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

Elisabetta De Angelis, Rosa Pilolli, Linda Monaci*

Institute of Sciences of Food Production, National Research Council of Italy (ISPA-CNR), Bari, Italy

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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

* Corresponding author.
E-mail address: Linda.monaci@ispa.cnr.it (L. Monaci).

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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 3000*g* 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 9400*g* 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 1000*g*). 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

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