

Technical Brief

Determination of Residual Egg White Proteins in Red Wines during and after Fining

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Abstract: Methods are needed for detecting allergenic proteins in wine after fining processes, both for legal requirements and to estimate any risk for allergic individuals. One of the critical allergens in this case is the protein fraction from egg white. The efficacy of use of a commercial egg protein detection kit was evaluated using wines spiked with egg protein. For calibration, a whole egg powder reference material was used. There were no matrix effects and the detection limit was 0.27 mg whole egg powder/L. The intra- and interassay coefficients of variation were 7.3% and 11.9%, respectively. Recoveries of different egg preparations in spiked red wine were between 80 and 110%. A field study showed different concentrations of egg during fining, but no detectable or low values of ≤ 1 mg/L in the bottled wine.

Key words: allergens, egg white, ELISA, wine, fining, residues

Fining processes during winemaking are used to clarify must and wine or to remove substances which otherwise would negatively influence the flavor of the wine. Traditionally, proteins from milk and egg have been used for a “fine-tuning” of wine flavor because of their capacity for selective tannin adsorption. Whereas milk and its constituents are used for white wine fining, egg is applied for red wine. In case of egg, the whole raw egg white (consisting of ovalbumin, ovomucoid, ovotransferrin, and lysozyme) could be used.

In recent years, reasonable concern has emerged that residues of proteins used for fining processes can remain in wine, which might elicit an allergic reaction in sensitive consumers. One study described a double-blind placebo-controlled wine challenge using fined and nonfined wines (Rolland et al. 2006). Unfortunately, the number of participants was very low (five patients with allergy to egg and only one to milk), and the few specific reactions to fined wines were not significant compared to reactions on control wines (nonfined wines). This low incidence is not remarkable, because allergies to milk and egg are rare (Kanny et al. 2001, Savage et al. 2007, Lam et al. 2008) but severe in adults, as shown for milk proteins (Lam et al. 2008) and egg (Hu et al. 2007). Minimum eliciting doses for egg proteins are as low as 0.13 mg (Taylor et al. 2002). Values of casein and egg proteins in fined wines in a recent study using a noncommercial enzyme-linked im-

munosorbent assay (ELISA) were below the detection limit (1 μ g ovalbumin/L and 8 μ g casein/L) (Rolland et al. 2008).

Nevertheless, according to the EU directive 2007/68/EC, all wines labeled after 31 May 2009 must declare if allergens like egg and milk were used during production (European Directive 2007). An extension of the term until 30 June 2012 was granted by the Standing Committee on the Food Chain and Animal Health of the European Union. Therefore, methods for the detection of proteins from these fining agents in wine are needed to determine whether the exception should be permanent, threshold values should be set, or the use of allergens irrespectively of the amount in the wine should be declared. Nevertheless, the detection limit should be as low as possible for sensitive individuals.

The aim of the study was to measure residues of egg white proteins in red wine during and after fining in the bottled wine, using a commercially available sandwich ELISA Ei/Egg protein kit manufactured by R-Biopharm.

Materials and Methods

Commercial wines and spiking materials. Commercially available red wines from different regional origins and cultivars were used for determination of the analytical background (nonspecific binding) and for spiking experiments. The following three egg protein containing materials were used as spiking materials.

Whole egg powder. The spray-dried whole egg powder from the National Institute of Standards and Technology (NIST, RM 8445) is intended for use in evaluating test kits for determination of the presence of allergenic egg proteins. The powder was produced by Henningsen Foods (Omaha, NE) and has a certified total protein content of $48 \pm 1\%$ (determined by the Dumas method) and no additives such as silicates.

Egg white powder. The P-11 egg white powder (Henningsen Foods) had a protein content of 83.8% (Kjehldahl determination) and no additives.

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Extract of cooked egg white. One gram of milled cooked egg white was extracted with 20 mL preheated allergen extraction buffer (provided with the Ei/Egg protein test kit described below; R-Biopharm AG, Darmstadt, Germany) for 10 min at 60°C. The extract was cooled to room temperature, centrifuged 10 min at 2500 g, and filtered. The protein content was measured by the BCA method (calibrated against the protein content of the egg white powder P-11) and resulted in a concentration of 2.8 mg/mL. Theoretically, the extraction of 1 g cooked egg white would result in a protein concentration of 5.5 mg/mL (1 g egg white contains 110 mg protein; Souci et al. 2000). Approximately 50% of heat-denatured egg white proteins could be solubilized by using the emphasized extraction procedure.

Field study. The experiment began in fall 2007 at a winery near Geisenheim (Rheingau, Germany). St. Laurent grapes were harvested by handpicking on 14 Oct 2007 and destemmed. Pinot noir were harvested by handpicking on 24 Oct 2007 and destemmed. St. Laurent grape must was inoculated with the dry active yeast AWRI 350 (Mauri Yeast Australia, Toowoomba, Australia) or Siha 8 (Begerow, Langenlonsheim, Germany) in open tanks at ambient temperatures (15 to 20°C) until alcoholic fermentation was completed. Pinot noir grape must was inoculated with ANAferm rot (Weinlabor Vogel, Geisenheim, Germany) in open tanks at ambient temperatures (10 to 18°C). To ensure a homogenous extraction, the must was manually stirred four times a day. The must was pressed using an automatic press (Rotapress PLE 1800, Howard, Germany) and stored in stainless-steel tanks for 14 days at 16°C to allow solid particles to settle. After this period they were pumped at 16°C in old oak barrels, which had been previously used three times. After storage over 15 months (ambient temperature 10 to 16°C), the wine was filtered over diatomaceous earth and stored for one night in a stainless-steel tank at 10°C for the subsequent fining process.

Three liters of commercially available food-grade liquid egg white (Eifix Eiweiss, Wiesenhof, Germany, 99 g protein/kg as determined by Kjeldahl) each were added to 3200 L Pinot noir or St. Laurent wine, stirred for two hours, and allowed to settle overnight. The following morning, wines were filtered over diatomaceous earth at 10°C, stored in a stainless-steel tank at 10°C after addition of sulfite, and bottled two days later using a fine filter with a 6 µm pore size. Samples were withdrawn at each step during the treatment (see Figure 1 for time points) and stored at 4°C until allergen extraction and ELISA analysis.

Theoretically, the addition of 3 L egg white with a protein content of 9.9% will result in concentrations of 297 g egg white protein/3200 L (92.8 mg/L). To convert this amount to whole egg powder (the calibrant of the ELISA), the following values were taken from the literature: The water content of whole egg is 74.4% and the egg white content is 63% (Souci et al. 2000). As stated above, the protein content of egg white is 9.9%. In summary, 92.8 mg/L of egg white protein is equivalent to 937.4 mg egg white/L, which is equivalent to 1487.9 mg whole egg/L, which is finally equivalent to 381 mg whole egg powder/L.

Analytical parameters after fining for St. Laurent and Pinot noir, respectively, were 10.7% v/v and 14.2% v/v ethanol, 5.7 g/L and 5.7 g/L titratable acidity (expressed as tartaric acid), 22.1 g/L and 27.3 g/L sugar-free extract, and 78 mg/L and 105 mg/L total sulfur dioxide.

Sample preparation for ELISA. One mL of wine was diluted with 19 mL preheated (60°C) allergen extraction buffer, which was provided with the RIDASCREEN FAST Ei/Egg Protein test kit (R6402, R-Biopharm AG, Darmstadt, Germany). The extract was heated for 10 min at 60°C and centrifuged for 10 min at 2500 g. After filtration, 100 µL of the extract was used in the sandwich ELISA.

ELISA for detection of egg white proteins. For quantification of proteins from egg, the Ei/Egg protein sandwich ELISA was used. The assay was calibrated using a spray-dried whole egg powder for allergen detection (RM 8445) from NIST ranging from 25 to 675 µg/L. Including the dilution factor of 20 during sample preparation, the result is a calibration range of 0.5 to 13.5 mg/L. The set of polyclonal antibodies specifically detects the egg white proteins ovalbumin and ovomucoid and shows cross-reactivities to ovotransferrin. For testing, 100 µL standard or sample solutions were pipetted to the wells, incubated for 10 min, and washed three times. Next, 100 µL enzyme-antibody conjugate was added, incubated for 10 min, followed by three washing steps before pipetting 100 µL substrate/chromogen solution to each well. The color development was stopped after 10 min with 100 µL stop solution and absorbance measured at 450 nm.

Validation of the assay. To characterize the specificity of the antibodies, a panel of approximately 60 different foodstuffs was treated like a sample (1 g sample extracted with 20 mL extraction buffer) and tested for cross-reactivity in the egg protein assay. The limit of detection (LOD) was determined by measuring four egg-free matrices (wine, salad dressing, biscuit, pasta) 10-fold and extrapolating the results from the calibration curve. The mean plus the three-fold

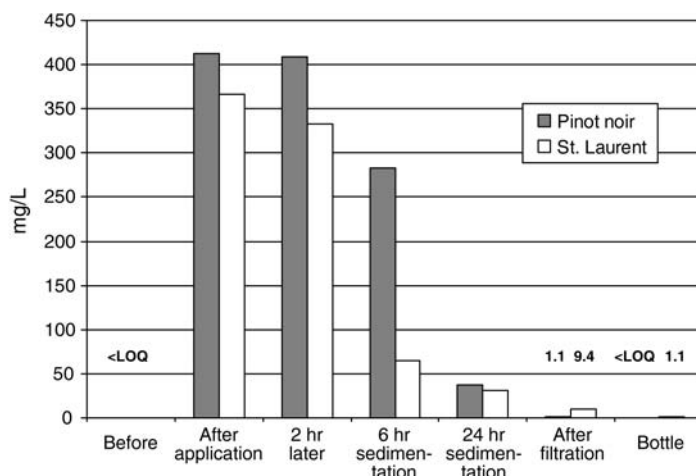


Figure 1 Measurement of egg content (calculated as mg whole egg powder per liter of wine) in two red wines at different stages during the fining process after addition of egg white. Low values of 1.1 mg/L, 9.4 mg/L, and values below the limit of quantification (LOQ) are given directly in the figure.

standard deviation was defined as the LOD. The determination of the limit of quantification (LOQ) was estimated by low-level spikes ~0.5 mg/L (results not shown). The start of the calibration curve was therefore derived from a practical point of view and covered unknown samples. Therefore, the starting point of the calibration was the point that the manufacturer guarantees. (A qualitative differentiation of blank wines from wines below 0.5 mg/L is possible, but not within the scope of this work.) For determination of recoveries in red wine, an Italian wine (Cabernet Sauvignon) was used and spiked with different egg protein containing preparations (the three spiking materials) at three different concentrations (Table 1). To characterize the precision of the test system, intraassay coefficient of variation (CV) was determined by measuring a whole egg powder-spiked red wine (1.5 mg/L) six times. For interassay CV, the spiked red wine was measured in four

Table 1 Recovery experiments in an Italian red wine using three different spiking materials (whole egg powder, egg white powder, and extract of cooked egg white).

Material	Spike ^a (mg/L)	Target value ^b (mg/L)	Found egg ^b (mg/L)	Recovery (%)
Whole egg powder	1.5	1.5	1.3	87
	4.5	4.5	4.9	109
	13.5	13.5	14.1	104
Egg white powder	1.0	4.2	4.5	107
	3.0	12.5	13.8	110
	9.0	37.4	36.5	98
Egg white, cooked (extract)	2.0	8.4	7.4	88
	6.0	25.2	19.2	76
	18.0	75.6	61.8	82

^aGiven as mg powder per liter (whole egg powder) or mg protein per liter (extract of egg white or egg white powder).

^bCalculated as mg whole egg powder per liter (for conversion factor see Materials and Methods; field study).

Table 2 Measurement of whole egg powder concentrations in different commercial red wines. OD readings at 450 nm are given for standards and the red wine samples.

Standard	OD (450 nm)	Egg concn (mg/L)
Calibrator 1	0.101	0.0
Calibrator 2	0.421	0.5
Calibrator 3	0.544	1.5
Red wine		
Sicilia forte (Italy)	0.121	<LOQ
Trentino (Italy)	0.578	1.7
Cabernet Sauvignon (Germany)	0.256	<LOQ
Cabernet Veneto (Italy)	0.120	<LOQ
Montepulciano Abbruzzo (Italy)	0.202	<LOQ
Birgi Marsala Cabernet (Italy)	0.169	<LOQ
Rosso Puglia (Italy)	0.285	<LOQ
Cantine la Rosa Merlot (Italy)	0.217	<LOQ
Duce di Scanzano, Vino Rosso (Italy)	0.102	<LOQ
St. Laurent (Germany)	0.122	<LOQ

independent runs in duplicate. The spike was prepared in extraction buffer and diluted before addition to the wine.

Results and Discussion

ELISA quality parameters. The specificity of the Ei/Egg protein assay was tested with more than 60 common compounds that occur as normal food constituents. No cross-reactivities against tree nuts, cereals, poultry meat, and legumes were observed. The LOD was 0.27 mg whole egg powder per liter of wine, the LOQ was 0.5 mg/L (lowest calibrator). The recoveries for an extract of cooked egg white, whole egg powder, and egg white powder in red wine showed good recoveries for each type of spike irrespective of the concentration (Table 1). A red wine spiked with 1.5 mg whole egg powder per liter revealed an intraassay CV of 7.3%. Using the same sample in duplicate, the interassay CV was 11.9% (n = 4).

The calibrator should be chosen very carefully since there can be differences between NIST reference materials and could be related to γ -radiation and the addition of silica aluminates (Lacorn and Immer 2010). Furthermore, the spiking of wine should be handled with care since a total solubility of the spike in wine is necessary.

Commercial red wine samples. The egg content in different red wines was measured (Table 2). Only one of the 10 wines analyzed was above the LOQ of the method. A broader screening is necessary to estimate the percentage of egg protein-containing wines.

Field study. The courses of concentrations for egg white protein in both red wines (Pinot noir and St. Laurent) revealed comparable results (Figure 1). Before fining, both wines had values below the LOQ of 0.5 mg/L. As expected, concentrations increased to values between 350 and 410 mg/L after adding the liquid egg white and remained at these high levels during stirring of the wine. These concentrations are close to the expected value of 381 mg/L and also show that even precipitates, which could be visually detected, are solubilized by the extraction procedure. After six hours of sedimentation, there is a clear difference of >200 mg/L between the two red wines. This difference is not observable after 24 hr of sedimentation when egg protein concentrations are <40 mg/L. The first filtration over diatomaceous earth led to concentrations of 1.1 mg/L for Pinot noir and 9.4 mg/L for St. Laurent. After filtration over a 0.6 μ m filter and bottling, the concentrations were below the LOQ for Pinot noir and 1.1 mg/L for St. Laurent. This was comparable to recently published results using highly sensitive ELISAs with detection limits of 4 μ g/L for casein and 1 μ g/L for egg white (Liffrani et al. 2009); that study also found detectable amounts at the beginning of the fining process but no residues after the final third filtration. Therefore, it could be speculated that a further filtration step in our study would lower the minimal residues of egg proteins in the St. Laurent below the LOQ as for the Pinot noir.

Conclusion

The described assay is suitable for high-throughput analysis of egg proteins in red wine. Only one of 10 commercial wines analyzed displayed an LOQ for egg white protein.

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