

Effect Of Various Treatments And Filtration Methods On Eliminating Casein Fining Agent Residues Of Wine

Manuella Webber-Witt¹, Mariana Deckwart², Carsten Carstens², Lisa Eichhorn³, Knut Brockow³
Volker Schaefer¹, Angelika Paschke-Kratzin², Monika Christmann^{1*}

Author affiliation(s)

¹Research Center of Geisenheim, Center of Wine Research & Process Engineering of Beverages
Blaubachstr. 19, D-65366 Geisenheim, Germany.

²University of Hamburg, Institute for Food Chemistry. Grindelallee 117, D-20146 Hamburg, Germany,

³Munich Technical University- Department of Dermatology and Allergology. Biedersteiner Str. 29, D-80802 Munich, Germany

***Corresponding author** Monika Christmann (monika.christmann@hs-gm.de) ph. +49 (0)6722 502 171

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Abstract: Fining materials derived from milk that are used during wine making processes may present the possibility to trigger allergic reactions. Depending on the legislation of each country, these substances should be declared on the wine label if present in the final product. Label information enables consumers with allergies to avoid food and beverages that may trigger their allergic symptoms. This study investigated whether technological procedures could be used to avoid allergic residues remaining in wine, after the use of fining agents containing milk protein. As there is no standard for the methodology of winemaking practices, a fining trial was conducted to study the influence of various filtration methods, along with other treatments, and its efficiency on reducing these possible allergens, even in a worst case scenario. The method of detection used was ELISA, and in vivo tests were also conducted. Casein is removed from both red and white wines to undetectable levels by all methods of filtration used in this study. Further methods evaluated in this study are just as efficient, apart from

flash-pasteurization or silica sol when an exaggerate dosage is applied on worst case scenario. However, this study shows that an additional sterile filtration after flash-pasteurization or silica sol fining decreases casein proteins to undetectable levels as well.

Key words: wine fining; casein; allergens; hidden allergens, filtration; flash-pasteurization.

Introduction

Together with egg albumin, cow's milk is one of the oldest proteinaceous agents used to fine wines (Babo 1872, Windisch 1908). Approximately 82% of cow's milk protein is casein (Belitz and Grosch 1982). Nowadays, casein and potassium caseinate are the main oenological fining agents derived from milk which are applied in the wine industry. Casein is employed in large amounts for wine production in many countries. In Germany up to 20% of the wines are fined with casein depending on the vintage (Deutscher Weinverband 2010), while in France, over 20.5 million hectolitres of wine are fined with casein annually. This corresponds to around 40% of all French wines produced (Weber et al. 2007). All milk-related fining agents are described to be used for color and flavor enhancement primarily, but not exclusively, for white wines. The presence of large amounts of phenolic compounds in wine increases the susceptibility to oxidation, possibly altering the color, aroma, and flavor; decreasing visual and sensorial qualities of wine (Silva Ferreira et al. 2003). Casein is known to have a "refreshing" effect on wine flavor and color, due to the nature that this protein acts by removing phenolic bitterness and harshness. Removing these compounds helps soften the palate, lighten wine color, and potentially remove off-flavors. Casein has a broad application ranging from grape must, finished wine to fortified wine, such as sherry. It may also be used preventively and curatively on yellowing and maderisation of white wines (Würdig and Woller 1989, Troost 1988, Ribereau-Gayon et al. 2006, Flanzky 2000, Margalit 2004, and Rankin 2004).

59 In a detailed study on the effect of various proteins on different molecular weight (MW)
60 proanthocyanidin fractions of red wine during fining, the authors show that casein reduces around 35%
61 of the monomeric flavan-3-ol, which is generally associated with bitterness, whereas potassium
62 caseinate reduces it to around 15%. Compared against ovalbumin, this study shows that casein is more
63 likely to react with monomeric proanthocyanidins. This study may help to understand the specificity of
64 each fining agent together with the form it is applied to wine (Cosme et al. 2009).

65 Caseins are characterized by sequences of hydrophobic and hydrophilic amino acids, resulting in an
66 ambiphilic character, thus micelle-forming properties. The casein component of milk is relatively heat-
67 stable, capable of surviving pasteurisation at ~62-71 °C (Horne 2002). In milk, caseins have a stable net
68 negative charge whereas in wine, they have a net positive charge, i.e. they reach their isoelectric point
69 (IEP). Casein reaching its IEP results in neutralization of the negative charges on the outer surface of the
70 micelle (by protonation of phosphate groups), and the neutral protein precipitates (Horne 2002, Weber et
71 al. 2007). Finally, casein protein will have a net positive charge at wine's pH due to the pKa values of
72 the amino acid side chains (Horne 2002, Fox et al. 1998).

73 Most wines worldwide have a pH around 3.5 and any pH over 3.8 is undesirable. Casein has an IEP of
74 approximately 4.6, consequently being insoluble in low pH. Due to its phosphorus content, it will
75 coagulate or precipitate at wine pH. The proteins also have hydrophobic or non-polar regions that are
76 exposed when caseins denature within the pH range of wine. The fining effects of this protein are
77 attributed to this bipolar feature (Stocké and Ortmann 1999). These hydrophobic regions can interact
78 with phenolic compounds along with other components, and studies have shown that casein significantly
79 reduces the total polymeric phenolic compounds and absorbance at 520 nm of muscadine wines (Sims et
80 al. 1995).

81 Due to casein's precipitation properties, it rapidly fluctuates when added to wine, and curds may be
82 visually seen directly after addition. Casein has been always known to be removed from wine after
83 settling and racking (Zoecklein 1990, Ribereau-Gayon et al. 2006). Nevertheless, the prevalence of
84 allergic reactions to milk proteins, and particularly to casein, among the general population of adults has
85 been reported to be around 0.5-1.0 % (EFSA 2011). Recent labelling law to protect consumers with milk
86 allergies has raised the necessity of detailed studies in this area, due to possible allergic reactions in
87 sensitive consumers. Only a few studies on the residues of casein on finished wine relative to its allergic
88 potential have been published (Rolland et al. 2006 and 2008, Weninger and Görtges 2005, Weber et al.
89 2007 and 2009a 2009b, Fischerleitner and Eder 2006, Lacorn et al. 2009, EFSA 2011, Christmann et al.
90 2012, Wigand and Decker 2011, Webber-Witt and Christmann 2012).

91 Recent European law (Regulation 2010/1266/EC) permitted the labelling and marketing of wines
92 without declaring allergen substances up to the 30th of June 2012, or until these stocks are exhausted
93 (Teissedre 2011). Any wine produced, bottled or commercialized in Europe after this date must declare
94 on the label if it contains 0.25 mg/L or greater of egg or milk allergens (EU Regulation 579/2012).

95 Casein is already in force of declaration in some countries like Australia and New Zealand, where
96 winemakers have been obligated to display the use of milk and egg products on their wine labels since
97 December 2012 (FSCANZ 2011).

98 This study researched whether technological procedures could be used to avoid allergic residues
99 remaining in wine, after the use of fining agents containing milk protein. These techniques include
100 filtering by different methods, together with further enological technologies, to test their efficiency on
101 removing the residues.

102 **Materials**

103 Two young wines from grapes of Rheingau region of Germany, made in the research winery of
104 Geisenheim vintage 2009, a white *Vitis vinifera* Mueller-Thurgau; pH 3.4, residual sugar 1.3, TA 4.5,
105 alcohol 12% v/v, total phenols 167 mg/L, 68 mg/L free and 148 mg/L bound sulfite. The red wine was a
106 hybrid Regent pH 3.6 residual sugar 2.4, TA 5.3, alcohol 12.3 % v/v, total phenols 1240 mg/L, 59 mg/L
107 free and 83 mg/L bound sulfite.

108 The two wines were fined in two different concentrations of potassium caseinate (Kal-CasinLeicht
109 löslich®) (Erbsloeh, Geisenheim Germany) and two different concentrations of a mixture fining agent
110 containing potassium caseinate with non-organic agent polyvinylpyrrolidone (PVPP) and silicate
111 (SensoVin®) (Erbsloeh, Geisenheim Germany), both milk based fining agents. The first concentration
112 “A” was based on the highest dose recommended by the fining agent manufacturer (solely casein 400
113 mg/L and mix agent 500 mg/L). The second dosage “B” was the double of the first concentration (solely
114 casein 800 mg/L and mix agent 1000 mg/L), to simulate a worst-case scenario.

115 **Methods**

116 The process of fining has been carried out in oversize 110 liters round-bottom flasks.
117 Wines were fined separately with both different doses A and B. After 24 hours further treatments took
118 place with the same wine, including different filtrations, separation, heat and finings - here used as
119 reduction strategies shown on Table 1, when a second fining took place as reduction strategy for
120 allergen fining agent, the amount of second fining agent, i.e. bentonite/silica sol, was not doubled, to
121 keep the worst-case scenario. For each one of the treatments a new batch of wine was fined. The control
122 wines were not fined at all, and were equally filtered and treated (Table 1). Samples of wine were taken
123 after each one of these sequence of events: after fining (sample 1), after each treatment (sample 2), and
124 after final sterile filtration (sample 3) as displayed on (Figure 1).

125 Finally, these wines were analyzed by using a sensitive indirect ELISA done according to Deckwart et
126 al. (2014) method, which is based on Weber et al. (2007 and 2009b) with some modifications. Method
127 validation of developed ELISA in both red and white wine was according to Lifrani et al. (2009), and
128 the measurement occurs in 1:10 diluted white wine and 1:20 dilute red wine. The limit of detection
129 (LOD) used for white wines is 0.1 mg/L and lower limit of quantification (LLOQ) 0.1 mg/L calibrated
130 against casein, while the indirect ELISA for red wines has a LOD 0.2 mg/L and LLOQ 0.5 mg/L
131 calibrated against casein as well. The International Organization of Vine and Wine (OIV) defines
132 thresholds for LOD and LLOQ when ELISA methods are applied on wine analysis. For the method to be
133 recognized by OIV it prerequisites to ensure a LOD of ≤ 0.25 mg/L allergen and a LLOQ of ≤ 0.5 mg/L.

134

135 The laboratory of the Department of Dermatology and Allergology in the Technical University of
136 Munich was in charge of allergic patients' recruitment for in vivo test of wines presenting potentially
137 allergenic residues of fining agents from chicken egg and/or cow's milk, which was the final step for
138 this study. It is important to mention that it is extremely challenging to recruit patients for allergy
139 testing, maybe being one of the leading obstacles on allergy studies. The Department has a database of
140 the preceding five years before the beginning of this study, in which food allergy was detected and
141 tested with positive serum IgE assay. Out of 16,461 patients, 1,931 were children and adolescents (0-18
142 years), and 14,530 were adults. From those only 331 were positive IgE for egg or milk, and from them
143 only 161 were allergic to cow's milk. Of these 331 patients, only 32 adult patients suffered a clinically
144 evident food allergy to egg and/or milk (only one person presented both an allergy to hen's egg and
145 cow's milk). Finally, only 10 patients, from the 32 were included in the test, due to lack of time,
146 pregnancy, strict alcohol abstinence, among others. Each one from these 10 reported a positive
147 anamnesis after ingestion of chicken egg or cow's milk, as well as for skin prick test. The patients were

eight women and two men ranging from 26-63 years of age (mean age 42). Relevant for this study are three women and two men which have an allergy to cow's milk.

Prick skin test (PST) and double-blind, placebo-controlled food challenge (DBPCFC) trial were performed to determine whether any allergic reactions followed consumption would be triggered by wines fined with casein. The wines were a white and a red wine fined with 400 mg/L Kal-CasinLeicht löslich® after being sterile filtered, or sample 3 of Figure 1. Blood samples were taken to detect sensitization to food allergens, and to test the allergenicity of fined wine (30mL whole blood in adults). For the PST to be considered a positive result in this study a wheal has to have a diameter greater than 3 mm with accompanying erythema greater than 5 mm compared to the negative control. The positive control was carried out with 0.1% histamine and the negative control containing 0.9% NaCl.

The oral provocation with fined (*verum*) and no fined (placebo) wine was conducted on two days, the time interval between treatment and placebo testing was at least 48 hours. It takes place in two "test blocks" for each one day. On the first day patients ingest the wine to be tested in an ascending amount, starting with a single drop, 0.1 mL, after 30 minutes a very small volume of 1 mL, if no symptoms are felt, after 60 minutes, a larger amount of wine is given (10 mL). If after another 30 minutes no symptoms occur, then the remaining 189 mL of wine is given for women and the remaining 289 mL for men. Thus, the total dose was 200 ml in women and 300 ml in men. In case of possible responses, the patient was in observation for roughly 2 more hours. On the second day patients repeated the procedure, but with another wine.

Results and Discussion

ELISA results for casein agent Kal-CasinLeicht löslich®: The majority of white wines fined with casein show a negative signal directly after being fined and settled for 24 hours, without any filtration. All red wine samples fined with casein show not detectable or negative results for all three steps, i.e. after

171 fining, after treatments (Table 1) and after sterile filtration. Only wines that have any positive signal or
172 values over LOD are shown on Table 2. Five white wines show actual low values, ranging from 0.3 up
173 to 1.3 mg/L, directly after fining, i.e. without any filtration. No residues were detected in these five
174 white wines when they are filtered (Table 1). Casein is removed from both red and white wines to
175 undetectable levels by all methods of filtration used in this work (Table 1). Further methods used, which
176 are not included in filtration, are just as efficient on removing to undetectable levels, apart from flash-
177 pasteurization on white wine. Flash-Pasteurization show positive values of 2.83 mg/L and 9.48 mg/L for
178 simple and double applied amounts, respectively. These positive results might be the result of possible
179 protein denaturation during heating, leading to changes on the physical structure of the protein, in which
180 possible additional epitopes/antibodies binding sites originates, and thus the ELISA reading values are
181 higher. Nonetheless an additional sterile filtration after flash-pasteurization decreases casein proteins to
182 no longer being detectable.

183

184 ELISA results for potassium caseinate in mixture SensoVin®: relatively similar results are found for this
185 mixture as for pure potassium caseinate (Table 3). When wines are sterile filtered, no residues of casein
186 are found in white wines. The same result is observed when the wine passes through any of the filtering
187 methods used. When white wine is only fined with bentonite, after protein fining, low amounts (0.1
188 mg/L) under the LOD is found. Further low, but positive results were found when wines were fined
189 only with silica sol after protein fining. Finally, both positive results are removed from wine when a
190 sterile filtration is applied.

191 Flash-Pasteurization: almost all flash-pasteurized wines of this study show higher reading by ELISA
192 than before the pasteurization. A study demonstrated that denaturing or partially denaturing treatments
193 have an influence on immunochemical reactivity of tests such as ELISA. A rise in reactivity could lead

to an over or underestimation of the actual protein level as seen by previous studies. A study conducted by Rumbo et al. (1996) observed that only denaturing treatments had an influence on immunochemical reactivity in ELISA. This might likewise apply to this presented study and could explain why the values are slightly higher or positive when wine is pasteurized.

A review on oenological fining agents' purity revealed that the majority of tested fining agents showed other milk proteins present, even if advertised only as potassium caseinate (Deckwart et al. 2011 and 2014). Included on this proteins are alpha and beta-lactoglobulin, which are whey proteins and are more thermolabile than casein, i.e. its thermolability lies around 60°C (Genkina and Koltysheva 2000).

Nonetheless, methods used in this work are not only able to detect casein, but also other milk allergens, since the allergen content was determined not only for casein but for the fining agent itself 'Kal-Casin' (Deckwart et al. 2014).

No positive PST was observed and no anaphylaxis was induced by wine consumption, nor were there any clinical reactions to protein-fined wines by any of the five milk allergic patients at the Technical University of Munich, Department of Dermatology and Allergology. Similar results have been reported by Rolland et al. 2006 and 2008.

Conclusion

Casein is removed from both red and white wines to undetectable levels by all methods of filtration used in this work. Further methods used are as efficient, apart from flash- pasteurization, bentonite, and silica sol, this last one only on the worst case scenario. Nevertheless, an additional sterile filtration after flash-pasteurization decreases casein proteins to no longer being detectable. More studies on denaturing occurrence and its influence on immunochemical reactivity are necessary for a better understanding of these results. The majority of red and white wines fined with casein already had a negative signal after fining and sedimentation of 24 hours without any treatments. However, some wines in this work fined

217 with casein and not given any other treatment did show low positive values, up to 1.4 mg/L. Therefore,
218 wines that have been fined with casein and not filtered may not automatically have negative signals.
219 These wines should be tested, as they might need to carry a label declaration. Even though the medical
220 results presented were without clinical reaction, the sample size of patients was limited and every human
221 being might react differently than another when it comes to allergic reactions. In conclusion of this
222 study, when appropriate practices, i.e. filtration of 0.45µm are followed for wines fined with casein,
223 there should be no detectable protein (LOD 0.25 mg/L) from the allergen left behind in the wine.

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304 **Tables**

305 **Table 1 Separation treatments after fining**

Treatment	Properties	Commercial name
Filtration	Filter pads - Ø 0,45µm	“SEITZ EK” Cellulose (size 200x200 mm) ⁽¹⁾
	Membrane cartridge -Ø 0,45µm	“SEITZ-MEMBRACart” type 419A, grade B (Beverage version) ⁽¹⁾
	Cross-flow - one module – defined within microfiltration rates	“Sartflow Compact” pump: 15-20 m ³ /h against 2.5 bars ⁽²⁾
	Diatomaceous earth -fine kieselgur	Fine-Kieselgur ⁽¹⁾
Separation	Centrifuge – 7500 rpm (1500 L/h against 3,5 bar)	“Tellerseparator SB14” 7500 rpm at 1500 L/h against 3.5 bars ⁽³⁾
Heat	Flash-pasteurisation – 20s at 72°C	Plate heat exchanger
Fining	Bentonite – Mix (Na ⁺ Ca ²⁺) 200g/hL (12 hours swelling in water)	“Aktivit [®] ” ⁽⁴⁾
	Silica sol - (SiO ₂) 50ml/hL	“Klar-Sol 30 [®] ” ⁽⁴⁾

⁽¹⁾Pall Corporation ⁽²⁾Sartorius ⁽³⁾Westphalia ⁽⁴⁾Erbisloeh

306

307 **Table 2 Casein (Kal-Casin) residues by ELISA on white wine**

Casein (Kal-Casin) residues ELISA measurements [mg/L] of white wine Mueller-Thurgau			
Casein (Kal-Casin) added	Sample 1 After fining	Sample 2 After Treatment	Sample 3 After EK*
Diatomaceous earth			
400 mg/L	LOD < x < LLOQ	< LOD	< LOD
800 mg/L	0.9 (5.7) ¹	< LOD	< LOD
Non-fined wine	< LOD	< LOD	< LOD
Centrifuge			
400 mg/L	< LOD	< LOD	< LOD
800 mg/L	0.3 (10.5) ¹	< LOD	< LOD
Non-fined wine	< LOD	< LOD	< LOD
Flash-Pasteurisation			
400 mg/L	< LOD	2.83 (20.2) ¹	< LOD
800 mg/L	LOD < x < LLOQ	9.48 (36.2) ¹	< LOD
Non-fined wine	< LOD	< LOD	< LOD
Silica sol			
400 mg/L	LOD < x < LLOQ	< LOD	< LOD
800 mg/L	1.4 (43.8) ¹	LOD < x < LLOQ	< LOD
Non-fined wine	< LOD	< LOD	< LOD
Bentonite			
400 mg/L	0.4 (5.6) ¹	< LOD	< LOD
800 mg/L	LOD < x < LLOQ	< LOD	< LOD
Non-fined wine	< LOD	< LOD	< LOD

EK*: Sterile filtration- Ø 0.45 µm; LOD Limit of detection. LLOQ Lowest Limit Of Quantification

¹Coefficient of variation (% n=9)

308

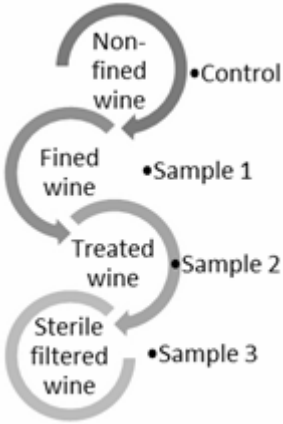
309 **Table 3 Casein (SensoVin) residues by ELISA on white wine**

Casein (SensoVin) residues ELISA measurements [mg/L] of white wine Mueller-Thurgau			
SensoVin added	Sample 1 After fining	Sample 2 After Treatment	Sample 3 After EK*
Flash-Pasteurisation			
500 mg/L	< LOD	1.4 (10.8) ¹	< LOD
1000 mg/L	< LOD	1.4 (43.9) ¹	< LOD
Non-fined wine	< LOD	< LOD	< LOD
Silica sol			
500 mg/L	0.5 (41,7) ¹	< LOD	< LOD
1000 mg/L	1.8 (40,8) ¹	0.6 (61.5) ¹	< LOD
Non-fined wine	< LOD	< LOD	< LOD
Bentonite			
500 mg/L	1.4 (37,7) ¹	0.1 (18.7) ¹	< LOD
1000 mg/L	0.3 (7,3) ¹	0.1 (13.3) ¹	< LOD
Non-fined wine	< LOD	< LOD	< LOD

EK*: Sterile filtration- Ø 0.45 µm; LOD Limit of Detection. ¹Coefficient of variation (% n=9)

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311 **Figures**

312 **Figure 1 Sampling chart**



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