Implementation of an Enzyme Linked Immunosorbent Assay for the Quantification of Allergenic Egg Residues in Red Wines Using Commercially Available Antibodies

Carole Koestel, Céline Simonin, Sandrine Belcher, and Johannes Rösti

Abstract: Since the early 2000s, labeling of potentially allergenic food components to protect people who suffer from food allergies is compulsory in numerous industrialized countries. In Europe, milk and egg components used during the winemaking process must be indicated on the label since July 1, 2012. Several ELISA procedures have been developed to detect allergenic residues in wines. However, the complexity of the wine matrix can inhibit the immunoenzymatic reaction. The aim of this study was to implement an ELISA assay for the detection of ovalbumin in red wines using commercially available antibodies. The specificity of the acquired antibodies and the absence of cross reactivity were assessed by immunoblotting and ELISA. An ELISA assay with a LOD of 14.2 µg/L and a LOQ of 56.4 µg/L of ovalbumin in aqueous solution was obtained. Differences in ELISA signals were observed when analyzing various fining agents, although reproducible conformation of the antigen could be reached for the comparison of ovalbumin and Ovicolle. The differences between samples in terms of pH could be leveled but the inhibition of the ELISA signal, positively correlated to the tannin content of the wines, could not be suppressed. Thus, standard curves of ovalbumin in several wines were obtained by relative quantification. The control steps and the difficulties encountered presented in this study should be considered by anybody working toward the development of ELISA assays for the detection of allergenic residues in complex food matrices.

Keywords: allergen, egg, ELISA, method validation, wine

Practical Application: Development of an analytical method to detect egg residues in red wines. Several control points and encountered difficulties are described and should be considered by those developing similar methods for the detection of allergens in complex food matrices.

Introduction

Food allergies are an important public health concern, estimated to affect 5% of young children and 3% to 4% of adults in industrialized countries (Sicherer and Sampson 2010). Allergic people need a strict control of their diet to avoid the specific allergenic food (Sicherer and Sampson 2010), even if this can be difficult. Minimum eliciting doses can be determined at the individual and population levels but knowledge about thresholds for food allergens is still limited (Crevel and others 2008; Crevel 2015). To protect people with allergies, several political directives and regulations have been adopted in Switzerland (Federal Dept. of Home Affairs 2005, 2013), in Europe (European Parliament 2000; European Commission 2012), and on other continents (FDA 2004; Minister of Justice 2012), in Europe (European Parliament 2000; European Commission 2012), and on other continents (FDA 2004; Minister of Justice 2012). Allergic people need a strict control of their diet to avoid the specific allergenic food (Sicherer and Sampson 2010), even if this can be difficult. Minimum eliciting doses can be determined at the individual and population levels but knowledge about thresholds for food allergens is still limited (Crevel and others 2008; Crevel 2015). To protect people with allergies, several political directives and regulations have been adopted in Switzerland (Federal Dept. of Home Affairs 2005, 2013), in Europe (European Parliament 2000; European Commission 2012), and on other continents (FDA 2004; Minister of Justice 2012). Most of these regulations have been adopted in Switzerland (Federal Dept. of Home Affairs 2005, 2013), in Europe (European Parliament 2000; European Commission 2012), and on other continents (FDA 2004; Minister of Justice 2012). Allergic people need a strict control of their diet to avoid the specific allergenic food, even if this can be difficult. Minimum eliciting doses can be determined at the individual and population levels but knowledge about thresholds for food allergens is still limited. To protect people with allergies, several political directives and regulations have been adopted in Switzerland (Federal Dept. of Home Affairs 2005, 2013), in Europe (European Parliament 2000; European Commission 2012), and on other continents (FDA 2004; Minister of Justice 2012).

Proteinous components may be found in the final product (Uberti and others 2014; Stockley and Johnson 2015).

Analytical methods are needed to detect and quantify the residues or to certify the absence of allergenic residues in fine wines, allowing to overcome the labeling obligation. Enzyme-linked immunosorbent assays (ELISA) are commonly used for the detection of allergenic residues in food products (Poms and others 2004; Koppelman and Hefle 2006; Kirsch and others 2009; Schubert-Ullrich and others 2009; Taylor and others 2009; Baumert 2014). With detection limits generally in the mg/L range, the sensitivity ensures the safety of allergic consumers (Baumert 2014). For the detection of allergenic residues in wine, the international organization for vine and wine (OIV) has also recommended the use of ELISA with a limit of detection (LOD) of ≤0.25 mg/L and a limit of quantification (LOQ) of ≤0.5 mg/L (OIV 2014).

Since 2007, various ELISA methods for the detection of proteinaceous fining agent residues in wines have been published (Weber and others 2007b; Lifrani and others 2009; Deckwart and others 2014a, 2014b). Most of these assays are based on specific in-house antibody production and are therefore difficult to implement elsewhere. Consequently, laboratories for wine analysis and control use commercially available kits specifically developed for the analysis of wine (Restani and others 2009, 2010) or adapted for wine from general use in food (Lacorn and others 2009).
The aim of this study was to implement an ELISA for the detection of ovalbumin in red wines using commercially available antibodies less expensive than whole kits when applied to a large number of samples. Nevertheless the method must be sufficiently robust and reproducible so that it can be easily used by staff experienced in analytical techniques and in laboratories with limited equipment.

For this purpose, the assay published by Rolland and others (2008) that is based on commercially available antibodies was adapted and implemented focusing on a number of critical steps. The acquired antibodies must show high specificity to avoid false-positive results, especially because the ELISA is dedicated to the detection of commercial oenological fining agents and not only purified ovalbumin. For this work the specificity and absence of cross-reactivity was individually tested for each of the 2 acquired antibodies. Moreover, the antigen conformation can vary according to the origin and production process of the fining agent and may depend on the characteristics of the wine matrix. In this study the response of the ELISA to different fining agents was investigated and the sample preparation adapted in order to reduce variability due to different antigen conformations. Finally, the food matrix can have an important inhibitory effect on the detection of allergenic residues by ELISA (Koppelmann and Hefle 2006; Taylor and others 2009). In the case of wine, low pH, alcohol and the polyphenol content can significantly affect the antibody–antigen binding (Weber and others 2007b; Rolland and others 2008; Monaci and others 2013). In this work further investigation of such inhibitions, with a focus on the wine pH and the polyphenol content of red wine, was conducted to improve the reproducibility of the ELISA reaction conditions. As a result, we propose an ELISA using commercially available antibodies and a sample preparation method that allows the detection of ovalbumin residues in red wine in a large number of samples for research purposes.

Materials and Methods

Reagents, buffers, wines, and fining agents

All chemicals used were of analytical grade or as specified. Rabbit anti-ovalbumin polyclonal antibody was obtained from Fitzgerald Industries International (Acton, Mass., U.S.A.). Alkaline phosphatase-labeled goat anti-rabbit IgG antibody was obtained from Kirkegaard & Perry Laboratories (Md., U.S.A.). Mouse anti-chicken egg-albumin monoclonal antibody (clone OVA-14), alkaline phosphatase-labeled goat anti-mouse IgG antibody, albumin from chicken egg white (≥98%), lysozyme (≥98%), ovomucoid, α-casein (≥70%), β-casein (≥98%), β-lactoglobulin (≥90%), bovine serum albumin (≥95%), p-Nitrophenyl phosphate (pNPP), gelatin from cold water fish skin were obtained (ovaclote and Ovocoll (Sofralab, Epernay, France), Albuvin (Erbsloeh, Geisenheim, Germany), Blancoil (Enartis, Trecate, Italy), Albumine d’oeuf poudre (Lamothe-Abiet, Bordeaux, France) hereafter named Albumin LA, and Ovoclaryl (Laffort, Bordeaux, France). Casesol® was from Martin Vialatte Oenologie (Sofralab, Epernay, France).

SDS-PAGE and Coomassie staining

Wine samples were precipitated in ice cold ethanol and the pellet was resuspended in PBS. Samples were diluted 1:1 in Laemmli buffer. Using a Mini protein II system (Bio Rad), proteins were separated with 10% to 15% acrylamide gels under denaturing conditions at 200 V (Model 200/2.0 power supply, Bio–Rad) for 45 min in Tris-Glycine buffer. The SDS-PAGE gels were developed in Coomassie Brilliant Blue following the manufacturer’s instructions.

Immunoblot

Immunoblots were performed on a Mini Trans-Blot cell® (Bio–Rad) using a nitrocellulose membrane with a 0.45 μm pore size (Schleicher and Schuell, BioScience GmbH, Dassel, Germany). Transfer was performed at 30 V during 1.5 h. TBS-3% gelatin was used as a blocking agent. Mouse anti-chicken ovalbumin monoclonal antibody was diluted 1:10000 in TBS-1% gelatin and rabbit anti-ovalbumin polyclonal antibody was diluted 1:1500 in TBS-1% gelatin. Detection was performed using alkaline phosphatase-labeled anti-mouse IgG antibody or alkaline phosphatase-labeled anti-rabbit IgG antibody (diluted 1:2000 and 1:1000 in TBS-1% gelatin). The free binding sites on the wells were blocked with 200 μL/well of PBS-0.1% gelatin for 1 h and subsequently washed. The wells of a polystyrene microtiter plate (Maxisorp™, Roskilde, Denmark) were coated with 50 μL/well of antigen conformation can vary according to the origin and production process of the fining agent and may depend on the characteristics of the wine matrix. In this study the response of the ELISA to different fining agents was investigated and the sample preparation adapted in order to reduce variability due to different antigen conformations. Finally, the food matrix can have an important inhibitory effect on the detection of allergenic residues by ELISA (Koppelmann and Hefle 2006; Taylor and others 2009). In the case of wine, low pH, alcohol and the polyphenol content can significantly affect the antibody–antigen binding (Weber and others 2007b; Rolland and others 2008; Monaci and others 2013). In this work further investigation of such inhibitions, with a focus on the wine pH and the polyphenol content of red wine, was conducted to improve the reproducibility of the ELISA reaction conditions. As a result, we propose an ELISA using commercially available antibodies and a sample preparation method that allows the detection of ovalbumin residues in red wine in a large number of samples for research purposes.

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Sample preparation and ELISA procedure

The pH of the wines was increased by adding 0.8% (v/v) NaOH (8 M) prior to a 1:10 dilution in mPBS. Standards in wine, PBS or mPBS were spiked with solutions of ovalbumin in PBS or mPBS to the desired concentration. Samples were heat-denatured in a water bath at 85 °C for 5 min and allowed to cool prior to analysis, and were analyzed in triplicate. Plates were washed 3 times with PBS–TWEEN 20® between each incubation period. The wells of a polystyrene microtiter plate (Maxisorp™, Roskilde, Denmark) were coated with 50 μL/well of anti-ovalbumin polyclonal antibody diluted 1:1000 in bicarbonate buffer. After 1 h of incubation at 30 °C, the plate was washed and the free binding sites on the wells were blocked with 200 μL/well of PBS-0.1% gelatin for 1 h at 30 °C and subsequently washed. Following this, 100 μL/well of samples were added and incubated for 1 h at 30 °C. After the washing steps, the plate was incubated with the egg albumin monoclonal antibody diluted 1:10000 in PBS-0.1% gelatin (100 μL/well) for 1 h at 30 °C and washed.
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again. Thereafter, the plate was incubated for 1 h at 30 °C with the alkaline phosphatase-labeled goat anti-mouse antibody diluted 1:2000 in PBS-gelatin (100 μL/well) and washed again. Finally, 150 μL of pNPP diluted to 1 mg/mL in substrate buffer were added to each well. Incubation was performed in the dark, at room temperature, for 60 min. The optical density (OD) was recorded at 405 nm after 15, 30, and 60 min of incubation using a microtiter plate reader (Dynex Technologies GmbH, Denkendorf, Germany). ODs are reported as the mean of 3 replicates for each determination. Normalized ELISA signals (Norm OD) were obtained by dividing the mean OD of each sample by the mean OD of the highest ovalbumin concentration. Standard curves were obtained by plotting normalized ELISA signals against the logarithmic concentration of ovalbumin in the samples and were fitted to a 4 parameter logistic function using Sigmaplot 12 (Systat Software Inc., San Jose, Calif., U.S.A.). The detection limit (LOD) and the quantification limit (LOQ) were calculated according to the OIV (2014). The inhibition percentage was calculated to evaluate the loss of ELISA signal due to the wine matrix, according to the following formula:

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\text{Inhibition of the ELISA signal [%]} = 1 - \left[ \frac{\text{Norm OD}_{\text{spiked wine}} - \text{Norm OD}_{\text{unspiked wine}}}{\text{Norm OD}_{\text{spiked PBS}} - \text{Norm OD}_{\text{unspiked PBS}}} \right] \times 100.
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Wine analysis

Total tannins content was measured by acid butanolyse (Govindarajan and Mathew 1965) and anthocyanins were measured by SO₂ bleaching (Ribèreau-Gayon and Stonestreet 1965), using an Evolution 160 UV-Vis Spectrophotometer (Fisher Scientific AG, Reinach, Switzerland) for absorbance measurements. pHs were measured using a 691 Metrohm pH-meter (Zofingen, Switzerland).

Statistical analysis

Statistical analysis of the data was performed using the XLStat for Windows (version 2014.2.07, Addinsoft, Paris, France). A Student’s t-test was used to compare differences between 2 specific means whereas one-way ANOVA permitted the comparison of several groups of data. Tukey’s test was performed to find means that were significantly different from each other. Spearman correlation coefficients were calculated to examine possible relationships between the analytical parameters.

Results and Discussion

Characterization of the antibody specificity

When developing an ELISA assay, the specificity and the absence of cross-reactivity of the applied antibodies have to be tested for the validation of the method according to the criteria stated by the OIV for the quantification of allergenic residues in wines (OIV 2014). Therefore, the specificity of the 2 commercial anti-ovalbumin antibodies used in the assay was verified. Proteins of 1 wine as well as purified ovalbumin and a commercial fining agent containing ovalbumin (Ovicolle) were separated by SDS-PAGE followed by immunoblotting (Figure 1). Wine proteins were separated into 1 band around 66 kDa and several bands between 21 and approximately 35 kDa, corresponding to the 4 characteristic wine

![Figure 1](image1.png)

![Figure 2](image2.png)
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protein groups (grape invertase, β-glucanases, chitinases, and thiamine-like proteins, respectively) (Dufrechou and others 2012). The migration profile of purified ovalbumin shows 1 major band near 45 kDa and lighter ones around this weight. A smeared band can also be observed between 78 and 120 kDa, likely corresponding to aggregated forms of the protein caused by the coagulation and purification process of this product. Ovalbumin was also detected in Ovicolle, as well as other bands such as ovotransferrin (78 kDa) (Desert and others 2001) because Ovicolle consists essentially of whole egg white. The immunoblotting results indicate that both antibodies show good specificity on purified ovalbumin (Figure 1). No wine proteins or other egg white proteins were detected by the antibodies. The smeared bands attributed to aggregated forms of ovalbumin were detected by both antibodies in ovalbumin and Ovicolle, but should not be considered as an unspecificity.

Absence of cross-reactivity must be tested to avoid false-positive results caused by several proteins that can be found in fining agents or wine samples. Immunoblot and ELISA were used to investigate the cross-reactivity of both antibodies with various egg proteins (ovomucoid and lysozyme), milk proteins (α-casein, β-casein, and β-lactoglobulin), bovine serum albumin and a commercial fining agent containing casein (Casesol®), all diluted in PBS. The ELISA signals of the proteins mentioned above were measured in both unheated and heat-denatured samples. The band revealed on the SDS-PAGE for each protein was reported in Figure 2, with indication of the migration range. None of the proteins tested was detected by immunoblotting apart from ovomucoid, which was detected only by the polyclonal antibody. It was not detected by ELISA, showing the double specificity conferred by the sandwich assay. No cross-reactivity was observed by ELISA with unheated samples; the ELISA signals measured were all lower than the LOD. After heat-denaturation of the proteins, only 1 cross-reactivity was observed with high concentrations (≥100 mg/L) of heat-denatured lysozyme. Lower concentrations of heat-denatured lysozyme as well as the other proteins tested were not detected by the ELISA. Heat denaturation also led to a significant increase in the ELISA signals obtained for ovalbumin (Figure 3). Considering these results, we supposed that the reactivity observed for high concentrations of heat-denatured lysozyme (purity >98%) was due to a contamination of lysozyme by ovalbumin from the production process, rather than to a real cross-reactivity. The results for the cross-reactivity are in accordance with those published by Rolland and others (2008).

Sandwich ELISA evaluation in aqueous buffer

Once these quality control steps were performed, the ELISA procedure for the detection of egg residues in food developed by Hefle and others (2001) and modified by Rolland and others (2008) was technically adapted for the use in our laboratory. Alkaline phosphatase was used as a detection system instead of horseradish peroxidase. The wells were filled with higher volumes of antibody solutions and samples to increase the robustness of the test. Finally, the incubations were performed at 30 °C to guarantee a better reproducibility. A standard curve ranging from 1 to 1×10^6 μg/L of ovalbumin in PBS with a LOD of 14.2 μg/L and a LOQ of 56.4 μg/L was obtained (Figure 4). The LOD is in accordance with the requirements fixed by the OIV (2014). The use of commercial antibodies, rather than the expensive, time consuming, and high-skilled in-house antibody production, is quite accessible for laboratories specialized in wine analysis, thanks to the availability of standardized products in sufficient quantities. Thus, providing that the specificity and the absence of cross-reactivity of the antibodies have been tested, this assay can be reproduced easily in any laboratory.

Influence of the antigen conformation on the variation of the ELISA signal

In addition to crude egg white, a large panel of commercial fining agents containing ovalbumin, obtained from pasteurized or spray- or freeze-dried egg white, is available for wine producers. Consequently, ovalbumin is not introduced in the wine alone but

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**Figure 3**—Impact of the heat-denaturation of the samples on the ELISA signal. ELISA standard curves obtained for different concentrations of unheated and denatured ovalbumin and Ovicolle diluted in PBS. Data are reported as mean ± standard deviation, n = 3.

**Figure 4**—ELISA standard curve of ovalbumin in PBS. Semi-log representation of the Normalized ELISA signal as a function of the ovalbumin concentration. Dynamic range of the standard curve is 1 μg/L to 1×10^6 μg/L. Data are reported as mean ± standard deviation, n = 3.
as a mixture of proteins containing different amounts of ovalbumin, although little information is available from the manufacturers about the protein content of the products and the production process. Therefore, such products should yield an ELISA signal lower than or equal to the signal obtained with purified ovalbumin. Different oenological products were analyzed by ELISA in comparison to ovalbumin to consider this assumption (Figure 5). Solutions of commercial products (unheated) diluted in mPBS led to higher ELISA signals than purified ovalbumin analyzed under the same conditions. The difference was statistically significant for 3 of the 5 products tested. Exactly the same trend was observed for unheated commercial products and ovalbumin diluted in red wine, but ELISA signals were lower than the signals of samples prepared in mPBS due to the inhibition of the ELISA signal caused by the red wine matrix. As a possible explanation of these results, and in relation those published by Kumbho and others (1996), we assumed that the drying part of the manufacturing process of these powdered products induces a partial denaturation of the protein content, leading to an increase of their immunological reactivity. However, the antibodies used in the ELISA need to recognize a reproducible conformation of the antigen in the sample solution. Therefore, heat-denatured samples of purified ovalbumin and commercial products were also analyzed by ELISA to confirm that the increase of the ELISA signal was due to heat denaturation of the protein. ELISA signals measured for heat-treated ovalbumin were significantly higher than the signals observed for unheated samples (Figure 5). Heat-denatured commercial products showed also a rise in ELISA signals which was significant for 3 of 5 products tested. Moreover, a greater increase was observed for the 2 products with an ELISA signal similar to purified ovalbumin, under untreated conditions. These results confirm our hypothesis of different denaturation stages of the proteins in commercial oenological products. Further investigation of the impact of the heat denaturation of the samples was conducted with a focus on purified ovalbumin and Ovicolle by ELISA analysis of both products diluted in PBS. Signals obtained for samples of unheated ovalbumin were significantly lower (ANOVA, \( P = 0.03 \)) than signals obtained for samples of heat-denatured ovalbumin, unheated and heat-treated Ovicolle, between which no significant difference in ELISA signal was observed (Figure 3). Thus, the systematic heat treatment for the sample preparation adopted in this study resulted in a reduction of the differences in ELISA signal between the commercial products, but did not suppress them completely. Nevertheless, for the comparison of purified ovalbumin and Ovicolle, the conditions of the heat treatment completely leveled the differences in ELISA signals and produced a reproducible conformation, and thus, immunochemical reactivity.

The variability in immunological reactivity observed on powdered oenological products obtained from egg white was also highlighted by Weber and others (2010) when studying isinglass and fish gelatin. The production process of these fining agents include the use of heat, acid extraction, and enzymatic hydrolysis, leading to commercial products containing proteins that range from a native to a highly denatured stage. More generally, the importance of food processing on the detection of allergens in food by ELISA has been reported by several authors (Diaz-Amigo 2010; Downs and Taylor 2010; Garber and Perry 2010; Khuda and others 2012a, 2012b). Antibody–antigen binding may be affected by a modification of the antigen structure occurred during food processing. In addition, antibodies recognize specific epitopes on the antigens that can experience different levels of modification or changes in accessibility.

### Interference of the red wine matrix with the ELISA signal

To use this ELISA assay for the analysis of wine samples, the sample preparation procedure had to be modified in order to guarantee that the properties of the ELISA reaction solution are reproducible and not dependent on the wine matrix. Notably, wine has variable acidic pHs and buffering capacities that have to be balanced in order to reach the most favorable pH of the reaction solution, since preliminary experiments showed that samples with an acidic pH can inhibit the ELISA signal (results not shown). Thus, we fixed an acceptable limit of ±0.2 around a targeted pH of 7.4. The pH of 6 red wines was measured and showed small variations between samples, around a mean of 3.86 (Table 1). A 10-fold dilution of the wine in PBS was chosen as the initial sample preparation procedure as it was used in other similar studies (Weber and others 2007a, 2007b; Deckwart and others 2014a) and considered more quantitative than the protein precipitation used by Rolland and others (2008), although no results seem to validate the sample preparation procedure regarding to a targeted pH. However, the pH of the samples remained below the target (mean pH 5.95) and an increase of the standard deviation was observed, highlighting the different buffering capacities of wines. A modified PBS (mPBS) containing 5 times more buffering components was prepared and used as a diluent. Fewer differences between the samples were measured but the pH remained out of the acceptable limits (mean pH 7.05). Neutralization of the wine samples pH with sodium hydroxide was studied as this method is used prior to some enzyme-based analytical methods. An addition of 0.8% (v/v) of NaOH 8 M to the wine was adopted, as the pH shift induced is sufficient to reach the set target after the dilution with PBS (mean pH 7.23) without changing the volume of the sample more than 1%. However, the use of PBS as a diluent was abandoned as some samples were still under the acceptable pH limit and because the differences between samples were too high. The best results...
were obtained when using mPBS as a diluent. The average pH was close to the target (mean pH 7.36) with a very low standard deviation. Therefore, the wine sample preparation procedure was modified to include the addition of NaOH and the use of mPBS as a diluent.

The complexity of the wine matrix (Weber and others 2007b; Rolland and others 2008; Monaci and others 2013), and particularly the high polyphenol content of red wines (Weber and others 2007b), are suspected to disturb the antibody–antigen binding, causing the inhibition of the ELISA signal. Thus, 9 red wines with different polyphenol contents were spiked with ovalbumin, diluted and analyzed by ELISA. Tannin and anthocyanin concentrations were measured for each wine. Inhibition percentages show the loss of the ELISA signal calculated as a percentage of the ELISA signal measured in wine in comparison to the ELISA signal measured in PBS. The higher the inhibition percentage, the more of the signal observed. Linear correlation coefficients were calculated between the inhibition of the ELISA signal and the total tannin content (Table 2). However, at similar tannin concentrations, the inhibition of the ELISA signal observed with the 2 Gamaret wines was lower than for the other wines. Anthocyanins appear to have a very limited impact on the inhibition of the ELISA signal.

Several sample preparation techniques such as solid phase extraction, protein precipitation and polyvinyl polypyrrolidone treatment were tested to reduce the impact of polyphenols on the ELISA signal. All led to a loss of ovalbumin due to the strong interactions between polyphenols and proteins (results not shown). Rolland and others (2008) used ethanol precipitation, but no spiking and recovery studies seem to validate this method. Protein precipitation and dialysis were also found to be unsuitable for quantitative protein analysis by Weber and others (2009) due to incomplete protein precipitation or potential adsorption of the proteins on the dialysis membrane. As the wine matrix is complex and because of the strong interactions between proteins and polyphenols, an efficient separation method should induce the separation of the protein–polyphenol complexes before the retention of polyphenols.

### Optimized ELISA assay and standard curves of ovalbumin in wine

The robustness of the assay was improved by the modification of the sample preparation procedure. The modified protocol, including pH leveling with NaOH, the use of a modified buffer (mPBS) to dilute the samples and a step of heat denaturation of the samples prior to analysis, requires little additional equipment. Although the inhibition of the signal caused by the wine matrix could not been suppressed, the modified ELISA procedure is usable by any laboratory for the relative quantification of ovalbumin by analyzing standard curves of ovalbumin in untreated red wines. Following the new version of the ELISA protocol, standards of ovalbumin in several red wines with different polyphenol contents were analyzed. Spiked wines with concentrations of ovalbumin between 5×10⁻² and 5×10⁻¹ μg/L were used as internal standards for each calibration. Standard curves were obtained using a 4 parameter-logistic regression for each curve after semi-log representation of the data (Figure 6). The regression was used to express the LOD in equivalent of ovalbumin (μg/L). Differences between the standard curves in terms of shape can obviously be linked to the variability of the wine matrix, especially the tannin content, and its inhibition on the ELISA signal. The ELISA assay could then be used to investigate successfully the effect of several winemaking practices on the presence of ovalbumin residues in the wine after fining, in the laboratory and at preindustrial scale. Despite the modifications of the procedure reported in this study, the assay could not be used in routine as a large-scale rapid detection method. Relative quantification requires the access to unfinned wine in order to obtain a valid standard curve. In addition, a very strong inhibition of the ELISA signal could lead to the impossibility to conclude about differences between small concentrations of ovalbumin, due to the shape of the standard curve. Moreover, the entire procedure is quite long compared to commercial detection kits using pre-coated plates and requiring less incubation steps.

#### Table 1–Changes in the sample preparation for ELISA analysis: sample pH and buffering capacity of the diluent. The pH values were initially measured in neat wine and again after a 10-fold dilution of the wine in PBS and mPBS. The pH values were then measured after the addition (0.8% v/v) of NaOH (8 M) to the wines prior to the dilution in the 2 different buffers.

| Dilution       | Wine 10x PBS | Wine 10x mPBS | Wine + NaOH 10x PBS | Wine + NaOH 10x mPBS |
|----------------|--------------|---------------|---------------------|----------------------|
| Gamay A        | 3.97         | 6.22          | 7.10                | 8.66                 |
| Gamaret A      | 3.95         | 5.80          | 7.02                | 5.98                 |
| Gamaret B      | 3.84         | 5.66          | 7.01                | 5.73                 |
| Red Blend A    | 3.92         | 6.03          | 7.10                | 6.60                 |
| Red Blend B    | 3.77         | 6.02          | 7.05                | 6.65                 |
| Red Blend C    | 3.72         | 5.97          | 7.03                | 6.51                 |
| Mean           | 3.86         | 5.95          | 7.08                | 6.69                 |
| Standard deviation | 0.10       | 0.20          | 0.04                | 1.03                 |

### Table 2–Inhibition of the ELISA signal by the red wine matrix related to phenolic compounds. The inhibition percentage of the ELISA signal of the different wines is calculated in relation to the ELISA signal of the PBS buffer (no inhibition). Wines and buffer were spiked with 10 mg/L ovalbumin. Linear correlation coefficients were calculated between the inhibition of the ELISA signal and the 2 phenolic compounds. An asterisk indicates a significant correlation.

| Inhibition of the ELISA signal | Tannins [g/L] | Anthocyanins [mg/L] |
|-------------------------------|--------------|---------------------|
| Gamay A                       | 34.4%        | 0.68                | 259.4               |
| Gamaret A                     | 40.0%        | 2.02                | 528.8               |
| Pinot Noir B                  | 44.4%        | 1.00                | 167.4               |
| Red Blend A                   | 44.8%        | 1.42                | 253.3               |
| Gamay B                       | 49.3%        | 1.03                | 341.3               |
| Gamaret B                     | 58.6%        | 2.55                | 620.6               |
| Red Blend B                   | 61.3%        | 1.93                | 411.9               |
| Red Blend C                   | 81.4%        | 2.46                | 203.3               |
| Gamay A                       | 90.7%        | 2.66                | 469.9               |

Linear correlation coefficients: 0.781* 0.759
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To overcome the drawbacks of immunoenzymatic methods several alternatives are now available. Real-time polymerase chain reaction kits are available from several manufacturers. A recent study reported the use of quantitative western blot for the detection of ovalbumin and casein in white wine (Meyer and Zanetti 2015). Finally, several mass-spectrometric (MS) based methods have been published for the detection of allergenic protein residues in food (Heick and others 2011; Monaci and others 2014) and also in wines (Monaci and others 2010, 2011, 2013; Tolin and others 2011, 2012; Losito and others 2013). Although the MS-based methods are promising, the cost of the employed materials and the complexity of these methods make them unsuitable for routine use at present.

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Author Contributions

Carole Koestel conducted the analysis, interpreted the results and drafted the manuscript. Céline Simonin conducted the analysis, interpreted the results and revised the manuscript. Sandrine Belcher planned the research and revised the manuscript. Johannes Rösti supervised the research, provided suggestions and technical advice and revised the manuscript.

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Figure 6—Standard curves of ovalbumin in different red wines. The wines were spiked with increasing concentrations of ovalbumin after a 10-fold dilution and were analyzed independently on 4 different ELISA plates. Data are reported as mean ± standard deviation, n = 3.

Conclusion

ELISA is the most widely used analytical technique for the detection of allergens in food because of its sensitivity and specificity (Poms and others 2004; Immer and Lacorn 2015). Enabling the analysis of many samples simultaneously, ELISA can be run quite easily by personnel with experience in laboratory techniques and require only little equipment.

We propose an ELISA using commercially available antibodies and a sample preparation method that allows the detection of ovalbumin residues in red wine in a large number of samples for research purposes. The specificity of the antibodies used in the assay and the absence of cross-reactivity have been tested by immunoblotting and ELISA showing satisfactory results. An ELISA assay with an LOD of 14.2 μg/L and a LOQ of 56.4 μg/L of ovalbumin in aqueous buffer was obtained. Differences in ELISA signals were observed when analyzing several oenological products obtained from egg white. The variability in terms of antigen confirmation was minimized by adding a step of heat denaturation to the sample preparation procedure. Modifications of the ELISA procedure were proposed to ensure reproducible reaction conditions in terms of pH. Inhibition of the ELISA signal, which was proved to be linked to the tannin content of the wine, could not be suppressed. Using relative quantification, standard curves of ovalbumin in several wines were obtained and used for research purpose. As observed in this study and reported by several authors (Koppelman and Hefle 2006; Weber and others 2007a; Rolland and others 2008; Taylor and others 2009; Monaci and others 2010), the complexity of the food or wine matrix as well as the impact of processing can hinder the detection of allergens by ELISA. This study describes several control points and obstacles that can be encountered when developing an ELISA dedicated to the use on complex food or wine matrices, using commercially available or in–house produced antibodies. When using a commercial test kit, these control and optimization steps have to be performed by the manufacturer, ensuring that the sold kit shows high performance.
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