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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Abstract

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

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

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

1. Introduction

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

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

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

Accurate and reliable methodologies enabling the detection and quantitation of allergen traces in foodstuffs are urgently needed to support the risk assessment. Mass Spectrometry (MS) is one of the most promising techniques that proved to be successfully applied to allergen detection, identification, quantification, and characterization for over a decade now, and has much promise as a reference method for food allergen analysis [Monaci 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 compared different aspects of food allergen quantification using advanced MS techniques, highlighting the main gaps that need to be addressed in terms of harmonization and results comparability across independent laboratories [Monaci 2018].

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

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

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

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

2. Materials and Methods

2.1. Materials

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

extraction (SPE) columns (1 cc, 50 mg; WAT054955) and Strata-X polymeric reversed phase (33 µm; 30 mg; 1 mL; 8B-S100-TAK) were purchased from Waters (Milford, Massachusetts, USA) and Phenomenex (Torrance, California, USA), respectively, and used for peptide purification and enrichment.

2.2. Model food matrix

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

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

2.3.1. UHPLC-MS/MS parameters and MRM selection

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

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

Additional experiments were carried out on a UHPLC LX-50 system coupled with a QSight® 220 triple quadrupole mass analyzer (Perkin Elmer). Chromatographic 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 10% to 35% B; 33.0–33.2 from 35% to 90% B, constant at 90% B for 10 min, from 43.2 to 43.4 min from 90% to 10% of solvent B, column equilibration for 16 min. Timed-MRM acquisition in positive ion mode was set up at unit resolution in both Q1 and Q3, and with 2 min wide acquisition windows. Electrospray source parameters were set as follows: drying gas (nitrogen): 120 (arbitrary units), hot-surface induced desolvation (HSID™) Temp: 250

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

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

2.4. Sample preparation protocol – optimization

2.4.1. Sample preparation

2.4.1.1 Chocolate pre-treatment

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

a) Grinding

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

b) Melting

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

c) Melting and defatting

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

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 µm 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

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

2.4.1.5 Peptide purification and sample extract concentration

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

evaporation under nitrogen flow (N_2; 40 °C) up to dryness. The dried extract was solubilized in 5% acetonitrile in 0.1% formic acid in water solution (100 µL), vortexed, and centrifuged (4660 g; 5 min; 10 °C). The supernatant was transferred into an injection vial and analyzed by UHPLC-MS/MS.

2.4.1.6 Statistical treatment

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

3. Results and Discussion

In this investigation, the optimization of a sample preparation workflow for multi-allergen detection in chocolate has been carried out. The accomplishment of this task is very important to understand and compensate for the effects that the specific matrix composition may have on the reliability and sensitivity of the LC-MS based detection [Croote 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.

3.1. Optimization of MS parameters

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

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

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

3.2. Sample preparation optimization

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

3.2.1. Chocolate pre-treatment

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

In addition, a dual step preparation including melting and defatting of chocolate sample, was also compared with the grinding procedure. The defatting step might contribute to reduce the matrix complexity, by removal of the lipophilic components potentially interfering with the enzymatic proteolysis and/or with the peptide detection [New 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 peptides), peanut (two peptides), hazelnut (one peptide) and soy (one peptide), appeared to be promoted by the melting-defatting procedure and two promoted, on the contrary, by the grinding procedure (see Figure S3 for details). However, the melting-defatting procedure suffers from several drawbacks, being time-consuming and neither user nor environmentally

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), NH_4HCO_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 β-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].

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

3.2.3. SEC protein purification

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

3.2.4. Protein digestion with trypsin

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

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

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

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

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

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

3.2.5. Tryptic peptides purification

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

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

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

3.3. Summary of the sample preparation workflow and final considerations

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

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

4. Conclusions

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

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

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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 (α = 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 (α = 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 1

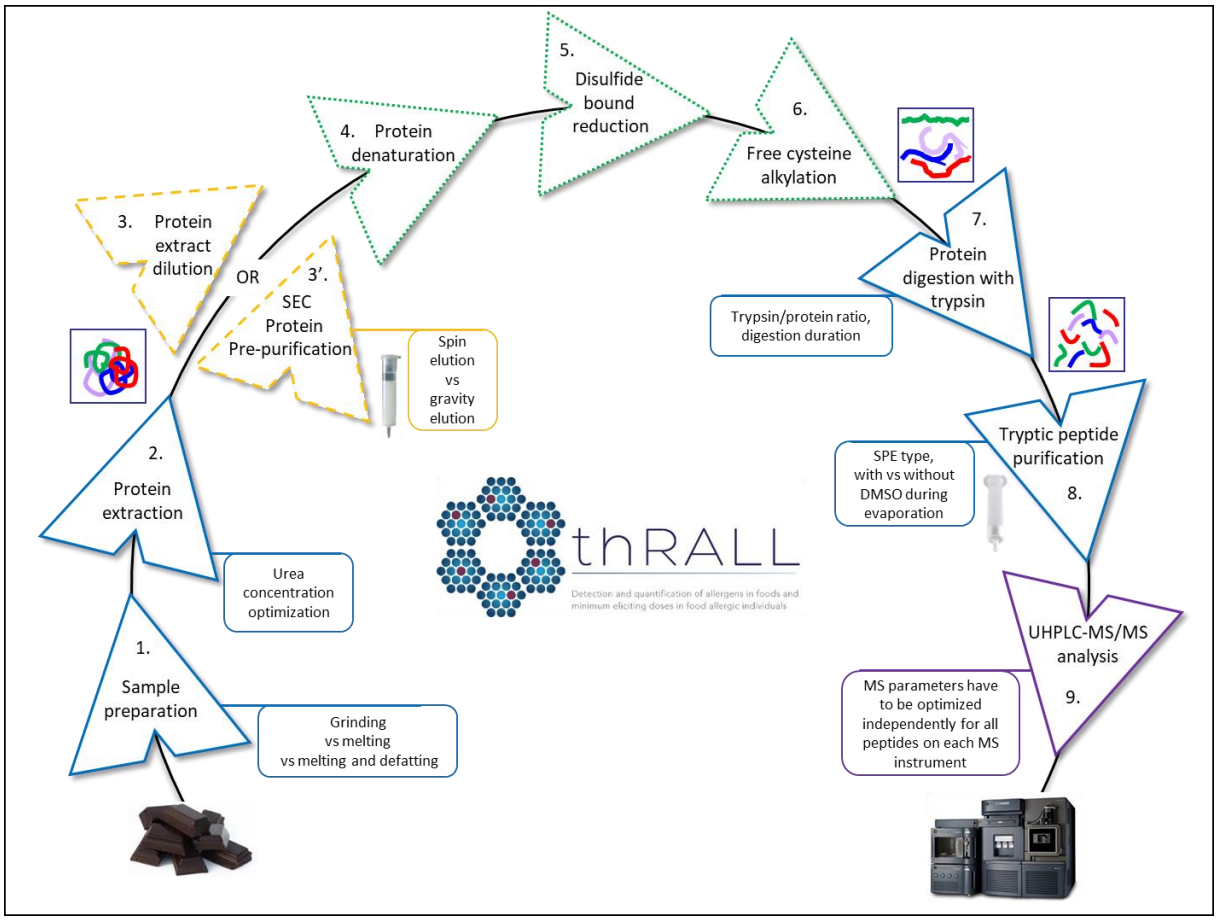
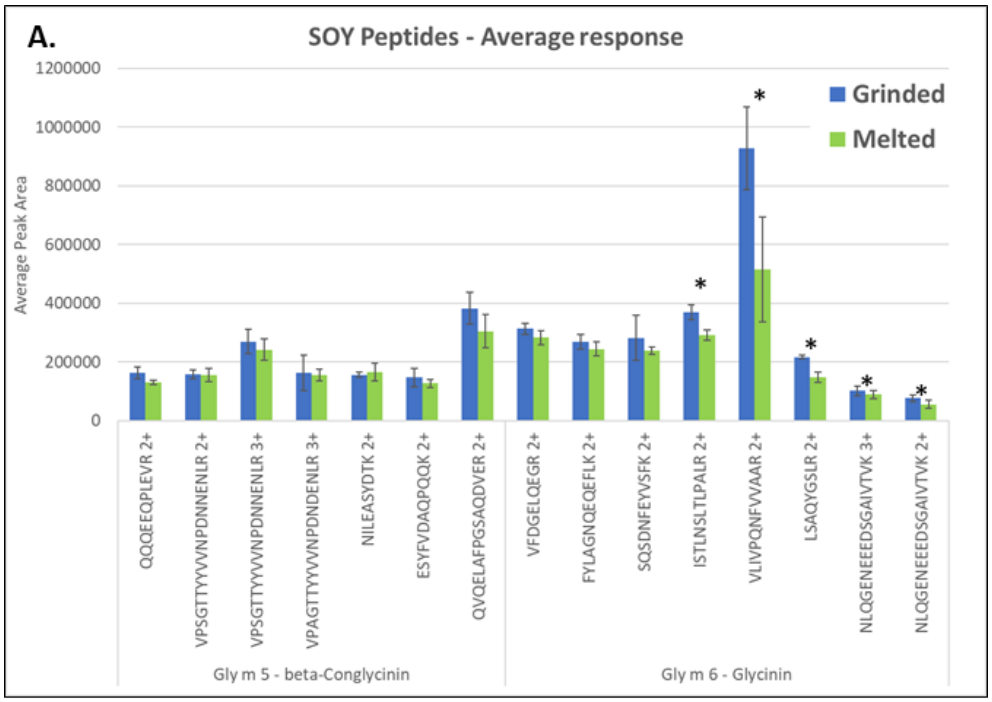

1. Sample preparation
Grinding vs melting vs melting and defatting
2. Protein extraction
Urea concentration optimization
3. Protein extract dilution
OR
3'. SEC Protein Pre-purification
Spin elution vs gravity elution
4. Protein denaturation
5. Disulfide bound reduction
6. Free cysteine alkylation
7. Protein digestion with trypsin
Trypsin/protein ratio, digestion duration
Tryptic peptide purification
8.
SPE type, with vs without DMSO during evaporation
UHPLC-MS/MS analysis
9.
MS parameters have to be optimized independently for all peptides on each MS instrument
thRALL
Detection and quantification of allergens in foods and minimum eliciting doses in food allergic individuals

Figure 2


A.
SOY Peptides - Average response
Average Peak Area
1200000
1000000
800000
600000
400000
200000
0
Grinded
Melted
*
QQQEEQPLEVR 2+
VPSGTTYYVVNPDNNENLR 2+
VPSGTTYYVVNPDNNENLR 3+
VPAGTTYYVVNPDNDENLR 3+
NILEASYDTK 2+
ESYFVDAQPQQK 2+
QVQELAFPGSAQDVER 2+
VFDGELQEGR 2+
FYLAGNQEQEFLK 2+
SQSDNFEYVSFK 2+
ISTLNSLTLPALR 2+
VLIVPQNFVVAAR 2+
LSAQYGSLR 2+
NLQGENEEEDSGAIVTVK 3+
NLQGENEEEDSGAIVTVK 2+
Gly m 5 - beta-Conglycinin
Gly m 6 - Glycinin

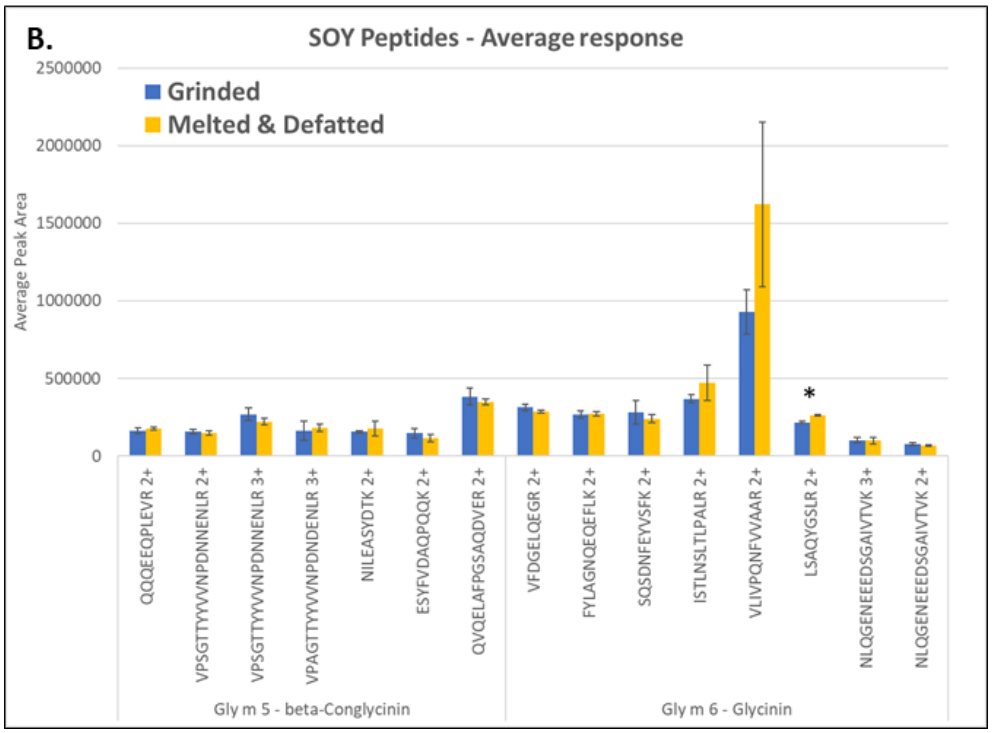

B.
SOY Peptides - Average response
Average Peak Area
2500000
2000000
1500000
1000000
500000
0
Grinded
Melted & Defatted
*
QQQEEQPLEVR 2+
VPSGTTYYVVNPDNNENLR 2+
VPSGTTYYVVNPDNNENLR 3+
VPAGTTYYVVNPDNDENLR 3+
NILEASYDTK 2+
ESYFVDAQPQQK 2+
QVQELAFPGSAQDVER 2+
VFDGELQEGR 2+
FYLAGNQEQEFLK 2+
SQSDNFEYVSFK 2+
ISTLNSLTLPALR 2+
VLIVPQNFVVAAR 2+
LSAQYGSLR 2+
NLQGENEEEDSGAIVTVK 3+
NLQGENEEEDSGAIVTVK 2+
Gly m 5 - beta-Conglycinin
Gly m 6 - Glycinin

Figure 3

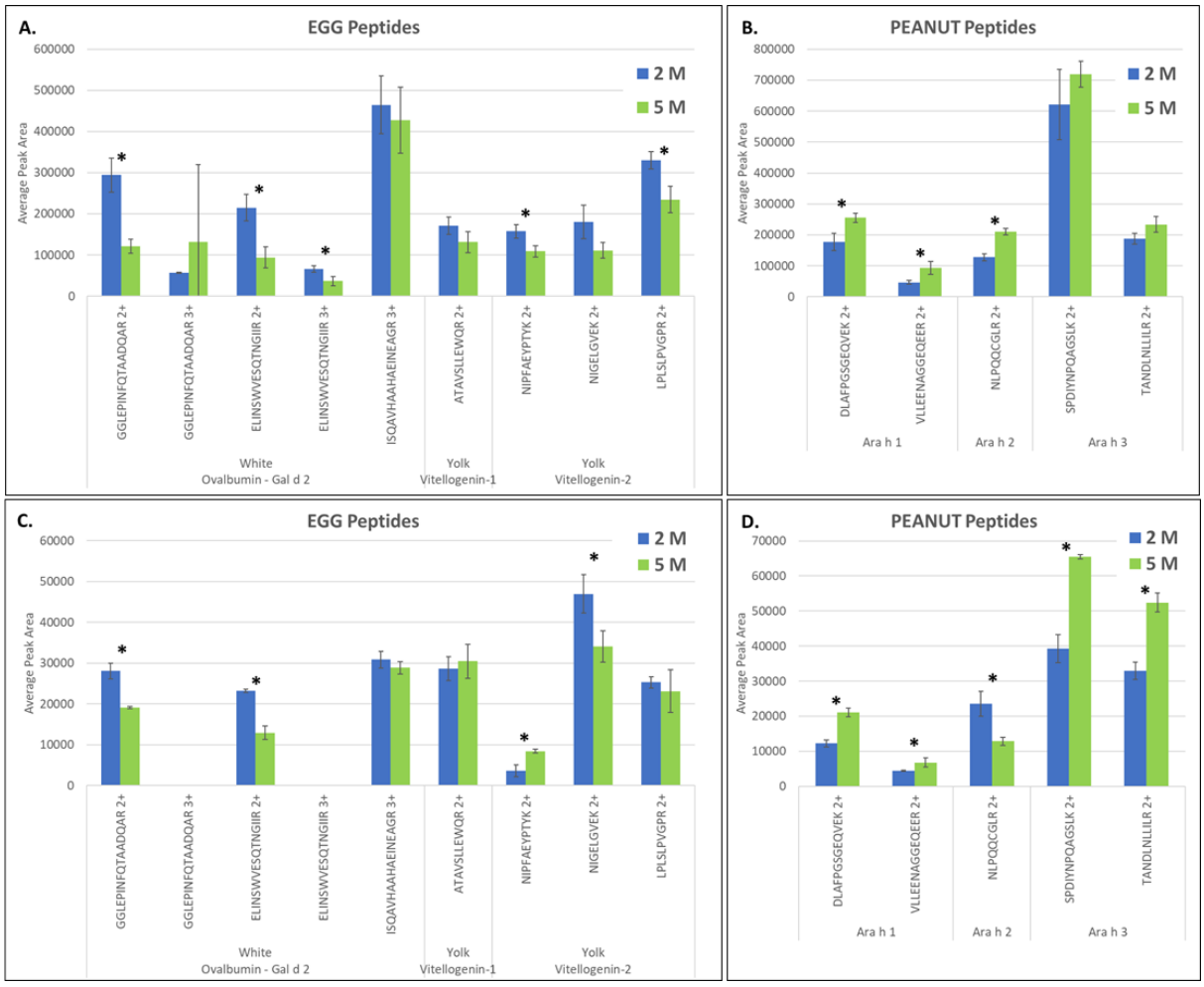

A.
EGG Peptides
Average Peak Area
2 M
5 M
GGLEPINFQTAADQAR 2+
GGLEPINFQTAADQAR 3+
ELINSWVESQTNGIIR 2+
ELINSWVESQTNGIIR 3+
ISQAVHAAHAEINEAGR 3+
ATAVSLLEWQR 2+
NIPFAEYPTYK 2+
NIGELGVEK 2+
LPLSLPVGPR 2+
White
Ovalbumin - Gal d 2
Yolk
Vitellogenin-1
Yolk
Vitellogenin-2
B.
PEANUT Peptides
DLAFPGSGEQVEK 2+
VLLEENAGGEQEER 2+
NLPQQCGLR 2+
SPDIYNPQAGSLK 2+
TANDLNLLILR 2+
Ara h 1
Ara h 2
Ara h 3
C.
EGG Peptides
D.
PEANUT Peptides

Figure 4

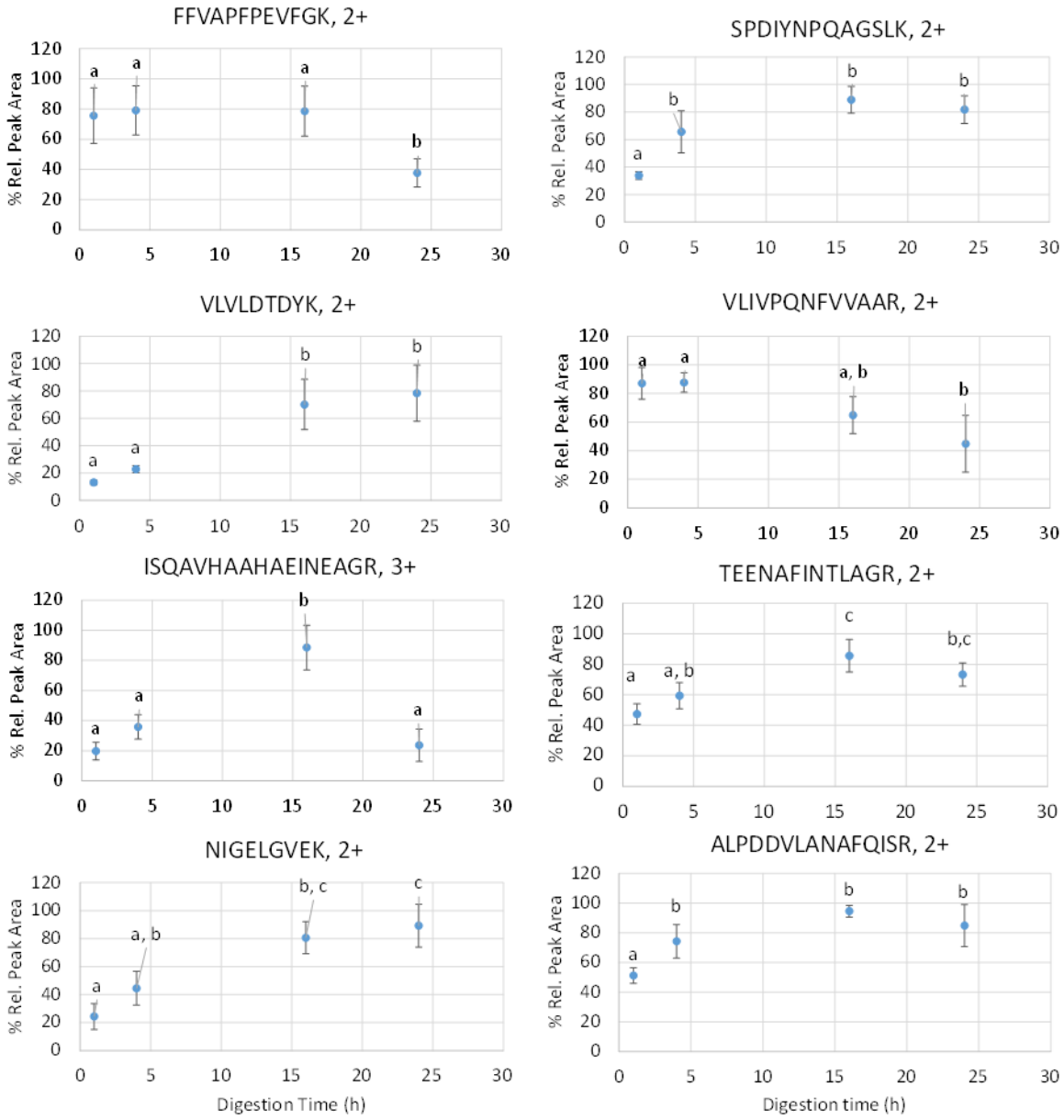

FFVAPFPEVFGK, 2+
SPDIYNPQAGSLK, 2+
VLVLDTDYK, 2+
VLIVPQNFVVAAR, 2+
ISQAVHAAHAEINEAGR, 3+
TEENAFINTLAGR, 2+
NIGELGVEK, 2+
ALPDDVLANAFQISR, 2+
% Rel. Peak Area
Digestion Time (h)
Digestion time (h)

Figure 5

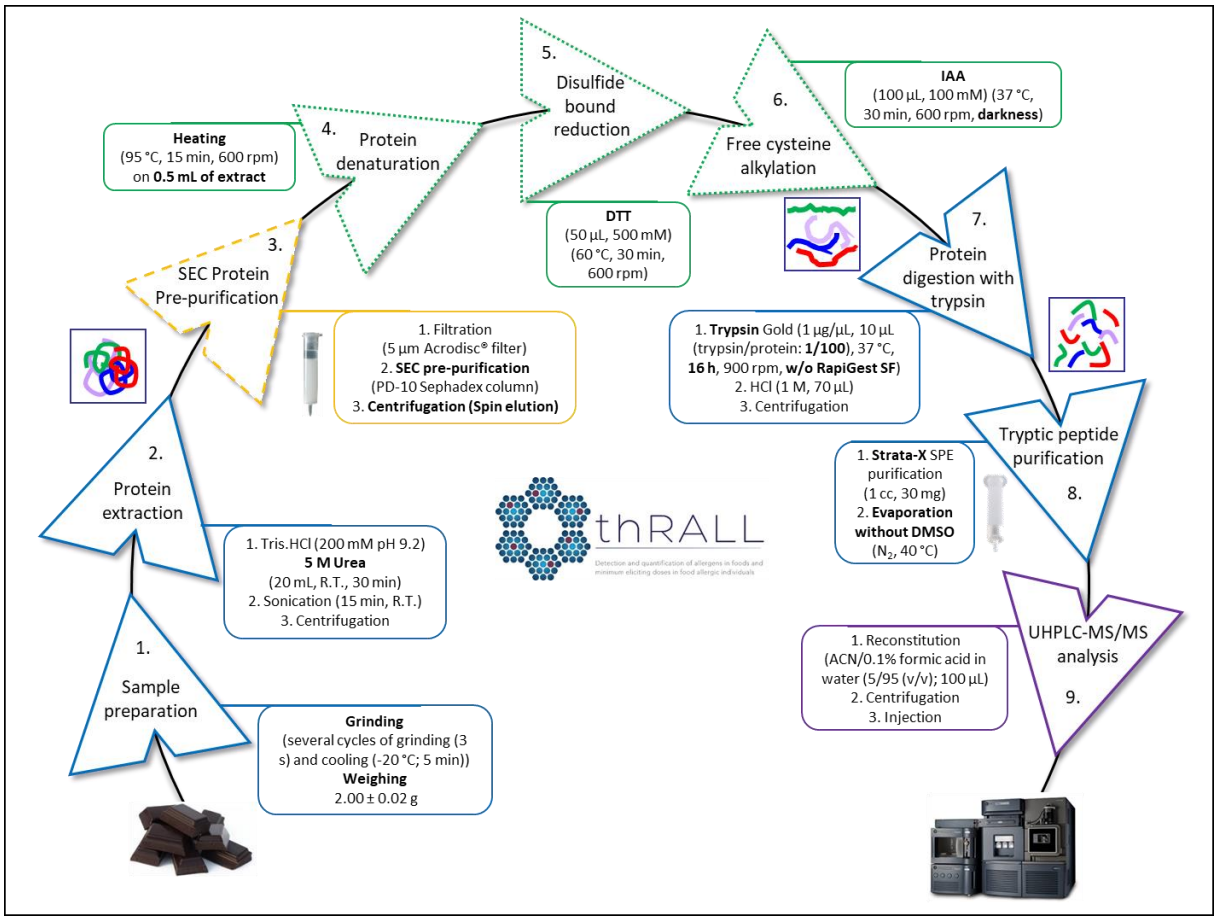

1. Sample preparation
Grinding
(several cycles of grinding (3 s) and cooling (-20 °C; 5 min))
Weighing
2.00 ± 0.02 g
2. Protein extraction
1. Tris.HCl (200 mM pH 9.2)
5 M Urea
(20 mL, R.T., 30 min)
2. Sonication (15 min, R.T.)
3. Centrifugation
3. SEC Protein Pre-purification
1. Filtration
(5 µm Acrodisc® filter)
2. SEC pre-purification
(PD-10 Sephadex column)
3. Centrifugation (Spin elution)
4. Protein denaturation
Heating
(95 °C, 15 min, 600 rpm)
on 0.5 mL of extract
5. Disulfide bound reduction
DTT
(50 µL, 500 mM)
(60 °C, 30 min, 600 rpm)
6. Free cysteine alkylation
IAA
(100 µL, 100 mM) (37 °C, 30 min, 600 rpm, darkness)
7. Protein digestion with trypsin
1. Trypsin Gold (1 µg/µL, 10 µL (trypsin/protein: 1/100), 37 °C, 16 h, 900 rpm, w/o RapiGest SF)
2. HCl (1 M, 70 µL)
3. Centrifugation
Tryptic peptide purification
8.
1. Strata-X SPE purification
(1 cc, 30 mg)
2. Evaporation without DMSO
(N2, 40 °C)
UHPLC-MS/MS analysis
9.
1. Reconstitution
(ACN/0.1% formic acid in water (5/95 (v/v); 100 µL)
2. Centrifugation
3. Injection
thRALL

Table 1

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

Sample preparation step modified/optimized	Milk			Egg			Peanut	Hazelnut	Soy	Almond	**Number of SD and not SD peptides over total number of detected peptides**
	Casein	β-lactoglobulin	*Total*	White	Yolk	*Total*					
Grinding	0	0	*0*	0	1	*1*	0	1	4	0	**6/47**
Melting	0	1	*1*	0	0	*0*	1	1	0	0	**3/47**
Not SD	5	3	*8*	3	3	*6*	4	7	9	4	**38/47**
Grinding	0	0	*0*	0	0	*0*	1	0	0	1	**2/47**
Melting & Defatting	0	0	*0*	2	0	*2*	2	1	1	0	**6/47**
Not SD	5	4	*9*	1	4	*5*	2	8	12	3	**39/47**
2 M Urea*	1	0	*1*	2	2	*4*	0	2	1	1	**9/47**
5 M Urea*	0	0	*0*	0	0	*0*	3	1	0	0	**4/47**
Not SD*	4	4	*8*	1	2	*3*	2	6	12	3	**34/47**

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

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

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