

High-resolution mass spectrometry using Orbitrap-based technology for multi allergen screening in wine

L. Monaci, I. Losito[†], B. Introna[†], E. De Angelis, <u>M. Godula⁽¹⁾</u> and A. Visconti





Overview

Purpose: Development of a LC-HRMS based method for the simultaneous detection of food allergens like milk and egg proteins in fined white wine

Methods: HPLC separation and HRMS(MS/MS) detection using an Exactive LC-MS system.

Results: Sensitive multi-allergen method optimized for a rapid screening of egg and milk allergen in fined wine.

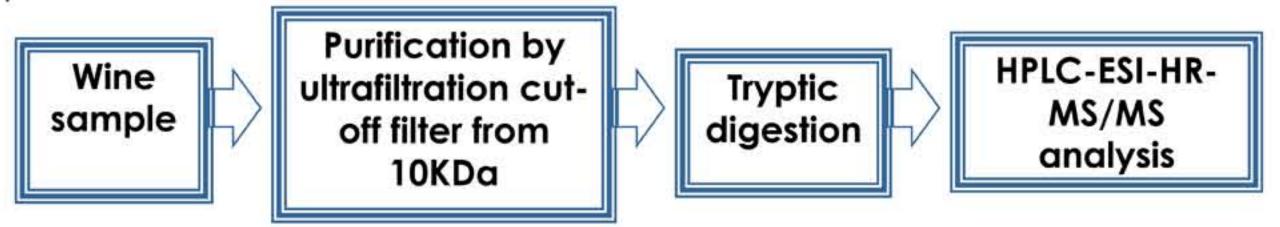
Introduction

Food allergy represents an increasing health problem especially affecting industrialized countries. In order to protect allergic consumers from the inadvertent ingestion of allergens through foods, legislation has been issued in different countries to regulate the allergen labeling on the respective food products. Among potential hidden allergens related to food or beverage processing for technological purposes, proteins adopted in wine clarification have recently received considerable attention.

Milk and egg proteins are allergens typically used by the winery industry for wine clarification to remove undesirable substances, like tannins or polyphenols. Although it is assumed that fining agents are nearly quantitatively removed during the manufacturing process, to date there is no evidence that the consumer ready product is truly free of residues. According to the new legislation issued, European wine producers are now obliged, likewise producers in Australia and New Zealand^{1,2} to indicate egg and milk derived products on the wine label whenever they are used for wine fining.

In this new legislative frame the development of reliable and sensitive methods enabling the detection and/or confirmation of milk and egg proteins in foods and beverages might open new perspectives for the producers. Indeed, the methods could help in the quantification of the residual amounts of fining agents in the finished and ready-to-use products.

High resolution mass spectrometry using Orbitrap technology³, implemented by the use of labeled peptides, could represent a very promising basis for a multi-allergen screening of milk and egg allergenic proteins in wine. In the present paper the development of a HR-MS-based method able to detect simultaneously food allergens like milk and egg proteins, potentially contaminating a white wine fined with caseinate or egg white powder, is presented.



Methods

Sample Preparation

A white wine from *Falanghina* vineyard was purchased form a local retailer and first checked for the absence of residual milk and egg allergens. The sample pre-treatment is briefly schematized above.

Liquid Chromatography

HPLC separation was accomplished on C12 Jupiter Proteo 90 Å column, 150 mm x 2 mm x 4μm (Phenomenex, Castel Maggiore, Bo, Italy) using a binary elution gradient (H₂O + 0.1% HCOOH and CH₃CN + 0.1% HCOOH) at a flow rate of 150 μL/min.

Mass Spectrometry

UHPLC system Accela equipped with gradient pump and thermostated autosampler was combined with an Orbitrap based mass spectrometer Exactive LC-MS comprising heated electrospray ionization source (HESI II) and High-energy Collisional Dissociation (HCD) cell (Thermo Scientific, San Jose, CA, USA). Data acquisition was performed using full scan experiments combined with HCD fragmentation at collision energy of 32V.

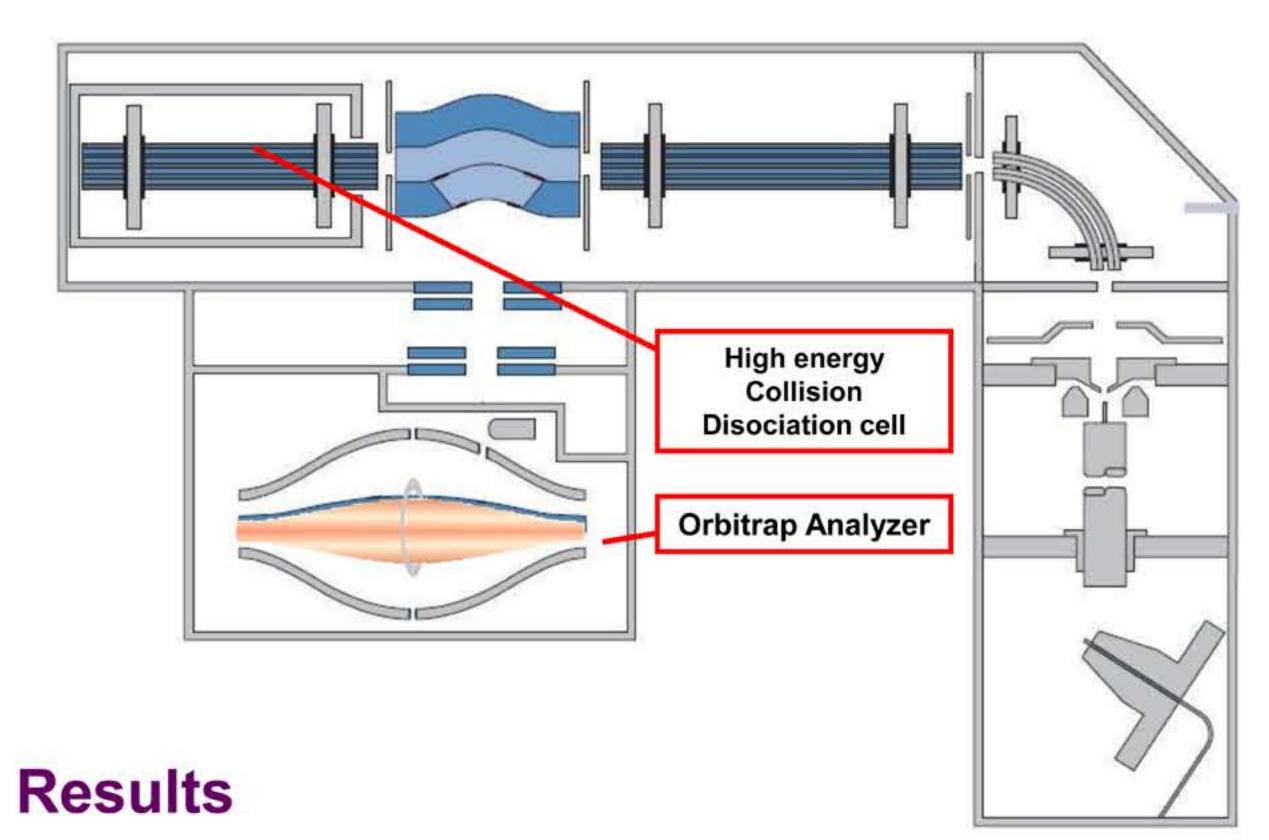
Data were processed using Xcalibur software. The principle of Exactive MS is described in Figure 1.

Mass spectrometer parameters:

Scan range (m/z); 200-2000; Resolving power 100.000 FWHM (m/z 200), Mircoscans 1, AGC 1*E6; Maximum IT 150 ms

Spray parameters: Sheath gas 15 au, Auxiliary gas 5 au, Spray voltage 4kV, Capillary temp: 250 C, Capillary Voltage 32.5V, Tube lens 130V, Skimmer Voltage 30V, Heater Temperature 30 C

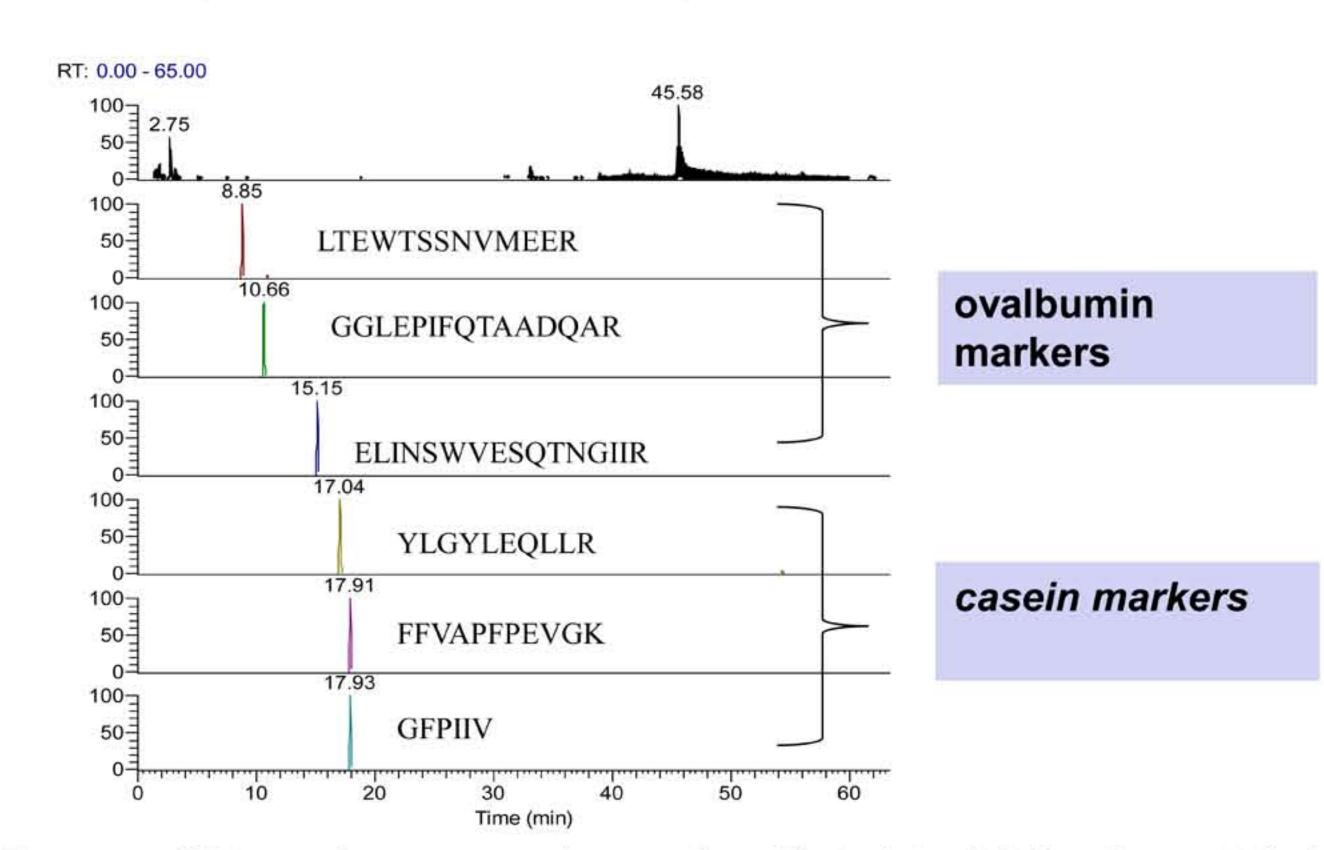
FIGURE 1. Schematics of the Exactive LC-MS system comprising of Orbitrap MS analyzer for HRMS data acquisition and HCD cell for fragmentation experiments



Identification of peptide markers for ovalbumin and caseins

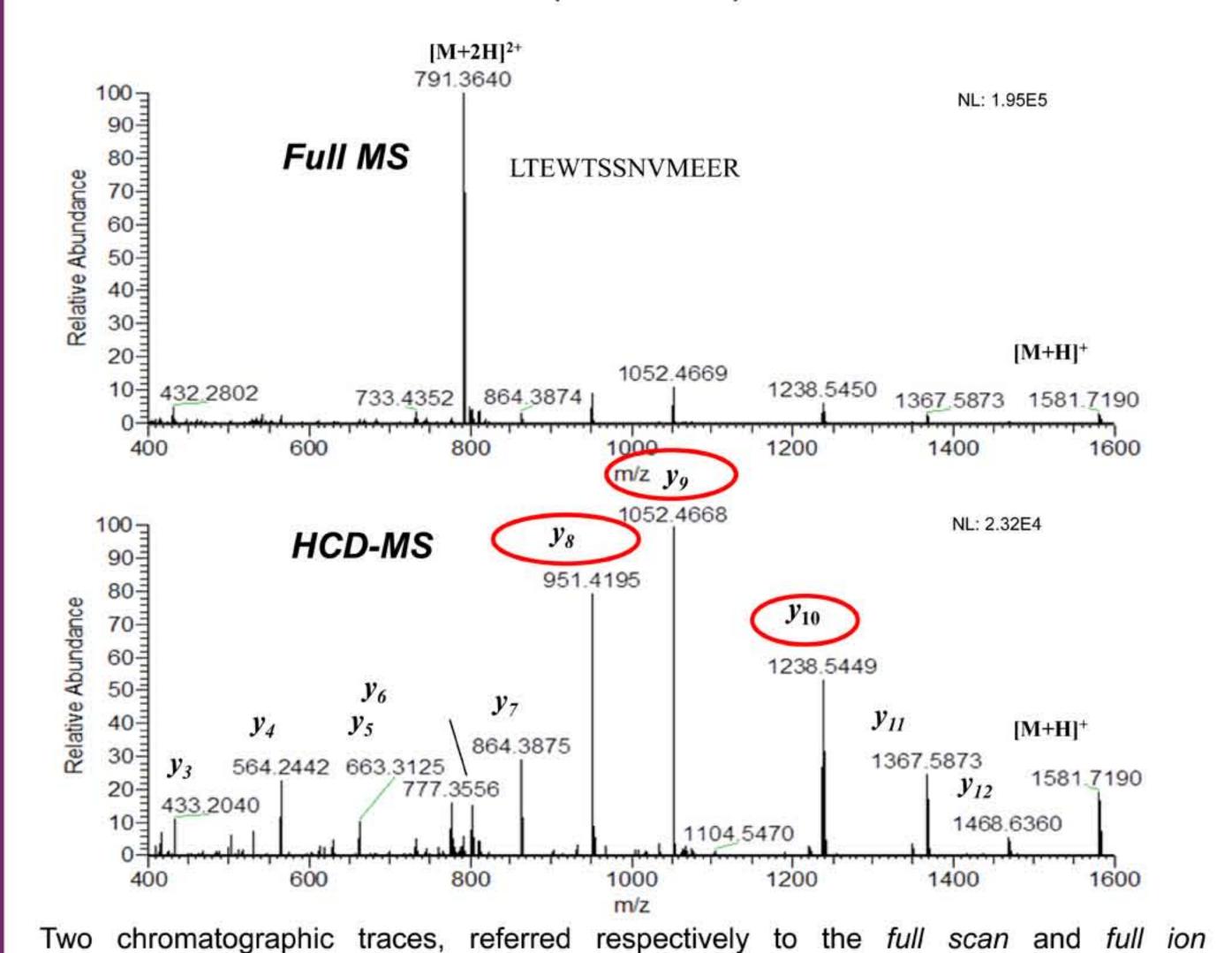
LC-MS (MS/MS) analyses of caseinate and egg-white powder tryptic digests were performed to investigate both the identification and the quantification capabilities of the Orbitrap mass spectrometer for the detection of egg and caseinate peptides in wine extracts. The high mass accuracy and mass-resolving power of the Orbitrap technology, working at a resolution power of 50000 in positive mode, enabled a reliable identification of casein and ovalbumin related peptides already using a database search based on the m/z values of their ions, since low mass tolerances (< 5 ppm) could be applied as shown in Figure 2.

FIGURE 2. Overlay of total ion current chromatogram (top trace) and extracted ion chromatograms (XICs) (bottom figure) relevant to a restricted pool of ovalbumin and casein (from αs -1, αs -2 and β - caseins) peptides in fined white wine.



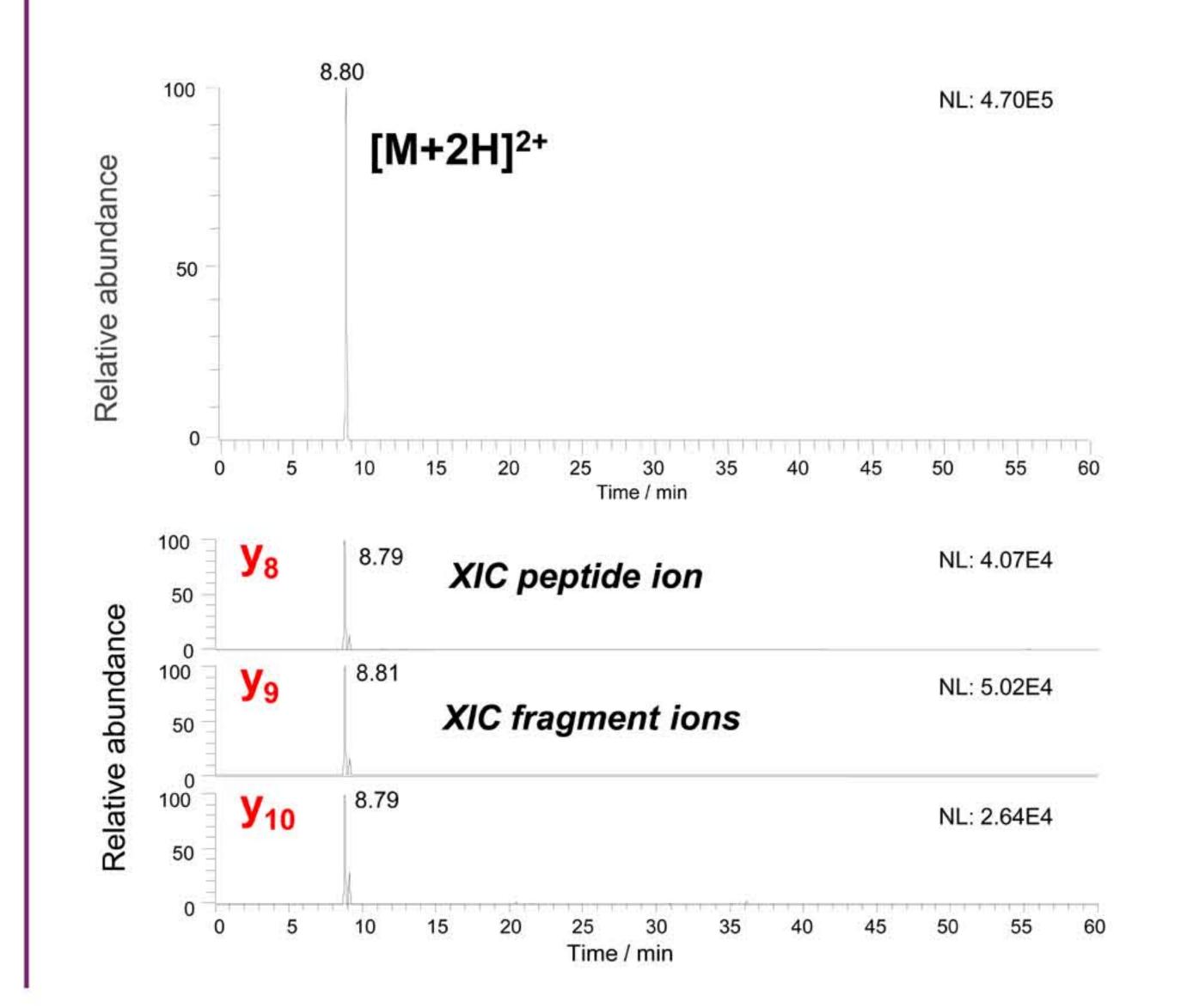
These candidate markers were subsequently subjected to "all ion fragmentation" acquisition mode by activating the collision chamber. The feature, provided by the presence of a HCD cell and allowing the acquisition of MS/MS spectra (alternated to MS ones), enabled to obtain a good fragmentation pattern of the selected peptide markers as shown in Figure 3. The list of product ions obtained usign collision energy 32V can enter the database searching software to strengthen peptide sequence identification.

FIGURE 3. Comparison between full scan MS and HCD-MS spectra referred to the ovalbumin marker LTEWTSSNVMEER ($t_R = 8.85 \text{ min}$).



fragmentation mode, can be generated. In the first case ions arriving from the source are directly diverted into the Orbitrap analyzer using a C-trap; in the second case ions are first driven into the HCD cell and then their eventual product ions are transferred into the Orbitrap using the C-trap. The association of a product ion to a specific precursor ion can be obtained by extracting ion currents (XIC) of the corresponding m/z ratios (in this case using extraction windows compatible with a 10 ppm mass accuracy) and evaluating the alignment, in terms of retention times, of the peaks detected in the two XIC traces. An example is shown in Figure 4.

FIGURE 4. Extracted chromatograms (XICs) of the precursor ion m/z 791.3640 and three fragment ions belonging to the y series.



Evaluation of ultra-filtration recoveries and digestion yields for allergenic proteins
In order to assess the linearity of the method, aliquots of the *Falanghina white wine were* fortified with both type of fining agents and four concentration levels, covering the 1-10

The calibration data obtained from the different peptide markers showed good R², LOD and LOQ values, calculated as 3x/10x the standard deviation of the intercept of the calibration lines and referred to the amount of fining agent added to the original wine are reported in Table 1.

TABLE 1. LOD and LOQ calculated for each peptide marker.

Fining agent/ marker	R ² of calibration curves	LOD (µg/mL)	LOQ (µg/mL)
Egg white powder			
LTEWTSSNVMEER	0.9934	0.5	1.8
GGLEPINFQTAADQAR	0.9902	0.7	2.2
ELINSWVESQTNGIIR	0.9953	0.5	1.6
Caseinate			
FFVAPFPEVFGK	0.9955	0.6	2
GFPIIV	0.9957	0.6	2
YLGYLEQLLR	0.9853	1.1	3.6

Method efficiency

µg/mL range, were prepared.

Based on the LOD values, peptides FFVAPFPEVFGK from aS1-casein and LTEWTSSNWMEER from ovalbumin were chosen as the best markers for caseins and egg-white powder respectively and they were used for quantification purposes. In order to calculate the digestion yield, the ultra-filtration extract of a blank wine aliquot was spiked with a known amount of ovalbumin or αS1-casein and then the concentration expected in the final digests for each of the two peptides was calculated (assuming 100% recovery). The amounts of the isotopically labeled peptides required to reproduce those concentrations were then added to the final digests. The digestion yield could be estimated from the ratio between the peak areas obtained from the XIC traces relevant to the unlabelled and the isotopically labeled peptides, respectively, provided that no interference on the signal of the labeled peptides arose from specific isotopologues of the natural peptides. But in this case, an interference due to the M+2 and to the M+1 isotopologues naturally occurring for the unlabelled peptides FFVAPFPEVFGK and LTEWTSSNVMEER had to be considered. In particular, once the peak areas for the M isotopologues of the non labeled peptides were known, the contribution due to the cited isotopologues to the peak areas of the labeled peptides could be calculated, using the isotopic patterns of the unlabelled peptides. The digestion yields calculated according to the present procedure were found to be 55% for ovalbumin and 80% for αS1-casein.

With the aim to experimentally estimate method efficiency, a wine sample fortified with ovalbumin at 2 µg/mL concentration was processed according to the entire procedure and the final digest was spiked with the isotopically labeled marker of ovalbumin, the peptide LTEWTSSNV*MEER, at a concentration corresponding to that expected for the natural peptide by assuming 100% method efficiency. The isotopic pattern observed for the peptide [M+2H]²⁺ ion in the resulting MS spectrum averaged under the peptide chromatographic peak is depicted in Figure 5. The presence of the first isotopologue (M+0) of the labeled peptide [M+2H]²⁺ ion (m/z 791.8627) is apparent. The method efficiency was then estimated by rationing the XIC peak areas obtained for the natural and the isotopically labeled peptide after correcting the latter for the contribution due to the [M+2H] 2+ ion of the M+1 isotopologue of the natural peptide. The same procedure was adopted for a wine sample spiked with αS1-casein at 2 µg/mL concentration. In this case the final digest was spiked with the peptide FFV*APFPEV*FGK, labeled on the two valine residues. As a result, method efficiencies of 20 and 16%, were estimated respectively for ovalbumin and α-S1casein.

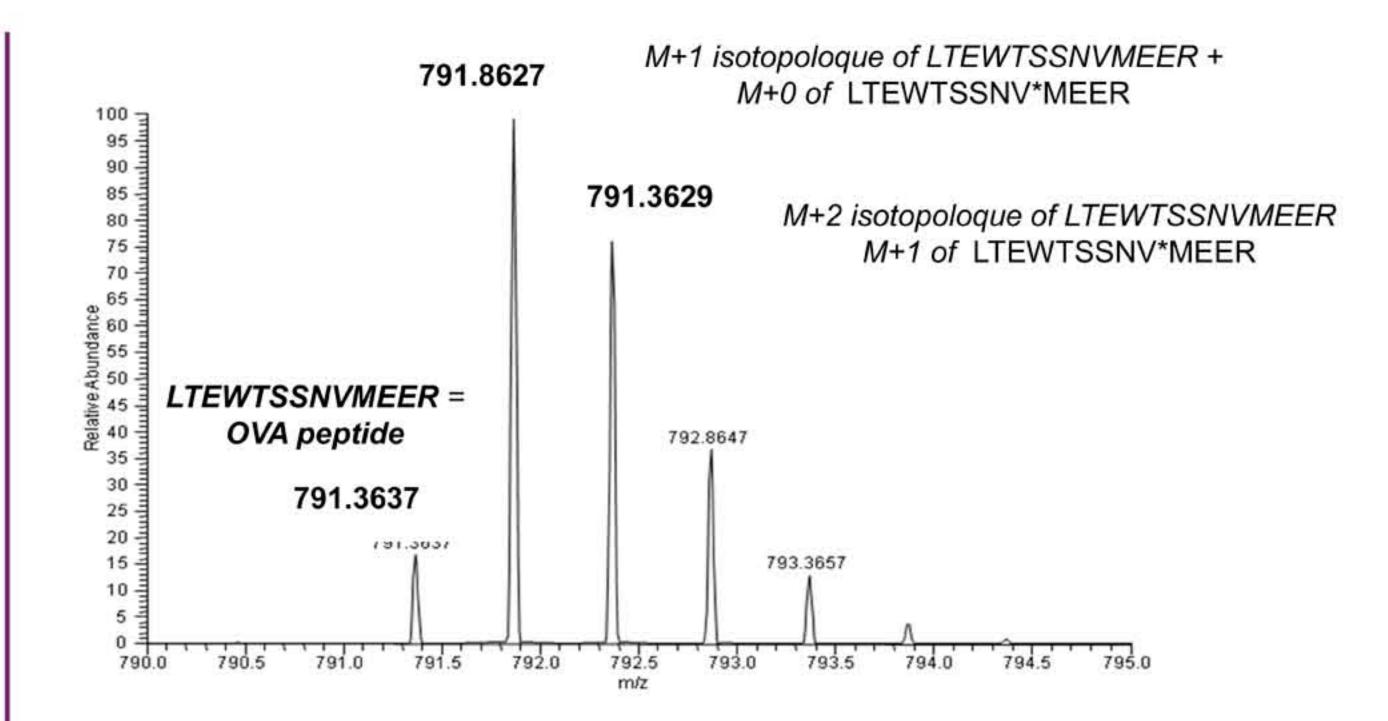


FIGURE 5. White wine sample fortified with ovalbumin at 2 μg/mL and subsequently added with an equimolar amount of the corresponding ¹⁵N-isotopically labeled peptide to estimate the total yield.

As last step the limits of detection achieved with the multi-allergens method for milk or egg-derived fining products were assessed in terms of compliance with threshold values to date available for milk and egg allergens. Sensitivity of the developed method proved to fall in the concentration range considered an hazard for allergic consumers.

Conclusions

- The coupling between ultra-filtration, tryptic digestion and LC-ESI-HRMS analysis enabled the development of an analytical method able to simultaneously detect caseins and ovalbumin potentially remaining in white wines after fining treatments with caseinate and egg-white derived products.
- LOD values of 0.6 and 0.5 mg/L were obtained for the two products using the MS responses of specific peptide markers obtained from the digestion of ovalbumin and a_{S1}casein, respectively.
- Targeted experiments, performed through the implementation of isotopically labeled variants of the peptide markers selected for ovalbumin and a_{S1}-casein, showed that the tryptic digestion yield for such proteins was higher than 80% whereas the whole method yields was 20 and 16% respectively.

References

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