

Analysis of allergenic residues in wines by triple quadrupole LCMS

F.R. Spinelli^{1,2,a}, G.J. Cargnel², A.P. Drehmer^{1,2}, C. Blatt^{3,b}, M. Baptista^{3,c}, and R. Vanderlinde¹

¹ University of Caxias do Sul, Institute of Biotechnology, Rua Francisco Getúlio Vargas, 1130, 95070-560 Caxias do Sul, RS, Brazil

² Brazilian Wine Institute (IBRAVIN) – LAREN/SEAPI – Laboratory of Oenologic Reference Evanir da Silva, Av. da Vindima, 1855 Caxias do Sul, RS, Brazil

³ Agilent Technologies Brazil, Alameda Araguaia, 1142, Barueri 064555-940, SP Brazil

Abstract. During the winemaking are used technology coadjuvants, between them: albumin, caseinates and lysozyme. These compounds have great oenological properties, however, the presence of their residues can represent risks to people who are allergic to them because they are derived from eggs and milk. Mass spectrometry methods enables unambiguous determination of allergenic proteins at low levels in wines. Therefore, the aim of this study was to determine the concentrations of ovalbumin, alpha-casein, beta-casein and lysozyme in experimental wines treated with different concentrations of them by triple quadrupole liquid chromatography mass spectrometry with Jet Stream Electrospray Ionization (ESI). The wines were elaborated and treated with different concentrations of albumin, lysozyme and potassium caseinate. Bentonite and decantation were used for the removal of the fining agents. The quantification limits (LOQ) for ovalbumin, a-casein, b-casein and lysozyme were: 0.002 mg/L, 0.24 mg/L, 0.75 mg/L and 0.04 mg/L, respectively. Non residues of the proteins were identified in the experimental wines treated with the different amounts of potassium caseinate, albumin and lysozyme, analyzed in this study. These results provide an evidence of the absence of residues of caseinate, albumin and lysozyme in the concentrations tested in the wines if good treatment practices are followed.

1. Introduction

In EU Regulation 1308/2013 [1] in combination with OIV-COMEX 502–2012 [2] labeling is mandatory when there is presence of egg or milk residues above the limit (0.25 mg/L); in addition, according to the Commission Implementing Regulation (EU) N° 579/2012 [3], terms or a pictogram can be used to highlight the presence of allergens.

The OIV adopted in 2014 a Code of good fining practices for wine to be applied in the use of proteinaceous wine fining agents with allergenic potential (casein and egg white) (Resolution OIV-OENO 520-2014) [4], specifying the steps to carry out the clarification and filtration process, avoiding residues of fining agents, with potential allergenic effect, which may be present. Because the wines have different compositions, they react differently with the same clarifying agent. Therefore, the efficacy of the fining agent will depend on the product used, the preparations, the method of addition, the dosage, pH, metal content, temperature, dissolved CO₂ level and previous treatments in wines.

According to [5], the possible traces of casein, isinglass and eggs (ovalbumin) in clarified wines presents a low

risk for consumers allergic to these proteins and labeling can generate a negative impact both for non- allergic consumers as well as for the allergic ones. According to this author, if a person allergic to albumin ingest a liter of wine, considering limit of detection of this protein, would be consuming less than 0.06 mg of egg white.

Different analytical methods are proposed in the literature to quantify allergenic residues in wines based on immunoassay techniques and mass spectrometry [6–16].

According to [16], immunoassays have the advantage of being quick and simple to perform, however, they are not as accurate as mass spectrometric assays. ELISA tests have some disadvantages due to the presence of interfering compounds in the matrix [8], such as polyphenols in red wines, which interact with proteins and antibodies [6].

To avoid these problems, methods for the direct detection of food allergens by mass spectrometry (MS) were developed [14]. Due to the high sensitivity, precision and reproducibility, mass spectrometry allows the detection of traces of proteins and makes identification independent of the structure of allergens [17].

Thus, this study aims to determine the presence of residues of allergenic oenological coadjuvants in experimental wines treated with different concentrations of casein, albumin and lysozyme, by a triple quadrupole (QQQ) ESI-LCMS.

^a e-mail: frespine@ucs.br, fernanda.laren@ibravin.org.br

^b e-mail: celso.blatt@agilent.com

^c e-mail: mariana.baptistao@agilent.com

2. Material and methods

2.1. Reagents, standards, glassworks and materials

All reagents used in this work were purchased from the brand Sigma-Aldrich, as well as the certified analytical standards for alpha-casein, beta-casein and ovalbumin. The analytical standard of lysozyme was purchased from Worthington Biochemical Corporation.

2.2. Experimental wines

The elaboration of experimental wines was carried out at the Laboratory of Oenologic Reference Evanir da Silva (LAREN) of the Secretariat of Agriculture, Livestock and Irrigation of the State of Rio Grande do Sul (SEAPI), linked to the Brazilian Wine Institute (IBRAVIN) located in Caxias do Sul, Rio Grande do Sul, Brazil.

2.2.1. Red wine elaboration

The microvinifications were performed in duplicates, in stainless steel tanks with a capacity of 10 liters. The grape samples (115 kg) of Merlot variety, from the city of Antônio Prado – RS – Brazil, were destemmed and crushed, totaling 14 tanks, containing approximately 8 kg of grapes each one. The grapes were microvinified by the traditional process with maceration of the film. After destemming, sulfur dioxide was added to the must at a concentration of 40 mg/L and after 30 minutes *Saccharomyces cerevisiae* (Maurivin brand) yeast was added at a concentration of 300 mg/L. The removal of the peels was determined by the relative density (approximately 1020), and the wine finished the fermentation in 4.5 L glass bottles, closed with Muller's valve. The wine undergoes spontaneous malolactic fermentation. After the end of the alcoholic and malolactic fermentation, the wines were transferred to another glass bottle and corrected to 40 mg/L of free sulfur dioxide for preservation. The tartaric stabilization was carried out at 0 °C for 20 days and then a new transfer for the removal of bitartrate crystals. After, different concentrations of albumin (30, 150 and 200 mg/L) and potassium caseinate (50, 125 and 250 mg/L) of the brand AEB Improvement Through Biotechnology were added in the bottles. A week after, bentonite was added for the removal of the fining agents and the wines decanted during four days, followed by a new transfer to 750 ml bottles (totaling 4 bottles per experiment), remaining stored in ideal conditions for subsequent analysis.

2.2.2. White wine elaboration

Chardonnay grape samples (120 kg) from the city of Antônio Prado were destemmed and crushed. The extraction of the must was carried out in press, not taking part of the peels in the fermentation. Microvinifications were performed in duplicates in 4.5 liter capacity glass bottles, totaling 14 bottles containing approximately 8.5 kg of must. Sulfur dioxide was added to the must at 40 mg/L and after 30 minutes the albumin and lysozyme (brand AEB Improvement Through Biotechnology) were added, at the concentrations of 200, 400 and 600 mg/L and 200, 400 and 500 mg/L, respectively. After an hour and

30 minutes, *Saccharomyces cerevisiae* (Maurivin brand) yeast was added at a concentration of 400 mg/L. The wine finished the fermentation in 4.5 L bottles, closed with Muller valve. After the alcoholic fermentation, the wines were transferred and the free sulfur dioxide was corrected to 40 mg/L. Subsequently, the bentonite was added for the removal of the fining agents and the tartaric stabilization (and decantation) was performed at 0 °C for 20 days. After, the wines were bottled in 750 mL bottles (totaling four bottles for each type of treatment), remaining stored in ideal conditions of temperature and humidity, for subsequent analysis.

2.3. Analysis

The analyses were performed according to the adaptation of the methods of [14] and [16]. The treatment of the wines prior to the analysis was done in the Laboratory of Oenologic Reference (Caxias do Sul – RS – Brazil), following the method of [14] with some adaptations and the analysis were performed in a triple quadrupole ESI-LCMS at Agilent Technologies laboratory, in Barueri – São Paulo – Brazil.

2.3.1. Treatment of samples

Day 1: Proteins were recovered by the Potassium Dodecyl Sulfate (KDS) method [18], starting from 50 mL of wine. 10% SDS was added to the wine at a final concentration of 0.2%. Samples were heated at 100 °C for 10 minutes. After cooling, 10 mL of 2M KCL were added and the samples were gently mixed for 45 minutes at 4 °C. Protein pellets were recovered by centrifugation at 4300 rpm for 60 minutes at 4 °C, the supernatant was discarded and the pellets were maintained in 1 mL of 20% trichloroacetic acid overnight.

Day 2: Samples were centrifuged at 4300 rpm for 60 min, the supernatants were discarded and, to further remove the SDS, the protein pellets were washed with cold acetone three times (5 mL of acetone each time). Samples were centrifuged at 4300 rpm for 30 min, supernatants were discarded and the protein pellets were let dry. Protein pellets were dissolved in 1 mL of NH₄HCO₃ 50 mM containing 8M of urea. For reduction step, dithiothreitol (DTT) was added to a final concentration of 10 mM and mixture was incubated at 37 °C for an hour. For alkylation step, iodoacetamide (IAM) was added to a final concentration of 30 mM and the mixture was left an hour at room temperature, in the dark. 1 µg of sequencing grade modified trypsin was added after the dilution of the samples with 50 mM NH₄HCO₃ to reach a final concentration of 0.4 M of urea, and digestion was carried out overnight at 37 °C.

Day 3: The samples were desalted with C18 cartridges (Econo-Pac 10DG Columns, Bio-rad) and the extracted peptides were dissolved in 40 µL of 10% formic acid to stop the digestion reaction before the analysis.

2.3.2. Triple quadrupole ESI-LCMS parameters

The analysis were performed in a 6400 Series Triple Quadrupole LCMS (Agilent Technologies) using a Jet Stream Electrospray Ionization (ESI) and the software Mass Hunter. LC separation was performed in a HPLC 1260 series using a 50 mm × 2.1 mm, C18

Table 1. Precursor ion, product ion, collision energy and polarity for each peptide.

Protein	Precursor ion	Product ion	Collision energy (v)	Polarity
Alpha-casein	692.9	295.1	26	Positive
		267*	38	
Beta-casein	1054.7	1151.7*	45	Positive
		648.7	45	
Albumin	635.7	235	42	Positive
		207*	50	
Lysozyme	715	804.6	30	Positive
		249.1*	30	

* Most abundant.

Poroshell 1120 column, thermostated at 45 °C and a flow rate of 0.400 mL/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 under the following optimized gradient: solvent B was initially set at 5% for 1 min, delivered by a linear gradient from 5% to 10% in 5 min, and then 3 min of rebalance for the next sample injection. Each sample was loaded 10 µL. Mass spectrometric conditions were set as follows: electrospray voltage 4 KV; gas temperature of 300 °C, gas flow 9 L/min, sheath gas heater and sheath gas flow set to 250 and 11 arbitrary units. The system was operated in positive ion mode. All analysis were performed in triplicates.

3. Results and discussion

Before the analysis of the wines, the conditions of the method were optimized and the precursor ions and their respective products were investigated. Both full scan and multiple reaction monitoring acquisition (MRM) modes were performed. Most abundant ions of each protein were selected as protein markers to investigate the presence of the protein residues.

For lysozyme, the precursor ion found was the same used in the study of [16], as well as for alpha-casein, the same precursor ion as the study of [10]. For the other proteins (beta-casein and albumin), the ions found in this research are distinct from those reported in some studies [14,16]. The precursor ion and the product ion MRM transition monitored for each peptide are reported in Table 1.

The detection (LOD) and quantification (LOQ) limits were calculated on blank and on wine and were expressed as the concentration of the analyte giving a signal that is 3s and 10s above the mean blank signal, respectively, where is the standard deviation of the blank signal obtained from independent blank measurements.

Regarding the limits of detection and quantification, satisfactory values were found when compared with other studies that presented values between 0.4–50 mg/L [10,19], except for the beta-casein that the LOQ was considered higher than expected. As expected, differences were observed in limits calculated on the blank matrix and the wine matrix. To assess recovery, a Merlot wine and a Chardonnay wines, without any protein treatment, were fortified with alpha and beta-casein, albumin and lysozyme

Table 2. Proteins curve correlations, LOD and LOQ.

Protein	R ²	LOD (mg/L)		LOQ (mg/L)	
		Blank	Wine	Blank	Wine
Alpha-casein	0.9901	0.04	0.07	0.12	0.24
Beta-casein	0.9780	0.16	0.22	0.54	0.75
Albumin	0.9976	6.10 ⁻³	7.10 ⁻⁴	0.02	2.10 ⁻³
Lysozyme	0.9999	0.02	0.01	0.07	0.04

Table 3. Recovery of proteins in wines.

Protein	Concentration added (mg/L)	Average Recovery (%)
Alpha-casein	5.00	167
Beta-casein	5.00	114
Albumin	0.65	63
Lysozyme	5.00	158

before sample extractions and measurements were carried out by monitoring the most abundant transition for the peptide (Table 3).

A peak displacement was observed when addition tests were performed on wines, when comparing with the standards retention times. This displacement was attributed to the possible existence of SDS residues in the samples. As cited by [20], SDS dissolves proteins and prevents interactions (e.g., with phenols) that could occur during protein concentration. The addition of potassium ions induces the protein-detergent insolubilization and allows their recovery. KDS method has been proven useful for protein recovery from fermented beverages. Detergent addition, such as urea or SDS might help protein recovery, but chaotropic agents persist in the samples and could affect further analyses. The SDS could co-elute with the analyte of interest, potentially causing ion suppression. Therefore, calibration curves should be performed in the matrix.

Finally, applying the method to the experimental wines elaborated in this work, non-residues of alpha-casein, beta-casein, albumin and lysozyme were identified. The results of this study are in accordance with the results obtained by [13] that analyzed, by ELISA test, experimental wines elaborated with the addition of 200–500 mg/L of caseinate and did not detect the presence of allergenic residues in the experimental wines.

4. Conclusion

Non-residues of the proteins were identified in the experimental wines treated with the different amounts of potassium caseinate, albumin and lysozyme, analyzed in this study. These results provide an evidence of the absence of residues of potassium caseinate, albumin and lysozyme in the concentrations tested in the wines if good treatment practices are followed. As perspectives of this work, tests will be carried out in the preparation of the samples to optimize the recovery of the proteins.

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