           A peptide acting as a reliable quantifier must be fully released. Therefore, the  
510 rate of protein digestion was investigated by monitoring peptide release from its constituent  
511 protein using a time course experiment [Nitride 2019, Korte 2019]. A single batch of the 40  
512 ppm allergen incurred chocolate sample was extracted with Tris-HCl containing 5 M urea,  
513 subjected to SEC protein purification, and aliquoted to carry out independent digestions at  
514 fixed trypsin to protein ratio (1/100) and different time course: 1, 4, 16, and 24 h (Figure 4  
515 and Supplementary Data Figure S10). The effect of digestion time on the measured peak  
516 intensity is markedly different depending not only on the specific protein but also on the  
517 peptide sequence. For example, the peptides monitored for the soybean allergens *Gly m 5* and  
518 *Gly m 6*, or almond allergen *Pru du 6* displayed peculiar kinetics depending on the specific  
519 sequence (Figure 4 and Supplementary Data – Figure S10). This experimental evidence can  
520 likely be ascribed to the accessibility of the peptide bonds specifically cleaved by trypsin. For  
521 some of the selected markers, the resulting digestion rate was very high, and the peptide

522 appeared to be fully released after only 1 h digestion (see, for example, FFVAPFPEVFGK<sup>2+</sup>  
523 and YLGYLEQLLR<sup>2+</sup> from  $\alpha$ -S1 casein (*Bos d 9*); VLIVPQNFVVAAR<sup>2+</sup> and  
524 SQSDNFEYVSFK<sup>2+</sup> from soy *Gly m 6*; GNLDFNVQPPR<sup>2+</sup> and ALPDEVQNAFR<sup>2+</sup> from  
525 almond *Pru du 6*; and ADIYTEQVGR<sup>2+</sup> and INTVNSNTLPVLR<sup>2+</sup> from hazelnut *Cor a 9*).  
526 However, significantly different trends were observed for several other markers, which  
527 required much longer incubations (*e.g.*, 16 h) to achieve maximum release from the intact  
528 protein. For example, the peptides NAVPITPTLNR<sup>2+</sup> and FALPQYLK<sup>2+</sup> from  $\alpha$ -S2 casein,  
529 *Bos d 10*; VYVEELKPTEGDLEILLQK<sup>3+</sup> and VLVLDTDYK<sup>2+</sup> from  $\beta$ -lactoglobulin *Bos d*  
530 *5*, GGLEPINFQTAADQAR<sup>2+</sup> and ISQAVHAAHAEINEAGR<sup>3+</sup> from ovalbumin *Gal d 2*,  
531 VLLEENAGGEQEER<sup>2+</sup> from peanut *Ara h 1*, NILEASYDTK<sup>2+</sup> from soybean *Gly m 5*,  
532 TEENAFINTLAGR<sup>2+/3+</sup> and ADIFSPR<sup>2+</sup> from almond *Pru du 6*, and  
533 ALPDDVLANAFQISR<sup>2+</sup> from hazelnut *Cor a 9* accomplished full release after 16 h of  
534 incubation. These results confirm previous data from similar investigations carried out from  
535 independent research groups on milk, egg [Nitride 2019], peanut, and tree nuts [Korte 2019].  
536 It is also worth noting that while most of the peptides fully released after 1 hour remained  
537 stable over an incubation of 16 hours, a very limited number of peptides were not stable over  
538 this longer digestion duration (16 h) and slightly degraded. This can be observed for  
539 VLIVPQNFVVAAR<sup>2+</sup> from soy *Gly m 6* (Figure 4), QVQELAFPGSAQDVER<sup>2+</sup> from soy  
540 *Gly m 5*, NLPQQCGLR<sup>2+</sup> from peanut *Ara h 2*, GNLDFVQPPR<sup>2+/3+</sup> and  
541 ALPDEVLQNAFR<sup>2+</sup> almond *Pru du 6*, and (Supplementary Data – Figure S10). This must  
542 be taken into account in the final peptide selection in the validation, as technically these  
543 peptides would lead to underestimation whenever long incubation time are applied.  
544 Accordingly, digestion for 16 h was chosen for the final optimized method to ensure that the  
545 molar amount of all the peptide markers can be representative of the moles of protein present  
546 in the extract (Supplementary Data – Figure S10).

547

## 548 3.2.5. Tryptic peptides purification

549 Due to the high complexity of the chocolate matrix, a further step of  
550 purification has been considered, applied to the peptide pool generating from the trypsin  
551 digestion. Solid phase extraction (SPE) with disposable cartridges was tested, with the dual  
552 aim of (i) removing polar interfering compounds from the matrix, potentially co-extracted  
553 with the target proteins and (ii) concentrating the peptide pool in a smaller volume of solvents  
554 suitable for the LC-MS analysis. According to the information available in the literature, two  
555 types of SPE columns were compared, namely the polymeric phase Strata-X® [Korte 2016,  
556 2019, Hoffman 2017] and a classical reverse phase C18 [Huschek 2016, Planque 2016,  
557 Monaci 2020]. In general, both formats performed equally well (Table 1 and Supplementary  
558 Data – Figure S11). Some exceptions were two peptides from milk casein  
559 (HQGLPQEVLNENLLR<sup>2+/3+</sup> and NAVPITPTLNR<sup>2+</sup>), one from egg white  
560 (ISQAVHAAHAEINEAGR<sup>3+</sup>), and one from soy *Gly m 6* (ISTLNSLTLPALR<sup>2+</sup>) which  
561 showed a significant improvement with the Strata-X SPE. In contrast, the signal intensity was  
562 significantly improved for only one peptide from hazelnut (ALPDDVLANAFQISR<sup>2+</sup>) and  
563 another from soy *Gly m 5* (VPSGTTYVYVNPDPNNENLR<sup>2+</sup>) when using the C18 SPE  
564 column. Although both SPE formats could be used (since they lead to very similar results), it  
565 was decided to use Strata-X SPE cartridges for the final optimized protocol.

566 After tryptic peptide purification on the SPE column, the solvent was  
567 evaporated to allow peptide concentration. Noteworthy, solvent evaporation to dryness might  
568 result in a partial loss of peptides due to their adsorption on the vial walls, therefore, it is  
569 recommended to use low-binding vials. An option to prevent this effect, might be to add the  
570 SPE eluate with a small volume of DMSO, which does not evaporate under these conditions  
571 and so avoids complete dryness of the peptides pool. This option was tested and compared



572 with the complete solvent evaporation, and as a fact most of the detected peptide markers  
573 (43/47) did not show any significant impact on their detection when DMSO is present  
574 (Supplementary Data – Figure S12), while four peptides (LPLSLPVGPR<sup>2+</sup> from egg yolk  
575 protein, TANDLNLLILR<sup>2+</sup> from peanut, ALPDDVLANAFQISR<sup>2+</sup> from hazelnut, and  
576 ISTLNLSLTLPALR<sup>2+</sup> from soy *Gly m 6*) (Supplementary Data – Figure S13) were even  
577 significantly better detected when evaporation was performed in the absence of DMSO.  
578 Given this results, the use of DMSO was not included during the evaporation step in the final  
579 optimized method even though this solvent may facilitate crude extract solubilization.

580

### 581 **3.3. Summary of the sample preparation workflow and final considerations**

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

597 including the purification of tryptic peptides on SPE cartridge and their concentration, is  
598 applied to improve the sensitivity of the final method under development.

599

#### 600 **4. Conclusions**

601           The paper describes the development of an analytical protocol for the  
602 extraction and quantification of six allergens in a complex food like chocolate by using low  
603 resolution mass spectrometry. In the present study, different parameters influencing protein  
604 and peptide recoveries were investigated and duly optimized in order to maximize the  
605 detection sensitivity. Fifty peptide markers tracing for the six allergenic ingredients have  
606 been validated in two independent laboratories, irrespective of the instrumental set-up (MS-  
607 platforms and chromatographic conditions) and operators involved, thus assessing the  
608 robustness of the method under development.

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

612

#### 613 **Acknowledgments**

614           The authors gratefully acknowledge the European Food Safety Authority for  
615 the financial support received as part of the ThRAI project (Grant  
616 GP/EFSA/AFSCO/2017/03). The present study, however, is the sole responsibility of the  
617 authors. The positions and opinions presented here are those of the authors alone and do not  
618 necessarily represent the views, official position, or scientific works of EFSA. The ThRAI  
619 project is also co-funded by the Belgian Federal Agency for the Safety of the Food Chain  
620 (FASFC) and the UK Food Standards Agency FS101209.

## Figures captions

**Figure 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).

**Figure 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 ( $\alpha = 5\%$ ).

**Figure 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 (Perkin Elmer).

\* 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 (Perkin Elmer), or in two charge states (2+ and 3+) on a Xevo TQ-S (Waters) instrument.

**Figure 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.

**Figure 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).

## References

Alves 2017

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 2013

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 2018

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 2019

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 2017

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 2015

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., Kenna, F., Kruizinga, A. G., Lack, G., Madsen, C. B., Mills, E. N. C., Papadopoulos, N. G., Alldrick, A., Regent, L., Sherlock, R., Wal, J. M., & Roberts, G. (2015). Precautionary allergen labelling: perspectives from key stakeholder groups. *Allergy*, 70, 1369–1051. <https://doi.org/10.1111/all.12614>.

DunnGalvin 2019

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 & Experimental Allergy*, 49, 1446-1454.

<https://doi.org/10.1111/cea.134793>.

EU Regulation – 1169-2011

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>

Codex STAN

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

Gavage 2020

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*, 127413.  
<https://doi.org/10.1016/j.foodchem.2020.127413>.

Gu 2018

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://dx.doi.org/10.1016/j.foodcont.2017.07.033>.

Hands 2020

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 2011

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 2019

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 2017

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 2009

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. *Journal of Allergy and Clinical Immunology*, *123*(2), 452–458. <https://doi.org/10.1016/j.jaci.2008.09.034>.

Huet 2022

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 2016



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 2011

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. *Journal of Allergy and Clinical Immunology*, 128(3), 673–675.  
<https://doi.org/10.1016/j.jaci.2011.04.025>.

Johnson 2016

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>

Kauzmann 1959

Kauzmann, W. (1959). Some factors in the interpretation of protein denaturation. *Advances in Protein Chemistry*, 14, 1–63. [https://doi.org/10.1016/S0065-3233\(08\)60608-7](https://doi.org/10.1016/S0065-3233(08)60608-7).

Khuda 2015

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 2016

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

Korte 2019

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 2020

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 Bioanalytical Chemistry*, 412, 8249-8267. <https://doi.org/10.1007/s00216-020-02959-0>.

Mattarozzi 2014

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>.

Mattarozzi 2019

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 Bioanalytical Chemistry*, 411, 4465-4480. <https://doi.org/10.1007/s00216-019-01642-3>.

## Mills 2019

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 (ThRAI). *Journal of AOAC International*, *102*(5), 1346–1353. <https://doi.org/10.5740/jaoacint.19-0063>.

## Monaci 2008

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>.

## Monaci 2013

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*, 2009–2018. <https://doi.org/10.1002/rcm.6662>.

## Monaci 2014

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 2018

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>.

New 2018

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 2020

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 2019

Nitride, C., Nørgaard, J., Omar, J., Emons, H., Estes, 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 2015

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 2017a

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 2017b

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 2018

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 2020

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*, Article 108747. <https://doi.org/10.1016/j.foodres.2019.108747>.

Pilolli 2021

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, Article 128533.

<https://doi.org/10.1016/j.foodchem.2020.128533>.

#### Planque 2016

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/http://doi.org/10.1016/j.chroma.2016.08.033>.

#### Planque 2017a

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

<https://doi.org/10.5772/intechopen.69361>.

#### Planque 2017b

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 2019

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>.

#### Remington 2020

Remington, B. C., Westerhout, J., Meima, M. Y., Blom, W. M., Kruijzinga, 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*, 111259. <https://doi.org/10.1016/j.fct.2020.111259>.

Sagu 2021

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 2016

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. *Analyst*, *141*(13), 4130–4141. <https://doi.org/10.1039/C6AN00359A>.

Sayers 2018

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>.

Shefcheck 2006

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 Agriculture and Food Chemistry*, *54*, 7953–7959. <https://doi.org/10.1021/jf060714e>.

Taylor 2014

Taylor, S. L., Baumert, J. L., Kruizinga, A. G., Remington, B. C., Crevel, R. W. R., Brooke-Taylor, S., Allen, K. J., The Allergen Bureau of Australia & New Zealand, & Houben, G., (2014). Establishment of Reference Doses for residues of allergenic foods: Report of the VITAL Expert Panel. *Food and Chemical Toxicology*, 63, 9–17. <http://dx.doi.org/10.1016/j.fct.2013.10.032>.

Viswanatha 1955

Viswanatha, T., & Liener, I. E. (1955). The inhibition of trypsin-III. Influence of urea. *Journal of Biological Chemistry*, 215(2), 777–785.

WHO 2006 INFOSAN

World Health Organization—International Food Safety Authorities Network (INFOSAN), *INFOSAN Information Note No. 3/2006 – Food Allergies*.

Xiong 2021

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>.



Figure 1

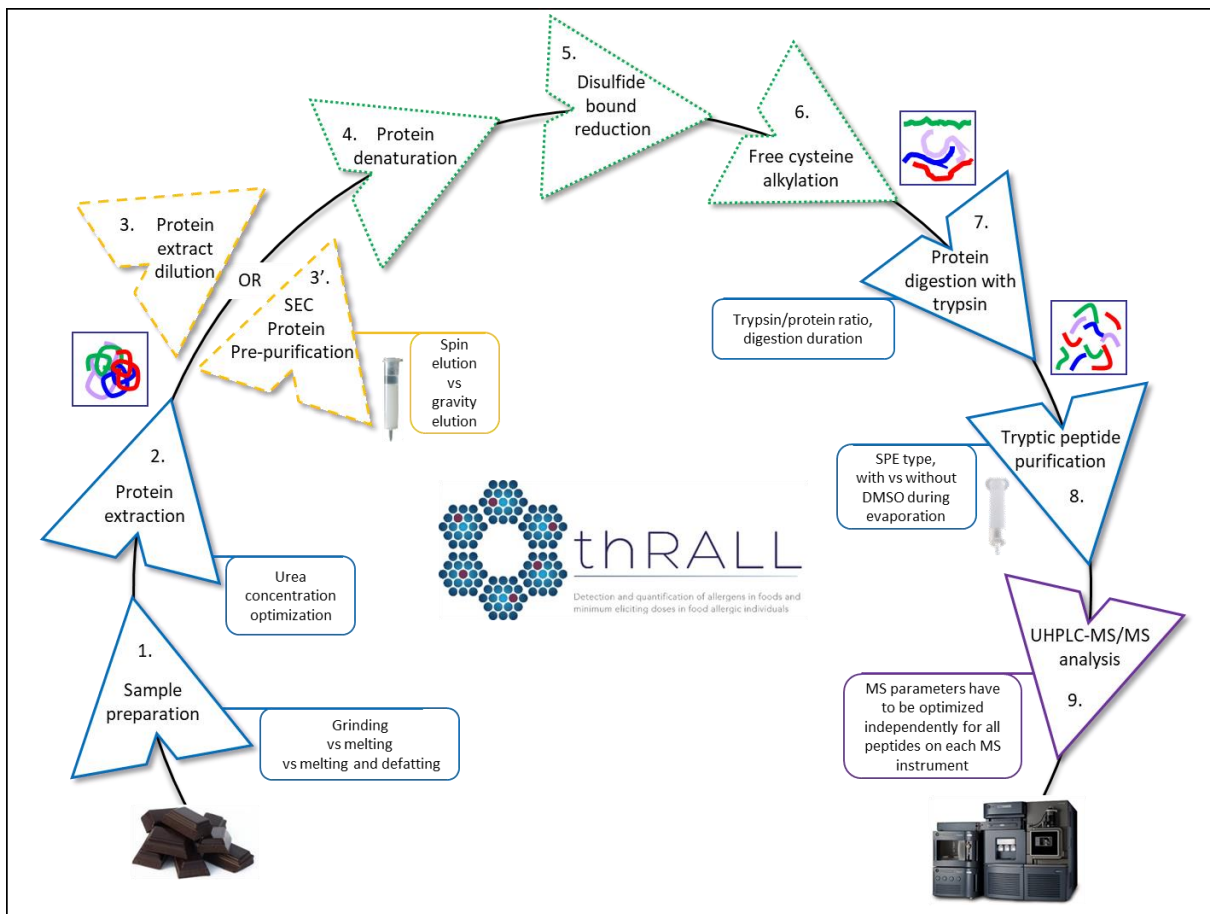


Figure 2

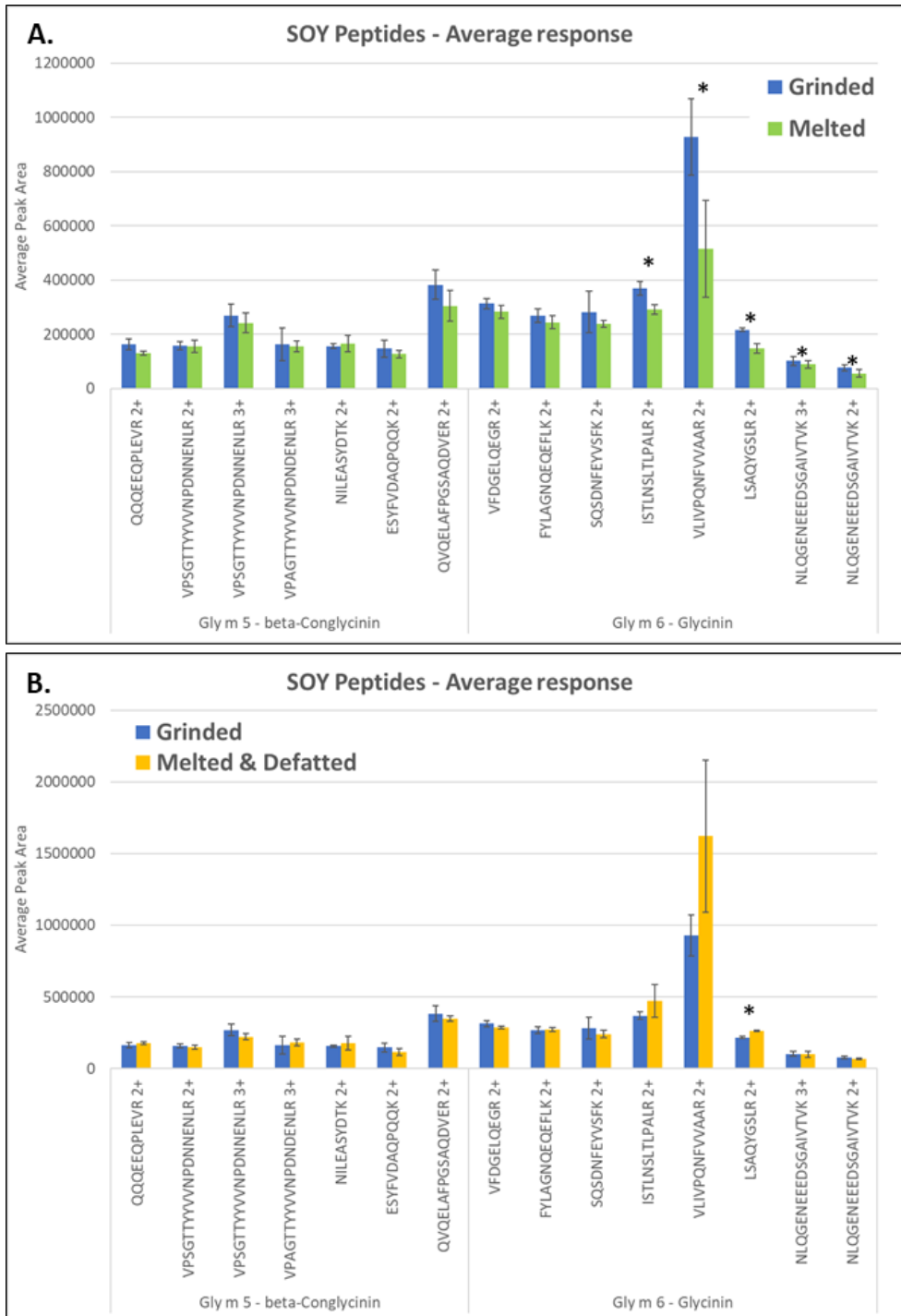


Figure 3

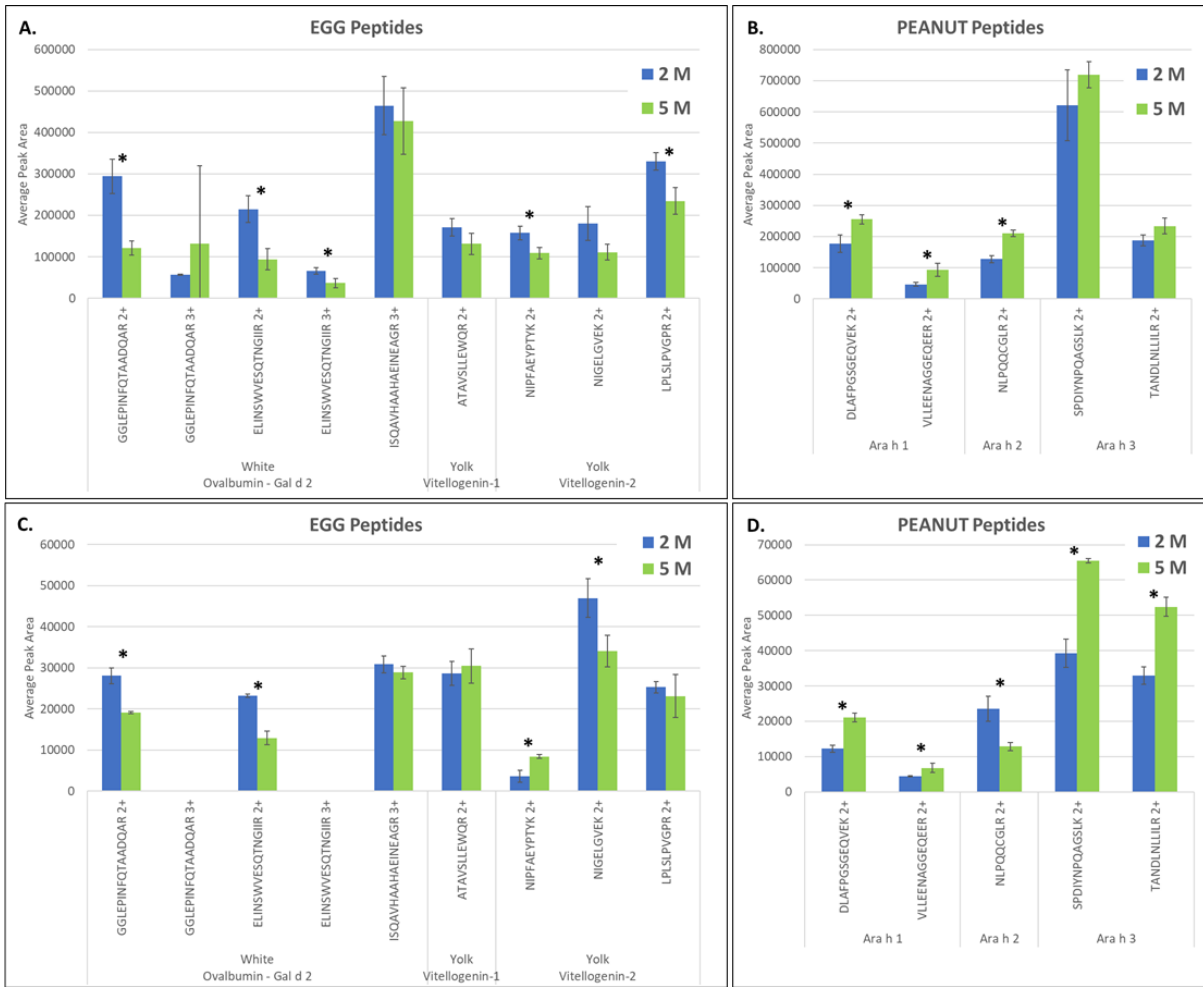


Figure 4

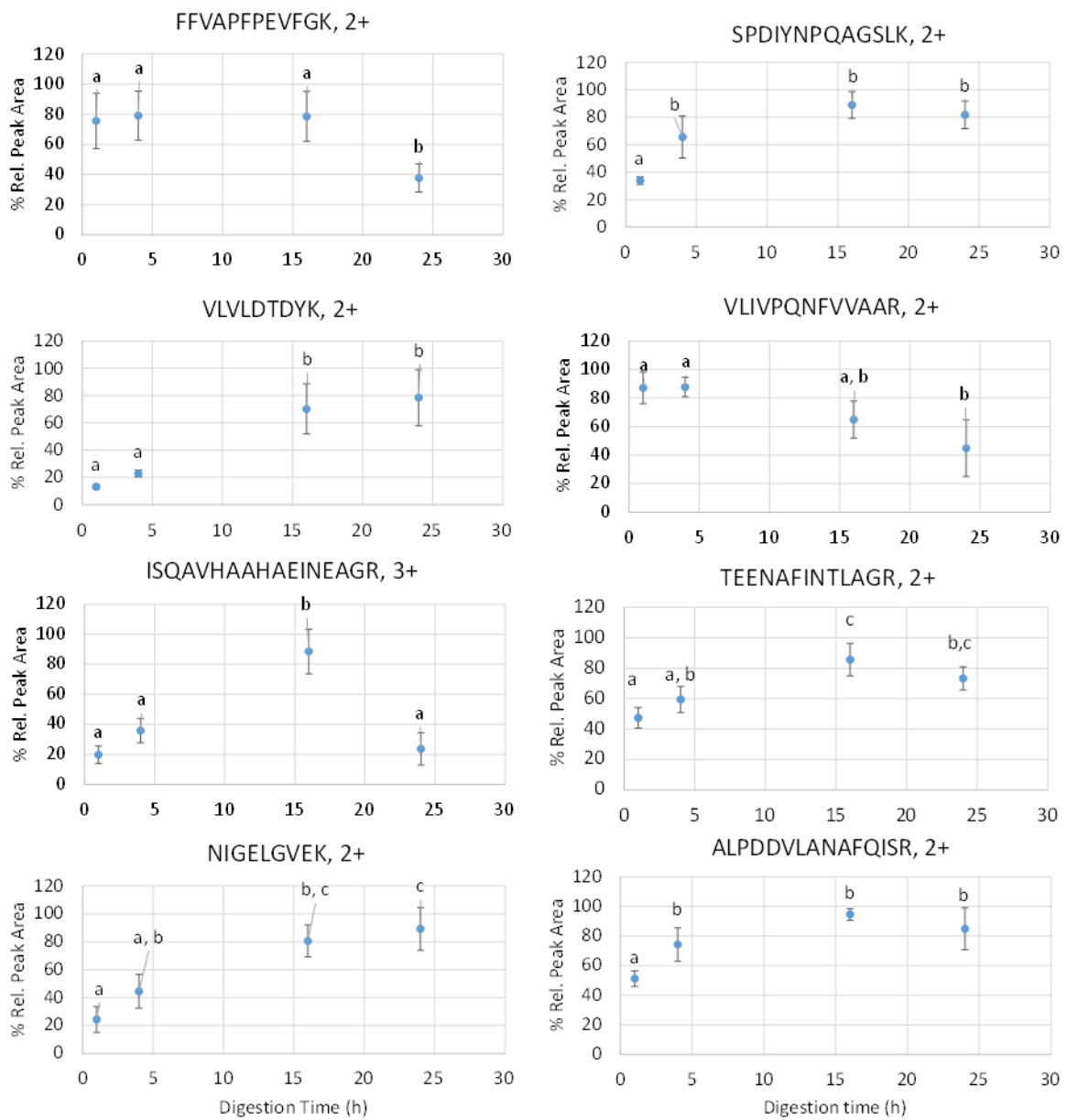
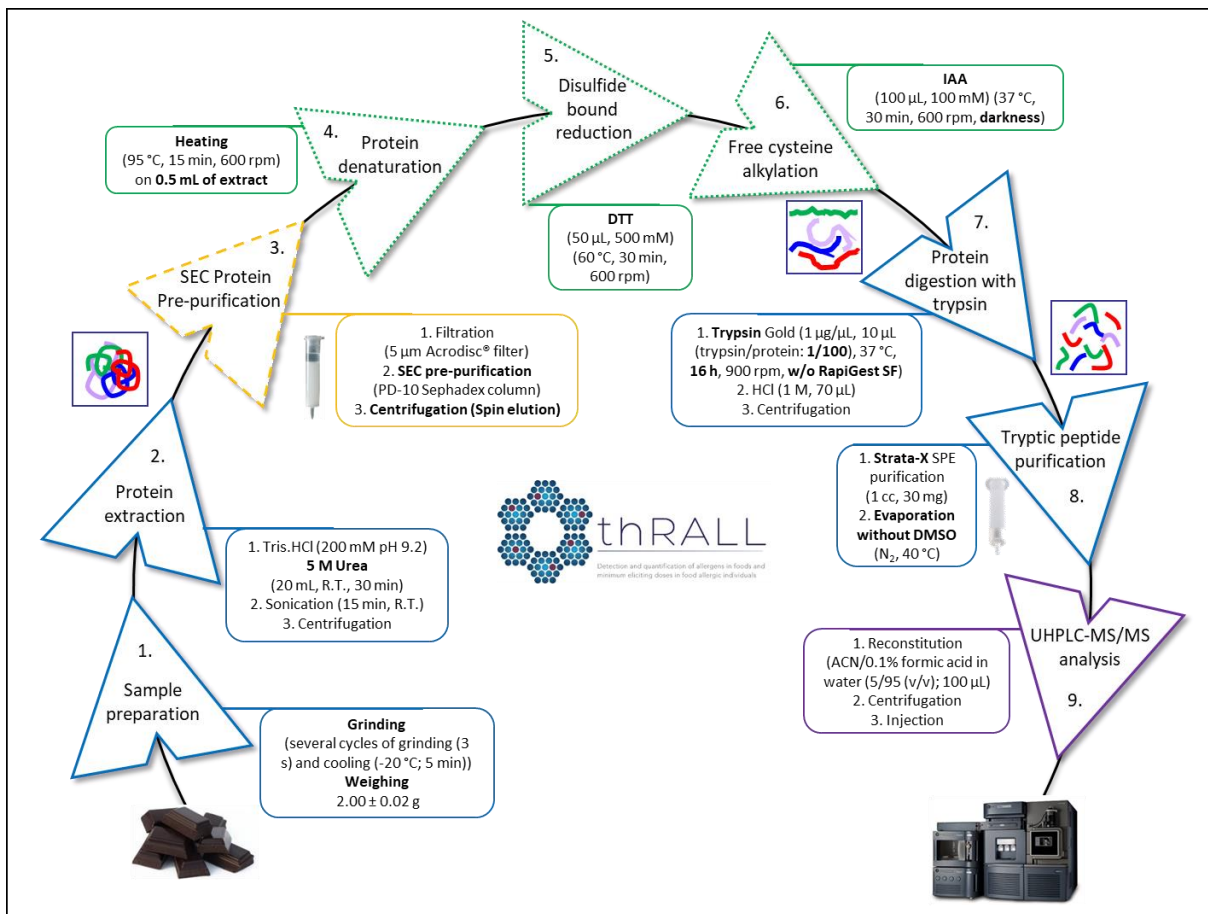


Figure 5



**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	$\beta$ -lactoglobulin	Total	White	Yolk	Total					
	Grinding	0	0	0	0	1					
Melting	0	1	1	0	0	0	1	1	0	0	<b>3/47</b>
Not SD	5	3	8	3	3	6	4	7	9	4	<b>38/47</b>
Grinding	0	0	0	0	0	0	1	0	0	1	<b>2/47</b>
Melting & Defatting	0	0	0	2	0	2	2	1	1	0	<b>6/47</b>
Not SD	5	4	9	1	4	5	2	8	12	3	<b>39/47</b>
2 M Urea*	1	0	1	2	2	4	0	2	1	1	<b>9/47</b>
5 M Urea*	0	0	0	0	0	0	3	1	0	0	<b>4/47</b>
Not SD*	4	4	8	1	2	3	2	6	12	3	<b>34/47</b>

2 M Urea**	0	1	1	2	1	3	1	1	0	1	<b>7/38</b>
5 M Urea**	0	0	0	0	1	1	4	1	0	0	<b>6/38</b>
Not SD**	5	3	8	1	2	3	0	5	6	3	<b>25/38</b>
with SEC (gravity)	5	1	6	1	3	4	1	7	6	3	<b>27/47</b>
without SEC (dilution)	0	1	1	1	0	1	0	0	2	0	<b>4/47</b>
Not SD	0	2	2	1	1	2	4	2	5	1	<b>16/47</b>
Gravity SEC elution	0	0	0	0	0	0	0	0	1	0	<b>1/49</b>
Spin SEC elution	4	4	8	2	4	6	6	9	11	4	<b>44/49</b>
Not SD	1	0	1	1	0	1	0	0	1	1	<b>4/49</b>
with RapiGest SF**	0	1	1	0	0	0	1	0	0	0	<b>2/38</b>
without RapiGest SF**	5	1	6	2	0	2	2	4	0	2	<b>16/38</b>
Not SD**	0	2	2	1	4	5	2	3	6	2	<b>20/38</b>
Trypsin/protein ratio 1/50	0	2	2	2	2	4	0	2	4	0	<b>12/49</b>
Trypsin/protein ratio 1/100	0	0	0	0	0	0	0	0	1	0	<b>1/49</b>
Not SD	5	2	7	1	2	3	6	7	8	5	<b>36/49</b>
Trypsin/protein ratio 1/50	3	4	7	2	2	4	1	4	5	0	<b>21/49</b>

Trypsin/protein ratio 1/200	0	0	0	0	1	1	0	2	2	0	<b>5/49</b>
Not SD	2	0	2	1	1	2	5	3	6	5	<b>23/49</b>
Trypsin/protein ratio 1/100	3	3	6	2	2	4	1	4	4	1	<b>20/49</b>
Trypsin/protein ratio 1/200	0	0	0	0	1	1	0	0	2	0	<b>3/49</b>
Not SD	2	1	3	1	1	2	5	5	7	4	<b>26/49</b>
StrataX SPE	2	0	2	1	0	1	0	0	1	0	<b>4/47</b>
C18 SPE	0	0	0	0	0	0	0	1	1	0	<b>2/47</b>
Not SD	3	4	7	2	4	6	5	8	11	4	<b>41/47</b>
with DMSO	0	0	0	0	0	0	0	0	0	0	<b>0/47</b>
without DMSO	0	0	0	0	1	1	1	1	1	0	<b>4/47</b>
Not SD	5	4	9	3	3	6	4	8	12	4	<b>43/47</b>

SD: Significantly different; \* Xevo TQ-S (Waters); \*\* QSight<sup>®</sup> 220 TQ (Perkin Elmer).