



Mass Spectrometry Determination of Fining-Related Allergen Proteins in Chilean Wines

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Abstract

Casein (milk powder) and ovalbumin (egg white powder) are often used as wine fining agents with the objective to promote interactions with undesirable compounds, e.g., polymeric phenols. This kind of proteins may trigger allergic reactions in susceptible individuals; therefore, their occurrence in wines could become a human health risk, moreover when their presence is not reported. The objective of this work was to establish a mass spectrometry method to determine casein and ovalbumin in Chilean wines. To the best of our knowledge, the present work reports for the first time the presence of these proteins in Chilean wines. Proteins were extracted combining the use of ultrafiltration membranes and protein precipitation with organic solvents. Thereafter, proteins were digested with trypsin during 7 h with a 1:10 enzyme protein ratio. Chromatographic separation was carried out on a Kinetex XB C₁₈ (100 × 4.6 mm, 2.6 µm) column set at 35 °C using an acidified (0.1% v/v formic acid) mobile phase composed of ultrapure water (A) and acetonitrile (B). For a highly selective evaluation, quantification was carried out by mass spectrometry applying multiple reaction monitoring (MRM). Validation was established according to the International Conference Harmonization guidelines. Calibration data ($n = 6$) fitted a linear regression model with determination coefficients (R^2) higher than 0.99. Repeatability ($n = 6$) and intermediate precision ($n = 3$) showed RSD values $\leq 1.36\%$ and $\leq 1.53\%$, respectively. Recovery ($n = 3$) at three levels ranged from 86.40 to 106.98%. Detection and quantification limits ranged from 4.70 to 8.50 µg L⁻¹ and 10 to 20 µg L⁻¹, respectively. Twenty Chilean wine samples were analyzed; 18 samples showed quantifiable levels, from which three samples presented a total casein and ovalbumin concentration (0.24–0.26 mg L⁻¹) closer or higher to the European limit for mandatory labeling (0.25 mg L⁻¹).

Keywords Casein · Ovalbumin · Mass spectrometry · Wines, fining agents

Introduction

Wine grape production is a very dynamic sector of Chile's economy in terms of productivity, exports, and employment. Chile is located within the ten principal worldwide wine producers with over 10 mhl produced during 2016, which are equivalent to almost 4% of the world's produced volume. It ranks in the fourth position of largest wine exporter with 9.1 mhl exported in 2016, which represents almost €1.7

billion (Organization Internationale de la Vigne et du Vin (OIV) 2017). Chilean wines have international recognition due to its particular sensorial and chemical profiles, both extensively investigated (Bridi et al. 2014; Mendoza et al. 2011). During winemaking, some fining agents are used with the aim of promoting interactions with undesirable compounds, especially polymeric phenols and polyphenols (Dordoni et al. 2015; Losito et al. 2013; Restani et al. 2014). Commonly, caseinate or milk powders are used for white wine fining, and ovalbumin or egg white powders proteins are used for red wines (González-Neves et al. 2014). The use of allergenic proteins in wines and their unlabeled presence in the final product has been subject of debate in the last years due to the health risk for susceptible consumers. Thus, its determination in wines is highly desirable to ensure consumer's health safety. For this reason, the European Union (EU) implemented the Directive 89/2003/EC and its latest version 579/2012/EC, which contain a list of allergenic substances including egg and milk derivatives (European Commission (EU) 2012). This

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document indicates the obligation of declaring the presence of egg and milk proteins when the concentration in wines is higher than the analytical limit described in OIV/COMEX/502/2012 resolution (Organization Internationale de la Vigne et du Vin (OIV) 2012). The OIV official method for egg and milk derivative determination in wines is based on enzyme-linked immunosorbent assay (ELISA), which present detection and quantification limits of 0.25 mg L^{-1} and 0.50 mg L^{-1} , respectively. Immunoassay analyses are simple and fast (Kerkaert et al. 2010) but present some drawbacks and limitations. In red wines, the high content of polyphenols could cause detection problems due its interaction with proteins and antibodies (Patrick et al. 2009). Also, allergen adsorption to solid matrix could produce some kind of alteration of protein epitopes modifying its recognition or accessibility, thus impairing the assay sensitivity (Kaul et al. 2007). Due to that, proteomic analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Mainente et al. 2014) and liquid chromatography (Mattarozzi et al. 2014) has become a valued option, moreover considering that protein and peptide separation is a key step for this kind of analysis. 2D-PAGE is a labor-intensive technique with some limitations in detecting hydrophobic and alkaline proteins (Chambery et al. 2009). This technique has not achieved an important automatization, forcing researchers to search other alternatives for protein separation. In this scenario, systems like liquid chromatography coupled to mass spectrometry (LC-MS) have become the most suitable technique for protein analysis. Currently, the techniques of choice to determine food allergen proteins are LC-electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), both in tandem mass spectrometry (LC-ESI or MALDI-MS/MS). These techniques offer several advantages, including multi-allergen detection and unambiguous identification and characterization of food allergen (Cristina et al. 2016). Due to the diversity of molecular weights and the different peptide and protein properties, there is no unique workflow suitable for all LC-ESI or MALDI-MS/MS analyses (Zhang and Jian 2014). Proteomic analysis can be performed applying two different strategies: *top-down* and *bottom-up*. The first one involves direct analyses of intact proteins without prior digestion (Monaci and Visconti 2009), for which the most convenient and used ionization technique is MALDI. In bottom-up approach, identification is performed comparing MS/MS spectra of protein digestion products (peptides) against protein sequence databases (Monaci and Visconti 2009). This is the most preferred strategy to perform proteomic studies because the obtained spectra are much easier to interpret than those obtained by top-down. For quantitative purposes, bottom-up experimental workflow is generally based on multiple reaction monitoring (MRM) detection of selected parent and fragmentation ions (Mattarozzi et al. 2014). The present work reports a fast and highly selective method to determine casein and ovalbumin in wines using LC

separation coupled to ESI-triple quadrupole (TQ) mass spectrometry. To the best of our knowledge, this work reports for the first time the evaluation of fining-related allergen proteins in Chilean wines. Prior to LC/MS/MS analysis, proteins are commonly extracted by ultrafiltration and then digested using trypsin enzyme. Several sample preparation approaches have been reported with the aim to reduce the analysis time (Careri et al. 2007; Gallardo et al. 2013; Heick et al. 2011; Monaci et al. 2014). In particular, protein digestion is considered the bottleneck of bottom-up proteomic analyses due to the long time required for digestion (12–24 h). Trypsin is the most used enzyme because it exclusively cleaves basic residues to arginine and lysine (Loziuk et al. 2013), generating peptides with an adequate mass range, which facilitate the sequence detection. In order to reduce digestion time, the present work optimized trypsin digestion conditions applying a central composite design based on reported digestion conditions (Calleri et al. 2005; Chiva et al. 2014; Fremout et al. 2010) and optimized protocols (Proc et al. 2010; Walmsley et al. 2013).

Material and Methods

Reagents, Standards, and Samples

Formic acid (98–100%), trichloroacetic acid (TCA, > 99%), ammonia solution (> 25%), ammonium hydrogen carbonate (NH_4HCO_3 , 99%), ethanol absolute, LC-grade acetonitrile, and Amicon ultracentrifugal filter devices (10-kDa cutoff membrane) were purchased from Merck (Darmstadt, Germany). Casein from bovine milk, albumin from chicken egg white, iodoacetamide (IAM, > 99%), and DL-dithiothreitol (DTT, > 99%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Trypsin from bovine pancreas was obtained from BDH Biochemicals (Poole, England). Buffered solutions and mobile phase were prepared with ultrapure water ($18.2 \text{ M}\Omega \text{ cm}$) produced by means of Simplicity system from Millipore (Bedford, MA, USA). Casein and ovalbumin stock solutions were prepared in $50 \text{ mM NH}_4\text{HCO}_3$ (pH 7.8) for a given concentration of 12 mg L^{-1} . Standard solutions were obtained by aliquot dilution from stock solution. All solutions stored refrigerated were stable for at least 20 days. A total of 20 Chilean commercial wine samples without allergen labeling were analyzed. All samples were purchased directly from supermarkets and specialized stores without contact with wineries.

Sample Preparation

Allergen proteins were extracted and purified from wine samples following the method proposed by Mattarozzi et al. (2014) with slight modifications. Briefly, 12.5 mL of wine was centrifuged using a Hettich (Tuttlingen, Germany)

centrifuge at $5433\times g$ for 40 min at 20 °C into 10-kDa cutoff membrane ultrafiltration tube, previously conditioned with 10 mL of distilled water. Recovered were 2.5 mL from which proteins were precipitated for 2 h on ice after addition of eight volumes of 15% TCA in ethanol, pH 4.5. Thereafter, the mixture was centrifuged at $8981\times g$ for 10 min at 4 °C using a Hettich refrigerated centrifuge. Ethanol/TCA residues were evaporated under a gentle flow of nitrogen. The obtained pellet was solubilized in 1 mL of 50 mM NH_4HCO_3 , pH 7.8. Trypsin digestion of both sample and standards was performed after protein reduction and alkylation. The first one was carried out by addition of 12 μL of 100 mM DTT and incubation at 95 °C for 5 min. After cooling down, the cysteine SH groups, formed during the reduction step, were irreversibly alkylated adding 24 μL of freshly prepared aqueous solution of 100 mM IAM; the mixture was left stand protected from light for 15 min at room temperature. Enzymatic digestion was performed adding 4 μL of trypsin solution (0.5 mg mL^{-1} in 50 mM of NH_4HCO_3 , pH 7.8) obtaining an enzyme/protein ratio of 1:10; the mixture was incubated at 37 °C for 7 h. The reaction was stopped by addition of 10 μL of formic acid (0.1% v/v) aqueous solution. All digested samples and standards were filtered through 13-mm PVDF syringe filter (0.22 μm) before LC injection.

Liquid Chromatography-Tandem Mass Spectrometry

For LC-MS analyses, a Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system consisted of LC-30 AD pump, DGU-20A5R degassing unit, SIL-30 AC autosampler, CTO-20 AC column oven, CBM-20A communication module, SPD-M20A diode array detector (DAD), and LCMS-8030 triple quadrupole (TQ) mass spectrometer (MS) was used. The system was controlled by Shimadzu LabSolution 5.8 software. Peptide separation was carried out on Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm $\times$ 4.6 mm, 2.6 μm), connected to a guard column of the same chemistry, both thermostated at 35 °C, using an acidified (0.1% v/v formic acid) mobile phase composed of ultrapure water (A) and acetonitrile (B). The following gradient program was applied at a flow rate of 0.6 mL min^{-1} : 0–19 min 10–40% B, 19–20 min 40–10% B, with 5 min for column conditioning. MS analysis was performed in positive mode using the following conditions: ESI voltage 4.5 kV; collision energy –30.0 V for casein and –40.0 V to ovalbumin; nebulizer gas (N_2) 3 L min^{-1} , desolvation gas (N_2) 18 L min^{-1} ; desolvation line temperature 250 °C; and heat block temperature 400 °C. Full-scan spectra were acquired from m/z 100 to 1000. All ions with signal intensity greater than 100 counts (range 10^1 to 10^6) and charge state +2 (or unknown charge (+1 to +3)) were submitted to fragmentation and product ion scan. MS/MS data were searched in SwissProt online database using Mascot Peptide Mass Fingerprint (Matrix Science Ltd.,

London, UK) as a bioinformatic tool applying the following stringent criteria: (i) only one missed cleavage was allowed for trypsin digestion, (ii) cysteine carbamidomethylation as fixed amino acid modification, and (iii) variable modifications: methionine oxidation and threonine and tyrosine phosphorylation. For MRM method, the most intense signals from the product ion spectra were chosen for MRM transitions, i.e., for α -casein $634.6 \rightarrow 991.8$, for β -casein $390.9 \rightarrow 258.5$, and for ovalbumin $929.5 \rightarrow 1116.5$.

Statistical Analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD), and relative standard deviation (RSD)]. Peptide calibrations were established applying linear regression analysis. Calibrations with and without matrix were compared using F test. All above tests were done with a significance level (α) of 0.05 using GraphPad (San Diego, CA, USA) Prism 6.0 software. Central composite design was prepared and analyzed by means of Statgraphics Centurion XV version 15.1.02 software (Rockville, MD, USA) and SAS (Marlow, Buckinghamshire, England) JMP 8 statistical software.

Results and Discussion

Selection of Marker Peptides

Selection of marker peptides is one of the key steps to develop quantitative MS-based method. In this work, peptide and fragment markers were established using pure standard and red wine samples (free of allergen proteins) spiked with 100 mg L^{-1} of each protein (caseins and ovalbumin). Both were digested with trypsin using general conditions and studied/analyzed by LC-MS/MS. As expected, several m/z signals were observed along the entire mass chromatogram. To limit the exploration, the search was based on already published data (Fremout et al. 2010; Heick et al. 2011; Losito et al. 2013; Mattarozzi et al. 2014; Monaci et al. 2011, 2010, 2014; Patrick et al. 2009; Tolin et al. 2012a) finding the following parent (precursor) ions: YLGYLEQLLR (m/z 634.6; +2, t_R 15.1 min), VLPVPQK (m/z 390.9; +2, t_R 4.0 min), and ELINSWVESQTNGIIR (m/z 929.5; +2, t_R 13.5 min) for α -casein, β -casein, and ovalbumin, respectively. After establishing precursor ions, a MS/MS approach was performed in product ion scan mode using the LC-MS/MS conditions described in “Liquid Chromatography-Tandem Mass Spectrometry” section. Fragment ion selection from each precursor was carried out applying the same criteria indicated in “Liquid Chromatography-Tandem Mass Spectrometry” section plus the following aspects: (i) matching with precursor peptides

already reported in literature (Heick et al. 2011; Mattarozzi et al. 2014; Monaci et al. 2014; Tolin et al. 2012a) and (ii) quality of product ion spectra matches (Matrix Science Ltd., London, UK). This approach allowed the selection of casein and ovalbumin peptides and fragment ions present in every digestion and fragmentation. Thus, it was possible to define peptide markets (precursor and fragment ions) to detect α -casein, β -casein, and ovalbumin in MRM mode. Figure 1 shows the product ion spectrum of YLGYLEQLLR peptide from α -casein after fragmentation into collision-induced dissociation cell set up at -35.0 V. Following the early described criteria, the two most abundant and stable fragments from the product ion spectrum were chosen for each peptide (protein), one for qualitative/confirmatory and one for quantitative purposes (Table 1). Thus, the MRM transitions established for quantification were m/z 634.6 $\rightarrow$ 991.8 for α -casein, m/z 390.9 $\rightarrow$ 258.5 for β -casein, and m/z 929.5 $\rightarrow$ 1116.5 for ovalbumin.

Optimization of Enzymatic Digestion

A face-centered central composite design with two central points was selected to optimize the enzymatic digestion. This type of design was chosen because it has a high efficiency with a reduced number of experiments (Dejaegher and Vander Heyden 2011). The optimization was done for each marker peptide in MRM mode. Since detection capability is one of the most relevant aspect for wine allergen protein determination, peak area was chosen as the critical variable (response) looking for lower detection limits. From the factors that possibly affect the critical variable, two were chosen: digestion time (X_1) and enzyme-to-protein ratio (X_2). Considering previous experiments and the intervals described in literature, the following ranges were established for digestion time (2.0 to 12.0 h) and enzyme-to-protein ratio (1:10 to 1:100),

resulting in an experimental plan with 10 runs (Table 2). All experiments were randomly conducted in duplicate ($n=2$) in order to minimize the effects of uncontrolled factors. All experiments were performed using a red wine sample (blank), free of allergen proteins, obtained from a winery that does not use casein or ovalbumin as fining agents. Wine sample was spiked with 1.2 mg L^{-1} of each protein and then submitted to the different digestion conditions according to the experimental plan. An analysis of variance (ANOVA) with a significance level of 0.05 was carried out to determine which experimental factors significantly affect the yields (peak area) obtained for each MRM transitions. According to the observed results, digestion time affected α -casein ($P=0.01$) yields; enzyme-to-protein ratio affected α and β -casein ($P=0.01$ and $P=0.02$); and digestion time (quadratic coefficient) affected α -casein ($P=0.002$), β -casein ($P=0.008$), and ovalbumin ($P=0.04$) yields. For all cases, lack of fit test for quadratic models showed P values higher than 0.05, and adjusted $R^2 > 75\%$, which is adequate in terms of model suitability (Derringer 1980). The corresponding digestion yields obtained for each MRM transition for each experimental run are shown in Table 2. Using the individual optimum, a multiple response optimization was done in order to determine the optimal conditions for all responses (desirability conditions). β -Casein and ovalbumin shared the same optimal conditions, while α -casein showed an optimal yield value with a digestion time of 6 h. The response surface plots for each transition are shown in Fig. 2. Optimal trypsin digestion conditions to simultaneously determine α -casein, β -casein, and ovalbumin in wines were 7 h as digestion time and 1:10 as enzyme-to-protein ratio. The optimum digestion time is ca. 42% lower than the most reported conditions (12 h) (Fremout et al. 2010; Heick et al. 2011; Mattarozzi et al. 2014; Tolin et al. 2012a). Regarding trypsin-to-protein ratio, it is clear

Fig. 1 Product ion spectrum of α -casein peptide YLGYLEQLLR (m/z 634.6)

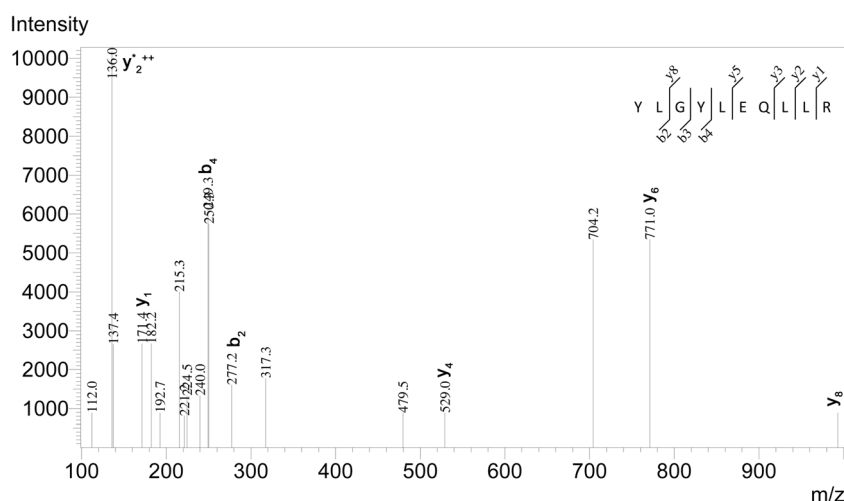


Table 1 MRM transitions for α -casein, β -casein, and ovalbumin determination in wines

Protein	Precursor ion sequence (m/z ; charge state; MW [Da])	Product ion sequence (m/z ; charge state)
α -Casein	YLGYLEQLLR (m/z 634.6; +2; 1267.2)	GYLEQLLR (m/z 991.8; +1) ^{a,b} LEQLLR (m/z 771.4; +1) ^c
β -Casein	VLPVPQK (m/z 390.9; +2; 779.8)	QK (m/z 258.25; +1) ^{a,b} PVPQK (m/z 284.6; +2) ^c
Ovalbumin	ELINSWVESQTNGIIR (m/z 929.5; +2; 1857.9)	VESQTNGIIR (m/z 1116.5; +1) ^{a,b} ESQTNGIIR (m/z 1017.5; +1) ^c

^a Most consistent MS/MS transition^b m/z transition monitored for quantification^c m/z transition monitored for confirmation

that enzyme concentration is crucial to accomplish adequate digestion yields, and therefore, higher concentration might result in higher digestion yields; however, this concentration must be controlled because autolysis could occur decreasing enzyme activity (Hustoft et al. 2011). This phenomenon can be avoided using a “modified trypsin” which is manufactured to provide maximum specificity through reductive methylation of lysine residues, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion (Finehout et al. 2005). This makes the enzyme very attractive for proteomic analyses; however, its price is much higher than the traditional one. In this case, the optimum enzyme-to-substrate ratio for maximum yields and therefore highest signal intensities were found to be the highest enzyme ratio assayed (1:10). Higher enzyme levels were not assayed to avoid autolysis and because the obtained signals were more than suitable for allergen protein determination in wines (ca. 60 times lower than OIV limit). Under these optimal conditions, the percentage of sequence coverage (SQ) increased from 18% to values above 23% for each marker peptide, which is adequate in terms of yield of proteolytic digestion (Hustoft et al. 2011).

Method Validation

The optimized method was validated following the International Conference on Harmonization recommendations (International Conference on Harmonization (ICH) 2005). Calibration curves of all proteins were established with and without wine matrix in order to evaluate a possible matrix effect. Since slopes were not statistically different ($P > 0.05$), calibrations were established with pure standards at six levels in triplicate from 0.10 to 1.20 mg L⁻¹. Calibration data fit a linear regression model with determination coefficients (R^2) = 0.99 for all proteins. Method precision was evaluated through repeatability and intermediate precision. Repeatability was studied injecting in sextuplicate ($n = 6$) a spiked wine sample (0.25 mg L⁻¹) showing RSD values from 0.51 to 1.36%. Intermediate precision was determined measuring in triplicate a spiked wine sample (0.25 mg L⁻¹) during three different days ($n = 3$), showing RSD values from 0.48 to 1.53%. Method accuracy was determined via recovery evaluation. Wine samples spiked at three levels (0.15, 0.25, 0.50 mg L⁻¹) were prepared and analyzed daily during 3 days. All proteins showed recovery yields between 86.40% and 106.98% with RSD values < 2.80%. Detection and

Table 2 Experimental runs for a central composite design with the corresponding responses (means) for each MRM transition (peak area)

Runs	Factors		Responses (peak area, m/z)*		
	Time (h)	Enzyme/protein ratio	634.6 → 991.8	390.9 → 258.25	929.5 → 1116.5
1	2	1:10	675.00 ± 4.59	1954.00 ± 2.47	23.00 ± 2.12
2	7	1:20	740.00 ± 0.35	2945.25 ± 0.70	263.32 ± 7.42
3	12	1:10	391.00 ± 2.82	2175.00 ± 3.53	47.00 ± 3.88
4	7	1:10	845.12 ± 4.24	4604.00 ± 1.76	188.25 ± 1.06
5	12	1:20	233.00 ± 2.82	1350.00 ± 0.35	56.00 ± 3.18
6	2	1:20	493.25 ± 1.41	1546.25 ± 3.18	104.15 ± 4.94
7	7	1:100	704.00 ± 5.30	2059.00 ± 0.70	98.00 ± 4.24
8	2	1:100	311.00 ± 0.00	1458.00 ± 1.06	167.00 ± 2.47
9	7	1:20	598.00 ± 0.00	2764.75 ± 0.36	261.15 ± 3.18
10	12	1:100	152.70 ± 1.41	863.00 ± 1.06	43.00 ± 1.41

*Mean ± standard deviation ($n = 2$)

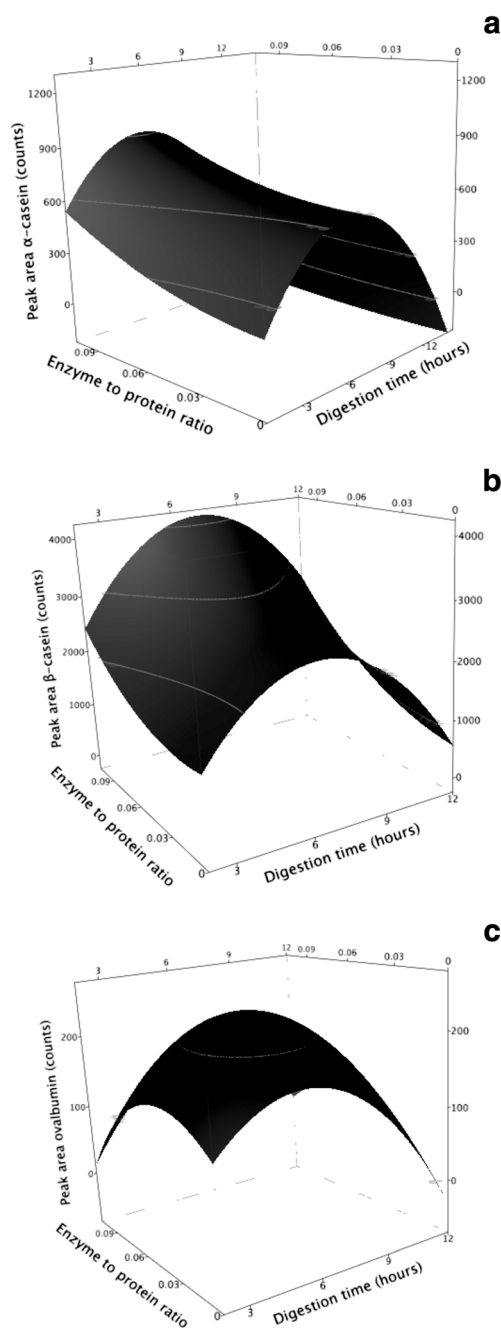


Fig. 2 Response surface plots for α -casein (**a**), β -casein (**b**), and ovalbumin (**c**) transitions

quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10, respectively. Considering the use of a core-shell column and an injection volume of 50 μL , the detection and quantification limits ranged from 4.7 to 8.5 $\mu\text{g L}^{-1}$ and 10.0 to 20.0 $\mu\text{g L}^{-1}$, respectively. Comparatively, this optimized method showed similar validation results than other methods for allergen protein determination in wines using LC-MS/MS. Linear range (0.10–1.20 mg L^{-1}) is lower than reported by Mattarozzi et al. (2014) (50–200 mg L^{-1}) and Chen et al. (2015) (1–100 mg L^{-1}). Recovery results (> 80%) are

similar to those reported by Chen et al. (2015) (98.8 to 106.7%), Mattarozzi et al. (2014) (82 to 105%) and Losito et al. (2013) (80 to 85%). In terms of precision, RSD values from 0.51 to 1.36% are slightly lower than reported by Mattarozzi et al. (2014) (10 to 19%) and Chen et al. (2015) (3.9 to 8.9%). Detection and quantification limits were lower than those reported by Restani et al. (2014) using ELISA method (LOD 0.15 mg mL^{-1} ; LOQ 0.38 mg mL^{-1}) and Mattarozzi et al. (2014) (LOD = 0.01 to 0.80 mg L^{-1} and LOQ = 0.03 to 2.00 mg L^{-1}). Regarding analysis time, some improvements were incorporated to decrease the required time to separate the proteins under study, i.e., shorter and lower particle size core-shell column, higher flow rates, and gradient program adjustments. Thus, a complete separation was achieved in less than 20 min (Fig. 3), which is slightly lower than the reported by Heick et al. (2011)—30 min—and Mattarozzi et al. (2014)—25 min, and at least 65% lower than the most published analysis time (60 min). Losito et al. (2013) developed a LC-ESI-IT-MS method for caseinate quantification in Italian commercial white wines in 75-min runs. Monaci et al. (2010) identified allergen milk protein markers in fined white wines by capillary LC-ESI-MS/MS with 60-min runs. Tolin et al. (2012a) detected egg proteins in red wines by LC-MS method with 50-min runs, and Wang et al. (2016) published a corn peptide identification method based on HPLC-diode array detection and HPLC-ESI-MS/MS that required ca. 80-min runs.

Sample Analysis

As indicated above, allergen protein detection in wines by ELISA (OIV official method) presents some limitations that impair the assay sensitivity. Detection and quantification limits of commercial ELISA test kits are 0.25 mg L^{-1} and 0.50 mg L^{-1} , respectively. These limits allow the quantification of ovalbumin and casein at low concentrations; however, the complexity of wine matrix as well as the impact of processing can hinder the allergen detection by ELISA. Koestel et al. (2016) proposed an ELISA test using commercially available antibodies capable of ovalbumin residue detection in red wines. It was reported that variability in terms of antigen conformation and ELISA signals depends on the sample pH and preparation protocol. Further, it was proved that inhibition of ELISA signal is associated with tannin content, which could not be avoided. Contrarily, the proposed LC-MS/MS method is highly sensitive capable of detecting concentrations as low as 0.01 mg L^{-1} (Table 3). Applying this optimized and validated method, 20 Chilean commercial wine samples without allergen labeling were analyzed. All wine sample labels described the wine type/category, i.e., young (without aging) or reserve (higher quality and/or aged), cv. (one grape variety represents at least 75% of the blend), harvest year, and geographical origin (denomination of origin). According to that, 15 samples were reserved and five young

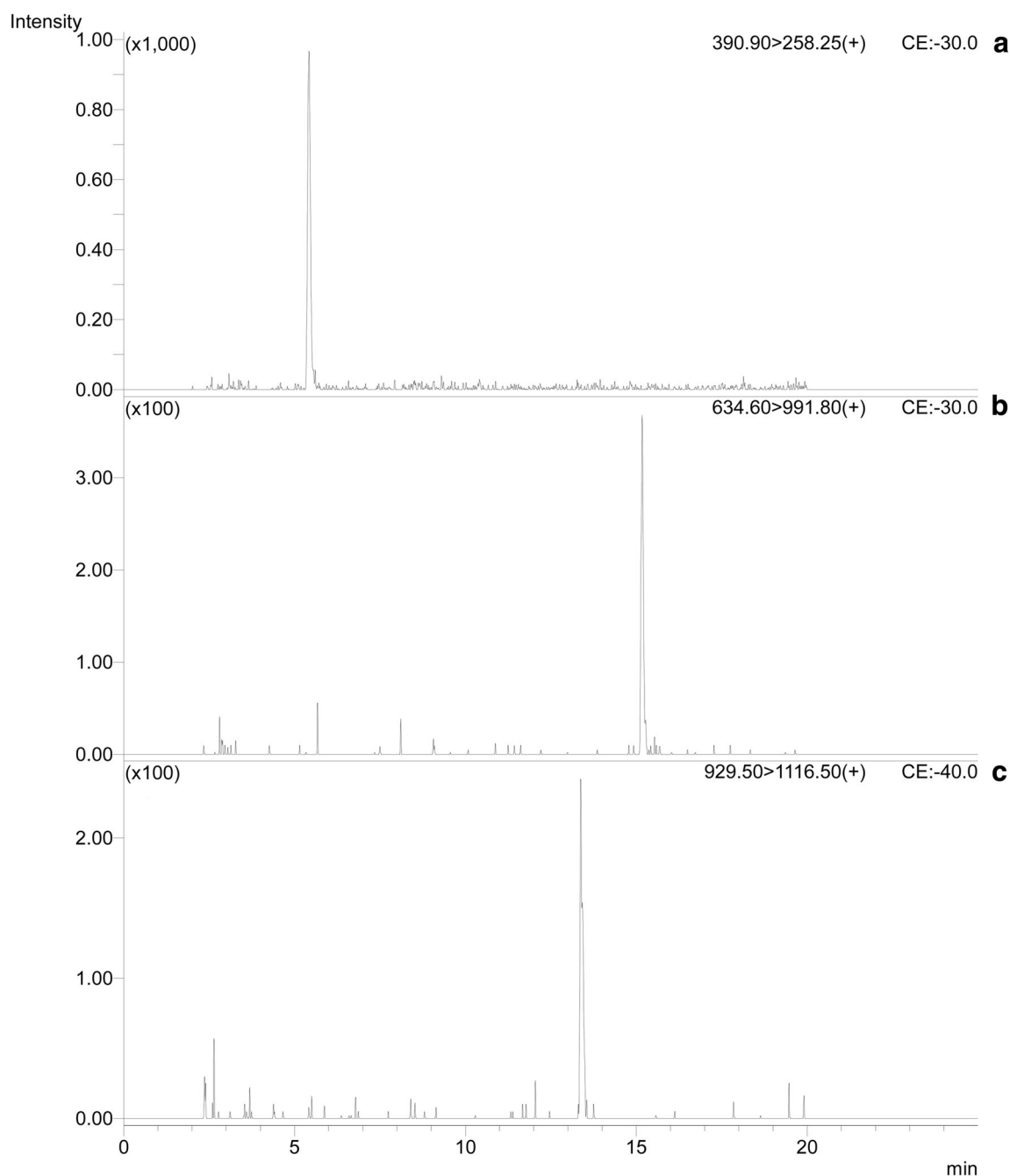


Fig. 3 LC-ESI-MS/MS chromatograms of Cabernet Sauvignon sample analyzed in MRM mode for β -casein (**a**), α -casein (**b**), and ovalbumin (**c**) detection

wines, belonging to six valleys and 14 wineries. Eight samples were Cabernet Sauvignon, four Sauvignon Blanc, four Carménère, and four Merlot. Two independent extractions from each sample were analyzed in duplicate. All samples, except two, showed quantifiable levels of allergen proteins, the level found ranged from non-detected to 0.21 mg L^{-1} (Table 3). One Merlot sample (1/20; 5%) showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}), and other two showed very close values (0.24 mg L^{-1}). Even when

a reduced number of samples were analyzed, the percentage of positive samples was high (18/20; 90%), which demonstrated the prevalent use of casein and ovalbumin as fining agents in Chilean wineries. This result is much higher than international reports. Lifrani et al. (2009) found residual contents of ovalbumin and casein in 11% (44/400) of commercially available French wines analyzed. Hoxey et al. (2013) reported detectable levels of casein (from 0.27 to 5 mg/L) in 0.6% (3/521) of Australian wines sampled. Rolland et al. (2008) found detectable concentrations of ovalbumin ($1 \text{ } \mu\text{g L}^{-1}$) in 1.3% (2/153)

Table 3 Allergen proteins content (mg L⁻¹) in Chilean wine samples

	Wine samples	Valley	Year ^a	α-Casein	β-Casein	Ovalbumin	Total
1	S. Blanc	Leyda	2016	0.06 ± 0.001	0.03 ± 0.000	Traces	0.09
2	S. Blanc	Rapel	2015	0.11 ± 0.000	0.09 ± 0.001	0.04 ± 0.002	0.24
3	S. Blanc	Central	2015	0.14 ± 0.002	0.10 ± 0.002	ND	0.24
4	S. Blanc	Maipo	2016	0.05 ± 0.002	0.03 ± 0.000	ND	0.08
5	C. Sauvignon	Central	2015	0.03 ± 0.002	0.01 ± 0.000	Traces	0.04
6	C. Sauvignon	Maipo	2015	0.03 ± 0.002	0.01 ± 0.000	0.02 ± 0.001	0.06
7	C. Sauvignon	Maipo	2013	0.01 ± 0.000	Traces	0.11 ± 0.003	0.12
8	C. Sauvignon	Central	2015	ND	Traces	Traces	Traces
9	C. Sauvignon	Colchagua	2015	ND	ND	0.06 ± 0.006	0.06
10	C. Sauvignon	Central	2014	ND	ND	0.09 ± 0.000	0.09
11	C. Sauvignon	Central	2013	ND	ND	0.09 ± 0.006	0.09
12	C. Sauvignon	Central	2014	Traces	Traces	ND	Traces
13	Carménère	Colchagua	2016	0.05 ± 0.001	0.03 ± 0.001	Traces	0.08
14	Carménère	Colchagua	2015	0.02 ± 0.000	0.05 ± 0.001	ND	0.07
15	Carménère	Central	2015	0.01 ± 0.001	0.01 ± 0.000	0.03 ± 0.003	0.06
16	Carménère	Curicó	2014	0.08 ± 0.002	0.06 ± 0.001	0.02 ± 0.002	0.17
17	Merlot	Maipo	2015	0.03 ± 0.000	0.02 ± 0.000	0.21 ± 0.080	0.26
18	Merlot	Maipo	2013	0.01 ± 0.001	0.01 ± 0.000	0.02 ± 0.002	0.04
19	Merlot	Curicó	2015	0.05 ± 0.005	0.04 ± 0.002	Traces	0.09
20	Merlot	Rapel	2014	0.02 ± 0.000	0.02 ± 0.000	0.02 ± 0.002	0.06

Two independent extractions from each sample were analyzed in duplicate, results are expressed as mean ± standard deviation

ND not detected (< LOD)

^a Year indicated in the label. Traces (> LOD; < LOQ)

of Australian wines. Tolin et al. (2012b) informed the presence of casein and ovalbumin in 32% (8/25) of Italian commercial wines evaluated. According to the European Food Safety Authority (EFSA) studies (EFSA 2007), only 0.3% of the world's adult population is allergic to casein and ovalbumin, and there are no reports of anaphylaxis at concentrations as low as those found in this work. Rolland et al. (2006) established a panel of five egg and one milk-allergic patients to study the allergenic potential of casein and ovalbumin in Australian wines. Although no anaphylaxis or symptoms of serious adverse reactions attributable to the consumption of wine containing allergens proteins were detected, three mild clinical reactions were observed. Cases of wine allergy have been reported particularly in the Mediterranean area, but a direct association between those adverse reactions and wine proteins has not been established yet as well as its relations with intolerances to compounds such as sulfites or histamine (Weber et al. 2007). Following the mandatory European Union allergen labeling for wines, other countries have established also regulatory frameworks. For example, in Australia and New Zealand, it is mandatory to declare the presence of these proteins in wines (FSANZ 2015). The United States implemented a mandatory allergen declaration in the Food Allergen Labeling and Consumer Protection Act 2004 (Food and Drug Administration). This

document established the obligation to declare the eight major food allergens if they are employed as wine fining agents. Unfortunately, Chilean sanitary authorities have not yet established an official limit for allergen protein content in wines; hence, casein and ovalbumin determination is not a common assay in wineries or in national reference laboratories. Therefore, the information obtained with this study could be useful in terms of food safety, decreasing the possible health risk for sensible consumers, and thereby preventing future sanitary problems that can negatively change the current worldwide status of Chilean wine. Even when clarification and filtration processes are commonly applied after fining process, residues of both proteins at nanograms or micrograms per liter levels are still present as it was demonstrated in the present study. This could become a serious problem for sensitive individuals that consume wines without allergen labeling since the lowest observed adverse effect level ranged from 100 to 200 µg of allergen protein (Stockley and Johnson 2015). Further, other allergen-related compounds such as biogenic amines (e.g., histamine and tyramine) could be present in Chilean wine increasing the possibilities to suffer some kind of adverse effects in sensitive consumers (Henríquez-Aedo et al. 2012; Pineda et al. 2012). Finally, attention should be paid considering also the Chilean per capita wine consumption of 23.5 L per year (Henríquez-Aedo et al. 2012).

Conclusions

This work showed for the first time the evaluation of allergen proteins in Chilean wines, including the analysis of white and red wines from four different grape varieties produced by 14 wineries. The enzymatic digestion time was reduced in 42%, which together with shorter chromatographic analysis (20 min) resulted in an important reduction in the analysis time required for casein and ovalbumin determination in wines. This highly selective method was capable of detecting casein and ovalbumin at ppb ($\mu\text{g L}^{-1}$) levels, which is more than 60 times lower than the European and OIV limit. Method suitability for casein and ovalbumin quantification in wines was demonstrated through validation results. Regarding allergen protein presence in Chilean wines, from 20 samples analyzed, 90% showed quantifiable levels ($>0.01 \text{ mg L}^{-1}$) and one showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}). According to results, it seems appropriate that Chilean sanitary authorities established an official limit for mandatory labeling of allergen proteins in wines in order to avoid a toxicological risk.

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Compliance with Ethical Standards

Conflict of Interest Jessy Pavon declares that she has no conflict of interest. Karen Henriquez-Aedo declares that she has no conflict of interest. Mario Aranda declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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