

Development of a Sensitive ELISA for the Detection of Casein-Containing Fining Agents in Red and White Wines

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ABSTRACT: Fining of wine with proteinogenic fining agents such as casein from cow's milk is a traditional and commonly used technique all over the world. Casein and other proteins from cow's milk are well-known food allergens, which pose a risk for allergic consumers. Temporary regulations exempting the labeling of milk and products thereof in wine expired. Since July 1, 2012, these fining agents have to be declared on the wine label under Regulation (EU) No. 579/2012 in conjunction to article 120g of Regulation (EU) No. 1234/2007 if exceeding the threshold of 0.25 mg/L allergenic protein. The aim of the presented study was to develop sensitive ELISA methods for the detection of casein in white and red wines and to investigate the risk of allergenic residues in fined wines. In this context it was shown that the used substance for calibration is highly relevant. Casein wine fining agents of different commercial producers were investigated by LDS-PAGE and immunoblot. In addition to casein, they contain other milk proteins, which are potentially allergic and therefore have to be incorporated in the development of antibodies for an ELISA method to be set up. An indirect ELISA for the investigation of white wine was developed. The LOD is 0.1 mg/L. For red wine the LOD is 0.2 mg/L in an indirect sandwich ELISA setup. The LOD of the indirect sandwich ELISA for white wine depends on the calibration standard. It is 0.1 mg/L for the fining agent casein and 0.01 mg/L for casein from a chemical trader. It is also shown that the use of different technological procedures during winemaking leads to no detectable amounts of casein in various wine samples.

KEYWORDS: wine, fining agents, food allergy, allergen, hidden allergen, casein, ELISA

INTRODUCTION

Well-known common food allergens, such as casein and caseinates, are traditionally used all over the world as wine fining agents. They are applied for clarifying and removing undesirable effects on organoleptic qualities by binding phenolic compounds in a noncovalent way.^{1,2} The current winegrower's belief is that the formed complexes are sufficiently removed by common enological techniques, which is not scientifically proven.

Casein and the other milk proteins are well-known food allergens, which have to be avoided by allergic people. Most allergic reactions to cow's milk occur against the casein fraction named Bos d 8 and the major whey proteins α -lactalbumin (Bos d 4) and β -lactoglobulin (Bos d 5).³ Cow's milk allergy (CMA), along with hen's egg allergy, is the most common food allergy in infants and young children.^{4,5} In contrast, CMA in adults is rare, but according to Lam et al.⁶ may be fatal for the patient. Depending on different studies the prevalence of CMA in children ranges from 1 to 7.5% and from 0.1 to 0.5% in adults.^{3,7–11} Most of the children outgrow their CMA by the age of 3 years and become CMA tolerant.^{5,11}

To protect sensitive consumers from "hidden allergens", which are allergens present in complex food products and would not be expected or recognized by an average consumer, European Regulation orders the declaration of potential allergenic

food ingredients, as well as processing aids used in food production. Therefore, Directive 2003/89/EC amending 2000/13/EC was legislated. According to Rolland et al.¹² there should be negligible residual protein in the final product if processing aids were used and removed considering good manufacturing practice. In the case of casein, Weber et al.² reported that caseins are almost completely coagulated and sedimented, because of their insolubility at the pH of wine and the formation of insoluble complexes with phenolic compounds from grapes. In contrast, Mezdoor et al.¹³ investigated the combined effects of alcohol concentration and pH on the solubility of sodium caseinate in water–ethanol mixtures. In conclusion, casein solubility is a function of pH, which can be increased by the volume of ethanol. Because of the lack of scientific data concerning potential allergenic residues of these fining agents in wine, the European parliament temporarily exempted the labeling of wines processed with milk and products thereof until June 30, 2012, by Regulation 1266/2010/EC, an amendment of the former Directive of the EC, to allow scientific research on the allergenicity of fining agents including casein. The question

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of whether allergic reactions can be elicited in allergic patients by residues of fining agents in wine is still unanswered. Kirschner et al.¹⁴ investigated the tolerability of casein-, ovalbumin-, and isinglass-fined wines in 14 allergic patients by skin prick tests (SPT), as well as by double-blind, placebo-controlled food challenge (DBPCFC) with fined and filtered wines. The highly concentrated fining agents were allergenic in the SPT, but no patient reacted adversely to the oral challenge of the fined and filtered wines. However, in another study by Rolland et al.¹² after DBPCFC with fined and unfined wines, unspecific subjective, but no objective, symptoms occurred, which does not allow a clear interpretation. Because of this situation, since July 2, 2012, the application of fining agents such as casein and milk-derived products during wine processing has to be labeled if the final product contains 0.25 mg/L or more allergenic protein, which is a threshold given by Organisation Internationale de la Vigne et du Vin (OIV, Paris, France) regulations. This is a legal threshold under Regulation (EU) No. 579/2012 in conjunction with Regulation (EU) No. 1234/2007.

The method recommended by OIV for the detection of potentially allergenic residues of fining agents proteins in wine is ELISA with a limit of detection (LOD) of ≤ 0.25 mg/L and a limit of quantification (LOQ) of ≤ 0.5 mg/L. For this purpose various sensitive ELISA methods are described in the literature detecting ovalbumin, casein, and casein-derived products as well as dried hen's egg white.^{2,15–18} Drawbacks of ELISA methods in this case are interfering compounds in the matrix such as polyphenols in red wine, as described by Weber et al.¹⁵ and Monaci et al.¹⁹ In recent years also a number of MS-based methods have been reported for the detection of residual allergenic fining agents in red and white wines.^{19–24} With MS techniques LODs and LOQs in the lower milligrams per liter range up to 51 mg/L of fining agent residues in wine are achieved. A big benefit of MS analysis compared to ELISA is the possibility for simultaneous detection of, for example, casein and ovalbumin. However, a drawback of MS methods is that it is a nonimmunological method and the antigenicity of the target protein(s) is not considered, which might be important for allergen analysis.

The aim of the study was to develop very sensitive ELISA methods with detection limits in the micrograms per liter region to investigate possible casein fining agent residues in wine. These methods could then be used for risk assessment related to residues of allergenic wine fining agents and may avoid unnecessary labeling, which may lead to uncertainty and decreased quality of life in allergic consumers.^{15,25}

For this purpose it is necessary to study the heterogeneity of different casein fining agents and get information on the influence of different standard substances as calibration reference on the results of the assay.

The characterization of different casein wine fining agents was done by lithium dodecyl sulfate–polyacrylamide gel electrophoresis (LDS-PAGE) with silver staining and immunoblot. For the quantification of these described possible fining agent residues in wine an indirect ELISA as well as an indirect sandwich ELISA were developed. Therefore, polyclonal antibodies raised against a casein fining agent were used for immunoblotting and ELISA development to achieve a higher specificity by considering all residual allergenic milk proteins remaining from the fining procedure with a casein-derived product.

The LODs and lower limits of quantification (LLOQ) depend on the type of ELISA, the calibrating substance, and the

dilution of the analyzed wine and show the complexity of the wine matrix, especially for red wine.

The developed ELISA methods were used to investigate wines after using certain technological treatments to remove wine fining agents.

MATERIALS AND METHODS

Casein Standard and Fining Agents. Casein of analytical grade from Merck KGa (Darmstadt, Germany) was used as casein standard.

For matrix calibration the fining agent KalCasin was used, which is described as a potassium milk caseinate. It was obtained directly from the manufacturer, Erbslöh Geisenheim AG (Geisenheim, Germany), and had a protein content of approximately 76% by the Kjeldahl method.

Investigated and characterized fining agents were 12 casein products of different commercial European producers (Agrovin, Begerow, Enartis, Erbslöh, Laffort, Spindal, Vason) organized in OENOPPIA (International Association of Oenology Industrials for Oenological Products and Practice, Paris, France).

Antibodies. The capture antibodies for the sandwich ELISA, which were also used as primary antibodies for the indirect ELISA as well as for immunoblotting, were polyclonal antibodies raised against one fining agent, which is described as potassium caseinate. Antibody production was done by Eurogentec (Seraing, Belgium). For this three rabbits were immunized against this fining agent four times in four week intervals. The serum samples were taken 10 days after the second, third, and fourth immunizations and purified using affinity chromatography. Affinity chromatography was performed at CNBr Sepharose, and the antibodies were eluted with 100 mM glycine (pH 2.5). The antibodies were conserved with 0.01% thimerosal in phosphate-buffered saline (PBS), and the purity was >85%, determined by SDS-PAGE. The concentration of the purified antibodies was 1.1 mg/mL.

The primary antibodies for the indirect sandwich ELISA were produced by Immunology Consultants Laboratory (Newberg, OR, USA) against bovine casein in sheep. The concentration of the antibodies was 1.0 mg/L.

The secondary antibodies, used for the indirect ELISA as well as for the immunoblot, were HRP-conjugated goat anti-rabbit antibodies, obtained from Dako (Dako GmbH, Glostrup, Denmark). The concentration of the antibodies was 0.25 mg/L.

The secondary antibodies for the indirect sandwich-ELISA were produced by Immunology Consultants Laboratory against sheep, conjugated with HRP. The concentration of the antibodies was 1.0 mg/L.

Wine. Method development and validation were done with well-known experimental wines, produced by the Wine Research Institute Geisenheim in the context of project AiF 16330N, "Reducing the concentration of allergenic fining agents in the final wine by technological treatment". An unfined white wine of the grape variety Mueller Thurgau (vintage 2009) and an unfined red wine of the grape variety Regent (vintage 2009) were used as matrix for method development of the ELISAs. These wines were produced according to common winegrowers practice and finally bottled through a filter layer into 0.75 L bottles. The musts were inoculated with yeast (*Saccharomyces cerevisiae*) and fermented at 16–18 °C. No fining procedure was done before filling.

Within the project different wine fining procedures were investigated concerning their effectiveness in reducing the content of certain allergenic wine fining agents in the final product wine. For that the described red wine and the described white wine were fined with the maximal permitted dose of wine fining agent as well as with double the maximal dose to demonstrate a worst-case scenario. This is to protect allergic consumers also from an accidental overdosage by the winegrower. These wines were produced according to common winegrower practice and finally bottled through a filter layer into 0.75 L bottles.

Eight processes to remove the fining agent were performed in 120 L flasks to simulate a wine tank. The wine was fined with 40 (A) and 80 g (B) KalCasin/hL wine.

To investigate whether there is a decrease of potential allergenic wine fining agent residues during the production process and how efficiently the different fining procedures work, two different production steps of every produced wine were analyzed by ELISAs. These two investigated production steps were the first after removal of the fining agent with different techniques (I) and the second after sterile filtration into commercially available bottles (II), respectively. The investigated red and white wines are presented in Table 1.

Table 1. Investigated White (WW) and Red (RW) Wines

wine		technique
WW1	RW1	I. 0.45 μm filtration
		II. sterile filtration
WW2	RW2	I. addition of bentonite
		II. sterile filtration

The following two filtration techniques and enological procedures used for wine production are discussed in this publication: (1) 0.45 μm filtration, using a Type 419A, grade B (beverage version) SEITZ-MEMBRACart cartridge with 0.45 μm pore size from Pall Corp. (Port Washington, NY, USA); and (2) addition of bentonite, using Aktivit bentonite from Erbslöh. Immediately after these treatments, sterilizing filtration was performed on all wines, using SEITZ-EK 200 $\times$ 200 mm depth filter sheets from Pall Corp., and the wines were bottled into 0.75 L bottles. For example, the term WW1AII describes the white wine fined with 40 g/hL KalCasin, treated with process 1 to remove the fining agent, at processing step II, after sterile filtration.

Lithium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. For electrophoretic separation, the casein standard and different fining agents were prepared in 10–13 mg/50 mL solutions with 10 mM carbonate solution under shaking on a horizontal shaker for at least 30 min.

All chemicals were of analytical grade. The proteins were separated as described by Steinhoff et al.²⁶ Precast NuPAGE Bis-Tris 12% gels (Invitrogen GmbH, Karlsruhe, Germany) were performed under denaturing conditions at 200 V using NuPAGE 3-(N-morpholino)-propanesulfonic acid (MOPS) SDS running buffer (Invitrogen). For electrophoresis the protein solutions were diluted 1:2 with LDS sample buffer, which contained dithiothreitol (DDT) for reduction. Every lane was loaded with 20 μL of sample solution. The unpicked proteins were further silver stained or blotted on a nitrocellulose membrane.

Silver Staining. Detection of the separated proteins was done by a silver staining procedure according to Heukeshoven et al.²⁷ as described by Steinhoff et al.²⁶ At first, the proteins were fixed in the gel by treatment with 30% ethanol and 10% acetic acid in water for 30 min. Afterward, an incubation with a solution containing 0.1% sodium thiosulfate, 30% ethanol, and 6.8% sodium acetate in water occurred for 30 min. This was followed by a washing step with water. Then the gel was incubated in a silver nitrate solution (0.2% silver nitrate and 0.02% formaldehyde in water) for 20 min. Finally, development occurred with 2.5% sodium carbonate and 0.01% formaldehyde in water. Stop solution was prepared with 1.8% ethylenediaminetetraacetate (EDTA) in water.

Immunoblotting. All chemicals were of analytical grade. Immunoblotting was performed according to the method of Steinhoff et al.²⁶ By LDS-PAGE separated proteins were electrophoretically transferred onto a nitrocellulose membrane (Whatman, Maidstone, UK) via semidry western blotting. The dried membranes first were blocked with blocking solution (0.5% polyethylene-sorbitan monolaurate (Tween20), 0.9% sodium chloride, and 0.6% tris(hydroxymethyl)-aminomethane (Tris)) to inhibit nonspecific binding to the membrane. The membrane was incubated with the primary antibody, anti-potassium caseinate antibody (1/10000), followed by incubation with the secondary HRP conjugates anti-rabbit antibody (1/20000) at room temperature. Finally, staining was performed with staining solution containing 0.06% 3,3',5,5'-tetramethylbenzidine (TMB), 0.2% diocylsodium sulfosuccinate (DONS), 25% ethanol, 0.7% citric acid,

and 1% Na_2HPO_4 in water. The protein weight marker was stained by adding Ponceau red solution.

Quantitative Indirect Sandwich and Indirect ELISA. Fining agent and protein standard solutions were prepared by dissolving about 12.5 mg of the respective substance in 50 mL of 10 mM carbonate solution under shaking on a horizontal shaker for at least 30 min. Depending on the ELISA to be undertaken, the calibration solutions were prepared by dilution with carbonate buffer (indirect ELISA) or PBS–Tween20 buffer (indirect sandwich ELISA).

All chemicals were of analytical grade. Buffer preparation was done according to the method of Weber et al.¹⁵ Coating buffer (carbonate buffer, pH 9.6) contained 75 mM Na_2CO_3 and 175 mM NaHCO_3 in bidistilled water. Blocking solution (Tris–Tween20 buffer, pH 9.6) contained 50 mM Tris, 150 mM NaCl, and 0.5% Tween20 in bidistilled water. Washing solution (PBS–Tween20 buffer, pH 7.4) contained 10 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 70 mM Na_2HPO_4 , 150 mM NaCl, and 0.5% Tween20 in bidistilled water. Citric buffer (pH 4.0) contained 210 mM citric acid monohydrate and 300 mM KOH in bidistilled water. The substrate solution was freshly prepared with 5 mg of TMB, dissolved in 125 μL of acetone filled to 1 mL with methanol, 19 mL of citric buffer, and 200 μL of H_2O_2 (1%). The stop solution was made of 2 M H_2SO_4 in bidistilled water.

An indirect ELISA procedure was modified according to the method of Weber et al.² Two hundred microliters per well sample solution in coating buffer was coated to a polystyrene microtiter plate (MaxiSorp F96, Nunc, Roskilde, Denmark) overnight at 8–9 °C. The plate was washed three times with 300 μL /well blocking solution. Subsequently, free binding sites of the wells were blocked with 250 μL /well blocking solution for 2 h at room temperature by horizontal shaking to inhibit unspecific bindings of the antibodies. Afterward, the plate was washed three times with 300 μL /well washing solution and incubated with 200 μL /well primary antibody in PBS–Tween20 (1:10000) for 1 h by shaking at room temperature. Then the plate was washed three times with 300 μL /well washing solution. Finally, 200 μL /well secondary antibody in washing solution (1:2000) was added and incubated for 1 h by shaking at room temperature. The plate was then washed three times with washing solution and three times with citric buffer. The enzymatic colorimetric reaction was performed by adding 200 μL /well substrate solution (TMB). By the HRP-catalyzed oxidation of the TMB, a blue compound is generated. The reaction was stopped after 5 min by adding 100 μL /well stop solution, which leads to a color change to yellow. The measurement occurs at 450 nm with 630 nm as reference wavelength.

For method validation the measurement occurs in 1/10 diluted white wine and 1/20 diluted red wine.

For the indirect sandwich ELISA method, the assay protocol of Weber et al.² was also used as guideline. The procedure started by coating a polystyrene microtiter plate (MaxiSorp F96, Nunc) with 200 μL /well capture antibody in coating buffer (1/20000) overnight at 8–9 °C. Afterward, the plate was washed three times with 300 μL /well blocking solution. Subsequently, free binding sites of the wells were blocked with 250 μL /well blocking solution for 2 h at room temperature by horizontal shaking to inhibit unspecific bindings. Then the plate was washed three times with 300 μL /well washing solution again and incubated with 200 μL /well sample solution in PBS–Tween20 for 1 h by shaking at room temperature. After a reapplied washing step, 200 μL /well primary antibody in washing solution (1/20000) was added. Then the plate was washed three times with 300 μL /well washing solution again and incubated with 200 μL /well secondary antibody in washing solution (1/20000) for 1 h by shaking at room temperature. Finally, the plate was washed three times with washing solution and three times with citric buffer. The colorimetric reaction was performed by adding 200 μL /well substrate solution as described for the indirect ELISA. The measurement occurs at 450 nm with 630 nm as reference wavelength.

For method validation the measurement occurs in 1/10 diluted white wine and 1/10 diluted red wine.

Washing of the microwell plates was done using an eight-channel automatic microplate washer ELx50 (BioTek, Winooski, VT, USA). Optical density (OD) was read using a MRX microplate reader

(Dynex Technologies, Chantilly, VA, USA) using the software Revelation G 3.2 (Dynex Technologies). Analysis of the measured data was done with SoftMax Pro 5.4 software (Molecular Devices, Sunnyvale, CA, USA).

Evaluation. The evaluation of the ELISAs occurred on the basis of a logistic four-parameter regression using the software SoftMax Pro 5.4 (Molecular Devices). The four-parameter curve is one of the most reliable and most flexible evaluations for immunoassays. The equation of a four-parameter curve is $y = [(A - D)/(1 + (x/c)^B)] + D$ with y = extinction, A = lower asymptote, B = raising in the central point, D = upper asymptote, and x = concentration (Figure 1).

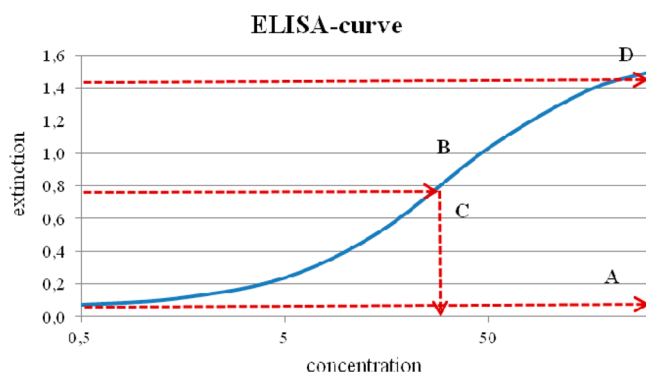


Figure 1. Sample diagram four-parameter regression.

The LOD was determined as the concentration of analyte for which the extinction of the blank value plus its standard deviation is lower than the extinction for the concentration of analyte minus its standard deviation. The LOD as well as the LLOQ are the most important statistical parameters for ELISA methods. A common practice is to use the signal-to-noise rate of the measured blank value for determination.^{28,29} This is estimated as nonreliable because only the blank value is considered.

Additionally, a precision profile according to Ekins³⁰ was developed. This precision profile takes the variance of the nonconformance above the curve progression (heteroscedasticity) into account. In this way the relative nonconformance for every analyte concentration can be defined, and by incorporating a threshold for this relative nonconformance, the measurement range with LLOQ and upper limit of quantification (ULOQ) can be determined.^{30–32} In this case the threshold for the relative nonconformance was defined with 20%.

Accuracy was checked by spiking unfined experimental wine samples with casein or KalCasin within the calibration range, respectively, and determining these recovery rates.

RESULTS AND DISCUSSION

Characterization of Different Casein Wine Fining Agents. A total of 12 commercially available casein wine fining agents of six European manufacturers were investigated by LDS-PAGE and silver staining as well as immunoblotting. These fining agents are denoted fining agents 1–12 in this publication, independent from their production and contained proteins.

The LDS-PAGE with silver staining (Figure 2) shows that every product contains not only casein (19–25 kDa) but also other milk allergens in different amounts, except for fining agent 7 (Figure 2, lane 7), which apparently mainly contains the whey proteins α -lactalbumin (ALA, 14.2 kDa) and β -lactoglobulin (BLG, 18.3 kDa). All other casein fining agents (Figure 2, lanes 1–6, 8–12) show similar protein band compositions, similar to the casein standard (Figure 2, lane Std), but with partially different band intensities. For electrophoresis, the fining agents, as well as the casein standard, were applied in concentrations from 200 to 250 mg/L. In two fining agents no bands were

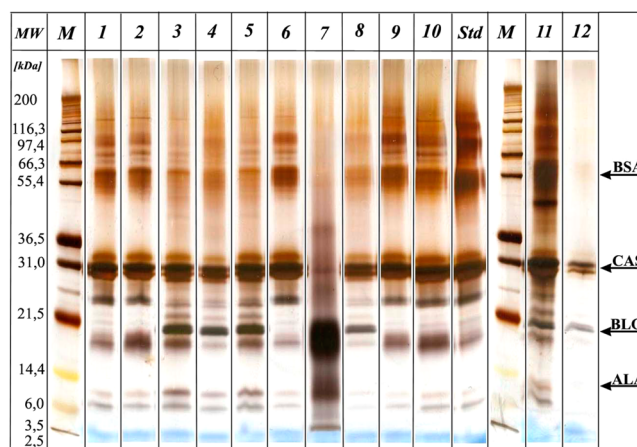


Figure 2. LDS-PAGE and silver-staining of the casein fining agents. MW, molecular weight (kDa); M, molecular weight marker; lanes 1–12, fining agents 1–12; Std, casein standard; BSA, bovine serum albumin (66.4 kDa); CAS, casein (19–25 kDa); BLG, β -lactoglobulin (18.3 kDa); ALA, α -lactalbumin (14.2 kDa).

detectable with this concentration; thus, the sample concentrations were increased to 2000 and 4000 mg/L. Degraded proteins were also detectable in the electropherogram of the casein products.

The described results of the unspecific silver staining are supported by specific immunochemical staining with an antibody raised against one commercially available fining agent. This antibody detects besides casein also the other milk proteins ALA, BLG, and bovine serum albumin (BSA, 66.4 kDa) as shown in Figure 3, whereas the antibody apparently has a low

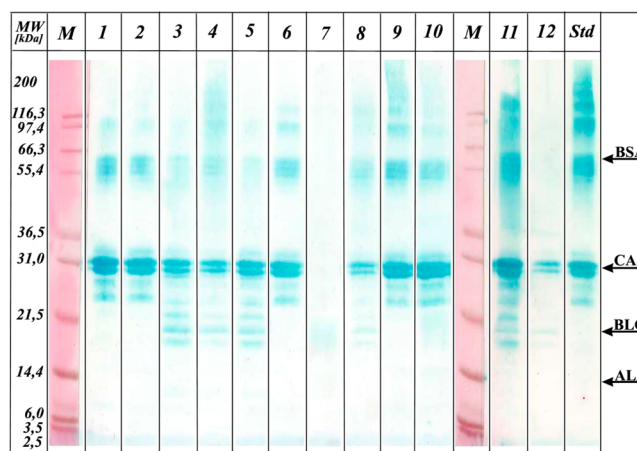


Figure 3. Immunoblot of the casein fining agents. MW, molecular weight (kDa); M, molecular weight marker; lanes 1–12, fining agents 1–12; Std, casein standard; BSA, bovine serum albumin (66.4 kDa); CAS, casein (19–25 kDa); BLG, β -lactoglobulin (18.3 kDa); ALA, α -lactalbumin (14.2 kDa).

affinity to the whey proteins ALA and BLG. Some of the degraded proteins are also detected with different intensity by the used antibody. With immunochemical staining the differences of the fining agents from one another as well as from the casein standard become more obvious. Most of the fining agents show more detectable protein bands than the casein standard (Figure 3, lane Std).

The demonstrated investigations of the different casein fining agents by LDS-PAGE and immunoblot show the necessity to

have analytical methods for determination of fining agent residues in wine, which are able to not only detect casein but also take into account the other milk allergens as well as the degraded proteins that are present in the fining agents and show detectable antigenicity as well.

ELISA Method Validation with a Casein Standard and a Casein Fining Agent. Validation of the developed ELISA methods was done in white wine as well as in red wine, although casein is mostly used for white wine, according to Lifrani et al.¹⁷ Four calibration curves of four stock solutions with seven standard concentrations and a blank value were measured on one microtiter plate respectively (intra-assay validation). Every standard concentration for calibration was measured in triplicate on every plate. For testing the repeatability, every calibration was repeated on three different days. Thus, for every standard solution 36 measured data were acquired. Precision profiles according to Ekins³⁰ with percental normed data were determined for the different ELISAs. Furthermore, wine samples were spiked within the calibration range for measuring the recovery rates. Described validations were done with a casein standard (Merck), as well as a casein fining agent (KalCasin), to investigate the variance of the results depending on the used calibration standard and to get some information about the need for standardized calibration substances. This is particularly important if a threshold is given by law.

Casein has to be declared on a wine label if ≥ 0.25 mg/L allergenic protein remains in the final product, given by the OIV regulations. Furthermore, the OIV defines thresholds for LOD and LLOQ concerning ELISA methods developed for the analysis of wine. Thus, an accepted ELISA method for wine analysis needs to have a LOD of ≤ 0.25 mg/L casein or rather potentially allergenic residues of fining agent proteins, as well as a LLOQ of ≤ 0.5 mg/L casein or rather potentially allergenic residues of fining agent proteins. It is extremely important to have legally defined thresholds for potentially allergenic residues of wine fining agents in the final product to protect allergic consumers as well as to give research establishments, producers, and inspection agencies the possibility to control these potential residues. In this context it is necessary to give clear definitions concerning the potential residues. The threshold given by OIV is referred to "potentially allergenic residues of fining agent proteins in wine". It is problematic when an analytical method, for example, an ELISA, detects only caseins, although the fining agent contains beside caseins other milk proteins or degraded parts of the concerning proteins. This is also a problem for ovalbumin-specific ELISA methods and fining agents derived from hen's egg white.

The aim of this study was to develop methods for risk assessment of allergenic casein wine fining agent residues in wine to protect sensitive consumers. Thus, methods should be developed with as low as possible LOD and LLOQ by using calibration standards already taken in the processing of the wine samples and for production of antibodies.

Indirect ELISA versus Indirect Sandwich ELISA. At first, the indirect ELISA was developed for white wine. In agreement with previous publications^{15,16} measuring casein in red wine by an indirect ELISA was unreliable. In unfined red wines, diluted 1/10 as the white wine, false-positive results were determined using the developed assay. Thus, an indirect sandwich ELISA was developed to investigate casein in red wine. Sandwich ELISA methods are supposed to be more specific because of the used capture antibody, which ideally binds only the target protein. Unfortunately, this assay also did not lead to satisfying

results for red wine with regard to the LLOQ and recovery rates when used in 1/10 dilutions. Finally, the indirect ELISA could be identified as a reliable method for use when 1/20 diluted red wine was analyzed. However, both assays, indirect ELISA and indirect sandwich ELISA, are applicable for white wine. These investigations show that wine is a complex matrix and not easily handled. Some reasons for this are its low pH and the existence of much higher levels of phenolic compounds in red than in white wine.¹⁵

Statistical Data of the ELISA Methods. The precision profiles of the different calibrations including the mean calibration curve of all calibrations, respectively, are shown in Figures 4–11. The presented diagrams of the precision profiles show the standardized calibration curve of the three performed calibrations as a black continuous line. The tolerable failure was ascertained at 20% (gray line), and the curve of the precision profile is shown as a black dotted line. For validation LLOQ and ULOQ were determined, giving the measurement range of the ELISA, respectively, defined as intersections of the

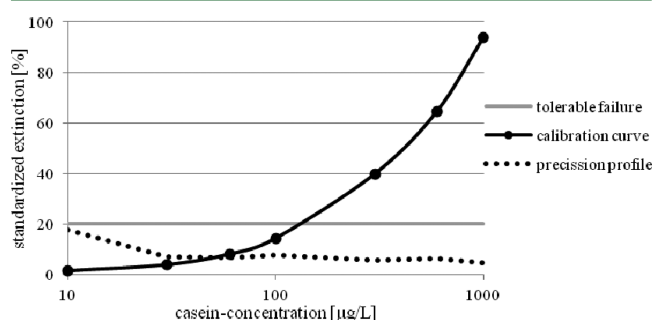


Figure 4. Precision profile of the indirect ELISA matrix calibration with casein in white wine.

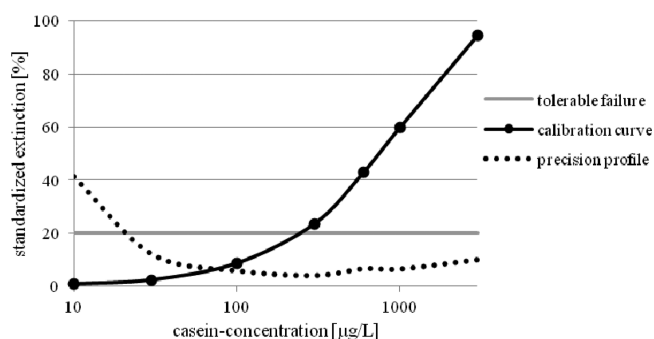


Figure 5. Precision profile of the indirect ELISA matrix calibration with casein in red wine.

Precision profile of the matrix calibration with KalCasin in white wine (indirect ELISA)

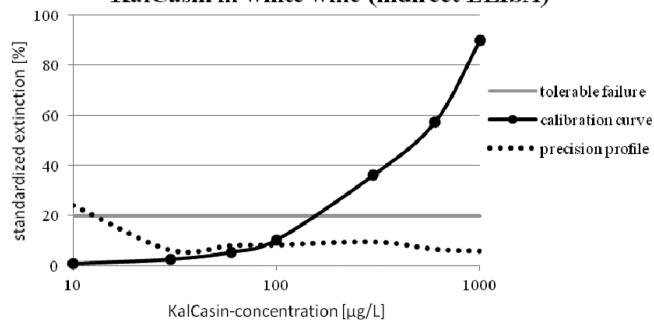


Figure 6. Precision profile of the indirect ELISA matrix calibration with KalCasin in white wine.

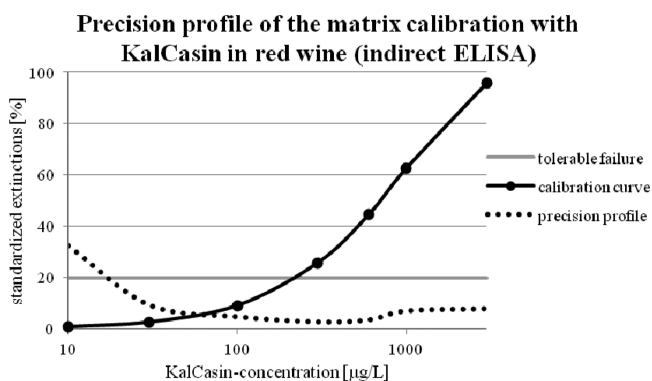


Figure 7. Precision profile of the indirect ELISA matrix calibration with KalCasin in red wine.

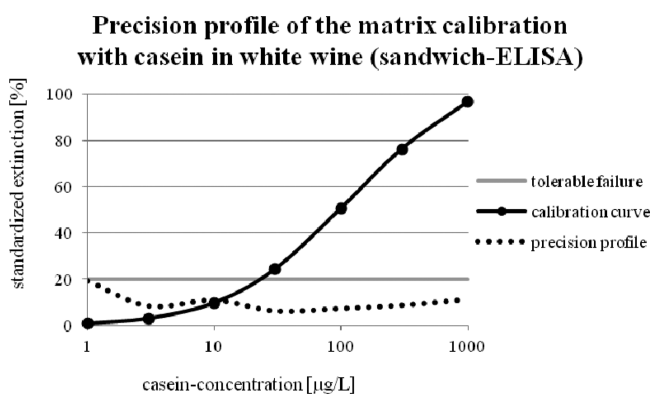


Figure 8. Precision profile of the sandwich ELISA matrix calibration with casein in white wine.

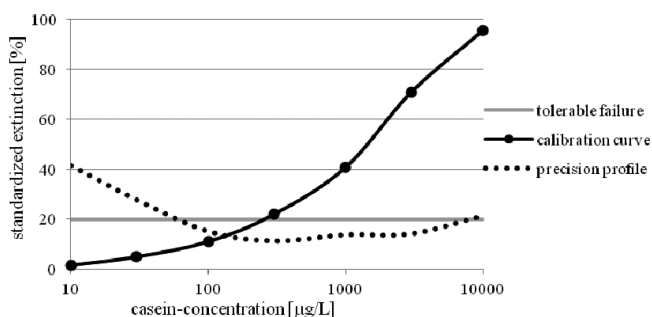


Figure 9. Precision profile of the sandwich ELISA matrix calibration with casein in red wine.

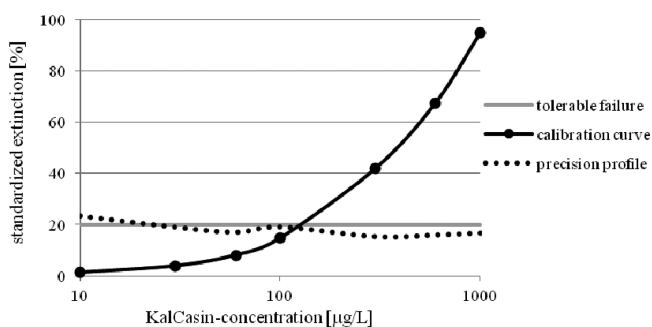


Figure 10. Precision profile of the sandwich ELISA matrix calibration with KalCasin in white wine.

precision profile curve with the tolerable failure line. All statistical data of the indirect and sandwich ELISA are mentioned in Tables 2–5.

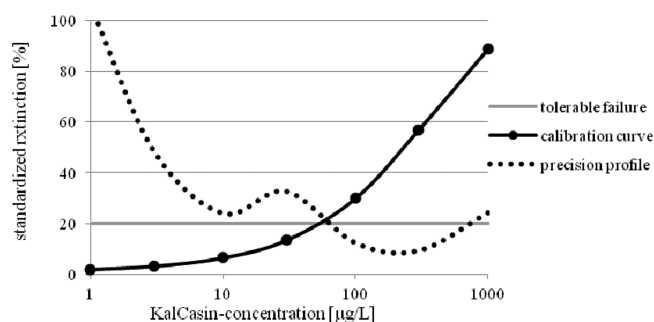


Figure 11. Precision profile of the sandwich ELISA matrix calibration with KalCasin in red wine.

In all developed assays, for white as well as red wine, the LOD of 0.25 mg/L (250 µg/L) accordingly requested by the OIV was reached for the detection of casein and the fining agent. The formerly described heteroskedasticity or the variance of the nonconformance is illustrated in the intra-assay coefficients of variation presented in Tables 2 – 5. The high data were determined for the standard solutions with the lower concentrations. For higher analyte concentration the intra-assay coefficients of variation are usually in the range of 10%. This discrepancy is caused through the sigmoidal curve progression. In the range of very low casein concentrations the curve reaches a plateau (see Figures 4–11). In this section small deviations in measured OD lead to comparatively huge deviations of the determined concentrations.

Accuracy was investigated by spiking the wine with casein and KalCasin at different amounts within the linear area of the calibration range and determining the recovery rates. Calibration curves were prepared with the same substance, which was used for wine spiking, respectively. For indirect ELISA the recovery rates ranged from 59 to 114% (casein) and from 64 to 84% (KalCasin) in white wine. Recovery rates determined with the indirect sandwich ELISA in white wine ranged between 66 and 78% for casein, whereas KalCasin showed no acceptable accuracy with recovery rates lower than 30%.

In red wine the recovery rates were achieved between 63 and 92% (casein) as well as 78 and 91% (KalCasin) for the indirect ELISA, determined in 1/20 diluted wine. In contrast, in 1/10 diluted red wine, for the indirect sandwich ELISA no satisfying recovery rates, <20% (casein) and <10% (KalCasin), could be achieved.

The concentration of casein in spiked wine with an OD significantly different from the blank value is defined as the LOD. For this, an unspiked and equally diluted wine was used for blank value. All presented assays were able to detect casein and KalCasin, respectively, according to the threshold of 250 µg/L allergenic protein, given by the OIV. For all developed ELISA methods the LODs are between 10 and 200 µg/L, usually just below the range of the first standard solution (Tables 2–5). The measurement range defined by the LLOQ and ULOQ can be determined from the precision profile (Figures 4–11). ELISA methods used for analysis of allergens in wine have to be able to quantify the allergen with a LLOQ of ≤500 µg/L. Looking at the statistical data, it can be seen that the indirect sandwich ELISA was not able to quantify casein as well as KalCasin in red wine under mentioned conditions with LLOQs around 730 µg/L. In contrast, the indirect ELISA is applicable for 1/20 diluted red wine with LLOQs of 491.20 µg/L (casein) and 414.56 µg/L (KalCasin). With both assays a quantification of casein and KalCasin in white wine is possible.

Table 2. Data of the Indirect ELISA Method Validation with 1/10 Diluted White Wine

	fining agent (KalCasin)	casein
standard curve		
calibration range ($\mu\text{g/L}$)	10–1000	10–1000
intraassay coefficients of variation (%)	3–80.7	3.2–68.7
recovery rate for spiked wine samples		
recovery rate (%)	64–84	59–114
statistical data		
LOD ^a in wine ($\mu\text{g/L}$) ($\text{blank} + \text{SD}_{\text{blank}} < x - \text{SD}_x$) ^b	100	100
measurement range in wine ($\mu\text{g/L}$) (LLOQ–ULOQ) ^c	146.26–10000	100–10000

^aLOD, limit of detection. ^bSD, standard deviation; x , value of solution x ; SD_x , standard deviation of solution x . ^cLLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

Table 3. Data of the Indirect ELISA Method Validation with 1/20 Diluted Red Wine

	fining agent (KalCasin)	casein
standard curve		
calibration range ($\mu\text{g/L}$)	10–3000	10–3000
intraassay coefficients of variation (%)	1.6–52.5	2.5–68.7
recovery rate for spiked wine samples		
recovery rate (%)	78–91	63–92
statistical data		
LOD ^a in wine ($\mu\text{g/L}$) ($\text{blank} + \text{SD}_{\text{blank}} < x - \text{SD}_x$) ^b	200	200
measurement range in wine ($\mu\text{g/L}$) (LLOQ–ULOQ) ^c	414.56–60000	491.24–60000

^aLOD, limit of detection. ^bSD, standard deviation; x , value of solution x ; SD_x , standard deviation of solution x . ^cLLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

Table 4. Data of the Indirect Sandwich ELISA Method Validation with 1/10 Diluted White Wine

	fining agent (KalCasin)	casein
standard curve		
calibration range ($\mu\text{g/L}$)	10–1000	1–10000
intraassay coefficients of variation (%)	5.9–46.4	1.2–47.6
recovery rate for spiked wine samples		
recovery rate (%)	<30	66–78
statistical data		
LOD ^a in wine ($\mu\text{g/L}$) ($\text{blank} + \text{SD}_{\text{blank}} < x - \text{SD}_x$) ^b	100	10
measurement range in wine ($\mu\text{g/L}$) (LLOQ–ULOQ) ^c	254.63–10000	10–10000

^aLOD, limit of detection. ^bSD, standard deviation; x , value of solution x ; SD_x , standard deviation of solution x . ^cLLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

Table 5. Data of the Indirect Sandwich ELISA Method Validation with 1/10 Diluted Red Wine

	fining agent (KalCasin)	casein
standard curve		
calibration range ($\mu\text{g/L}$)	10–10000	10–10000
intraassay coefficients of variation (%)	4.7–35.9	2.5–48.3
recovery rate for spiked wine samples		
recovery rate (%)	<10	<20
statistical data		
LOD ^a in wine ($\mu\text{g/L}$) ($\text{blank} + \text{SD}_{\text{blank}} < x - \text{SD}_x$) ^b	100	100
measurement range in wine ($\mu\text{g/L}$) (LLOQ–ULOQ) ^c	735.08–100000	732.58–100000

^aLOD, limit of detection. ^bSD, standard deviation; x , value of solution x ; SD_x , standard deviation of solution x . ^cLLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

With the LLOQ of 100 $\mu\text{g/L}$ (indirect) and 10 $\mu\text{g/L}$ (sandwich) quantification of casein are more sensitive than for KalCasin with LLOQs of 146.26 $\mu\text{g/L}$ (indirect) and 254.63 $\mu\text{g/L}$ (sandwich). Qualitative analysis of casein in white wine is more sensitive using the indirect sandwich ELISA with a LOD of 10 $\mu\text{g/L}$, whereas for KalCasin in the indirect sandwich ELISA and casein as well as KalCasin using the indirect ELISA a LOD of 100 $\mu\text{g/L}$ is achieved. Qualitative red wine analysis is more sensitive using

the indirect sandwich ELISA with a LOD of 100 $\mu\text{g/L}$ for casein as well as KalCasin in contrast to the indirect ELISA with a LOD of 200 $\mu\text{g/L}$ for casein as well as for the fining agent. However, as previously mentioned, the recovery rates for red wine analysis are <20% using the sandwich method.

Analysis of Fined Wines. The results of the investigated wines treated with two different processing steps are given in Table 6. The white wines, diluted 1/10, were investigated using

Table 6. Results of the Fined Red (RW) and White (WW) Wines Using the Developed ELISA Methods, Indirect ELISA for WW, and Indirect Sandwich ELISA for RW, with Casein and KalCasin as Calibration Substance (A:40g/hL KalCasin, B: 80g/hL KalCasin)

wine	processing step	allergen content ^a ($\mu\text{g/L}$)	below the value of 0.25 mg/L given by the OIV
allergen content determined as casein			
RW1AI	0.45 μm filtration	$x < \text{LOD}$	yes
RW1AII	sterilizing filtration	$x < \text{LOD}$	yes
RW1BI	0.45 μm filtration	$x < \text{LOD}$	yes
RW1BII	sterilizing filtration	$x < \text{LOD}$	yes
RW2AI	addition of bentonite	$x < \text{LOD}$	yes
RW2AII	sterilizing filtration	$x < \text{LOD}$	yes
RW2BI	addition of bentonite	$x < \text{LOD}$	yes
RW2BII	sterilizing filtration	$x < \text{LOD}$	yes
WW1AI	0.45 μm filtration	$x < \text{LOD}$	yes
WW1AII	sterilizing filtration	$x < \text{LOD}$	yes
WW1BI	0.45 μm -filtration	$x < \text{LOD}$	yes
WW1BII	sterilizing filtration	$x < \text{LOD}$	yes
WW2AI	addition of bentonite	$x < \text{LOD}$	yes
WW2AII	sterilizing filtration	$x < \text{LOD}$	yes
WW2BI	addition of bentonite	$x < \text{LOD}$	yes
WW2BII	sterilizing filtration	$x < \text{LOD}$	yes
allergen content determined as KalCasin			
RW1AI	0.45 μm filtration	$x < \text{LOD}$	yes
RW1AII	sterilizing filtration	$x < \text{LOD}$	yes
RW1BI	0.45 μm filtration	$x < \text{LOD}$	yes
RW1BII	sterilizing filtration	$x < \text{LOD}$	yes
RW2AI	addition of bentonite	$x < \text{LOD}$	yes
RW2AII	sterilizing filtration	$x < \text{LOD}$	yes
RW2BI	addition of bentonite	$x < \text{LOD}$	yes
RW2BII	sterilizing filtration	$x < \text{LOD}$	yes
WW1AI	0.45 μm filtration	$x < \text{LOD}$	yes
WW1AII	sterilizing filtration	$x < \text{LOD}$	yes
WW1BI	0.45 μm filtration	$x < \text{LOD}$	yes
WW1BII	sterilizing filtration	$x < \text{LOD}$	yes
WW2AI	addition of bentonite	$x < \text{LOD}$	yes
WW2AII	sterilizing filtration	$x < \text{LOD}$	yes
WW2BI	addition of bentonite	$\text{LOD} < x < \text{LLOQ}$	yes
WW2BII	sterilizing filtration	$x < \text{LOD}$	yes

^aLOD, limit of detection; LLOQ, lower limit of quantification.

the developed indirect ELISA. The red wines, diluted 1/10, were investigated using the developed indirect sandwich ELISA. The LOD of 100 $\mu\text{g/L}$ of these ELISA methods is lower than the required LOD of 250 $\mu\text{g/L}$ given by OIV.

The determined data show no detectable allergen content after the second processing step (sterilizing filtration) of the red wine as well as in the white wine using the developed ELISA methods. Thus, the value of 0.25 mg/L, given by the OIV, is complied with and labeling of the final wine is not necessary. An allergen content also is not detectable in the first processing step of the red wines. For the first processing step of the white

wine, only WW2BI, with 80 g/hL KalCasin fined white wine after the addition of bentonite, shows a detectable amount of allergen between LOD and LLOQ (100 and 146.26 $\mu\text{g/L}$) using KalCasin for calibration.

This indicates the higher sensitivity using the fining agent in contrast to casein for calibration, because using casein for calibration leads to no detectable residues of the fining agent.

Concluding Remarks. Some formerly published studies fulfill the requirements for ELISA methods detecting potentially allergenic residues of fining agent proteins in wine. Weber et al.² developed an indirect ELISA for the detection of total casein and casein-derived fining agents with LOD of 5 $\mu\text{g/L}$ casein, whereas a method for the quantification of caseinates in wine developed by Restani et al.¹⁷ with a LOD of 280 $\mu\text{g/L}$ and a LOQ of 760 $\mu\text{g/L}$ no longer conforms with the criteria established by the OIV, which were given in 2012. In addition to these immunological-based methods many MS-based methods for the detection of residual allergenic wine fining agents were recently published. Matarozzi et al.²³ investigated red wines treated with α -casein, β -casein, and ovalbumin by liquid chromatography–electrospray–tandem mass spectrometry. In this case for caseinate a LOD of 200 $\mu\text{g/L}$ was calculated. In 2013 Monaci et al.²² published a MS-based multiallergen quantification of fining-related egg and milk proteins in white wines. With this method LODs between 400 and 900 $\mu\text{g/L}$ are achieved for the detection of casein fining agent. A method published by Cereda et al.³³ is described to have a LOD of 1 $\mu\text{g/L}$ casein in white wine, and 3.8 $\mu\text{g/L}$ fining agent in red wine is described by D'Amato et al.²³ Summing up, ELISA as well as MS-based methods are very sensitive and able to detect residual allergenic wine fining agents. For this purpose it is beneficial that ELISA as an immunological-based method considers the antigenicity of the target proteins.

The method validation illustrates again that wine, especially red wine, is a complex matrix. This supports former publications and their results concerning allergen detection in wine.^{15,21} Here the validation of a method for a risk assessment consideration in a very low area of concentration ($\mu\text{g/L}$ and not mg/L) demonstrates very impressively these interferences by a complex matrix. Furthermore, it can be seen that results differ according to the standard used for calibration. Having homogeneous standard substances is important for the comparison of results acquired with different assays and in different laboratories. As shown by LDS-PAGE and immunoblot different casein fining agents also contain other milk allergens in different amounts, which might also be a problem for allergic consumers and have to be measured. As food allergy is very individual, people who are allergic to “milk” can be allergic to different milk proteins with variable intensity of allergic reactions.^{25,34} Thus, a method that takes into account all milk proteins is necessary to have a reliable method and to implement the legal request for thresholds as discussed previously. Furthermore, possible different processings of the fining agents might result in different affinities of the contained proteins to the used antibody, and consequently this also might yield altered allergenic potency for allergic people. With regard to the described results an underestimation of residual allergenic fining agents in the analyzed final wine is possible if common ELISA methods commercially available are used for the detection of for example casein.

In conclusion, ELISA methods for the sensitive quantification of casein fining agents in white and red wines were developed, also taking into account other possible milk allergens in the

casein fining agents by antibodies developed against a commercial casein fining agent. These methods enable a risk assessment for fining agent residues in casein-fined wines to protect allergic consumers. Within the research project “Reducing the concentration of allergenic fining agents in final wine by technological treatment” (AiF 16330N), the development of technological procedures that reduce the concentration of allergenic substances used in wine treatment was aimed for. For the evaluation of these reductions and the risk assessment of well-defined wines and well-known fining agents used in wine processing these developed highly sensitive ELISA methods were needed.

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ABBREVIATIONS USED

ALA, alpha-lactalbumin; BLG, beta-lactoglobulin; BSA, bovine serum albumin; CAS, casein; CMA, cow's milk allergy; DBPCFC, double-blind, placebo-controlled food challenge; DONS, dioctylsodium sulfosuccinate; EDTA, ethylenediaminetetraacetate; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LDS-PAGE, lithium dodecyl sulfate–polyacrylamide gel electrophoresis; LLOQ, lower limit of quantification; LOD, limit of detection; M, molecular weight marker; MW, molecular weight; OD, optical density; OENOPPIA, International Association of Oenology Industrials for Oenological Products and Practice; OIV, Organisation Internationale de la Vigne et du Vin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SPT, skin prick test; TMB, 3,3',5,5'-tetramethylbenzidine; Tris, tris(hydroxymethyl)-aminomethane; Tween20, polyethylene-sorbitan monolaurate; ULOQ, upper limit of quantification

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