



Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Coupling SPE on-line pre-enrichment with HPLC and MS/MS for the sensitive detection of multiple allergens in wine

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ARTICLE INFO

Article history:

Received 2 August 2016

Received in revised form

15 September 2016

Accepted 21 September 2016

Available online xxx

Keywords:

Food allergens

Milk

Egg

On-line SPE

SRM-MS/MS

Food

Wine

ABSTRACT

Mass spectrometry (MS) represents an essential tool in proteomics studies, in the last years also exploited for monitoring allergens contamination in food products. Milk and egg are renowned allergens often used as fining agents to promote clarification of wines, therefore any residual amount in the end-products could represent a menace for allergic individuals. In view of this, it is of paramount importance to have at disposal sensitive analytical methods able to detect traces of milk and egg allergens in food. In this work we describe the upgrade and the optimization of an analytical workflow based on the use of a pre-enrichment column coupled with HPLC separation and MS/MS detection for the selective and sensitive detection of milk and egg allergens in white wine. Two different sample pre-treatments based on the use of mass cut-off filters or size exclusion cartridges were evaluated and compared, before tryptic digestion and LC-SRM-MS/MS analysis of the resulting peptides mixture. The devised UF based method coupled with peptide on-line pre-enrichment enabled to reach the lowest LODs down at 0.036 µg/mL and 0.05 µg/mL for egg and milk allergens respectively, proving to be the most sensitive strategy for monitoring allergens contamination in wine.

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1. Introduction

Wine is a worldwide consumed beverage obtained from the fermentation of grape must by appropriate processing and use of additives. Vinification can deeply affect the final characteristics of wine causing various defects capable of influencing the appearance, flavor, color and composition (including the content of alcohol, acids, etc.) of the final end product and consequently impairing consumer acceptability. In order to limit drawbacks due to vinification and to improve wine quality, addition of several additives during winemaking is a common practice (Peñas, di Lorenzo, Uberti, & Restani, 2015). Milk and egg proteins, well known to be among the most important protein allergens, are also typically utilized by the winery industry as fining agents to promote wine clarity and to improve wine color, flavor and physical stability (Yokosuka & Singleton, 1995). The mechanism of action lies in their interaction with wine polyphenols to form complexes that can be further removed by decantation or filtration steps (Castillo-Sánchez, Mejuto, Garrido, & García-Falcón, 2006). Inorganic

agents like bentonite, are also successfully used to remove residual allergenic proteins by a secondary fining procedure although this treatment can change according to the general practice adopted by the winery (Sauvage, Bach, Moutounet, & Vernhet, 2010). Even if processing aids would not remain in the final product, their absence (also in trace amount) must be assessed in the end product to exclude any clinical reaction triggered by the ingestion of milk or egg allergens by sensitive subjects (Peñas et al., 2015). Since this menace is likely to exist as pointed out in scientific opinions issued by EFSA (EFSA, 2011), it is of paramount importance to have at disposal analytical methods capable of detecting with a high level of confidence and sensitivity traces of these allergens in wine.

In order to protect the health of allergic people, food labeling legislation has been put in place around the world, such as Food Allergen Labeling and Consumer Protection Act (FALCPA) in the United States (Food Allergen Labeling and Consumer Protection, 2004) and several Directives in the European Union mandating the labeling of certain allergenic ingredients whenever used for food manufacturing, irrespective of their level of inclusion. Notably, Directive 2007/68/EC provides a list of 14 groups of allergenic foods to be obligatorily labeled, including gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybeans, milk, nuts, celery, mustard, sesame seeds, lupine, mollusks, and sulfites (European

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Commission, 2007). In addition, according to the EU Commission Implementing Regulation No. 579/2012 wine producers are currently obliged to indicate egg (albumin and lysozyme)- and milk (caseins)-derived products on wine labels whenever used for clarification (European Commission, 2012). Concerning fining agents in wine, the International Organization of Vine and Wine (OIV), following an inter-laboratory trial based on an enzyme-linked immune-sorbent-assay (ELISA), published a document establishing the criteria for the methods of quantification of residual amounts of milk and egg fining agents in wines (OIV, 2012). In this document for the first time were indicated both the limit of detection and quantification at 0.25 mg/L and 0.50 mg/L to be fulfilled by the immunoassay. According to the Regulation 579/2012 these values represent the most restrictive thresholds established for the declaration of allergenic proteins on wine labels. Currently, ELISA is the most widespread and easy-to-use analytical tool for the detection of allergenic ingredients in food commodities. However antibody-based methods, such as ELISA, often suffer from limitations due to the complexity of the matrix under study and the presence of interfering compounds that might compromise the final results. As a valid alternative to immunochemical methods, mass spectrometry (MS) has been successfully applied to the detection of allergens in food, thanks to the several advantages offered by this technique including multi-target detection, unambiguous allergen identification and accurate quantitative data (Monaci & Visconti, 2010, 2012). Different analytical methods targeting milk and egg markers in wine by Mass Spectrometry have been reported in literature (Losito, Introna, Monaci, Minella, & Palmisano, 2013; Mattarozzi et al., 2014; Monaci, Losito, De Angelis, Pilolli, & Visconti, 2013; Monaci, Losito, Palmisano, & Visconti, 2010; Monaci, Losito, Palmisano, Godula, & Visconti, 2011; Pilolli, De Angelis, Godula, Visconti, & Monaci, 2014; Tolin et al., 2012) with the lowest detection limits comprised between 0.4 and 1 µg/mL in white wine. In the present work, a new analytical workflow using a multidimensional set up combining the on-line pre-enrichment with a chromatographic separation and MS detection for the simultaneous detection of milk and egg based fining agents in white wine is herein described. Our efforts were directed to simplifying and shortening the sample treatment procedure for time and cost-effectiveness method enabling challenging LODs for each target allergen. First, a SRM method for the detection and quantification of milk and egg allergens in white wine was optimized. Then to speed up sample handling, two different protocols based on cut-off filters and size exclusion purification cartridges were compared. After enzymatic digestion, the pool of peptides was injected into a C-18 one-cm-long trap-column whose function was to pre-enrich and partially purify the peptides of interest before the HPLC separation accomplished on the RP-C18 analytical column. The main advantages of this approach rely on the potential of i) independently optimizing the two steps by using a dual pump in order to decrease the sample complexity while eliminating the majority of matrix contaminants, ii) sample clean-up automation and iii) reduction of the analysis time (Barreiro, Luiz, Maciel, Maciel, & Lanças, 2015). To the best of our knowledge, this is the first report of a multi-target SRM based method for the detection of allergen contaminants in wine by exploiting an on-line pre-enrichment of the tryptic digests for quantitative purposes.

2. Experimental

2.1. Chemicals and reagents

Casein sodium salt from bovine milk (CN), egg-white powder (EP), acetonitrile (LC/MS grade), formic acid (FA), acetic acid, ammonium bicarbonate, hydrochloric acid, iodoacetamide (IAA)

and dithiothreitol (DTT) were provided by Sigma-Aldrich (Milan, Italy). Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA) and trypsin (proteomic grade) was purchased from Promega (Milan, Italy). Ultrafiltration (UF) tubes with 10 kDa cut-off membranes were provided from Millipore while disposable cartridges PD-10 were from GE Healthcare Life Sciences (Milan, Italy). Polytetrafluoroethylene (PTFE) syringe filters, 0.2 µm (size 4 mm) were obtained from Sartorius Italy S.r.l. (Muggiò, MB, Italy).

2.2. Standard solutions

Stock solutions of EP and CN were prepared in ammonium bicarbonate 50 mM at the final concentration of 1000 µg/mL and were diluted to produce working solutions used for wine fortification (comprised in the range 10–500 µg/mL).

2.3. Sample treatments and protein tryptic digestion

Experiments were carried out on white wine produced from *Pinot grigio* grape variety purchased from a local retailer. Two different sample treatment procedures were tested and for both of them matrix-matched calibration curves were produced in which each calibration point was prepared in two replicates. With reference to UF procedure (see Fig. 3), 5 mL of wine aliquots were fortified with CN and EP working solution at various concentration levels comprised between 0.1 and 2 µg/mL for each allergen, whereas in the case of SEC procedure, 5 mL of wine matrix were spiked within the range of 0.25 and 4 µg/mL. In both cases, the fortified wine was left under stirring to promote a complete dissolution of the added proteins into the wine matrix (15 min) and then was kept quiescent for 20 min at room temperature. In the UF procedure, 4 mL aliquot was successively withdrawn and centrifuged for 40 min at 3000g in 10 kDa cut-off tubes. The retained fraction was further submitted to dialysis a) against 1 mL of water to remove residual low molecular weight interferences eventually present in the extract and following dialysis b) against 1 mL of ammonium bicarbonate 50 mM for a further reduction of interfering compounds and to re-suspend sample proteins in a more appropriate solvent ready for the enzymatic digestion. A volume of 500 µL of wine extract underwent tryptic digestion according to the procedure already reported in another paper (Monaci et al., 2013). As final step, 4 µL of trypsin standard solution (0.1 µg/µL in acetic acid 50 mM) were added to the extract to have a final ratio higher than 1/50 (enzyme/protein, w/w). Samples were incubated at 37 °C under shaking for about 14 h in order to ensure a complete digestion. At last, 25 µL of a 1 M HCl were added to the digest to quench the trypsin activity. After digest centrifugation at 9400g for 10 min, the supernatant was carefully collected, filtered on 0.2 µm syringe filter and analyzed in LC/MS equipment.

In the SEC procedure (Fig. 3), a treatment on the size exclusion column was tested on the wine sample. Wine was loaded on a 5 kDa-size exclusion column PD-10 and the following steps were followed: column conditioning with 3 × 4 mL of deionized water and with by 4 × 4 mL of ammonium bicarbonate 50 mM, followed by centrifugation for 2 min (at 1000g). The eluted fraction (2.5 mL) was collected and 500 µL of purified sample were submitted to tryptic digestion according to the procedure previously described.

To assess recovery, two samples were prepared and compared each other: 1) allergen-free wine fortified prior to undergoing the whole sample treatment; 2) allergen-free wine fortified with both allergens after the clean-up procedure and before the enzymatic digestion. In both cases samples were spiked at the

concentration of 1 $\mu\text{g/mL}$ for each allergenic ingredient considered.

2.4. On-line pre-enrichment scheme coupled to HPLC and MS/MS analysis

Wine digests were analyzed on a fully automated on-line solid phase extraction (SPE) - reversed phase (RP) HPLC-MS/MS system consisted of a dual UHPLC pumps provided with an autosampler programmed to control a 2-position switching valve (10-ports) and a Dual Pressure Linear Ion Trap Mass Spectrometer Velos Pro™ equipped with an ESI interface (Thermo Scientific, San José, USA). Peptide enrichment and purification was accomplished on an Acclaim™ 300 C-18 cartridge ($2 \times 10 \text{ mm}$, $3 \mu\text{m}$, $300 \text{ \AA}$, Thermo Fisher-San José, US) while an Aeris peptide $3.6 \mu\text{XB-C-18}$ analytical column ($150 \times 2.10 \text{ mm}$, $3.6 \mu\text{m}$, $100 \text{ \AA}$, Phenomenex) was employed for the chromatographic separation. For both SPE and RP-HPLC analysis, the following mobile phase was employed: solvent A = $\text{H}_2\text{O} + 0.1\% \text{FA}$; solvent B = acetonitrile + $0.1\% \text{FA}$. The pre-enrichment/clean-up step was performed by loading 50 μL of wine digest onto the SPE column at 300 $\mu\text{L/min}$ by keeping the valve in position 1 (the trap column connected to the waste, Fig. 1A). The multistep gradient started with 100% of solvent A kept stable for 2 min, then a short washing step of the cartridge was performed by ramping solvent B from 0 to 8% in 1 min and then maintained at 8% for another minute. After 4 min the valve was switched to position 2 to allow the elution of the captures peptides and its transfer to the analytical column for the chromatographic separation (Fig. 1B). To this aim the following gradient was optimized: from 15% to 40% of solvent B in 24 min, then up to 80% in 1 min, kept stable for 10 min, and then back to 15% in 0.2 min. This

composition was maintained for 16 min to allow column equilibration. The flow rate was set at 200 $\mu\text{L/min}$.

2.5. MS/MS untargeted analysis

For the identification of allergen proteins, a list of marker peptides providing good ESI sensitivity and unambiguous attribution to the target protein is required. At this aim a tryptic digest of wine sample fortified with 1 $\mu\text{g/mL}$ for each allergen and submitted to both sample treatment investigated, was analyzed by running MS system in the acquisition scheme designed *Nth order double play* (Data Dependent™ Acquisition, DDA) mode by activating the dynamic exclusion. Recorded data was searched against a customized database (including egg and milk proteins) for allergenic protein identification by Proteome Discoverer™ (version 1.4) software (Thermo-Fisher-Scientific, San José, US). Instrumental set-up was detailed in our previous work (Monaci, Pilolli, De Angelis, Godula, & Visconti, 2014).

2.6. Multiple selected reaction monitoring (SRM) analysis

After selection of the candidate peptide marker, MS was operated in Selected Reaction Monitoring (SRM) MS/MS acquisition mode by setting two segments/eight events acquisition scheme, where the eight events were represented by acquisitions in which each selected peptide marker was isolated and activated by CID with a normalized collision energy of 35%, and ion current related to the three most intense transitions were recorded within a 3 m/z window. ESI parameters were detailed elsewhere (Pilolli et al., 2014).

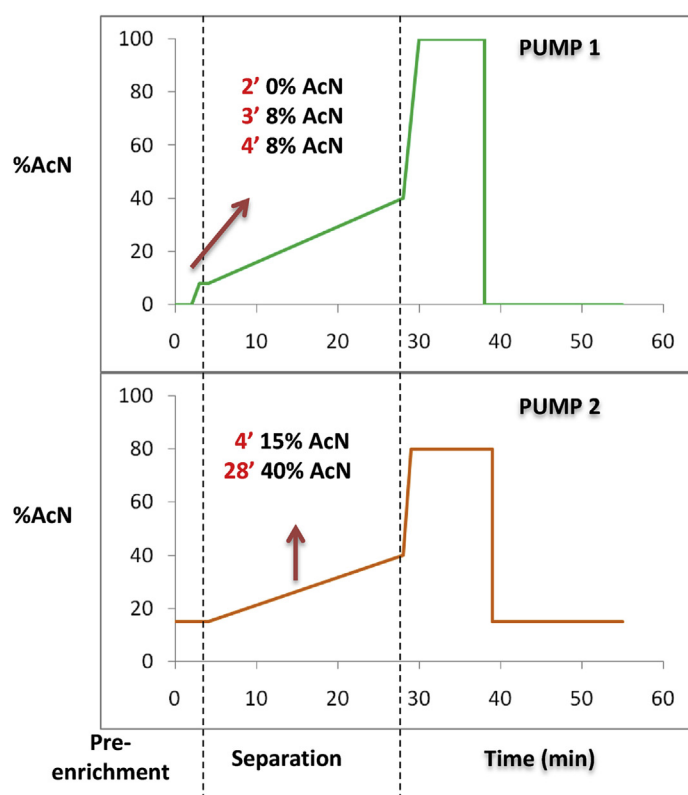
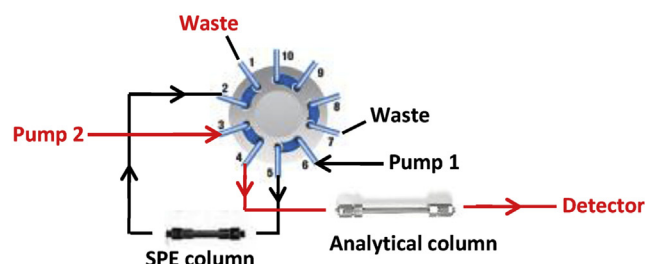
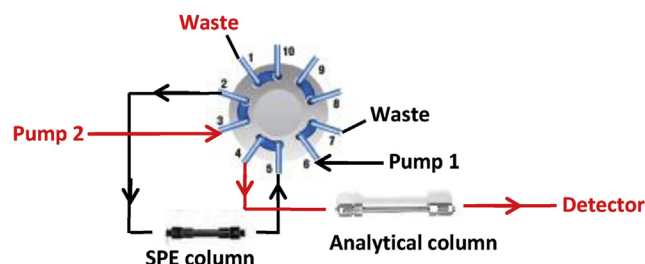


Fig. 1. Scheme of the optimized on-line pre-enrichment conditions before chromatographic separation and MS/MS analysis. The schematic representation of LC-LC system is also depicted. (A) pre-enrichment/washing position and (B) elution/separation position.

A) Pre-enrichment+clean up (valve position 1)



B) Separation (valve position 2)



3. Results and discussion

3.1. Selection of egg and milk allergen peptide markers

A new method based on on-line pre-enrichment coupled with HPLC separation and mass spectrometry detection for the simultaneous detection of milk and egg based fining agents in white wine is herein described. The first step in the development of a MS-based method for food allergen detection is the proper selection of peptide marker which should met specific criteria to be considered reliable for allergens detection (Johnson et al., 2011). Concerning milk and egg allergens, several studies have been reported in literature listing the most reliable peptides proved to be good allergen markers for confirmative and quantitative purposes in white wine (Losito et al., 2013; Monaci et al., 2013; Monaci et al., 2010; Monaci et al., 2011; Pilolli et al., 2014). Starting from the reported data, preliminary MS experiments were accomplished in order to confirm the suitability of the peptides markers identified in our previous work monitored for detecting milk and egg allergens by implementing this multidimensional MS-based analytical scheme. In particular, wine samples fortified at the level of 1 µg/mL for both allergens and treated with procedures UF and SEC reported in Fig. 3 were firstly acquired by MS-data dependent acquisition (DDA). Raw data were processed via commercial Proteome Discoverer™ software (version 1.4) and protein assignment was attained via Sequest scoring algorithm by searching MS/MS spectra against a small-size database compiled in house and restricted to food allergens and other contaminants (entries approximately total 200). MS-results confirmed that the peptides YLGYLEQLLR (YLG), FFVAPFPEVFGK (FFV) and EPMIGVNQELAYFYPELFR (EPM) arisen from alfa-S1casein digestion and the peptide DMPIQAFLLYQEPVLGPVR (DMP) obtained from beta-casein could be successfully used as good markers for milk detection in white wine. Concerning egg proteins, peptides LTEWTSSNVMEER (LTE) and GGLEPINFQTAADQAR (GGL) produced from ovalbumin and NTDGSTDY-GILQINSR (NTD) from lysozyme, were found to be the most suitable peptides for allergens detection in white wine. Beyond these markers, an additional peptide entered the list, namely peptide

AFKDEDTQAMPFR (AFK, derived from ovalbumin) was used as egg marker. This subset of peptides was employed to set up a tailored SRM acquisition method for the sensitive and selective detection of allergen markers in wines. Table 1 summarizes the list of monitored peptides along with the selected transitions included in the acquisition method. Finally, in order to design a proper MS acquisition scheme, parameters such as number of segments along the LC run, number of scan events per segment, acquisition scan rate (and consequently resolution) and acquisition range, were optimized. Based on the expected elution time for each peptide marker, the eight peptides included in the SRM method were separated in two segments with a maximum of four scan events per segment. Each selected peptide was isolated and activated by collision induced dissociation and ion current related to the three most intense transitions recorded within a 3 m/z window. A typical representation of a multi-target analysis restricted to only two peptides per allergen in SRM mode is reported in Fig. 2. It shows an overlay of four chromatographic traces referred to wine fortified at 2 µg/mL and submitted to SEC procedure along with the three transitions monitored for each peptide marker. Allergen-free wine was also analyzed in order to confirm the absence of any interfering peak at the expected retention times.

3.2. Sample pre-treatment

After confirmation of the most suitable peptide markers for qualitative/quantitative analysis, our work focused on the improvement of the purification protocol for target proteins in wine sample by exploring easy and rapid clean up strategies based on the combination between SPE on-line pre-enrichment and HPLC separation and MS/MS detection.

The first sample treatment protocol tested (designed as UF procedure) took advantage from our previous investigation in which a multi-allergen method for egg and milk proteins detection in white wine by using an Orbitrap™ technology-based mass spectrometer was developed (Monaci et al., 2013). The pre-treatment based on the use of UF tubes is schematized in Fig. 3 and included protein pre-concentration using ultra-filtration cut-off membranes followed by repeated dialysis step with de-ionized water and ammonium bicarbonate 50 mM in order to remove the majority of interfering compounds. Despite the long original procedure (approximately one hour and half), such streamlined workflow allowed to save time (the total time was reduced to one hour) with 8 folds pre-enrichment factor. The efficacy of the treatment was estimated by comparing peptide detection sensitivity obtained by using this procedure with that already published. A sensitivity gain of eight times was observed by using this workflow highlighting that, although proteins resulted less pre-concentrated compared to the original protocol (loss of pre-concentration factor of approximately five times), the exploitation of the on-line pre-enrichment scheme made possible to balance this drawback with an overall increase of the final sensitivity of the method.

Aiming to further speed-up sample handling, the first procedure tested was improved by replacing wine loading on UF cut-off membranes with its direct purification onto disposable size exclusion column (SEC). Although no protein pre-concentration occurred, good values of sensitivity for each peptide detected was observed, thanks to the pre-enrichment step feasible with this multidimensional set up employed. From the time-benefit point of view, given the rapidity and ease of execution (it takes only 20 min including column conditioning, loading and elution) this procedure was taken into consideration as clean up strategy for egg and milk protein analysis in white wine.

Table 1
Overview of m/z values and aminoacid sequences of the detected peptides along with the MS/MS transitions monitored for milk and egg proteins in wine.

m/z (charge)	Sequence	Peptide fragments	Target protein
519.24 (+3)	AFKDEDTQAMPFR	y ₃ ⁺ = 419.24 y ₄ ⁺ = 550.28 y ₅ ⁺ = 621.32	Ovalbumin
791.36 (+2)	LTEWTSSNVMEER	y ₅ ⁺ = 564.24 y ₉ ⁺ = 951.42 y ₁₀ ⁺ = 1052.47	Ovalbumin
877.42 (+2)	NTDGSTDYGILQINSR	y ₅ ⁺ = 489.28 y ₆ ⁺ = 617.34 y ₇ ⁺ = 730.42	Lysozyme
844.42 (+2)	GGLEPINFQTAADQAR	y ₇ ⁺ = 666.34 y ₁₁ ⁺ = 1121.53 y ₁₃ ⁺ = 1331.67	Ovalbumin
634.35 (+2)	YLGYLEQLLR	y ₆ ⁺ = 658.39 y ₇ ⁺ = 771.47 y ₉ ⁺ = 991.56	alfa-S1Casein
692.86 (+2)	FFVAPFPEVFGK	y ₈ ⁺ = 920.49 y ₉ ⁺ = 991.52 y ₁₀ ⁺ = 1090.59	alfa-S1Casein
1093.59 (+2)	DMPIQAFLLYQEPVLGPVR	y ₇ ⁺ = 737.45 y ₈ ⁺ = 866.51 y ₁₀ ⁺ = 1157.63	beta-Casein
1158.57 (+2)	EPMIGVNQELAYFYPELFR	y ₅ ⁺ = 661.37 y ₆ ⁺ = 824.43 y ₈ ⁺ = 1134.56	alfa-S1Casein

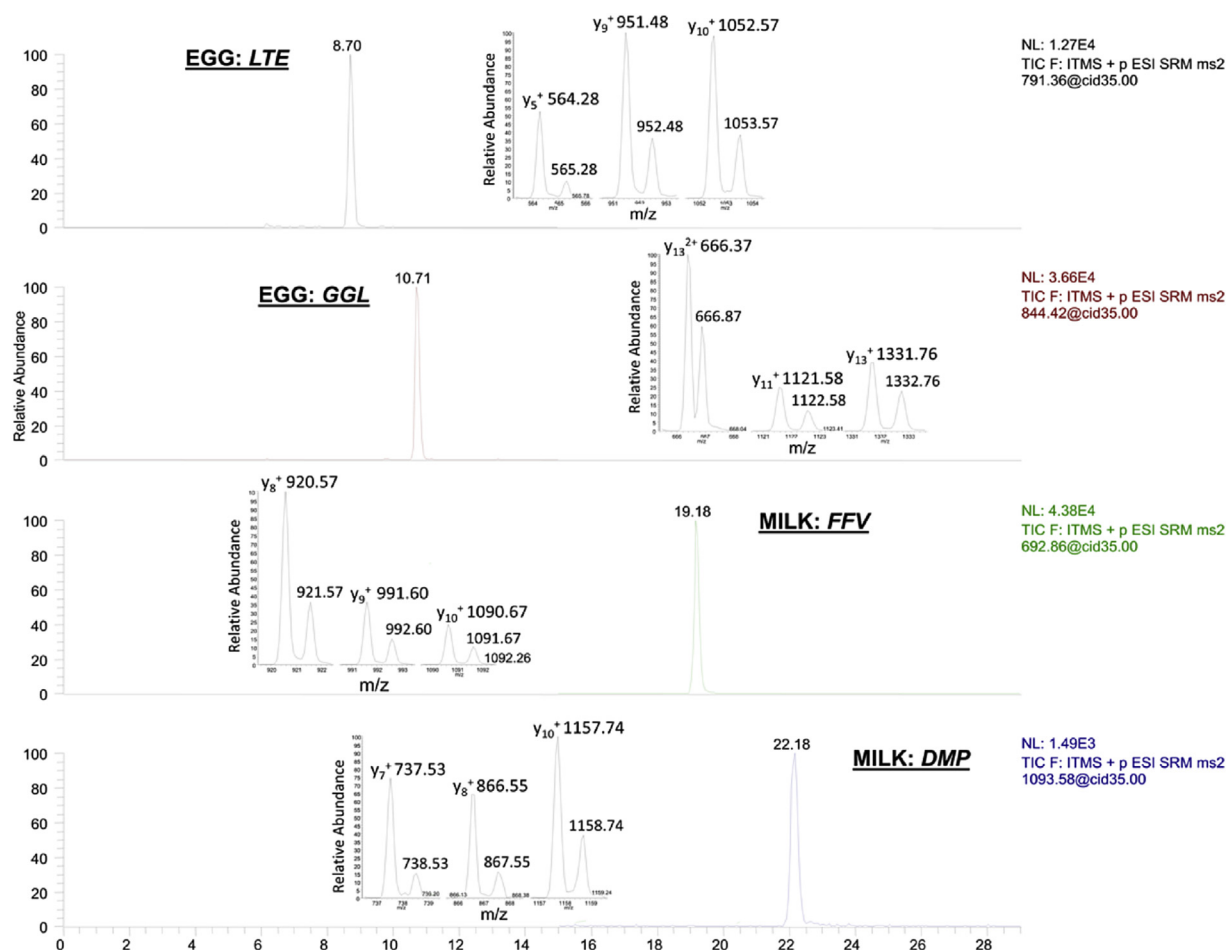


Fig. 2. Typical reconstructed ion chromatograms acquired in SRM-MS/MS mode along with the most relevant transition spectra for egg and milk quantitative peptides referred to SEC-purified wine sample fortified at 2 $\mu\text{g/mL}$.

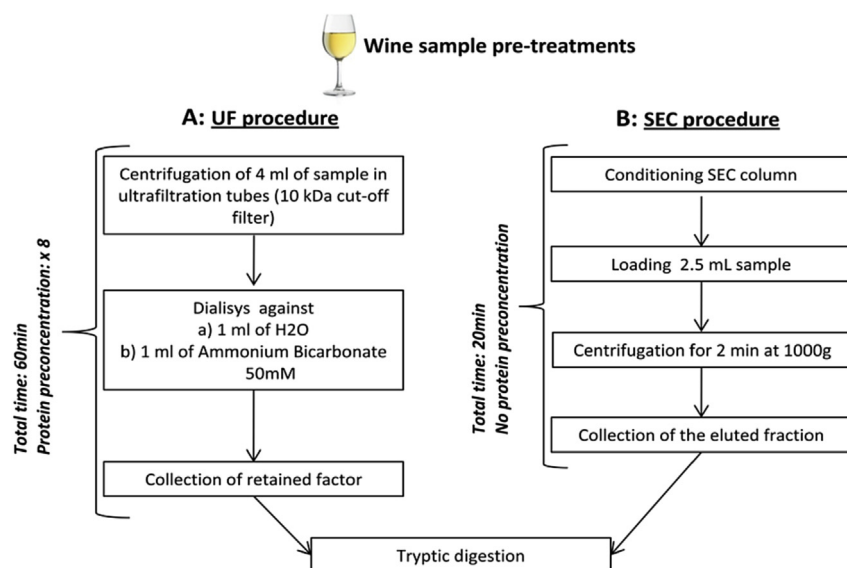


Fig. 3. Scheme of the different experimental workflows designed for multiplex detection of egg and milk allergens in white wine.

3.3. Optimization of pre-enrichment conditions for on-line SPE/RP-HPLC analysis

As inferring from above, the improvement of the analytical

workflow for egg and milk allergens detection in wine started from a sharper and straightaway sample pre-treatment procedure although a loss of protein was observed despite that expected. This unfavorable factor was successfully superseded by the

implementation of the multidimensional set-up available in our lab equipped with an HPLC system provided with two independent pumps and a 10-port-switching valve which allows devising an automated on-line selective enrichment of peptides on the C-18 trap column. On-line SPE-LC is increasingly becoming the tool of choice to facilitate and speed up purification for the analysis of complex matrices such as food and applications in different fields have been described so far in literature (Barreiro et al., 2015). Typically, it combines a short trap column with an analytical column allowing analytes trapping and separation. In particular, in this dual pump system, the loading pump transfers the injected sample on the trap column (position 1 in which the trap column is connected to the waste; see scheme in Fig. 1) and non retained compounds are transferred to the waste while the analytes of interest are trapped by the cartridge. Subsequently, thanks to the multi-ports valve, after valve switching (to position 2 connecting the trap-column directly to the analytical column) the analytical pump push the flow through the trap column allowing elution of the trapped compounds that are then sent to the LC column for their separation and final detection. The main advantages offered by this system rely on the higher selectivity, speed and easy automation that turns very useful when the system is coupled with a MS detector; hence, the on-line SPE clean-up step contributes to minimize the matrix effects improving method selectivity and sensitivity (de Mendoza et al., 2012).

In our work, this 2D set-up was used for peptide enrichment. In order to obtain the maximum trapping efficiency combined with the highest degree of matrix depletion, the proper composition of the mobile phase was investigated along the run and the intensity of peak areas of selected peptides was monitored and considered a distinctive factor for the appropriate tuning of the method. To this aim 50 μ L of tryptic digest was injected into the SPE C-18 column and different loading and washing conditions were tested. For the loading, 2 min in isocratic (100% solvent A: H₂O + 0.1% FA) was found to be the best condition for trapping target molecules. After 2 min loading, three solvent compositions were tested for removing the majority of other interfering compounds; in the first case the organic phase was increased up to 5% in 1 min (AcN + 0.1% FA) before switching the valve. In the second test after 2 min at isocratic condition, the organic phase was gradually increased up to 8% in 1 min; while in the last attempt the percentage of organic phase was gradually raised up to 8% in 1 min and this composition was kept stable for another 1 min. These last conditions provided the best results in terms of removal of interfering compounds combined with a minimum loss of analytical signal. The devised gradient extended the whole chromatographic run by only four minutes.

3.4. Evaluation of the performance of the method

The two different analytical methods developed, each based on a different sample treatment, namely UF and SEC, were finally compared in terms of linear dynamic range, limits of detection and

quantification, in order to select the one offering a good compromise between time-execution and sensitivity in peptide detection. Recovery values were calculated only for the selected procedure.

A set of artificially contaminated wine samples with increasing amount of each allergenic protein was prepared in the concentration range of 0.1–2 μ g/mL for UF method and 0.25–4 μ g/mL for SEC procedure. The whole set of samples were processed according to the procedure mentioned and the relevant chromatographic runs recorded.

UF procedure. Reporting integrated peak areas versus the spike concentrations, calibration curves with squared correlation coefficient (R^2) of 0.99 were obtained for egg peptides, while R^2 values comprised between 0.97 and 0.99 were calculated for caseinate depending on the specific peptide. Limit of detection (LOD) and quantification (LOQ) were then estimated at S/N equals 3 and 10, respectively (3^* and 10^* SD intercept/slope of the calibration curve). Among the list of eight peptide markers included into SRM-MS/MS instrument method, only the two most sensitive peptides for each allergenic food category were selected and assumed to be most reliable markers for quantitative purpose. Very challenging LODs were achieved for egg allergens, as low as 0.05 and 0.04 μ g/mL respectively referred to LTE and GGL peptides; as for LODs for milk allergens, similar values were found for FFV and DMP caseinate peptides, approximately 0.05 μ g/mL. Concerning LOQ, values ranging from 0.16 to 0.12 μ g/mL were calculated for egg and approximately 0.17 μ g/mL for milk allergen.

Intra-day reproducibility of the method was assessed by analyzing $n = 5$ white wine samples fortified with EP and CN at the final concentration of 0.25 μ g/mL each and injected twice in the multidimensional system. It was expressed as RSD% and good results were obtained, ranging from 3 to 11% for egg white marker peptides and from 11 to 18% for caseinate selected peptides.

SEC procedure. As mentioned above, linearity of the method based on SEC purification, was assessed by plotting peak areas of spiked white wine versus allergen concentrations in order to produced matrix-matched calibration curves. R^2 values comprised between 0.97 and 0.99 were obtained for milk and egg detection while LOD, calculated for peptides selected for quantitative purpose, were found to be 0.12 and 0.07 μ g/mL in the case of LTE and GGL egg peptides, while 0.3 μ g/mL and 0.27 μ g/mL were LODs calculated for caseinate peptides, FFV and DMP peptides, respectively. As for LOQ, values as low as 0.4 and 0.2 μ g/mL were found for peptides LTE and GGL respectively. By contrast, LOQ calculated for caseinate were found to be in the range from 0.9 to 1 μ g/mL for DMP and FFV peptides, respectively.

Concerning intra-day reproducibility, RSD% value was calculated to be approximately 20% for both egg peptides and 17 and 20% for FFV and DMP milk peptides, respectively.

Table 2 reports an overview of method performance for both treatments investigated. From the table appears that good sensitivities were obtained for both milk and egg peptides either using cut-off filter or size-exclusion column as sample pre-treatment. However, according to our findings the procedure based on the

Table 2
Comparison of matrix-matched calibration curves, with the parameters of the regression lines. LOD and LOQ values were obtained by monitoring the quantitative peptides selected for both procedures (UF and SEC) before SRM-MS/MS analysis.

Peptide	Sequence (allergen)	UF procedure				SEC procedure			
		Slope	R^2	LOD ng/ml	LOQ ng/ml	Slope	R^2	LOD ng/ml	LOQ ng/ml
791.36	LTEWTSSNVMEER	$(8.3 \pm 0.2) \cdot 10^5$	0.99	49	162	$(7.5 \pm 0.2) \cdot 10^4$	0.99	117	389
844.42	GGLPINFQTAADQAR	$(2.12 \pm 0.03) \cdot 10^6$	0.99	36	121	$(1.86 \pm 0.03) \cdot 10^5$	0.99	71	238
692.86	FFVAPPEVFGK	$(3.7 \pm 0.1) \cdot 10^6$	0.99	50	166	$(2.5 \pm 0.2) \cdot 10^5$	0.97	304	1015
1093.59	DMPQAFLLYQEPVLGPVR	$(1.58 \pm 0.04) \cdot 10^5$	0.99	53	176	$(1.25 \pm 0.1) \cdot 10^4$	0.98	267	889

use of cut-off filters coupled with a peptide pre-enrichment step before LC-MS/MS analysis, proved to be the most promising and sensitive approach with LODs reached much lower than that set by OIV. On the other hand aiming at shortening the whole procedure without losing peptide sensitivity, sample clean-up based on the use of SEC cartridges can represent a good alternative to the use of cut-off filters representing a good compromise between execution-time- and method performance. Notably, the adoption of the SEC based procedure, resulted in the sharpest protocol and shortest handling time in wine (LODs of 0.07 µg/mL for egg LODs of 0.27 µg/mL for milk). In addition, in order to evaluate recovery of the SEC based strategy for both allergens, *ad hoc* experiments were performed and two types of samples were compared: 1) allergen-free wine fortified with EP and CN at the final concentration of 1 µg/mL each and then submitted to the whole pretreatment procedure and 2) allergen-free wine processed in the same way and fortified after the purification step (by assuming 100% recovery). Both samples were then submitted to tryptic digestion and from the ratio of peak area for each peptide marker, the actual recovery percentage was calculated. Values ranging from 40 to 75% were calculated for both milk and egg markers, respectively. These results are in agreement with what was expected since alpha-caseins have an isoelectric point close to the wine pH and this can induce a partial precipitation of these proteins in such matrix (Peñas et al., 2015).

4. Conclusions

An analytical workflow based on minimum sample pre-treatment and on-line pre-enrichment LC-MS/MS analysis was described for the simultaneous detection of egg and milk allergens in white wines. The use of either size exclusion columns (SEC) or cut-off filters (UF) for sample clean up, combined with the selective peptides enrichment on a 1 cm-long C-18 trap column upstream the chromatographic separation, provided the best results in terms of analysis-time (SEC based procedure) and LODs reached (UF based procedure). However, despite the longer sample handling time required by the UF procedure, the most sensitive LOD was attained by this route enabling to reach LODs and LOQs down to 0.036 and 0.12 for egg and 0.050 and 0.17 for milk, respectively. The method proposed proved to be the most promising and was sensitive enough to identify and quantify egg allergens in white wines at levels equal or lower than those set by the OIV ($\leq 0.25/0.50$ µg/mL).

Acknowledgements

The authors are thankful to the Project S.I.Mi.S.A.: Innovative tools for the improvement of food safety: prevention, control and correction-P.O.N. Ricerca e competitività 2007–2013 per le Regioni della Convergenza (PON02_00657_00186_3417512/1) for funding this research.

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