



Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens

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ARTICLE INFO

Article history:

Received 24 February 2012

Received in revised form

1 May 2012

Accepted 9 May 2012

Keywords:

Allergy

Mass spectrometry

Wine

Egg

Milk

Fining

ABSTRACT

Fining agents of animal origin are commonly used in winemaking process to clarify and stabilize wines and to optimize their organoleptic properties thanks to the removal of phenolic compounds that cause bitterness and astringency. Considering the potential allergenicity of proteins used as fining agents, the need of label declaration for wines treated with such compounds is compelling. However, the difficulties in detecting proteins in a wine matrix by immunological assays have given rise to a search for alternative methods that can overcome the limitations of classical approaches. Mass spectrometry (MS) has recently emerged as a powerful and sensitive technique to detect residual fining proteins in wines. In this study we show that a simple and straightforward mass spectrometric approach can be used to reliably detect egg and milk allergens in commercial bottled wines. We tested 25 different wines by liquid chromatography coupled with tandem MS (LC-MS/MS) and found proteins of animal origin in 8 samples, so demonstrating that the proposed method allows to monitor the presence of potential allergenic proteins in fined commercial wine.

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1. Introduction

Milk and egg white proteins are commonly used as processing aids in winemaking. They remove undesirable substances such as phenolic compounds in order to prevent colloidal precipitation, reduce bitterness and astringency, thus resulting in clear wines with no flavor faults. These proteins are included in the list of allergenic substances drafted by the European Union (Directive 2003/89/EC; Directive 2007/68/EC) and must be declared on label when added in foods as ingredients. On the contrary, when used as processing aids in winemaking, these products have been temporarily excluded from the obligation to be declared in the labels, because of lack of scientific data concerning their real presence as residual proteins in fined wines (Commission Regulation EU 1266/2010; Directive 2000/13/EC; Directive 2003/89/EC; Directive 2005/26/EC; Directive 2007/68/EC). Also in other countries, such as Australia, New Zealand and the United States, a similar regulation has been introduced (Weber, Steinhart, & Paschke, 2007).

Nevertheless, the complete removal of fining agents after the fining process cannot be taken for granted. The availability of accurate and sensitive detection protocols for allergens is crucial to avoid risks of allergic reactions to sensitive consumers and to evaluate the necessity of declaration on wine label of the fining agents used.

The methods to detect residual allergens in wines rely mostly on enzyme-linked immunosorbent assay (ELISA) (Lacorn, Gößwein, & Immer, 2011; Lifrani et al., 2009; Rolland, Apostolou, De Leon, Stockley, & O'Hehir, 2008; Weber et al., 2007; Weber, Steinhart, & Paschke, 2009) and these systems are recommended by the Office International de la Vigne et du Vin (OIV) (Resolution OIV/OENO 427/2010, p. 8). In general, ELISA tests show good sensitivity, are easy and fast to perform, but have some drawbacks arising from the presence of interfering compounds in the matrix (Weber, Steinhart, et al., 2009), such as polyphenols in red wine, interacting with both proteins and antibodies (Weber et al., 2007). Moreover, in ELISA, the adsorption of the allergens on the solid matrices could alter the protein epitopes thus compromising the sensitivity of the assay (Kaul et al., 2007).

To overcome these problems, methods for the direct detection of food allergens by mass spectrometry (MS) have been recently developed. Thanks to its high sensitivity, accuracy and

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reproducibility (Picariello, Mamone, Addeo, & Ferranti, 2011), MS allows the detection of trace amounts of proteins and makes the identification independent from the structure of the allergens (Kaul et al., 2007; Kirsch et al., 2009). MS-based methods have been recently applied also to white and red wines to detect residues of milk proteins derived from the fining treatment (Cereda, Kravchuk, D'Amato, Bachi, & Righetti, 2010; D'Amato, Kravchuk, Bachi, & Righetti, 2010; Monaci, Losito, Palmisano, & Visconti, 2010; Monaci, Losito, Palmisano, & Visconti, 2011).

Moreover, a new and simple method based on the recovery and identification of proteins by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in a gel-free approach has been recently described for the detection of residual gluten and egg white proteins in wines fined in our laboratory (Simonato, Mainente, Tolin, & Pasini, 2011; Tolin et al., 2012).

The aim of the present study was the application of the previously described method (Tolin et al., 2012) for the extraction of proteins from wine and for their identification by a LC-MS/MS approach, to detect possible residual milk and egg white fining proteins in 25 commercial wines (12 red, 12 white and 1 rosé).

2. Materials and methods

Italian commercial wines of different brands (12 red, 12 white and 1 rosé wines from Veneto and Friuli-Venezia Giulia regions) were purchased at local supermarkets. All chemical reagents and solvents were from Sigma Aldrich (St. Louis, MO, USA) and of sequencing grade, unless otherwise specified.

2.1. Protein recovery from wine

Proteins were recovered by the Potassium Dodecyl Sulfate (KDS) method (Vincenzi et al., 2005) starting from 50 mL of wines. Briefly, 10% (w/v) SDS was added to the wines to a final concentration of 0.2% (w/v). Samples were then heated at 100 °C for 10 min. 2 M KCl was added to a final concentration of 400 mM and samples were gently mixed for 45 min at 4 °C. KDS-protein pellets were recovered by centrifugation at 21,000 g for 15 min at 4 °C and kept in 20% trichloroacetic acid overnight. Samples were then centrifuged at 21,000 g for 15 min, the supernatants were discarded and, to further remove SDS, protein pellets were washed three times with cold acetone. Samples were finally centrifuged at 14,000 g for 10 min, supernatants were discarded and the protein pellets were let dry under the hood.

2.2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses

Protein pellets were dissolved in 50 mM NH_4HCO_3 containing 8 M urea. Cysteines were reduced with 10 mM dithiothreitol (DTT) (1 h, 37 °C) and alkylated with 30 mM iodoacetamide (1 h at room temperature, in the dark). 1 µg of sequencing grade modified trypsin (Promega, Madison, WI, USA) was added after 1:10 dilution of the samples with 50 mM NH_4HCO_3 to reach a final concentration of 0.8 M urea, and digestion was carried out overnight at 37 °C. Samples were desalted with C18 cartridges (SepPack, Waters, UK). Extracted peptides were dissolved in 50 µL of 0.1% formic acid. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed with a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a chip-based chromatographic interface. A Large Capacity Chip was used and 2 µL of samples were injected into the enrichment column (C18, 9 mm, 160 nL volume) at a flow rate of 4 µL/min and peptides were separated in the C18 nano-column (150 mm × 75 µm) at a flow rate of 0.5 µL/min. Water/formic acid 0.1% and acetonitrile/formic acid 0.1% were used as eluents A and B, respectively. The

chromatographic separation was achieved by a linear gradient of eluent B from 3% to 50% in 50 min. Mass spectra were acquired in a data dependent mode: MS/MS spectra of the 3 most intense ions were acquired for each MS scan in the 350–2400 Da mass range. Scan speed was set to 4 MS spectra/s and 3 MS/MS spectra/s. Capillary voltage was set to 1750 V and drying gas to 5 L/sec. Raw data files were converted into Mascot Generic Format (MGF) files with MassHunter Qualitative Analysis Software version B.03.01 (Agilent Technologies) and analyzed using Proteome Discoverer Software (version 1.2, ThermoFisher Scientific). The software was connected to a Mascot Search Engine server version 2.2.4 (Matrix Science, London, UK). Spectra were searched against an in-house database obtained by a *Vitis vinifera* database (version 2010, 10,573,053 sequences, 3,710,354,253 residues) concatenated with the protein databases of *Saccharomyces cerevisiae*, *Oenococcus oeni*, *Botrytis cinerea*, and a database of contaminant proteins commonly found in proteomics experiments. Enzyme specificity was set to trypsin with 2 missed cleavages, precursor and fragment ions tolerance was set to 10 ppm and 0.05 Da, respectively. Carbamidomethylcysteine was selected as fixed modification and oxidation of methionine as variable modification. A False Discovery Rate (FDR) of 0.5% and 0.1% was calculated by Proteome Discoverer based on the search against the corresponding randomized database. Before the search, data were filtered to exclude MS/MS spectra containing less than 5 peaks and with a total ion count lower than 50. Identified peptides were classified as high (99%) and medium (95%) confidence, according to the corresponding FDR.

3. Results

A panel of 25 commercial wines produced in Italy were analyzed in order to monitor the occurrence of residual amounts of fining agents commonly used in winery, such as potentially allergenic proteins derived from egg and milk. Wines were purchased at local supermarket and were purposely selected among red and white wines of different brands and typology (i.e. dry, sweet, sparkling, etc.). By applying our previously optimized method (Tolin et al., 2012), we recovered proteins from 50 mL of wine, and analyzed them by LC-MS/MS. In general for every wine sample we identified several proteins, including proteins of the grape berry, wine yeast (*S. cerevisiae*), bacteria (*O. oeni*) and mold (*B. cinerea*). Importantly, 8 wines were found to contain peptides belonging to egg and milk proteins, all of them known as potential allergens (Table 1). In particular, ovalbumin, the major egg allergen, was detected in all 8 positive wines, and 2 of them also contained the milk allergens alpha- and beta-casein. The egg allergen ovotransferrin was also identified in 2 wines. Thus, these results demonstrated that this approach is well suitable for all types of wine (white, red and rosé wines). Except for lysozyme found in the rosé wine (which was identified with a single peptide), the identification of all the fining proteins was based on the detection of 2 or more peptides, indicating a good level of reliability. In Fig. 1 we show as an exemplificative case the extracted chromatograms of peptide ions deriving from 4 egg and milk proteins detected in the rosé wine (n. 3 of Table 1), together with the MS/MS spectra of two peptides, showing an almost complete match of b and y ions that indicate unequivocally the positive identifications. This example was chosen as representative of the good quality of the data. The complete information on all the MS/MS identifications is reported in Table 1S (Supplementary Material).

4. Discussion

The proteins commonly used in the process of wine fining are well-known allergens (milk caseins and proteins from egg white)

Table 1

Egg and milk residual allergenic fining proteins detected in commercial wines. The proteins are reported with their UniProt accession code, the number of identified peptides, and their sequences.

Sample	UniProt code	Residual fining protein (species)	# peptides	Peptide sequence
White wine (n. 1)	P01012	Ovalbumin (<i>G. gallus</i>)	4	HIATNAVLFfGR LTEWTSSNVmEER ELYRGGLEPINFQTAADQAR GGLEPINFQTAADQAR
White wine (n. 2)	P01012	Ovalbumin (<i>G. gallus</i>)	5	LTEWTSSNVmEER ISQAVHAAHAEINEAGR AFKDEDTQAMPFR VTEQESKPVQMMYQIGLFR VASMASEK
Rosè wine (n. 3)	P01012	Ovalbumin (<i>G. gallus</i>)	5	NVLQPSSVDSQTAMVLVNAIVFK GGLEPINFQTAADQAR LTEWTSSNVmEER ELINSWVESQTNGIIR VTEQESKPVQMMYQIGLFR
	P02666	Beta-casein (<i>B. taurus</i>)	3	DMPIQAFLLYQEPVLGPVR GPFPIIV VLPVPQK
	P02662	Alpha-S1-casein (<i>B. taurus</i>)	4	YLGYLEQLLR HQGLPQEVNLNENLLR FFVAPFPEVFGK FFVAPFPEVFGKEK
Red wine (n. 4)	P00698	Lysozyme (<i>G. gallus</i>)	1	FESNFNTQATNR
	P01012	Ovalbumin (<i>G. gallus</i>)	3	GGLEPINFQTAADQAR VASMASEK NVLQPSSVDSQTAmVLVNAIVFK
White wine (n. 5)	P01012	Ovalbumin (<i>G. gallus</i>)	3	GGLEPINFQTAADQAR LTEWTSSNVmEER VASMASEK
	P00698	Lysozyme (<i>G. gallus</i>)	3	NTDGSTDYIGILQINSR NLcNIPcSALLSSDITASVNCaK GYSLGNNVcAAK
	P02666	Beta-casein (<i>B. taurus</i>)	2	VLPVPQK AVPYPQR
	P02789	Ovotransferrin (<i>G. gallus</i>)	14	FFSAScVPGATIEQK GTEFTVNDLQGK GAIEWEGIESGSVEQAVAK ISLTcVQK ATYLDcIK TGTcNFDEYFSEGcAPGSPNSR TcNPSDILQMCSFLEGK WcTISSPEEK KGTEFTVNDLQGK NLQmDDFELLcTDGR DQLTPSPR LcQLcQGS GGIPPEK DSAlmLK AQSDFGVDTK
White wine (n. 6)	P01012	Ovalbumin (<i>G. gallus</i>)	4	GGLEPINFQTAADQAR LTEWTSSNVmEER VASMASEK LYAER
	P00698	Lysozyme (<i>G. gallus</i>)	3	NTDGSTDYIGILQINSR FESNFNTQATNR NLcNIPcSALLSSDITASVNCaK
Red wine (n. 7)	P01012	Ovalbumin (<i>G. gallus</i>)	3	LTEWTSSNVmEER VASMASEK GGLEPINFQTAADQAR
Red wine (n. 8)	P01012	Ovalbumin (<i>G. gallus</i>)	4	ILELPFASGTMSMLVLLPDEVSGLEQLESIINFEK DILNQITKPNDDVVSFSLASR NVLQPSSVDSQTAMVLVNAIVFK HIATNAVLFfGR
	P02789	Ovotransferrin (<i>G. gallus</i>)	3	TDERPASyFAVAVAR ATYLDcIK DQLTPSPR

and the presence of traces of them in bottled wine can represent a risk for the allergic consumers.

In this work we evidenced that some residual allergenic proteins, deriving from egg and milk sources used during wine-making processes, are still present in bottled wines. We applied a previously described method (Tolin et al., 2012) able to detect

residual fining proteins at the concentrations commonly used in winery, i.e. 5–15 g/hL. This method is very simple and rapid, being based on a first step of recovery of proteins by KDS precipitation (Vincenzi et al., 2005) and, after SDS removal, a second step of protein identification by LC-MS/MS. This analytical strategy overcomes the limitations of classical immunological approaches not

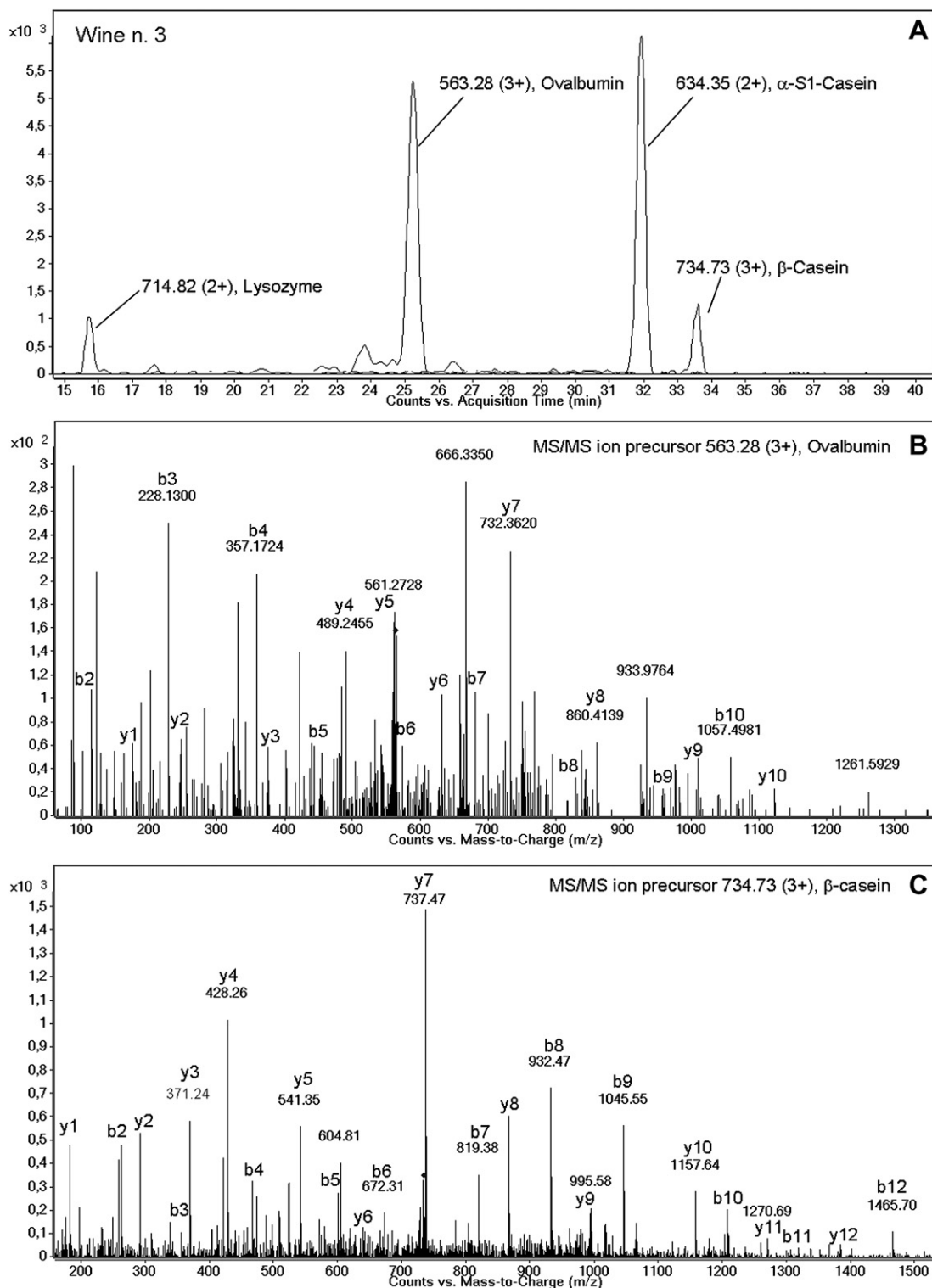


Fig. 1. MS results for egg and milk proteins detected in the rosé wine (sample n. 3 of Table 1). A: Extracted Ion Chromatograms of the peptide ions arising from lysozyme, ovalbumin, α -S1-casein, and β -casein. B: MS/MS spectrum of peptide ion 563.28 (3+) (GGLEPINFQTAADQAR) deriving from ovalbumin. C: MS/MS spectrum of peptide ion 734.73 (3+) (DMPIQAFLLYQEPVLGPVR) deriving from β -casein. The main matching b and y product ions are indicated in the spectra.

only in terms of sensitivity, but also because immunoassays are affected by the integrity of the labile 3D-structure of proteins (Kaul et al., 2007), while MS methods rely on data based on the chemical identity of the proteins, i.e. the amino acid sequence. Moreover, in this work we blindly analyzed the wine samples, without any information on the fining treatment to which they were subjected,

and we directly provided confirmative and unambiguous data on the identities of residual fining proteins still present in the final commercial product. This is another advantage over the immuno-based methods that require the use of specific antibodies to reveal the presence of a specific fining agent used during the winemaking process.

Recently, a method based on combinatorial peptide ligand libraries coupled to MS for identifying traces of proteins in white and red wines was reported (Cereda et al., 2010; D'Amato et al., 2010). In these studies, the authors started from larger volumes of wine (200–750 mL) and performed several experimental steps of sample preparation: a partial removal of phenolic compounds with polyvinylpyrrolidone overnight, the absorption of proteins to the beads (about 3 h), their subsequent desorption, an SDS-PAGE step followed by in-gel trypsin digestion, and finally LC-MS/MS analysis. This protocol seems to be quite complicated and hardly adaptable for routine monitoring of commercial wines. On the contrary, our approach is simpler, more rapid, and allows the recovery of proteins from white and red wines in a gel-free protocol compatible with MS analysis, overcoming the difficult step of removal of phenolic compounds. Moreover, surprisingly the authors of these studies found only caseins in white wines and also in the red ones, while it is commonly known that red wines are mainly treated with egg products. It may be that the beads used to recover proteins from wine have higher affinity for specific classes of proteins, such as caseins, than for ovalbumins or other egg proteins (Keidel, Ribitsch, & Lottspeich, 2010). Therefore, to date our work demonstrates for the first time the presence of egg proteins in commercial red wines. Notably, we started from a very small amount of wine, 50 mL, a quantity easy to handle for setting up a study at a laboratory scale. Considering that the amount of wine available as sample is not an issue, we could expect even better results starting from a larger volume. Our data are particularly relevant and represent a hot topic especially in view of recent European Food Safety Authority (EFSA) publications in which some studies were taken into consideration to analyze the presence of ovalbumin and casein/caseinate/milk products in wines. The results considered in these reports showed no detectable amounts of ovalbumin in any of the 77 commercial wines tested (EFSA Journal 2011; 9(10):2385), and no detectable amounts of casein in any of the 63 commercial wines analyzed from an Italian study (EFSA Journal 2011; 9(10):2384). In these documents it is evidenced that the improvement on the methods used for the detection of ovalbumin and casein in wine, and the new analytical data provided, are insufficient to change a previous conclusion about the likelihood of allergic reactions triggered by the consumption of fined wines. Therefore, the need of new and reliable analytical methods to detect traces of allergens in wines emerges and seems to be imperative.

In our study also lysozyme, that represents a major allergen from hen's egg (Mine & Zhang, 2002; Pérez-Calderón, Gonzalo-Garijo, Lamilla-Yerga, Mangas-Santos, & Moreno-Gastón, 2007), has been detected in three wines (n. 3, 5 and 6 of Table 1). Because of its antibacterial activity, lysozyme can be used as fermentation controller against spoilage and to lower the sulphite dosage during winemaking (Weber, Kratzin, et al., 2009; Weber, Steinhart, et al., 2009). Nevertheless, lysozyme could have also been added to the wines unawares (i.e. together with egg white proteins), as limited information are available on the purity of the commercial egg white albumin(s) used as fining agent for winemaking (EFSA Journal 2011; 9(10):2386).

Here we have presented a “small scale” screening of commercial wines, analyzing a relatively small number of samples, even if including different types of white and red wines. The quantification of the allergenic residues found in commercial wines has not been performed here, because our intention was to provide scientific evidence that residual egg white and milk proteins are still present after the clarification process in commercial wines and thus the risk for allergic people cannot be excluded. As a matter of fact, a threshold protein value for allergic disease cannot be established *a priori* (Weber, Kratzin, et al., 2009). However, we can provide an

estimate of the residual amount of detected fining proteins, based on the limit of detection of our instrument and on the quantity of wine analyzed. Considering that with our instrumental set up we can routinely identify as little as 1–5 fmol of tryptic digests of proteins and that wine is a very challenging matrix, we estimate that in 50 mL of wine there are at least 125 fmol of residual fining proteins (2.5 pmol per L). Therefore we can deduce a minimal residual concentration of ovalbumin and casein of about 100 ng and 60 ng per L of wine, respectively, based on their molecular weights. Obviously, considering that the amount of wine that can be analyzed is not an issue (unlike other sample types like, for example, human biopsies), the possibility to analyze larger volumes of wines, will allow to extract and concentrate larger amounts of residual fining proteins and therefore to detect them even if present in lower concentrations. Analogous considerations can be done if more sensitive mass spectrometers are used for the analysis. The next crucial step will be the setting up of a quantitative method able to accurately quantify residual amount of allergenic proteins. By extending the survey to a large number of wines, it would be possible to evidence the most common residual proteins detectable in fined wines. Therefore, an interesting option would be the development of a quantitative MS assay, for example a Selected Reaction Monitoring assay (SRM) based on the most detected peptides deriving from the allergenic proteins identified. This targeted approach would also have the advantage of being more sensitive than the method applied in our studies, allowing to detect possible allergenic residual proteins even at a lower level. This possibility would open the way to establish a reliable method to monitor the potential allergenicity of commercial wines in the context of the EU regulations on wine labeling.

Acknowledgments

The authors are grateful to Cassa di Risparmio di Padova e Rovigo Holding (Cariparo) for funding the acquisition of the Q-TOF mass spectrometer.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.foodcont.2012.05.015](https://doi.org/10.1016/j.foodcont.2012.05.015).

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