

Evaluation of the Efficiency of Enological Procedures on Lysozyme Depletion in Wine by an Indirect ELISA Method

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ABSTRACT: Potential residues of the potent allergen lysozyme used as a microbial stabilizing agent in wine production might pose a serious health threat to susceptible individuals. Therefore, EU legislation requires the labeling of the allergenic agent, if it is present in the final product. To allow for product testing, an indirect ELISA method to be specifically used in wine analysis was developed and validated. Furthermore, trial wines treated with defined amounts of lysozyme were subjected to an array of different filtration and other enological processing regimes in order to evaluate their potential to deplete the allergen content of the wines. By these means, processing methods ought to be identified that can be integrated in a good manufacturing practice guideline to enable wine producers to utilize lysozyme in their cellars and still provide wines free of allergenic residues. However, among the enological procedures under scrutiny, only bentonite fining proved to be capable of significantly reducing the allergenic residues.

KEYWORDS: lysozyme, allergenic residues, ELISA, depletion, wine

■ INTRODUCTION

Microbial control of wine fermentation greatly affects wine quality. To suppress disadvantageous microorganisms, wines and musts may be subjected to treatment with sulfur dioxide. However, since high concentrations of sulfur dioxide can lead to adverse reactions in sensitive consumers, winemakers strive to reduce the sulfur dioxide levels in their wines.¹ By the use of lysozyme, a 14.4 kDa protein commercially sourced from hen's egg white, the amount of sulfur dioxide required for preventing wine spoilage can be significantly reduced.² Lysozyme (E.C. 3.2.1.17) possesses antimicrobial activity toward Gram-positive bacteria, including lactic acid bacteria that could cause undesired malolactic fermentation. The cross-linked oligosaccharides forming the peptidoglycan cell wall of Gram-positive bacteria are hydrolyzed by the enzyme, leading to cell lysis.³ Showing no inhibitory activity toward Gram-negative bacteria or eukaryotic cells like yeasts,⁴ lysozyme has become widely employed as an agent for microbial stabilization of musts and wines and may be used in the EU up to a maximum permissible limit of 0.5 g/L.^{5,6}

However, hen's egg, especially egg white, is one of the foods to which allergies are most frequently reported.⁷ Along with three other proteins, namely, ovomucoid (Gal d 1), ovalbumin (Gal d 2), and conalbumin/ovotransferrin (Gal d 3), lysozyme (Gal d 4) has been identified as one of the major allergens of hen's egg white.^{8,9} It is believed that at least some 15–35% of individuals allergic to hen's egg white are sensitized to lysozyme,^{10,11} and recent findings even suggest reactivity toward lysozyme in up to 58% of sensitized individuals.¹² While it is sometimes alleged that patients allergic to egg white

proteins are predominantly children and tolerance is generally developed during adolescence, persistent egg white protein allergy in adults—and thus in potential consumers of lysozyme-treated wine—does occur. It is estimated that egg allergy affects 0.2% of the adult US population (compared to 1.3% in children)¹³ and that the patients most likely to develop a persistent egg allergy are also the ones with especially high egg specific IgE titers.¹⁴ Since it was found that egg white-specific IgE titers can be predictive of the severity of adverse reactions in a food challenge,¹⁵ it may be concluded that adult egg allergic subjects are particularly vulnerable if exposed to even minute amounts of egg white protein.

In order to protect sensitized consumers from potential health threats, EU Directive 2003/89/EC in combination with Commission Implementing Regulation (EU) No. 579/2012 requires the labeling of allergenic ingredients used in the production of foodstuffs, including enological agents used in wine processing, if they are present in the final product.^{16,17} However, wines treated with lysozyme during their fermentation may be subjected to several enological procedures prior to being bottled, and these procedures may significantly affect the amount of detectable lysozyme in the final product.²²

Lack of knowledge on the presence of allergenic residues may prompt wine producers to precautionarily label the presence of residues to circumvent product liability issues.

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Overcautious and unsubstantiated precautionary labeling however unnecessarily limits the freedom of choice for allergic consumers in a given product range and can thus negatively affect their quality of life.¹⁸

It is therefore important to have reliable and sensitive methods at hand to accurately determine the allergenic residues' concentration and the resulting health hazard. Recent analytical approaches toward detecting lysozyme include chromatographic methods with either fluorescence or mass spectrometric detection,^{19,20} DNA aptamer-based methods,²¹ and competitive ELISA methods for use in wine²² and cheese matrices.²³ Since all of these methods may be prone to severe matrix interactions,²⁴ a noncompetitive, indirect ELISA method to be specifically used in wine analysis was developed and validated.

Furthermore, in order to assess the contamination risk of the final product and to evaluate the possibilities for effective allergen management by technical measures, the lysozyme depletion efficiencies of an array of different filtration regimes and other enological procedures were compared. To gain knowledge about the fate of lysozyme throughout the wine making process, trial wines were treated with lysozyme, further processed according to the respective enological procedures, and successively analyzed for allergen content.

MATERIALS AND METHODS

Reagents and Materials. Antibodies were produced by immunizing rabbits with enological grade lysozyme by Eurogentec (Seraing, Belgium) as described by Weber et al.²⁵

Goat anti-rabbit antibody-horseradish peroxidase (HRP) conjugate was purchased from Dako (Glostrup, Denmark). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Serva (Heidelberg, Germany). Analytical grade lysozyme, ovomucoid, and ovalbumin were purchased from Sigma (St. Louis, MO, USA), and enological grade lysozyme used for treatment of the wines was purchased from Begerow (Langenlonsheim, Germany). Further enological grade lysozyme qualities from several European producers were supplied by Oenoppia (Paris, France). Gradient grade acetonitrile for HPLC-F analysis was purchased from VWR (Radnor, PA, USA). Citric buffer, pH 4.0 (210 mM citric acid monohydrate, 300 mM potassium hydroxide), carbonate buffer, pH 9.6 (75 mM sodium carbonate, 175 mM sodium bicarbonate), Tris/Tween20 buffer, pH 9.6 (50 mM tris(hydroxymethyl)aminomethane, 150 mM sodium chloride, 0.5% Tween 20), and phosphate buffered saline (PBS)/Tween20, pH 7.4 (10 mM sodium phosphate monobasic monohydrate, 70 mM sodium phosphate dibasic, 150 mM sodium chloride, 0.5% Tween 20), for ELISA were prepared according to Weber et al.²⁵ All other chemicals were of analytical grade if not stated otherwise.

Wines. Wine fining and processing was performed at pilot scale size at the Geisenheim cellars using a Rheingau region Müller-Thurgau as white wine and a Nahe region Regent as red wine. Musts were inoculated with cultured yeast strains and kept in stainless steel tanks after vinification. The physicochemical characteristics of the wines are given in Table 1.

Treatment with enological grade lysozyme was carried out at the maximum permissible concentration of 0.5 g/L and, to mimic the case of a potential accidental overdose, at a double concentration of 1.0 g/L. Twenty-four hours after initial lysozyme treatment, the following filtration regimes and enological procedures were employed to evaluate their respective lysozyme depletion potentials:

- sheet filtration using SEITZ-K 200 200 × 200 mm filter sheets from Pall Corporation (Port Washington, NY, USA)
- membrane filtration using a Type 419A, grade B (beverage version) SEITZ-MEMBRACart cartridge with 0.45 μm pore size from Pall Corporation (Port Washington, NY, USA)

Table 1. Analytical Characterization of the Trial Wines

	white wine (Müller-Thurgau)	red wine (Regent)
alcoholic strength by volume	90.7 g/L (11.5% vol)	97.2 g/L (12.3% vol)
total alcoholic strength	90.7 g/L (11.5% vol)	97.8 g/L (12.4% vol)
total dry extract	23.2 g/L	22.1 g/L
reduced extract	23.2 g/L	20.7 g/L
residual extract	8.8 g/L	8.5 g/L
reducing sugars	1.0 g/L	2.4 g/L
reducing sugars after inversion	1.0 g/L	2.4 g/L
pH value	3.5	3.6
total acidity	3.5 g/L	5.2 g/L
tartaric acid	1.7 g/L	2 g/L
lactic acid	1.4 g/L	1.8 g/L
malic acid	0.3 g/L	0.2 g/L
volatile acid	0.4 g/L	0.6 g/L
glycerol	6.6 g/L	8.5 g/L

- cross-flow filtration using a Sartoflow Compact system from Sartorius (Göttingen, Germany) at a flow of 15–20 m³/h at a pressure of 2.5 bar
- precoat/alluviation filtration using fine diatomaceous earth (kieselguhr) from Pall Corporation (Port Washington, NY, USA) as filter aid
- centrifugation using a SB14 disk separator from GEA Westfalia (Oelde, Germany) at 7500 rounds per minute, separating 1500 L/h at a pressure of 3.5 bar
- flash-pasteurization at 72 °C for 20 s
- fining with the inorganic fining agent silica, using Klar-Sol 30 silica colloid from Erbslöh (Geisenheim, Germany) at a concentration of 0.5 mL/L
- fining with the inorganic fining agent bentonite using Aktiv bentonite from Erbslöh (Geisenheim, Germany) at a concentration of 2 g/L.

Immediately after secondary treatments, sterilizing filtration was performed on all wines, using SEITZ-EK 200 × 200 mm depth filter sheets from Pall Corporation (Port Washington, NY, USA), and wines were bottled into 0.7 L screw cap bottles. Samples were drawn after every production step and kept at 8 °C until analysis.

Electrophoresis and Immunoblotting. Electrophoresis and successive immunoblotting were carried out following the protocol laid down by Steinhoff et al.²⁶ Proteins were separated with precast NuPAGE Bis-Tris 12% gels and MOPS SDS running buffer from Invitrogen (Karlsruhe, Germany) after reduction with dithiothreitol (DTT) and lithium dodecyl sulfate (LDS). For unspecific detection of the separated proteins, silver staining was carried out as described by Heukeshoven and Dernick.²⁷

For immunoblotting, proteins were transferred to a 2 mm nitrocellulose membrane from Whatman (Maidstone, U.K.) by semidry-Western blot and incubated overnight at room temperature with rabbit anti-lysozyme antibodies, diluted 1:20000 in Tris/Tween20 buffer, pH 9.6. Successively, the membranes were incubated with goat anti-rabbit antibody-HRP conjugate, diluted 1:20000 in Tris/Tween20 buffer, pH 9.6, and staining solution, as described by Steinhoff et al.²⁶

ELISA. For ELISA analysis, analytical grade lysozyme stock solutions of 5000 mg/L were prepared in citric buffer, pH 4.0, and diluted with carbonate buffer, pH 9.6, to the respective calibration standard concentrations. Wine samples were diluted with carbonate buffer, pH 9.6, at least 1:10 or higher, if required to match the calibration range. For matrix calibration and determination of repeatability and recovery rates, untreated red and white wines were diluted 1:10 and spiked with ascending lysozyme concentrations to reach the corresponding calibration standard concentrations. Measurements were performed on two separate assays on 3 consecutive days (*n* = 6). For the determination of dilution linearity, wines were spiked

with 300 mg/L and diluted 1:30, 1:300, and 1:3000. For ELISA analysis of wines, 3 measurements from 2 discrete bottles of each sample were performed on 3 consecutive days ($n = 6$) and values confirmed by HPLC-F analysis. The measured lysozyme concentrations were calculated as % of the theoretical lysozyme concentrations. All measurements were carried out in triplicate by pipetting $3 \times 200 \mu\text{L}$ per sample in Nunc MaxiSorb 96-well microplates (Thermo Fisher Scientific, Waltham, MA, USA) and leaving the plates for 16 h for incubation at 4°C . All samples and calibration standards were prepared immediately before incubation, and stock solutions were prepared freshly at least every 5 days and kept at 4°C .

After incubation, wells were washed three times with Tris/Tween20 buffer, pH 9.6, and blocked for 2 h with 300 μL of Tris/Tween20 buffer, pH 9.6, at room temperature under gentle agitation. After blocking, wells were washed three times with PBS/Tween20, pH 7.4, and 200 μL of rabbit anti-lysozyme antibodies diluted 1:10000 in PBS/Tween20, pH 7.4, was added to each well. After incubation for 1 h at room temperature under gentle agitation, wells were washed three times with PBS/Tween20, pH 7.4, and 200 μL of goat anti-rabbit antibody-HRP conjugate, diluted 1:2000 in PBS/Tween20, pH 7.4, was added to each well. Wells were left for incubation for 1 h at room temperature under gentle agitation again and washed three times with PBS/Tween20, pH 7.4, before rebuffing by washing three times with 300 μL of citric buffer, pH 4.0. All washing procedures were carried out using a Biotek Elx50 plate washer with 8-tube manifold (Biotek, Winooski, VT, USA).

For the detection step, HRP substrate solutions were prepared immediately before incubation as described by Holzhauser and Vieths,²⁸ resulting in a final organic solvent content of no more than 5%.

Incubation was started by adding 200 μL of substrate solution to each well, and color development was stopped by addition of 100 μL of stopping solution consisting of 2 M sulfuric acid after 2 min. Incubation times were intentionally kept short in order to minimize TMB autoxidation, especially with regard to the blanks and low concentration standards. Increased signal intensity deviations caused by autoxidation could otherwise augment detrimental effects on the assays sensitivity.

Photometric measurements were carried out on a Dynex MRX plate reader (Dynex, Chantilly, VA, USA) at 450 nm with a reference wavelength of 630 nm. Calculation of regression curves was accomplished using the nonweighted 4 parameter logistic regression logarithm of the SoftMax Pro v5.4 software (Molecular Devices, Sunnyvale, CA, USA).

HPLC-F. HPLC analysis was performed on a Agilent 1100 series HPLC equipped with a G1321A fluorescence detector (Agilent, Santa Clara, CA) using a Phenomenex Jupiter C4 column ($250 \times 4.6 \text{ mm}$), 5 μm particle size with 300 Å pore size (Phenomenex, Torrance, CA, USA), held at 36°C . Mobile phase was a gradient of solvent A (0.1% formic acid in bidistilled water) and solvent B (acetonitrile). The gradient program started with 18% solvent B for the first 1.5 min, then a ramp toward 40% solvent B at minute 3 and back to 18% at minute 4.5, with a gradient flow of 2.5 mL/min. Total run time was 7 min, and lysozyme retention time was at 0.8 min. Fluorescence detection was performed at 280 nm excitation wavelength and 340 nm emission wavelength. Wine samples were diluted 1:1 with a mix of solvent A and solvent B (82/18, v/v) prior to analysis.

RESULTS AND DISCUSSION

Antibody Characterization and Lysozyme Preparation Characterization. By choosing enological grade lysozyme actually intended for enological application as sensitizing agent instead of highly purified analytical grade lysozyme, it was facilitated to raise antibodies that show specificity not only against intact lysozyme but also against partially denatured, modified, or fragmented lysozyme molecules potentially formed during production and handling of the enological product,

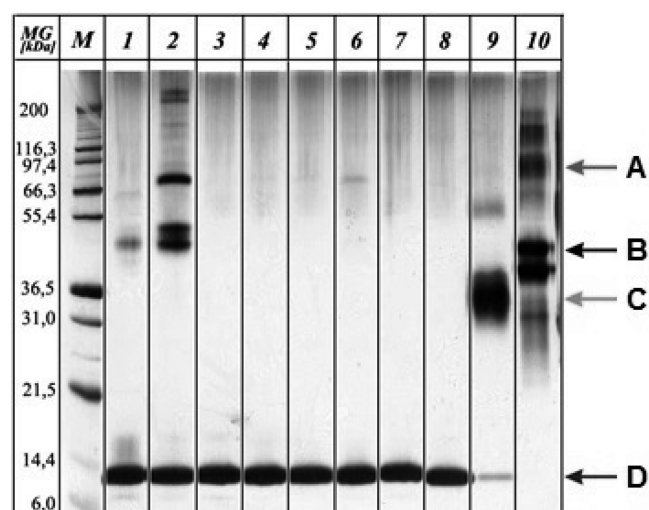


Figure 1. Antibody characterization, LDS-PAGE (M, molecular weight marker; 1–7, enological grade lysozyme preparations from different suppliers, with 7, enological grade lysozyme used for wine treatments; 8, analytical grade lysozyme; 9, analytical grade ovomucoid; 10, analytical grade ovalbumin; A, conalbumin (77–78 kDa); B, ovalbumin (42.7 kDa); C, ovomucoid (28 kDa); D, lysozyme (14.4 kDa)).

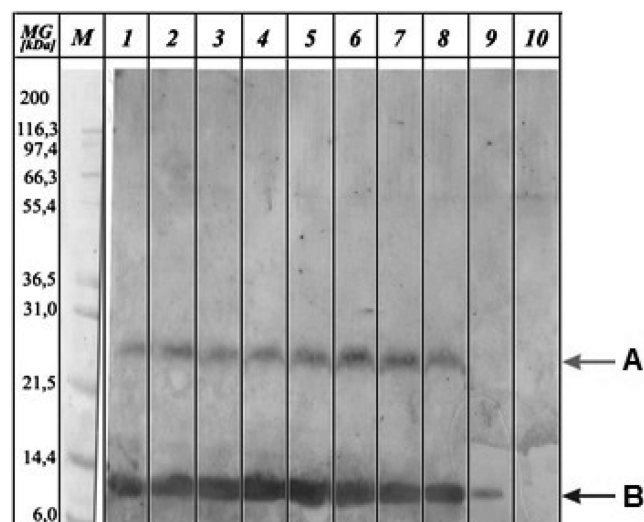


Figure 2. Antibody characterization, immunoblot (M, molecular weight marker; 1–7, enological grade lysozyme preparations from different suppliers, with 7, enological grade lysozyme used for wine treatments; 8, analytical grade lysozyme; 9, analytical grade ovomucoid; 10, analytical grade ovalbumin; A, unknown/artifact?; B, lysozyme (14.4 kDa)).

which could still possess allergenic properties, but potentially exhibit different solubility or depletion resistance patterns than intact lysozyme.

To show specific affinity and exclude potential cross-reactivities, antibodies were characterized by electrophoresis and immunoblotting of analytical grade lysozyme, enological grade lysozyme from different suppliers, and contiguous egg allergens ovomucoid and ovalbumin (Figures 1 and 2). It could be shown that the antibodies specifically detect lysozyme and show no cross-reactivity toward ovomucoid or ovalbumin (Figure 2, lanes 9 and 10). Furthermore, in all lysozyme preparations including analytical grade lysozyme, they are

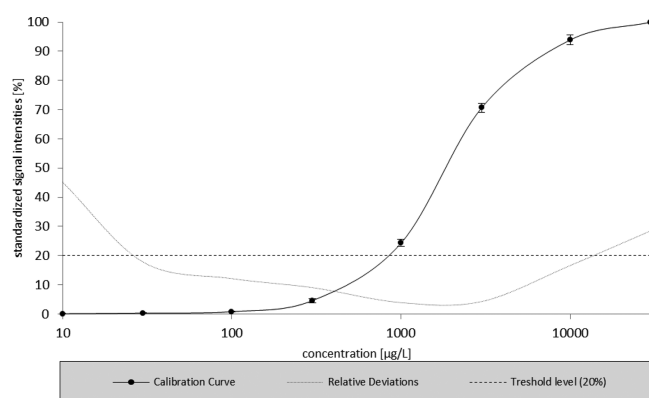


Figure 3. ELISA calibration curve and according precision profile.

Table 2. Statistical Characteristics of the Developed Indirect ELISA Method

LOD (blank + $3 \times SD_{\text{blank}}$)	5 $\mu\text{g/L}$
LOD (blank + $3 \times SD_{\text{blank}} < x - SD_x$)	6 $\mu\text{g/L}$
LLOQ (blank + $10 \times SD_{\text{blank}}$)	10 $\mu\text{g/L}$
LLOQ (precision profile)	30 $\mu\text{g/L}$
ULOQ (precision profile)	10 mg/L
repeatability ($n = 6$)	5.2–14.7%
recovery rate	69.8–105.1%

Table 3. Performance Characteristics of HPLC-F Methods

	HPLC-F method as described	OIV-MA-AS315-14 ³⁰
linearity range	5–400 mg/L	1–250 mg/L
correlation coeff	0.9998	0.9990
LOD	0.46 mg/L	0.18 mg/L
LOQ	1.38 mg/L	0.59 mg/L
repeatability ($n = 5$)		
buffer	0.98%	2.61%
white wine	1.78%	0.68%
red wine	2.54%	2.37%
recovery rate	94.4–101.3%	97.7–102.2%
retention time	0.8 min	25.5 min
total run time	7 min	40 min

capable of detecting an additional protein band of a higher molecular weight than native lysozyme (Figure 2, lanes 1–8, indicator A), possibly a lysozyme artifact or dimerized lysozyme. Since this protein has only been detected by immunoblotting and not by silver staining, specificity seems to be high and concentrations low. Immunostaining intensities suggest that the concentration is lowest in analytical grade lysozyme and may vary in enological grade lysozyme preparations, indicating a potential influence of the production protocol on its presence. Furthermore, residues of ovalbumin could be detected in some preparations (lanes 1, 2, and 6), but not in others. Low molecular weight lysozyme fragments could not be detected in either of the lysozyme preparations.

Development of the ELISA Method. Comparison of different enological grade lysozyme preparations showed a significant degree of variability in composition. In order to have a more reliably defined calibrator, calibration and validation of the ELISA method were carried out using analytical grade lysozyme. Signal response intensities of the analytical grade lysozyme used for calibration and the enological grade lysozyme used for treatments of the wines showed a correlation of 97.4–106.0% over the entire quantification range, averaging

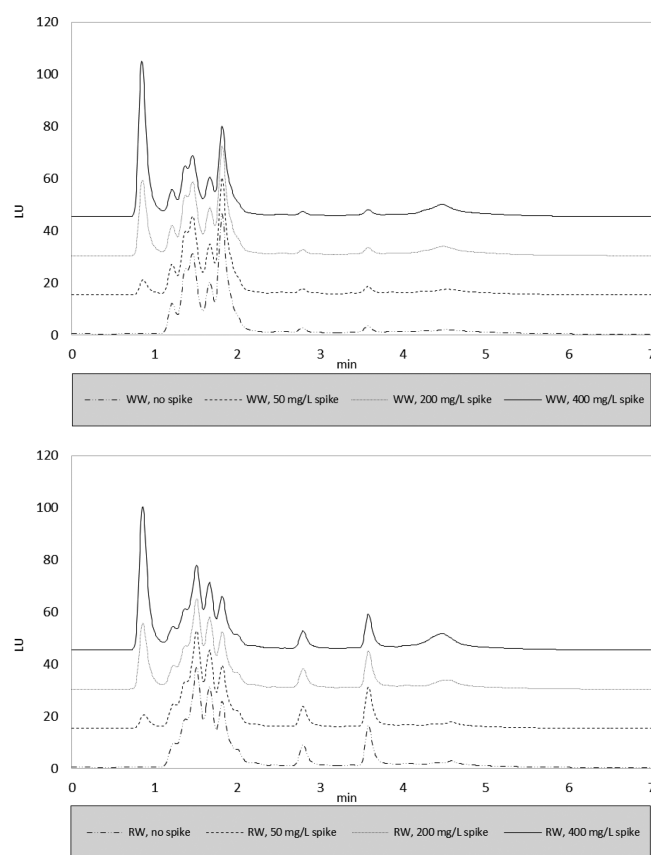


Figure 4. HPLC-F analysis of untreated white and red wines spiked with lysozyme.

101.1%. Therefore, signal responses of this enological grade lysozyme can be regarded as equivalent to the analytical grade lysozyme signal responses. In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), it is a well-established approach to calculate the concentration value corresponding to the matrix blank signal plus a respective multiple (usually $n = 3$ for the LOD, $n = 10$ for the LOQ) of the standard deviation of the matrix blank signal. This approach is based on the presumption of homogeneity of variances over the complete calibration range and does not take into account the actual signal variations of the calibration standards. However, in an analytical system like an ELISA, relying on a cascade of interrelated principles for signal generation and amplification, a heteroscedastic distribution of signal deviations over the calibration range may be assumed, even more so when employing a calibration range covering multiple orders of magnitude. The relevance of this consideration for determining the LOD/LOQ is further enhanced by the nonlinear, logistic nature of the signal curve in an ELISA. With the decreasing slope on both ends of the curve, it becomes increasingly difficult to reliably distinguish between discrete calibration standard concentrations on the fringes of the calibration range. Depending on the calibration range, this may lead to the necessity to determine not only a lower but also an upper LOQ.

To account for these characteristics, an alternative approach toward establishing potentially more meaningful LOQs for ELISA methods has been pursued by employment of a precision profile as proposed by Ekins.²⁹ This approach aims to take both the steepness of the slope and the standard deviation of the matrix calibration standard signal over the

Table 4. Analysis of Wines^a

EnP	matrix	dosage [mg/L]	before StF				after StF			
			c(LYZ) [mg/L]	SD [mg/L]	CV [%]	% (c_{theo})	c(LYZ) [mg/L]	SD [mg/L]	CV [%]	% (c_{theo})
ShF	WW	500.0	251.5	47.3	18.8	50.3	257.5	57.2	22.2	51.5
		1000.0	593.9	147.3	24.8	59.4	304.7	76.5	25.1	30.5
	RW	500.0	245.8	27.9	11.3	49.2	218.1	30.8	14.1	43.6
		1000.0	505.3	64.5	9.5	50.5	517.7	8.8	1.2	51.8
MeF	WW	500.0	297.7	49.2	16.5	59.5	304.0	78.6	25.9	60.8
		1000.0	586.8	196.6	33.5	58.7	642.8	164.0	25.5	64.3
	RW	500.0	297.3	28.6	9.6	59.5	223.1	14.1	6.3	44.6
		1000.0	596.0	68.6	9.9	59.6	602.0	114.6	14.1	60.2
CrF	WW	500.0	282.4	64.6	22.9	56.5	255.4	59.2	23.2	51.1
	RW	500.0	283.1	21.9	7.7	56.6	256.1	37.8	14.7	51.2
		1000.0	477.9	442.2	30.5	47.8	561.1	0.7	3.1	56.1
KiF	WW	500.0	325.8	72.7	22.3	65.2	265.9	85.2	32.0	53.2
		1000.0	622.7	140.0	22.5	62.3	776.4	147.0	18.9	77.6
	RW	500.0	199.7	9.6	4.8	39.9	198.0	8.4	4.2	39.6
		1000.0	492.8	36.9	8.8	49.3	540.0	183.9	16.6	54.0
Cen	WW	500.0	321.6	46.4	14.4	64.3	316.3	72.1	22.8	63.3
		1000.0	570.6	135.7	23.8	57.1	612.8	182.1	29.7	61.3
	RW	500.0	313.5	20.3	6.5	62.7	199.9	14.0	7.0	40.0
FIP	WW	500.0	666.3	0.2	1.1	66.6	670.6	29.8	3.9	67.1
		1000.0	292.8	60.4	20.6	58.6	256.3	79.0	30.8	51.3
	RW	500.0	586.1	149.9	25.6	58.6	672.9	167.5	24.9	67.3
		1000.0	276.8	8.9	3.2	55.4	170.8	16.1	9.4	34.2
SiF	WW	500.0	577.8	7.0	1.4	57.8	585.1	101.0	11.2	58.5
		1000.0	402.0	57.9	14.4	80.4	385.6	125.2	32.5	77.1
	RW	500.0	511.8	47.5	9.3	51.2	647.0	183.1	28.3	64.7
		1000.0	257.2	23.6	9.2	51.4	213.9	23.0	10.8	42.8
BeF	WW	500.0	574.8	33.4	3.5	57.5	588.4	34.4	3.4	58.8
		1000.0	36.0	8.1	22.5	7.2	n.d.			
	RW	500.0	101.8	25.1	24.6	10.2	90.8	27.2	29.9	9.1
		1000.0	57.6	8.2	14.2	11.5	n.d.			
		1000.0	221.7	46.6	6.8	22.2	212.3	12.0	3.5	21.2

^aWW: white wine. RW: red wine. EnP: enological procedure. c(LYZ): lysozyme concentration. SD: standard deviation ($n = 6$). CV: coefficient of variation ($n = 6$). % (c_{theo}): recovery of theoretical lysozyme concentration. StF: sterilizing filtration. ShF: sheet filtration. MeF: membrane filtration (0.45 μm). CrF: cross-flow filtration. KiF: kieselguhr filtration. Cen: centrifugation. FIP: flash-pasteurization. SiF: silica fining. BeF: bentonite fining. n.d.: not detected.

whole range of the calibration curve into consideration for determining a lower and an upper LOQ (LLOQ, ULOQ). Relative deviations of the calibration standard signal intensities are calculated by dividing the standard deviations of the calibration standard signals by the product of the calibration standard concentration and the steepness of the slope at the calibration standard concentration value in question. These relative deviations are plotted against a set threshold level that provides a cutoff value to warrant a defined quantitative significance of the signal read-out. Thus, the LLOQ and ULOQ are respectively considered the lowest and highest matrix calibration standard concentrations yielding a relative deviation below the threshold level. Figure 3 shows the calibration curve and the according precision profile.

Furthermore, for the estimation of an LOD taking both the standard deviation of the blank and the standard deviations of the calibration standards into account, the lowest calibration standard concentration for which the matrix blank signal plus 3-fold its standard deviation is smaller than the matrix calibration standard signal minus 3-fold its standard deviation is considered the LOD.

Table 2 summarizes the statistical characteristics of the ELISA with a relative deviation of 20% defined as the threshold

value determining the LLOQ and ULOQ. The proposed alternative approaches toward establishing an LOD and LLOQ bring forth higher values than with the commonly applied method. This comes as no surprise, taking into consideration that these alternative approaches aim to address more elements of uncertainty than only the standard deviation of the blank. With regard to the presumed higher reliability of these values, however, this appears acceptable. Recovery rates for matrix calibration standards calculated on the calibration curves obtained from calibration standards in buffer were between 69.8 and 105.1% over the entire quantification range. Repeatability is reflected in the coefficients of variation ranging from 5.2 to 14.7% for $n = 6$. Dilution linearity for dilutions 1:30, 1:300, and 1:3000 was considered satisfying with recovery rates of 86.7–99.8%.

For analysis of the wines, ELISA results were confirmed by additional HPLC-F analysis. With a LOD < 0.5 mg/L (calculated as blank + $3 \times \text{SD}_{\text{blank}}$), linearity range from 5 to 400 mg/L, and correlation coefficient of 0.9998, the HPLC method shows good correlation with the statistical characteristics defined in the official OIV method for the measurement of lysozyme in wine by HPLC OIV-MA-AS315-14,³⁰ but with a total run time of 7 min as compared to 40 min in OIV-MA-

AS315-14, it gives an almost 6-fold increase in sample throughput. Table 3 gives a brief comparison of the performance characteristics of the two methods. Chromatograms of untreated white and red wine spiked with ascending lysozyme concentrations are shown in Figure 4. Upon comparison, the congruency of the ELISA and the HPLC-F method was considered very satisfying. Lysozyme concentrations measured by HPLC-F in the spiked wines were on average 98% of the concentrations measured by ELISA.

Analysis of Wines and Comparison of Enological Procedures. For the assessment of the depletion efficiencies, the wines were subjected to the respective enological procedures and samples drawn directly after the procedure and after sterilizing filtration. As can be seen from Table 4, all enological procedures under scrutiny are capable of reducing the detectable lysozyme amount. However, except for bentonite fining, the detectable amounts are still very high. This shows that most of these procedures are not capable of satisfactorily reducing the lysozyme content and thus do not represent suitable tools for allergen depletion. This may at least in part be attributed to the good solubility and low molecular weight of lysozyme. For both red and white wines treated with 500 mg/L lysozyme, bentonite fining is effective enough to deplete the lysozyme amounts to levels below 100 mg/L. While the effect of sterilizing filtration on depletion efficiencies generally appears to be negligible, for bentonite-treated wines, this last filtration step serves to lower lysozyme levels below the LOD. Potentially, the remaining lysozyme adsorbs to bentonite particles still in suspension, forming aggregates big enough to be removed by sterilizing filtration. For wines treated with 1000 mg/L lysozyme, however, not even bentonite fining and successive sterilizing filtration proved sufficient for total depletion. This spotlights the general limitation of the capability of a given amount of bentonite to reduce a given amount of lysozyme present in wine. On the other hand, employed amounts of bentonite cannot be continuously increased, without severely affecting the sensory and organoleptic characteristics of the wine. Excessive use of bentonite may impair both the aroma profile and the color intensity of the wine, resulting in quality deterioration and thus economic loss. Differences in bentonite quality used for treatment of wines may effect different results regarding their respective potential to deplete lysozyme or to affect the overall quality and taste of the wine being treated.

Hence, it may be concluded that, depending on the production technique employed, lysozyme used as a microbial stabilizer in the wine making process may potentially be present in the final product at concentrations that could pose a threat toward sensitized individuals.

This is relevant information for winemakers intending to establish a good manufacturing practice guideline to be able to utilize lysozyme in their cellars and still provide wines free of allergenic residues. Since in the case of wine, the labeling of allergenic ingredients is only statutory if the wines were positively tested for allergenic residues, bentonite fining might offer a viable opportunity to enable lysozyme treatment of musts and wines, and still provide products that can safely be enjoyed by allergic individuals.

Overall, it can be concluded that allergen management of lysozyme in wine should always include analytical control for product safety. Regarding future developments, there are also intentions not to rely on dissolved lysozyme in the wine but rather to use lysozyme immobilized on supportive beads.³¹

Uncertainties regarding the durability of the attachment of the lysozyme to the beads and the release rates encountered in commercial application would of course create sufficient necessity for sensitive lysozyme analysis of the final product just as if the wine was treated with the native enzyme and then subjected to filtration or other further processing.

AUTHOR INFORMATION

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