

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

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1	Optimization of a sample preparation workflow based on UHPLC-MS/MS method for
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25 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.

33 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 34 development, namely the optimization of the sample preparation protocol for six allergens 35 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. Different steps of the sample preparation protocol have been taken into consideration: (i) 38 sampling, (ii) composition of the extraction buffer, (iii) protein purification, (iv) protein 39 enzymatic digestion, (v) peptide purification and pre-concentration, and some experiments 40 were carried out by two independent laboratories and two different MS platforms to provide a 41 first assessment of the robustness of the method under development. Fifty target peptides 42 were monitored in multiple reaction monitoring mode and validated in different laboratories 43 44 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 45 full analytical validation of a prototype reference method for MS-based allergen 46 47 quantification.

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49 Keywords: food allergen detection, sample preparation, reference method, mass50 spectrometry, ThRAll.

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52 **1. Introduction**

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

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

food operators. This inconsistent application of PAL has led to a loss of trust in allergic 74 consumers, which do not fully understand their purpose [DunnGalvin 2019, DunnGalvin 75 2015]. Irrespective of whether the allergen risk assessment is performed appropriately or 76 77 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 78 establishment of mandatory thresholds possible. Such thresholds have already been set at 10 79 mg of total ingredient protein /kg in Japan, and the VITAL initiative takes into account 80 clinical data to extrapolate thresholds currently implemented in Australia and New-Zealand 81 82 to support the PAL [Taylor 2014]. Accurate and reliable methodologies enabling the detection and quantitation of 83 allergen traces in foodstuffs are urgently needed to support the risk assessment. Mass 84

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,

2017b, 2018; Nitride 2019; Sayers 2016, 2018; Planque 2016-2017-2017bis-2019; Henrottin

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,

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

Under this frame, the present work addresses the development of a prototype 113 reference method as commissioned by EFSA and focus on the optimization of a reliable, 114 straightforward, reproducible, and harmonized sample preparation protocol for multi-allergen 115 detection in processed foodstuffs by MS analysis. This report describes the optimization of a 116 multiplex MS method including 50 marker peptides (from 5 to 13 specific peptides [and from 117 118 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 119 confirm the robustness and reliability of the protocol developed herein. Since the detection 120 121 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 122 of these peptides. Typical method transfer procedures were also described herein, allowing to 123

easily and universally transpose this harmonized multi-allergen MS-based detection method 124 to various MS platforms. Besides MS parameters, sample preparation workflow was also 125 duly optimized, including: composition of the extraction buffer (also including denaturation 126 agents), inclusion of technical aids for protein and peptide purification (*i.e.* several stationary 127 phases), and optimization of the tryptic digestion (incubation time, enzyme to protein ratio, 128 addition of chemical aids to improve proteolytic activity (e.g., RapiGest SF)). The impact of 129 each parameter/modification on the signature peptide detection was investigated in incurred 130 chocolate (as an example of complex food matrix) according to a systematic approach to 131 132 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 133 detection. 134 135 2. Materials and Methods 136 2.1. Materials 137 Tris(hydroxymethyl)aminomethane (Tris), urea, dimethyl sulfoxide (DMSO), 138 DL-dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate (ABB) were from 139 Sigma-Aldrich (Bornem, Belgium). Trypsin Gold (mass spectrometry grade) was from 140 Promega (V5280; Leiden, The Netherlands). Acetic acid was from Acros Organics (Geel, 141 Belgium), hydrochloric acid was from Fisher Chemical (Loughborough, UK), and RapiGest 142 143 SF was from Waters (Milford, Massachusetts, USA). Acetonitrile, water, methanol (ULC-MS grade), and formic acid were obtained from Biosolve (Valkenswaard, The Netherlands). 144 Acrodisc® syringe filter with Versapor® membrane (PALL laboratory; 5 µm, 25 mm) was 145 obtained from VWR (Leuven, Belgium; #28143-963). PD-10 desalting columns pre-packed 146 with Sephadex G-25 M resin used for protein extract pre-purification were purchased from 147

148 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.

- 153
- 154

2.2. Model food matrix

The model food matrix used for the optimization of the parameters was an 155 incurred chocolate bar prepared within the ThRAll project, in a food pilot plant in order to 156 157 mimic real production process [Huet 2022]. Briefly, chocolate refiner flakes were weighed and an appropriate amount of each targeted allergenic ingredient (cow's milk, hen's egg, 158 peanut flour (lightly roasted and partially defatted powder), full fat soy flour (non-toasted), 159 160 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 161 obtain a mixture with equal particle (final particle size of $20 \,\mu$ m). The obtained pre-mix of 162 chocolate refiner flakes was further diluted with blank chocolate refiner flakes. These 163 chocolate refiner flakes were melted in a dry heat chocolate melter (Mol D'Art) at 40°C to 164 45°C. Once the refiner flakes were liquid, 200 g of cocoa butter were added and gently 165 mixed. Ammonium phosphatide (20 g), used as emulsifier, was added and mixed until a 166 glazy mass was obtained. The liquid chocolate was then transferred to a pastry bag and was 167 168 dripped into pellets of about five grams. Pellets were kept overnight at 4°C, packed in sealed aluminum laminate and finally stored at 4°C. The chosen contamination level for method 169 development was 40 mg total protein of each allergenic ingredient/ kg of chocolate bar (40 170 171 ppm).

172

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

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

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

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

9

°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 2020 and 2021]. Since the MS settings (including detected MRM 203 transitions, collision energies, etc.) may vary across MS platforms, the experimental 204 optimization of the mass spectrometry parameters was carried out as first optimization step. 205 Optimal cone voltage and collision energies were determined for all peptide markers under 206 207 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 208 without any further purification to identify the optimal MRM transitions and collision 209 210 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; 211 https://skyline.ms/project/home/software/Skyline/begin.view) was used to this purpose. The 212 following options were selected: b or y fragments generated (with a minimum length of 3 213 amino acids, in order to guarantee the maximum specificity), fixed carbamidomethylation of 214 cysteines, precursor ion charge +2 or +3, and product ion charge +1. In addition, due to the 215 multi-target MS/MS method under development, the number of MRM transitions were 216 limited to a maximum of five for each selected peptide. The optimal parameters determined 217 218 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 219 transitions under these optimized conditions. 220

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	$(2.00\pm0.02~g$ of sample in 50 mL Falcon tube) and stored at 4 $^\circ C$ 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 ± 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 [®] syringe filter with a 5 μ m Versapor [®] 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 $^{\circ}$ C for 15 min (600 rpm) for denaturation. Second, once cooled
279	down on ice, DTT (50 $\mu L,500$ 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 $\mu L,100$ 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 μ g/ μ L in acetic acid 50
284	mM) was added (10 μ L; 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 μ L, 1 M) and centrifuged (14 800 rpm; 10 min).

287

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2.4.1.5 Peptide purification and sample extract concentration

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

evaporation under nitrogen flow (N₂; 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 centrifuged
(4660 g; 5 min; 10 °C). The supernatant was transferred into an injection vial and analyzed
by UHPLC-MS/MS.

303

2.4.1.6 Statistical treatment

In terms of method development, as shown in the scheme reported in Figure 1, 304 five steps of the sample preparation protocol were considered. For all selected procedures, 305 three independent replicates were carried out for statistical relevance of the comparisons (n =306 307 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% 308 significance level to evaluate the influence of each parameter on detection sensitivity. The 309 310 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 311 was performed for multiple comparisons of mean values. For each digestion time (t = 1, 4, 312 16, 24 h), mean values that are not significantly different will be marked with equal labels (a, 313 b, c, d) in relevant plots. 314

315

316 **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 2019, Korte 2019], especially when complex foodstuffs are going to be analyzed [Mattarozzi 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 2017, 2015]. 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 2013, Khuda 2015, Korte 2019, New 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.

330

3.1. Optimization of MS parameters

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

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

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

367

368

3.2. Sample preparation optimization

Each step of the sample preparation workflow displayed in Figure 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 ofrecorded mean values.

- 376
- 377 3.2.1. Chocolate pre-treatment

For a proper sampling of representative and homogeneous portions of the 378 chocolate bar, two physical approaches have been compared: grinding [Gu et al. 2018, Korte 379 et al. 2016, 2019] and melting [Huet 2022]. As for the grinding protocol special attention was 380 required to avoid unintended melting caused by overheating. Most of the detected peptides 381 382 (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 383 (Supplementary Data)). Some differences were disclosed only for soybean, since four 384 385 specific peptides (out of the 13 peptides selected) showed a higher sensitivity when grinding was used (Figure 2). All four peptides originated from the 11S globulin named glycinin 386 (known as the allergen Gly m 6), one of the major soy allergens associated with severe 387 allergic reactions to soybean in children [Holzhauser 2009, Ito 2011]. 388

In addition, a dual step preparation including melting and defatting of 389 chocolate sample, was also compared with the grinding procedure. The defatting step might 390 contribute to reduce the matrix complexity, by removal of the lipophilic components 391 potentially interfering with the enzymatic proteolysis and/or with the peptide detection [New 392 393 2018, 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 394 peptides), peanut (two peptides), hazelnut (one peptide) and soy (one peptide), appeared to be 395 396 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 397 suffers from several drawbacks, being time-consuming and neither user nor environmentally 398

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friendly. Therefore, as the impact of an additional hexane defatting step is limited to some 399 peptides, the grinding procedure was preferred and chosen for the preparation and 400 homogenization of the sample, being simple and environmental/user friendly, also confirming 401 the protocols previously applied by independent research groups on similar matrixes 402 [Bignardi 2013, Sayers 2018, Gu 2018, Korte 2016, 2019]. 403 404 405 3.2.2. Protein extraction – Extraction buffer The next step of the sample protocol (Figure 1) is protein extraction, which 406 407 usually requires the use of Tris-buffered saline (TBS), NH4HCO3, or Tris.HCl, at a pH value of 8.0 to 9.2 [Gu 2018, Martinez-Esteso 2020, Shefcheck 2006, Plangue 2016, Monaci 2014]. 408 These buffers can be used in combination with denaturing, reducing agents, and/or 409 410 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 411 [Martinez-esteso 2020, Monaci 2014, New 2018, 2020, Savers 2018, Sagu 2021, Xiong 412 2021]. However, some of these additives can interfere with the enzymatic digestion step (e.g., 413 proteases like trypsin are inhibited by urea concentrations higher than 1 M) or may adversely 414 affect the MS analysis (e.g., SDS is not MS compatible); therefore whenever added to 415 improve the protein extraction yield, such additives require proper removal steps in the 416 preparation workflow such as solid phase extraction, cut-off filtration or dilution down to 417 418 compatible concentrations, to avoid any interference with the final detection [Boo 2018, Croote 2019, Monaci 2020, Planque 2016-2019, Xiong 2021]. In addition, to further improve 419 the extraction yield, a sonication step may also be included as physical aid [Monaci 2014, 420 Pilolli 2017a, Planque 2016], particularly efficient in promoting the recovery of specific 421 allergenic proteins such as caseinate proteins from milk [Monaci 2014]. 422

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

446

447 3.2.3. SEC protein purification

When used as a chaotropic agent in protein extraction, a major drawback of 448 urea is that concentrations higher than 1 M can denature trypsin, reducing the effectiveness of 449 the digestion step. This can be overcome by diluting the extract with ammonium bicarbonate 450 buffer before the digestion step to give a theoretical urea concentration below 1 M [Croote 451 2019, Planque 2016-2017a-2017b-2019]. Alternatively, technical aids for buffer exchange 452 using disposable cartridge-based size exclusion chromatography (SEC) can be implemented 453 to remove urea. This option also has the additional advantage of simplifying the sample 454 composition by removing low molecular weight interfering compounds (5kDa cut-off of the 455 456 stationary phase), such as polyphenols, that might be co-extracted from the matrix, thus reducing the background signal from the chocolate matrix [Pilolli 2017b, 2018, 2021]. 457 Therefore, direct dilution and SEC protein purification procedures (using either a spin or 458 459 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 460 the detection of peptides from milk, egg, hazelnut, soy, and almond proteins compared to 461 dilution. For peanut, only one peptide (SPDIYNPQAGSLK²⁺) showed a significant 462 improvement in its MS detection after SEC protein purification (Supplementary Data -463 Figure S6). Of the SEC elution procedures (gravity *versus* spin protocols), the spin elution 464 significantly improved the detection of 90% of the peptides (44 out of 49 peptides), 465 irrespective of the allergen or protein fraction (Table 1; Supplementary Data – Figure S7), 466 467 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 468 centrifugation was chosen to be included in the final optimized protocol. 469

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471 3.2.4. Protein digestion with trypsin

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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 476 surfactant RapiGest SF, on the efficiency of in-solution enzymatic digestion [Johnson 2016, 477 Sayers 2018]. Unlike other commonly used denaturants, RapiGest SF does not modify 478 peptides or protease (trypsin) activity and is hydrolysable at acid pH (half-life 8 min at pH 2). 479 480 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 481 sample) just before proteins thermal denaturation (at 95°C), and hydrolyzed during protease 482 483 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 484 negatively affected by the RapiGest SF (Table 1 and Supplementary Data – Figure S8), 485 which was thus not included in further optimization steps and the final protocol. 486

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

effect was observed for soy and egg peptides. It is noteworthy that this influence is quite 497 different depending on the target allergen (egg or soy). Most of the egg peptides displayed a 498 significant improved detection when using a 1/50 trypsin to protein ratio, while for soy a 499 500 lower trypsin to protein ratio significantly favored detection of the most intense peptide VLIVPQNFVVAAR²⁺ (Supplementary Data – Figure S9). Based on these results, the 1/100 501 trypsin to protein ratio was found to be the best compromise, allowing effective protein 502 digestion at a lower cost. To reduce furtherly the analysis costs, the digestion of a lower 503 absolute amount of extract (0.5 mL instead of 1.0 mL) with this 1/100 trypsin to protein ratio 504 505 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 506 final optimized protocol and applied in the following optimization steps, confirming similar 507 508 protocols already described in the literature [Xiong 2021].

509 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 510 protein using a time course experiment [Nitride 2019, Korte 2019]. A single batch of the 40 511 ppm allergen incurred chocolate sample was extracted with Tris-HCl containing 5 M urea, 512 subjected to SEC protein purification, and aliquoted to carry out independent digestions at 513 fixed trypsin to protein ratio (1/100) and different time course: 1, 4, 16, and 24 h (Figure 4 514 and Supplementary Data Figure S10). The effect of digestion time on the measured peak 515 516 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 517 Gly m 6, or almond allergen Pru du 6 displayed peculiar kinetics depending on the specific 518 sequence (Figure 4 and Supplementary Data – Figure S10). This experimental evidence can 519 likely be ascribed to the accessibility of the peptide bonds specifically cleaved by trypsin. For 520 some of the selected markers, the resulting digestion rate was very high, and the peptide 521

522	appeared to be fully released after only 1 h digestion (see, for example, FFVAPFPEVFGK ^{$2+$}
523	and YLGYLEQLLR ²⁺ from α -S1 casein (<i>Bos d 9</i>); VLIVPQNFVVAAR ²⁺ and
524	SQSDNFEYVSFK ²⁺ from soy <i>Gly m</i> 6; GNLDFNVQPPR ²⁺ and ALPDEVQNAFR ²⁺ from
525	almond <i>Pru du 6</i> ; and ADIYTEQVGR ²⁺ and INTVNSNTLPVLR ²⁺ from hazelnut <i>Cor a 9</i>).
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 ²⁺ and FALPQYLK ²⁺ from α -S2 casein,
529	<i>Bos d 10</i> ; VYVEELKPTEGDLEILLQK ³⁺ and VLVLDTDYK ²⁺ from β -lactoglobulin <i>Bos d</i>
530	5, GGLEPINFQTAADQAR ²⁺ and ISQAVHAAHAEINEAGR ³⁺ from ovalbumin Gal d 2,
531	VLLEENAGGEQEER ²⁺ from peanut Ara h 1, NILEASYDTK ²⁺ from soybean Gly m 5,
532	TEENAFINTLAGR ^{2+/3+} and ADIFSPR ²⁺ from almond <i>Pru du</i> 6, and
533	ALPDDVLANAFQISR ²⁺ from hazelnut <i>Cor a 9</i> 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 ²⁺ from soy <i>Gly m</i> 6 (Figure 4), QVQELAFPGSAQDVER ²⁺ from soy
540	Gly m 5, NLPQQCGLR ²⁺ from peanut Ara h 2, GNLDFVQPPR ^{2+/3+} and
541	ALPDEVLQNAFR ²⁺ almond <i>Pru du</i> 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).

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 ^{$2+/3+$} and NAVPITPTLNR ^{$2+$}), one from egg white
560	(ISQAVHAAHAEINEAGR ³⁺), and one from soy <i>Gly</i> $m \delta$ (ISTLNSLTLPALR ²⁺) 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 $^{2+}$) and
563	another from soy $Gly m 5$ (VPSGTTYYVVNPDNNENLR ²⁺) 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

with the complete solvent evaporation, and as a fact most of the detected peptide markers 572 (43/47) did not show any significant impact on their detection when DMSO is present 573 (Supplementary Data – Figure S12), while four peptides (LPLSLPVGPR²⁺ from egg volk 574 protein, TANDLNLLILR²⁺ from peanut, ALPDDVLANAFQISR²⁺ from hazelnut, and 575 ISTLNSLTLPALR²⁺ from soy Gly m 6) (Supplementary Data – Figure S13) were even 576 significantly better detected when evaporation was performed in the absence of DMSO. 577 Given this results, the use of DMSO was not included during the evaporation step in the final 578 optimized method even though this solvent may facilitate crude extract solubilization. 579

580

3.3. Summary of the sample preparation workflow and final considerations

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

597 including the purification of tryptic peptides on SPE cartridge and their concentration, is

applied to improve the sensitivity of the final method under development.

599

600 **4.** Conclusions

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

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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).

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Figure 3





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)

		Milk			Egg						Number of SD and
Sample preparation step					00						not SD peptides
modified/optimized							Peanut	Hazelnut	Soy	Almond	over total number
incurred, optimized	Casein	β-lactoglobulin	Total	White	Yolk	Total					of detected
											peptides
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).