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Investigation of the Allergenic Potential of Wines Fined with Various Proteinogenic Fining Agents by ELISA

PATRICK WEBER, HANS STEINHART, AND ANGELIKA PASCHKE*

Department of Chemistry, Food Chemisry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

Hidden allergens are a common problem in food safety that has been known for many years. This is why the European Parliament adopted Directive 2003/89/EC amending 2000/13/EC. In addition to specific ingredients, Directive 2003/89/EC also requests the declaration of specific products that were used in the production and could be a risk for allergic individuals. This also includes the declaration of fining agents and lysozyme used in wines. In fact, it could be assumed that fining agents would be almost completely removed during the manufacturing process; however, until now there has been no necessity to analyze wine for these fining agents. By applying enzyme-linked immunosorbent assay (ELISA), residuals of fining agent proteins and the stabilizer lysozyme were investigated in various German wines. The results showed no detectable amounts of fining agents in wines, except for dried egg white and lysozyme, both derived from hen's egg white. For those products, adverse reactions against treated wines could not be excluded.

KEYWORDS: Wine; fining agent; allergen; allergy; isinglass; lysozyme; food allergy; hidden allergens; immunoassay; fish gelatin; hen's egg protein; ovalbumin; casein

INTRODUCTION

Hidden allergens are a common problem in food safety that has been known for many years (*1*). The main issue is that the average consumer does not expect these allergens in the food and, thus, they present a potential high risk for allergic individuals. The main reasons for hidden allergens in foods are contaminations from previously or simultaneously produced products ("cross-contact" or "carry-over"), the use of allergenic materials as processing aids, or simply the lack of or misleading labeling declaration of food products (*2*, *3*). Because newer studies suggest that the thresholds for allergens such as allergens from egg or milk range between 1 and 2 mg to trigger allergic reactions in sensitive individuals (*4*), the need for investigations became essential. For that reason, the European Parliament adopted Directive 2003/89/EC amending 2000/13/EC. In addition to specific foods, Directive 2003/89/EC also requests the declaration of specific substances that were used in the production and could present a risk for allergic individuals. Not only ingredients or contaminations but also processing aids are affected. Annex IIIa specifies a list of substances that are known to trigger allergic reactions for which no labeling exemptions are allowed. This list also includes products derived from egg, milk, or fish. Because no scientific data exist, a temporary exemption of labeling has been granted by the European Community to provide research data when it is investigated whether these processing aids can cause adverse reactions in allergic individuals (*5*).

Directive 2003/89/EC affects wine manufacturers not only within the European Community but also in several other countries, for example, Australia, New Zealand, or the United States, where similar regulations have been introduced or are already taken into consideration. Because egg, milk, and fish products, such as isinglass, are used as fining agents to clarify wines or as stabilizers according to **Table 1**, they need to be listed on the wine label. It is easy to understand that a phrase such as "contains fish products" on the label will contribute to the uncertainty of customers, especially those allergic to egg, milk, or fish, and the impairment of the product's image.

Fish products such as fish gelatin or collagen are used in wine production for the reduction of off-flavor ingredients such as polyphenolic compounds by adsorptive effects. Additionally, fish gelatin or collagen forms positive charged colloidal particles at the pH of wine, which aggregate and sediment with negative charged particles, such as yeast or most wine proteins. Gelatin is traditionally used to clarify wines and to reduce tannin levels. Since BSE became an important concern for gelatin products, many food producers have switched to fish gelatin. Gelatin is prepared by acidic denaturation of fish skin collagen. Thus, the amino acid composition is similar to that of collagen but the structure is different. However, the mode of action is similar to that of collagen but gelatin is considerably less selective and effective.

Special milk proteins, particularly caseins, are characterized by sequences of hydrophobic and hydrophilic amino acids,

* Author to whom correspondence should be addressed (telephone 0049 40 42838 4353; fax 0049 40 42838 4342; e-mail angelika.paschke@uni-hamburg.de).

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Table 1. Current Estimates of the Wine Volumes Fined with Ingredients Listed in Annex III of Directive 2000/13/CE, Produced Annually in France and Germany[a]

	volume (in millions of hL)			
wine	fined with casein	fined with dried egg white	fined with isinglass	medium national annual production
German	<1.96	0.2	0.7	9.8
French	20.6	5.3	8.2	50

[a] Sources: Deutscher Weinbauverband DWV and Union Française Des Laboratoires et Industries Œnologiques UFLIO.

resulting in an ambiphilic character with micelle-forming properties. At the pH of wine, caseins are insoluble, which leads to coagulation and sedimentation. Coevally, they bind and eliminate phenolic compounds that affect the color and taste of wines.

Egg proteins, such as ovalbumin or conalbumin, are the major proteins of egg white and are responsible for the fining effect of egg white. Like fish collagen, polyphenolic compounds are removed by adsorption and, thus, dried egg white is used for color and taste improvement. Additionally, they build positive charged particles at the pH of wine, which lead to aggregation and sedimentation with negative charged particles and proteins.

The enzyme lysozyme is another egg white protein. It has an antimicrobial activity against particular Gram-positive bacteria and, thus, it is used as a stabilizer for better control of the fermentation process and against spoilage. Additionally, it is used to increase the effect and to lower the dosage of sulfites.

So far, no cases of allergic reactions after wine consumption have been reported due to the content of fining agent residues. This could be due to the absence of allergenic amounts of fining agents in wines or because allergic reactions usually are not associated with the consumption of wine as the average consumer does not expect allergens such as egg, milk, or fish proteins in wines (*5*). Indeed, cases of allergy against wines have been reported particularly in the Mediterranean area, but it has also been proven that those adverse reactions were triggered by wine proteins or intolerances to compounds such as sulfites or histamine (*6*−*9*). Therefore, it could be assumed that fining agents are almost completely removed during the manufacturing process, for example, by filtration and adsorption to processing aids, such as bentonite, or by precipitation with tannins in wine. This has been confirmed for isinglass in beer clarification (*10*), but, until now, there has been no evidence for wine. Furthermore, some countries use fining agents after filtration or adsorption steps. Thus, the analysis of fining agent residues in fined wines is important to evaluate the possible risk of fined wines for allergic individuals and to evaluate the need of labeling according to Directive 2003/89/EC. First efforts have been published by Rolland et al. on Australian wines with double-blind, placebo-controlled food challenges (DBPCFC) and basophile activation analysis (*11*). There was no anaphylaxis or symptom or sign of an adverse reaction that could be attributable to the consumption of wine made using the food allergens fish or egg. However, this study considered a panel of 5 egg-allergic, 1 milk-allergic, and 10 fish-allergic patients, indicating problems of the statistical reliability especially for egg- and milk-allergic persons. Furthermore, regional differences in wine treatment may affect the transferability of those results and make the investigation of European wines necessary.

The determination of hidden allergens is an ambitious intention. In addition to the high specificity of those methods, sufficient sensitivity is essential to detect trace amounts in foods that could trigger adverse reactions. The enzyme-linked immunosorbent assay (ELISA) is a well-known and the most promising tool for this type of analysis (*12*). Although ELISAs are commercially available to detect major egg and milk allergens, no suitable ELISA kit was found to determine lysozyme or fish proteins, especially gelatin or collagen.

The detection limits of hidden allergens are described in some studies. On the basis of the comprehensive DBPCFC of Morisset et al. (*13*) with 125 egg-allergic and 59 milk-allergic humans, detection limits should be 2 ppm for egg proteins and 12 ppm for milk proteins to evaluate the risk for sensitive individuals based on the lowest observed adverse-effect level (LOAEL). However, other publications describe the sensitivity to be $\leq$10 ppm (*14*).

Although fish is a widely known and well-reported cause of food allergy (*15*), oral challenge studies especially for fish gelatin or collagen—which is the main protein of isinglass—are rare, and threshold doses have not been established yet. Minimal doses for codfish have been identified by Hansen and Bindslev-Jensen at 6 mg, indicating that the minimal eliciting dose for fish appears to be in the milligram range for the most sensitive patients (*16*) and, therefore, making a sensitivity of $\leq$10 ppm inevitable. But, generally, no adverse reactions especially to gelatin have been reported (*17*). Indeed, antibodies against fish gelatin were detected in fish-allergic patients (*18*−*20*), but an oral DBPCFC with 30 codfish-allergic patients showed no allergic reactions to doses up to 3.6 g of fish gelatin (*21*). However, because of the rare information about allergic reactions to fish gelatin or collagen, and to evaluate the possible risk for fish allergic patients, it is necessary to determine the fining agent residues in wine. This is particularly important when allergic reactions after the consumption of products containing gelatin or collagen such as wine, beer, juices, cider, or pharmaceutical products such as vaccines are observed but not diagnosed as such because these ingredients were not labeled. Consequently, those allergic reactions are less reported (*5*).

The aim of this study was to determine residues of proteinaceous fining agents and the wine stabilizer lysozyme in wines using an ELISA. Therefore, reliable methods were developed, validated, and used for the analysis of various German wines.

MATERIALS AND METHODS

Reagents, Buffers, and Instrumentation. Goat anti-rabbit biotin conjugate as secondary antibody and avidin−horseradish peroxidase (HRP) conjugate were purchased from Dako GmbH (Hamburg, Germany).

All chemicals used were of analytical grade. Phosphate-buffered saline (PBS) consisted of 10 mM $NaH_2PO_4 \times H_2O$, 70 mM Na_2HPO_4, and 150 mM NaCl in bidistilled water. Washing solution contained 50 mM tris(hydroxymethyl)aminomethane (TRIS), 150 mM NaCl, and 0.5% polyethylene-sorbitan monolaurate (Tween 20) in bidistilled water (*22*). Coating buffer, citric buffer, and substrate solution were prepared according to the method of Holzhauser et al. (*23*). Coating buffer, pH 9.6, contained 15 mM Na_2CO_3 and 35 mM $NaHCO_3$ in bidistilled water. Citric buffer, pH 4, consisted of 210 mM citric acid monohydrate and 300 mM KOH in bidistilled water. Substrate solution was freshly prepared with 5 mg of 3,3′,5,5′-tetramethylbenzidine (TMB), dissolved in 125 μL of acetone and made up to 1 mL with methanol, 19 mL of citric buffer, and 6.6 μL of H_2O_2 (30%). The stop solution was made of 2 M H_2SO_4 in bidistilled water.

Pipetting and washing were done using Eppendorf Research and Eppendorf Multipette Plus pipettes. Fining agent protein contents and ELISAs were read with an MRX microplate reader (Dynex Technologies, Chantilly, VA) using Revelation G 3.2 software (Dynex Technologies).

Table 2. Fining Agents and Lysozyme and Their Dosages in the Assayed Wines

fining agent	manufacturer's recommended dosage/hL	dosage/hL of wine
isinglass 1	25–100 mL	50 mL
		250 mL
isinglass 2	25–100 mL	50 mL
		250 mL
isinglass 3	25–100 mL	50 mL
		250 mL
potassium caseinate 1	2–20 g	6 g
		30 g
potassium caseinate 2	2–20 g	6 g
		30 g
lysozyme	25–30 g	25 g
		50 g[a]
fish gelatin	3–10 g	10 g
		50 g
dried egg white	4–16 g	4 g
		20 g

[a] Treatment according to the regulated threshold of 50 g/100 L within the European Community.

Fining Agents and Lysozyme. Seven commercially available and well-known fining agents and the wine stabilizer lysozyme were provided directly from the manufacturers BEGEROW GmbH (Langenlonsheim, Germany) and ERBSLÖH AG (Velbert, Germany) and were investigated in this study.

Isinglass. Three different isinglass samples were investigated with differences in geographical origin and manufacturer. The products were supplied as clear gel with 2% isinglass. Isinglass is a form of processed collagen derived from the swim bladder of caviar fish, mainly Hausen (Beluga whale), with a collagen content of 70−90% native collagen.

Potassium Caseinate. Two potassium caseinate samples from two different manufacturers were investigated. The products were supplied as yellow powder with a protein content of approximately 85%. Potassium caseinate consists of caseins, which form the major milk protein fraction.

Lysozyme. One commercial lysozyme sample for wine treatment was investigated in this study. The sample was supplied as white powder. The legitimate threshold within the European Community is 50 g/hL. Lysozyme was derived from hen's egg white.

Dried Egg White. One dried egg white sample was investigated in this study. The sample was provided as white powder with a protein content of approximately 78%. The dried egg white was derived from hen's egg white by pasteurization and spray-drying.

Fish Gelatin. One fish gelatin sample was investigated in this study. The sample was provided as yellow powder with a bloom number between 80 and 100. The gelatin was prepared by acidic denaturation of fish skin collagen.

Wine Samples. Four different and well-characterized German wines were prepared in cooperation with the Dienstleistungszentrum Laendlicher Raum (DLR) Mosel, Germany: Riesling Mosel, Riesling Rheingau, Pinot Blanc Pfalz, and Pinot Gris Baden. Briefly, the untreated wines were purchased from selected winemakers in 425 L quantities each. Those wines were prepared according to the usual commercial protocol for the proper cultivar, except fining and bentonite treatment. The wines were sulfurated, cross-flow filtered, and temporarily stored in 25 L carboys at 15 °C in the dark. No fining or related treatments were performed until this step. Afterward, the cross-flow filtered wines were treated with two different dosages of fining agents and the stabilizer lysozyme for a period ranging between 13 and 19 days: The usual dosage was within the manufacturer's recommended dosages, and the excess dosage was up to 5 times higher than recommended, according to **Table 2**. Afterward, the wines were separated from the fining precipitation and treated with bentonite for 3 days. Finally, the wines were cross-flow filtered and bottled through a membrane filter (0.45 μm pore size). The wines were bottled in green glass bottles with a volume of 0.7 L, sealed with screw caps, and stored at 15 °C in the dark. For each wine, unfined but bentonite-treated control wines were provided.

Apart from the fining agent dosage, the achieved wines were comparable to commercially available wines.

Primary Antibodies. Primary antibodies were produced by EUROGENTEC (Seraing, Belgium) against isinglass 1, dried egg white, potassium caseinate 1, and lysozyme. Briefly, three rabbits were immunized for each fining agent. Immunization was performed four times in 4 week intervals. Serum samples were taken 10 days after the second, third, and fourth immunizations and purified by affinity chromatography. Affinity chromatography was performed at CNBr Sepharose. Antibodies were eluted using 100 mM glycine (pH 2.5) and, finally, were conserved with 0.01% thimerosal in PBS. The purity was >85% for each antibody, discovered by SDS-PAGE. Concentrations of the purified antibodies by the ELISA assay are shown in **Table 3**.

Table 3. Antibody Concentrations in Rabbit Sera Used for Competitive ELISA after Affinity Purification

antibody against	concentration (mg/mL)
isinglass 1	1.9
dried egg white	2.2
potassium caseinate 1	1.1
lysozyme	2.4

Antibodies against isinglass 1 were also used for isinglasses 2 and 3 and fish gelatin. Antibodies against potassium caseinate 1 were also used for potassium caseinate 2.

Preparation of Fining Agent Protein Standards. Fining agents and lysozyme were dissolved with PBS by shaking on a horizontal shaker (Swip KL-2 shaker, Edmund Buehler, Tuebingen, Germany) for at least 1 h at 300 rpm. Isinglass and gelatin were dissolved in PBS by heating at 50 °C overnight and stirring at 500 rpm with a magnetic stirrer (MR 2002, Heidolph, Kelheim, Germany).

The protein content was determined after centrifugation, followed by a filtration, according to the Bradford method (*24*) in a polystyrene microtiter plate (F96, Nunc, Wiesbaden, Germany). The optical density (OD) was read with a microtiter plate reader.

Sample Preparation. Wine samples were diluted 1:10 in PBS to diminish the effect of tannins and acids and were directly adopted for the ELISA.

Spiking of Samples and Recovery Studies. Recovery experiments were performed by spiking unfined control wines with fining agent proteins or lysozyme at three different concentrations within the standard curve (**Table 4**). Afterward, the spiked samples were treated as described for sample preparation. All experiments were conducted in triplicate.

ELISA Procedure. For the competitive ELISA, 150 μL/well of 20 μg/mL fining agent proteins in coating buffer was coated to a polystyrene microtiter plate (Maxisorb F96, certified, Nunc) for 16 h at 4 °C. The plate was washed three times with 250 μL of washing solution. Afterward, free binding sites of the wells were blocked with 250 μL/well washing solution for 2 h at room temperature to prevent unspecific bindings of the antibodies. Finally, the plate was washed two times with 300 μL of washing solution and subsequently used for the ELISA procedure.

The competitive ELISA procedure was performed by adding of 75 μL/well sample solution and 50 μL/well primary antibody solution (dilutions were carried out according to **Table 5** in washing solution) to the coated wells in succession. To consider matrix effects and cross-reactivities, samples of control wines (B_0) were run and considered for all measured values (B). All experiments were performed in triplicate. After 1 h of incubation at room temperature, the plate was washed three times with 200 μL/well washing solution. Thereafter, 125 μL/well of anti-rabbit biotin conjugate solution (diluted 1:10000 in washing buffer) was added and incubated for 1 h at room temperature. After three washing steps with 200 μL/well washing solution, 125 μL/well avidin−HRP conjugate (1:4000 in washing buffer) was added and allowed to react for 1 h in darkness at room temperature. The wells

Table 4. Standard Curves, Recovery Rates, Coefficients of Variation, and Limits of Detection for Fining Agent Proteins and Lysozyme ELISAs

	standard curve		recovery rate		
fining agent	standard curve range (ppm)	coefficient of variation (%)	recovery rate for spiked wine samples (%)	coefficient of variation (%)	LOD (ppm)
isinglass 1	1–10	3.9–9.8	100–137	28–49	0.05
isinglass 2	1–10	0.5–3.5	111–121	4.4–10.8	0.28
isinglass 3	0.5–5	1.5–8.0	77–106	8.6–16.8	0.22
potassium caseinate 1	1–10	2.2–6.0	85–113	2.8–33.0	0.33
potassium caseinate 2	1–10	2.8–3.4	80–83	5.5–8.8	0.10
lysozyme	0.1–1	0.8–2.0	63–106	30.9–33.1	0.001
fish gelatin	0.05–0.5	1.1–5.4	107–124	4.2–11.3	0.005
dried egg white	0.1–1	1.1–5.4	114–130	20.9–46.8	0.02

Table 5. Dilutions of the Primary Rabbit Antibodies for Competitive ELISAs

fining agent	polyclonal antibody used	dilution in washing buffer
isinglass 1	isinglass 1	1:100000
isinglass 2	isinglass 1	1:10000
isinglass 3	isinglass 1	1:4000
potassium caseinate 1	potassium caseinate 1	1:27000
potassium caseinate 2	potassium caseinate 1	1:27000
lysozyme	lysozyme	1:800000
fish gelatin	isinglass 1	1:2000
dried egg white	dried egg white	1:3200000

were washed four times with 200 μL/well washing solution and finally filled with 125 μL/well substrate solution. The enzymatic colorimetric reaction was performed for 15 min at room temperature in darkness and stopped by adding 75 μL of stop solution. The OD values were measured at 450 nm against a reference wavelength of 630 nm using a microtiter plate reader. The plate was covered with a plate lid (Nunc) during every incubation.

Standard curves were derived from serial dilutions of fining agent proteins in PBS. The dilutions were performed in triplicate. The attained curves were evaluated by AssayZap Software (Biosoft, Cambridge, U.K.) using four-parametric regression. Therefore, the reduced ODs were plotted against the logarithm of the fining agent protein concentration. The reduced OD describes the ratio of the measured sample values (B) to the measured values of the unfined control wine (B_0) and was calculated as follows: $B/B_0 \times 100\%$ = reduced OD (%). Furthermore, the methods were validated with regard to accuracy, precision, and sensitivity.

RESULTS

ELISA Standard Curve and Method Validation. The reduced ODs for serial dilutions of the fining agent proteins and lysozyme are shown in **Figure 1**. The proper standard curves are summarized in **Table 4**. For each standard curve, the intraassay coefficients of variations were below 10% and ranged between 0.5 and 9.8%. Interassay coefficients of variations were not acquired because method validation and sample determination were performed on the same plate. Recovery rates were measured within the standard curve and were achieved between 63 and 137%. The limit of detection (LOD) was calculated as the protein concentration derived from the reduced OD of the unfined control wine (B_0) reduced by 3-fold the standard deviation of the mean B_0 value (*23*). The limit of quantitative determination was defined as the lowest concentration of spiked wine that yielded a recovery rate between 60 and 140% and a variation coefficient lower than 50%. The limit of quantitative determination was equal to the lowest concentration of the standard curve. The achieved values ranged between 0.001 and 0.33 ppm for the limit of detection and between 0.05 and 1 ppm for the limit of quantitative determination, as summarized in **Table 4**. Especially for lysozyme and gelatin, very low limits of detection were achieved.

Wine Assays. Results from the wine assays with treated wines were negative, except wines treated with lysozyme and dried egg white (according to **Table 6**). Positive results were obtained for lysozyme in all wines with a significant lysozyme content equal to the limit of detection (level of significance = 95%). After consideration of the sample dilution, this corresponded to an estimated amount of 0.01 ppm. However, because these amounts were significantly equal to the limit of detection, a reliable quantification is not possible. Only for Pinot Gris Baden, fined with the excess dosage, was the content significantly higher than the limit of detection but lower than the limit of quantitative determination. However, the content was estimated at 0.06 ± 0.02 ppm ($N = 3$).

For dried egg white, positive results were observed for Riesling Rheingau at the excess dosage. The content was significantly equal to the limit of detection, corresponding to a potential amount of 0.2 ppm after consideration of the sample dilution.

DISCUSSION

Method Development. Eight adequate methods for the quantification of various residual fining agent proteins and lysozyme in wines have been developed. However, the coefficients of variation and recovery rates showed that the wine matrix is not easy to handle. Especially the low pH and the content of tannins make the determination of proteins in wine difficult. Tannins are well-known to interact with proteins and are present in white wines at concentrations of <0.2 and $1-2.5$ g/L in red wines (*25*), but simple dilution of wines resulted in adequate results with coefficients of variation lower than 35% for six of eight fining agents and lysozyme. All coefficients of variation were lower than 50%, and the recovery rates ranged between 63 and 137%, according to **Table 4**. However, the determination of Dornfelder Rheinhessen red wine failed to produce acceptable results with this method. Probably, this was due to the distinctly higher content of polyphenols in red wines (*25*). Thus, results are not shown in this paper, and further sample preparation such as dialysis is strongly recommended to remove polyphenolic compounds from red wines for this kind of ELISA method.

Concerning the sensitivity, sensitive methods have been developed for all fining agents and lysozyme that fit the current requirements for analysis of hidden allergens. All LODs were <0.4 ppm and thus suitable also at dilutions of 1:10 as performed for wines. LODs for fish gelatin and lysozyme assays were ≤ 0.005 ppm, indicating the possibility of a highly sensitive detection of those proteins by competitive ELISA. Interestingly, the slope of the lysozyme curve was weak compared to those of the fining agents.

Results. The results of the wine investigations showed no detectable amounts of soluble fining agent proteins in wines in

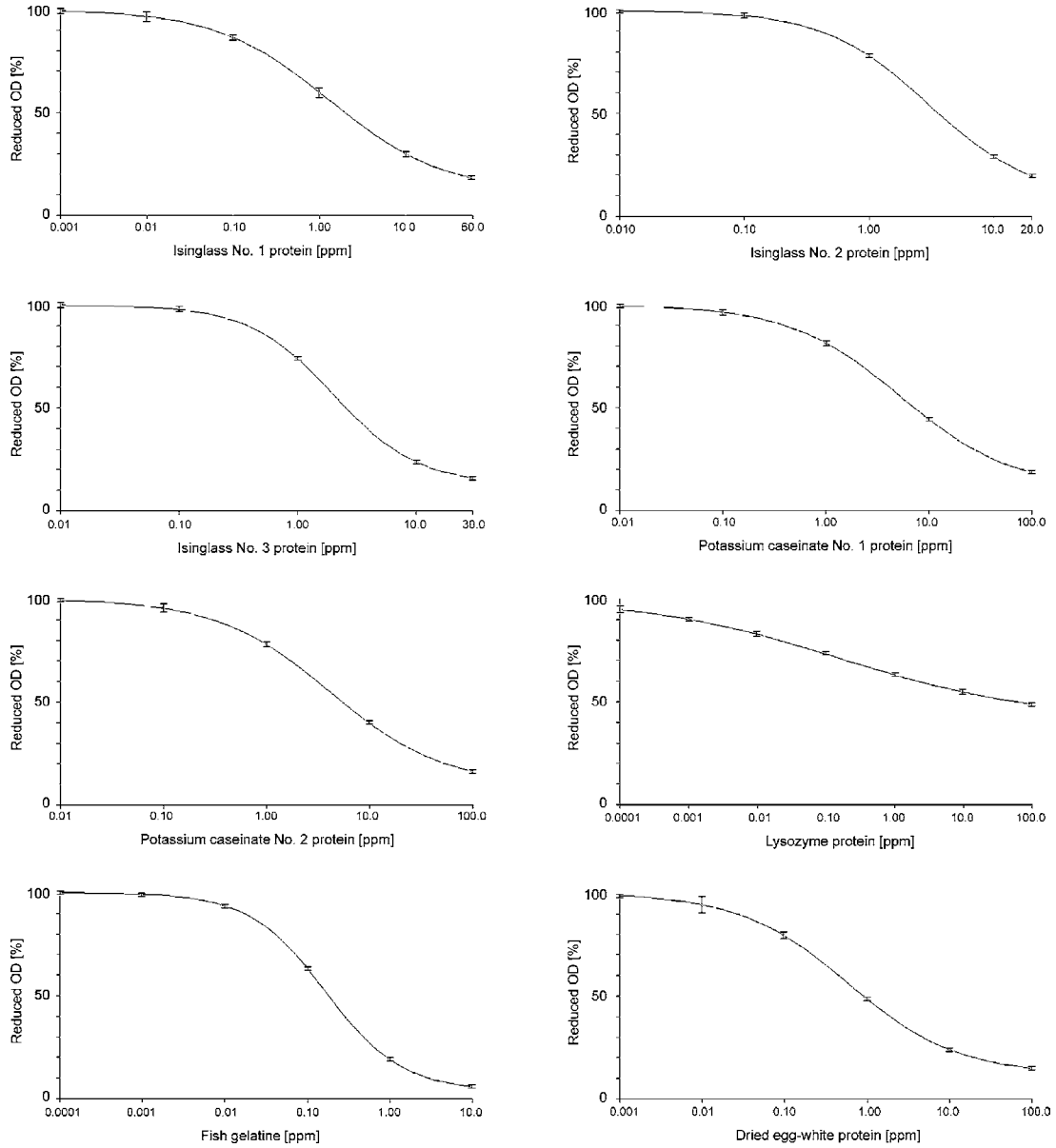

Figure 1. Reduced ODs for serial dilutions of fining agent proteins and lysozyme. Error bars indicate the standard deviation of the protein standard.

which the manufacturer's recommended dose was used. Residual soluble fining agent proteins were detected in only one of the four wines fined with an excessive 5 times higher dosage of dried egg white as described below, so it seems that fining agents used for wine treatment are almost removed during the manufacturing process or are almost insoluble and removed by filtration. Tannins and the treatment with bentonite could play an important role because they were both known to interact with proteins. Whereas tannins are known to form protein cross-links and lead to protein precipitation, insoluble bentonite adsorbs positively charged proteins (*25*). Consequently, affected proteins can be removed by filtration. Especially for fining agents derived from isinglass or gelatin, dissolubility was low. Dissolving of isinglass 1 in PBS at a dosage nearly 100-fold higher than the highest recommended dosage for wine treatment only led to a detectable protein content of maximal 120 ppm. For isinglasses 2 and 3 and fish gelatin, the detectable protein content in PBS was between 36 and 85 ppm for dosages about 330-fold higher than recommended for wine treatment. Neither treatment with 1 N sodium hydroxide or 0.5 N sulfuric acid solution nor heating at 100 °C for 1−2 h led to an appreciable increase of soluble protein content. Thus, it is very likely that these small amounts were removed during the wine processing, for example, adsorption onto bentonite or on filtration materials such as diatomite (*25*), and by interactions with tannins. Caseins are insoluble at the pH of wine, explaining why results were also negative for this kind of fining agent. Only dried egg white and lysozyme demonstrate good solubility in wine. Therefore, they were exclusively found in the assayed wines.

Positive results were obtained for lysozyme in all dosages and wines and for dried egg white in Riesling Rheingau, fined with the excess dosage of 20 g/hL. For lysozyme, which is not used as a fining agent but as a stabilizer against spoilage, small amounts in the range of approximately 0.01−0.06 ppm were detected. Dried egg white was estimated at a level of 0.2 ppm in Riesling Rheingau. Because this amount was equal to the limit of detection, it could be assumed that the other excess fined wines also contained dried egg white residues in slightly lower amounts that were not detected. Both materials are produced from hen's egg white. However, none of these findings corresponded to the content of any other wine compounds such as sugars, acids, pH, or alcohol content.

Because thresholds for egg white products are very low to trigger allergic reactions in sensitive individuals and lysozyme

Table 6. Results of the Wine Assays with Treated Wines

fining agent	dosage	Riesling Mosel (ppm)	Riesling Rheingau (ppm)	Pinot Blanc (ppm)	Pinot Gris (ppm)
isinglass 1	50 mL	nd[a]	nd	nd	nd
	250 mL	nd	nd	nd	nd
isinglass 2	50 mL	nd	nd	nd	nd
	250 mL	nd	nd	nd	nd
isinglass 3	50 mL	nd	nd	nd	nd
	250 mL	nd	nd	nd	nd
potassium caseinate 1	6 g	nd	nd	nd	nd
	30 g	nd	nd	nd	nd
potassium caseinate 2	6 g	nd	nd	nd	nd
	30 g	nd	nd	nd	nd
lysozyme	25 g	~0.01[b]	~0.01[b]	~0.01[b]	~0.01[b]
	50 g	~0.01[b]	nd	~0.01	~0.06[c]
fish gelatin	10 g	nd	nd	nd	nd
	50 g	nd	nd	nd	nd
dried egg white	4 g	nd	nd	nd	nd
	20 g	nd	~0.2[b]	nd	nd

[a] Not detectable (lower than the LOD). [b] Estimated content because the measured values were significantly equal to the LOD (dilution considered; level of significance = 95%). [c] Estimated content because the measured values were below the standard curve range (according to **Table 5**; dilution considered).

Table 7. Brief Composition of Crude Egg White (*27*)

protein content	10.6%
lysozyme	0.37%
dry mass	12.1%

is a well-known egg white allergen (*26*), allergic reactions to wines treated with lysozyme or dried egg white cannot be excluded. Known thresholds for allergic reactions to egg white range between 1 and 2 mg (*4*). Morisset et al. (*13*) reported the lowest oral dosages of approximately 2 mg of crude egg white in their comprehensive DBPCFC study with 125 patients sensitive to egg. In consideration of the composition of crude egg white (**Table 7**), this corresponds to 0.007 mg of lysozyme and 0.24 mg of dried egg white. Consequently, the consumption of approximately 0.1−0.7 L of lysozyme-treated wine or approximately 1.2 L of dried egg white treated Riesling Rheingau wine would probably be necessary to trigger allergic reactions in those sensitive humans. Only 5.6% of sensitive individuals reacted against a dosage inferior or equal to 15 mg of crude egg white, which corresponds to a consumption of 1−5.5 L of lysozyme-treated or 9 L of dried egg white treated wine. According to the International Center of Alcohol Policies (ICAP; Stockholm, Sweden) a moderate consumption range would be between 20 and 70 g of alcohol/day for men and between 10 and 50 g of alcohol/day for women within the European Community, corresponding to a possible daily wine intake of 200−700 mL for men and 100−500 mL for women. Consequently, the required consumption of dried egg white treated Riesling Rheingau is very high, and it must be considered that this wine was fined with an excess dosage 5 times higher than recommended. Thus, adverse reactions to Riesling Rheingau manufactured according to good manufacturing practice are unlikely because those excess dosages would affect color and flavor as well as be costly. In contrast, the volume of lysozyme-treated wine that could possibly trigger allergic reactions seems to be in the range of a moderate wine consumption. Indeed, it must also be considered that the lysozyme treatment was done 2-fold higher than recommended by the manufacturer but the ordinary treatment of 25 g/hL caused detectable residues of lysozyme in the assayed wines too. Furthermore, considering the regulated threshold of 50 g/hL (equal to the excess dosage) within the European Community and scientific recommendations up to 50 g/hL (*28*, *29*), there could be a problem, particularly for some white wines treated equally to the excess dosage. However, there were no oral challenge studies known that give thresholds, for example, LOAEL, especially for lysozyme, so there is no distinct evidence that lysozyme would trigger allergic reactions in such small concentrations as detected in wines, because egg white contains other allergens in larger amounts, such as ovalbumin or ovomucoid, that could be the reason for allergic reactions at low dosages of egg white in those patient studies.

In conclusion, the results show the need of further investigations and improvement of residual analysis not only for wines but for other products, such as beer, cider, or juices, which are fined with similar fining agents or at different dosages. Across the world, dosages used for food treatment are certainly different and could result in inconsistent hazards for allergic patients in different world regions. In this study, a risk has been proven for lysozyme. Therefore, method improvement and validation for routine analysis could be helpful for monitoring purposes in food safety, both for manufacturers and for consumers.

ABBREVIATIONS USED

B, signal of analyte; B_0, signal at zero dosage of analyte; DBPCFC, double-blind, placebo-controlled food challenge; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidise; LOAEL, lowest observed adverse-effect level; LOD, limit of detection; OD, optical density; PBS, phosphate-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; TRIS, tris(hydroxymethyl)aminomethane; Tween 20, polyethylene-sorbitan monolaurate.

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