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Determination of the Bovine Food Allergen Casein in White Wines by Quantitative Indirect ELISA, SDS−PAGE, Western Blot and Immunostaining

WEBER PATRICK, STEINHART HANS, AND PASCHKE ANGELIKA*

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

This study describes the characterization of allergic bovine casein and caseinate fining agents by SDS−PAGE analysis and the development of a quantitative indirect ELISA for the detection of these substances in wines. The ELISA was applied to various experimental wines that were treated with different caseinate dosages and went through different processing steps and to a panel of commercial wines. Positive results were assured by SDS−PAGE, Western blot, and immunostaining. Comprehensive literature research was done to evaluate the demanded sensitivity of the ELISA. The results showed that α- and β-caseins remain in some wines and are detectable. Estimated amounts were in the range or below an estimated no-observed adverse effect level (NOAEL) of 0.9 mg/L, but it was concluded that there is still an uncertainty about this NOAEL. Additional applied processing, referring to bentonite treatment and successive filtration, was determined to contribute to a significant decrease of casein residues in wines.

KEYWORDS: Wine; fining agents; casein; caseinate; alpha lactalbumin; beta lactoglobulin; bentonite; allergy; ELISA; SDS−PAGE; Western blot; LOAEL; NOAEL; immunoassay

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INTRODUCTION

Casein and caseinates derived from bovine milk are traditionally used in wine production in optimizing organoleptic properties due to the removal of phenolic compounds, such as tannins. Phenolic compounds are bound by noncovalent interactions, mainly hydrogen bonds, forming insoluble phenol−casein complexes. These complexes are removed in subsequent filtration and/or decantation steps.

Caseins are known as major food allergens of bovine milk (*1*, *2*). Thus, they are affected by Directive 2000/13/EC last amended by Directive 2007/68/EC of the European Community. According to this Directive, "any substance used in production of a foodstuff and still present in the finished product" must be labeled when it originates from an allergic material specified in Annex IIIa. Similar regulations have been established in nations such as Australia, New Zealand, Japan, and the USA. However, no clear evidence exists about the presence of casein residues in finished wines that are able to trigger allergic reactions. Caseins are nearly insoluble at the pH of wine and they form insoluble complexes with phenolic compounds from grapes as described earlier. Thus, caseins are considered to be almost completely coagulated and sedimented (*3*). Rolland et al. found that one adult male allergic to bovine milk repeatedly reacted with mild subjective symptoms to a wine that had been fined with milk proteins in a double-blind placebo-controlled food challenge (DBPCFC) (*4*). However, no signs of an objective clinical reaction were noted on either occasion. Two *in vitro* studies investigated various white wines fined with different amounts and preparations of caseinate by competitive enzyme-linked immunosorbent assay (ELISA) sensitive to total caseinate. No residues of caseinate were detected in these studies (*3*, *5*). However, limits of detection were achieved between 1 and 3 mg/L if the required 10-fold wine dilution is considered. This means that the assay's sensitivity appears improvable since published dosages of milk proteins that induce allergic reactions are reported in the range of a few milligrams or even lower (*6*, *7*). Rolland et al. applied a sensitive sandwich ELISA (sw-ELISA) and found no detectable amounts of α-casein in 75 commercial wines fined either with bovine milk or casein (*8*). Inconsistently, Lifrani et al. revealed positive results for casein in 13 of 400 commercial wines using a sw-ELISA (*9*). No quantitative information was given by Lifrani et al. Also, neither sw-ELISA study presented a method validation. Thus, it still remains unclear how sensitive and accurate these methods worked in the investigated wine matrices. Furthermore, neither study presented data about the water-soluble whey proteins which may have been present in casein isolates used for wine fining. α-Lactalbumin (ALA) and β-lactoglobulin (BLG) both together account for more than 70% of the whey protein fraction and are known as major milk allergens (*10*, *11*). Both allergens could possibly be present as impurities in casein preparations prepared for technical purposes such as the fining of wines. The introduction of these whey proteins into the wine could also present a risk to milk-sensitive humans.

After all, the demand for further sensitive and reliable data regarding residual casein and possible whey proteins in wines still

*Inquiries should be addressed to this author. Phone: 0049 40 42838 4353. Fax: 0049 40 42838 4342. E-mail: angelika.paschke@uni-hamburg.de.

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remains. Thus, a sensitive quantitative indirect ELISA for the detection of total casein and casein derived fining agents was developed. A comprehensive literature research was done to evaluate the demanded sensitivity for this assay by gathering and interpreting known lowest-observed adverse effect levels (LOAELs) of milk proteins in adults allergic to milk. A panel of various white wines fined with different caseinate dosages and preparations was investigated by this ELISA, in addition to a selection of 61 commercial European wines with unknown fining. Additionally, an important technological processing step was considered to have a significant influence on casein residues in wine as demonstrated earlier for other proteins: the bentonite fining (*12*, *13*).

MATERIALS AND METHODS

Literature Research. Publications presenting LOAELs for milk proteins in adults allergic to cow's milk were identified between July and September 2008. SciFinder discovery tool (American Chemical Society, Washington, DC) was used to extract respective publications from the MEDLINE and CAS/CAPLUS databases using 27 keywords or phrases specific for milk allergies in adults. Publications including any type of oral challenge that described LOAELs were thoroughly evaluated in the establishment of a general LOAEL.

Casein and Caseinate Samples. A total of three bovine casein and casein derived products were used in this study. One whole casein product with a protein content of 89% was purchased from Merck (Darmstadt, Germany) as a white powder. Two potassium caseinates (protein contents 77% and 76% respectively) were purchased as white powders from two different fining agent suppliers located in Germany. Potassium caseinate is derived from casein by dissolving in aqueous potassium hydroxide and spray drying. It is used in wine production rather than casein due to its higher solubility in the wine matrix.

Wine Samples. Four different and well characterized German wines were prepared in cooperation with the Dienstleistungszentrum Laendlicher Raum (Mosel, Germany): Riesling Mosel, Riesling Rheingau, Pinot blanc Pfalz, and Pinot gris Baden. These wines were treated with either potassium caseinate 1 or potassium caseinate 2 according to a previously published protocol (*3*). Briefly, untreated wines were supplied from different winemakers and were treated with 6 and 30 g/hL potassium caseinate, respectively, for a period ranging between 13 and 18 days. After that, the wines were filtered, treated with bentonite, and crossflow filtrated. The bentonite dosage was determined by two industrial standard methods: Bentotest and the caloric method at 65 °C. The Bentotest method consists of a specific reagent, mainly consisting of phosphomolybdic and hydrochloric acid, which is added to the wine sample. Unstable wine proteins are detectable due to the formation of a haze caused by the cross-linkage of molybdenum ions and proteins. Afterward, small samples of the unstable wine are treated with increasing bentonite dosages until no haze is detected. The caloric method depends on the formation of a haze due to the denaturation of unstable proteins by heating to 65 °C and then cooling in the refrigerator. The bentonite dosage is determined as in the Bentotest method (*12*). Results for the applied bentonite dosages are shown in **Table 1**. Lastly, the wines were bottled through a membrane filter and sealed with a screw cap. For each wine, caseinate free control wines and wines without bentonite treatment were prepared. Wines without bentonite treatment were bottled directly after separation from the fining precipitate and, thus, lacked the final cross-flow and membrane filtration step. Apart from the fining agent dosage, the achieved wines were comparable to commercially available wines.

Polyclonal Anti-Bovine Casein Antibodies. Polyclonal antibodies for the detection of bovine casein and casein derived products were produced by Eurogentec (Seraing, Belgium) against potassium caseinate 1 in New Zealand white rabbits, which is described elsewhere (*3*).

Casein, Caseinate, and Wine Sample Preparation. Casein and potassium caseinates were soluted in 10 mM sodium carbonate and were used for indirect ELISA or SDS−PAGE analysis.

Wine samples were directly used unless otherwise stated. A 150-fold increase in concentration of wines for SDS−PAGE analysis was obtained by freeze-drying. First, 150 mL of wine was freeze-dried and the residue was dissolved in 12−15 mL of a phosphate-buffered saline (PBS), pH 7.4. The solution was then injected to a Pierce 3500 MWCO dialysis chamber (Peribo Science, Bonn, Germany) and dialyzed three times at room temperature using 1 L of bidistilled water for a total duration of 48 h. Afterward, the dialysate was freeze-dried again, dissolved in 1 mL of PBS, and centrifuged at 6500*g* for 10 min. The supernatant was stored at −80 °C.

Table 1. Bentonite Dosages Applied in the Production of Experimental Wines[a]

wine treatment	bentonite dosage (g/hL)			
	Riesling Mosel	Riesling Rheingau	Pinot blanc	Pinot gris
6 g/hL pot. caseinate 1	50	0	250	0
30 g/hL pot. caseinate 1	50	0	200	0
6 g/hL pot. caseinate 2	50	0	200	0
30 g/hL pot. caseinate 2	100	0	200	0

[a] Bentonite dosages were determined by industrial standard methods as published elsewhere (*12*); pot. caseinate = potassium caseinate.

SDS−PAGE with Silver Staining. SDS−PAGE was performed on Invitrogen equipment (Karlsruhe, Germany) with a MOPS running buffer serving as the electrolyte: 2.5 mM 4-morpholinepropanesulfonic acid (MOPS), 2.5 mM tris(hydroxymethyl)aminomethane (TRIS) base, 0.005% SDS, and 0.05 mM EDTA, pH 7.7. The proteins were separated in 1 mm thick, 8 × 8 cm, precast NuPAGE Novex gels with 12% acrylamide and Bis-Tris buffer system (Invitrogen). Serva Mark12 (Invitrogen) was used as a molecular weight marker. Electrophoresis was performed at 200 V for 55 min. Gels were silver stained according to the procedure described elsewhere by Heukeshoven et al. (*14*).

Semidry Western Blot with Immunostaining. Semidry Western blot with immunostaining was performed according to an earlier published protocol (*12*). The membranes were incubated overnight with polyclonal anti-bovine casein antibodies diluted to 1:5000 in Tris solution, and polyclonal anti-bovine whey antiserum (Sigma, Missouri) diluted to 1:10000 in Tris solution. Goat anti-rabbit horseradish peroxidase conjugated antibodies (Dako GmbH, Hamburg, Germany) diluted to 1:2000 in Tris solution were used as secondary antibodies.

Quantitative Indirect ELISA. The following solutions were prepared from analytical grade chemicals: carbonate buffer, pH 9.6, contained 75 mM Na_2CO_3 and 175 mM $NaHCO_3$ in bidistilled water. PBS-Tween20 solution, pH 7.4, was composed of 10 mM NaH_2PO_4, 70 mM Na_2HPO_4, 150 mM NaCl, and 0.5% Tween 20, all in bidistilled water. Washing solution and substrate solution were prepared as described elsewhere (*3*).

For the indirect ELISA, 200 μL/well of the sample (diluted in carbonate buffer) was coated to a certified Maxisorp F96 polystyrene microtiter plate (Nunc, Wiesbaden, Germany) overnight at 8 °C. Wine samples were diluted 10-fold with the carbonate buffer. The plate was then washed with washing solution and free binding sites of the wells were blocked with the washing solution for two hours at room temperature. After washing with PBS-Tween20 solution, polyclonal anti-bovine casein antibodies (diluted 1:12000 in PBS-Tween20) were added and incubated for 1 h at room temperature. The plate was washed with PBS-Tween20 solution, and goat anti-rabbit horseradish peroxidase conjugated antibody solution (Dako GmbH, Hamburg, Germany) diluted to 1:2000 in PBS-Tween20 was added and incubated for another 1 h at room temperature. Substrate solution was added after a final washing step with the PBS-Tween20, and the enzymatic color reaction was performed in the dark for 15 min at 8 °C. The reaction was stopped by the addition of 2 M sulfuric acid. The optical density (OD) values were measured at 450 nm, with a reference wavelength of 630 nm, using a MRX microtiter plate reader (Dynex Technologies, Chantilly, VA).

The attained curves were evaluated by AssayZap Software (Biosoft, Cambridge, U.K.) using a 4-parametric regression. Outliers were eliminated by the Nalimov-Test (P = 95%). The limit of decision (LODC) was calculated from the blank values (B_0) plus 3-fold of the standard deviation (s) of the blank values: LODC = $B_0 + 3s(B_0)$. The limit of detection (LOD) was calculated from the blank values (B_0) plus 6-fold of the standard deviation (s) of the blank values: LOD = $B_0 + 6s(B_0)$ (*15*, *16*). All

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Table 2. Publications Available in MEDLINE and CAPLUS Databases That Present Reactive Dosages of Cow's Milk Allergens in Sensitized Adults[a]

author	year	no. of probands	age (years)	reactive dosage	LOAEL	equiv amt of total milk proteins[b]	equiv amt of caseins[b]	type of study
Bernstein et al. (*17*)	1982	2	28−29	14.1 g of milk powder (OR)	yes	3.5 g	3 g	DBFC
Carroccio et al.(*18*)	2006	4	30−52	5 g of whey (OR)	no	600 mg		DBPCFC
Kanny et al. (*19*)	1998	1	19	55 mg of bovine serum albumin (OR)	yes			DBPCFC
Koppelman et al. (*20*)	1999	1	30	10−50 mg of casein (OR)	no		10−50 mg	
Lam et al. (*6*)	2008	10	17−68	0.3 mg of low-fat milk powder (SR)	yes	0.1 mg	0.1 mg	DBPCFC
				300 mg of low-fat milk powder (OR)	yes	105 mg	90 mg	DBPCFC
Loveless et al. (*21*)	1950	6	>18	45 mL milk (OR)	no	1.6 g	1.35 g	DBPCFC
Norgaard et al. (*22*)	1992	11	29−44	5 g of milk (SR)	yes	180 mg	150 mg	DBPCFC
				50 g of milk (OR)	yes	1800 mg	1500 mg	DBPCFC
Olalde et al. (*23*)	1989	1	29	250 mg of casein (OR)	yes		250 mg	SBPCFC
Pastorello et al. (*24*)	1989	3	20−40	500 mg of milk powder (OR)	yes	125 mg	108 mg	DBPCFC
Sexto et al. (*25*)	1998	1	18	50 mL of milk (OR)	no	1.8 g	1.5 g	SBPCFC
Traencker et al. (*26*)	1993	1	17	200 mL of milk (OR)	no	7.2 g	6 g	DBPCFC
Wüthrich et al. (*27*)	1987	1	39	3.5 μL of milk (OR)	yes	130 μg	100 μg	OC
Wüthrich et al. (*28*)	1986	3	23−35	200 μL of milk (OR)	yes	7.2 mg	6 mg	OC

[a] DBFC = double-blinded food challenge; OR = objective allergic reaction; SR = subjective allergic reaction; OC = probable open challenge. [b] Calculations are based on a total protein content of 3.6% and a casein content of 3% in bovine milk.

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experiments were performed in triplicate unless otherwise stated and each positively tested wine sample was repeatedly assayed in quintuple to ensure reproducibility. Intraassay variations were calculated from six determinations on the same microtiter plate, whereas interassay variations were calculated from one determination on six separate microtiter plates.

RESULTS

Literature Research. In total, over 200 publications matched our search criteria. Information about reactive dosages of milk allergens in adults were found in 13 manuscripts (**Table 2**). Among these 13 manuscripts, eight publications presented LOAELs: Bernstein et al. observed a LOAEL of 14.1 g of milk powder in two adult females by DBPCFC (*17*). Serious doubts about the accuracy of these results are present due to the occurrence of delayed allergic reactions and to the inconsistent application of placebos. Kanny et al. found a young woman that reacted to a LOAEL of 55 mg of bovine serum albumin (*19*). Unfortunately, this was the lowest dose used in the DBPCFC. Thus, objective allergic reactions to lower dosages cannot be excluded. A more recent DBPCFC in adults allergic to milk proteins was published by Lam et al. in 2008 (*6*). Subjective allergic reactions were observed within a group of ten allergic persons at a LOAEL of 0.3 mg of low-fat milk powder. Unfortunately, this was the lowest dose used in the DBPCFC. Thus, subjective allergic reactions to lower dosages also cannot be excluded. Objective reactions were observed at a LOAEL of 300 mg of low-fat milk powder, corresponding to approximately 105 mg of milk proteins or 90 mg of casein. Norgaard et al. attained a LOAEL of 5 g of whole milk in their DBPCFC with three adults allergic to cow's milk (*22*). This LOAEL triggered subjective reactions while a dosage of 50 g of whole milk was necessary to trigger objective allergic reactions. A considerably lower LOAEL was discovered by Olalde et al. in a case study using a single-blind placebo-controlled food challenge (SBPCFC) (*23*). The adult reacted to a dosage of 250 mg of casein with objective allergic reactions. Pastorello et al. investigated three adults allergic to cow's milk in a DBPCFC and that resulted in a LOAEL of 500 mg of milk powder (*24*). Unfortunately, this was the lowest administered dose and, with that, objective allergic reactions to lower dosages cannot be excluded. Certainly the smallest LOAEL was found by Wüthrich et al. in two studies with four women allergic to cow's milk (*27*, *28*). A LOAEL with objective allergic reactions was observed in a 39 year old woman after the consumption of 3.5 μL of whole milk on the basis of an oral desensitization.

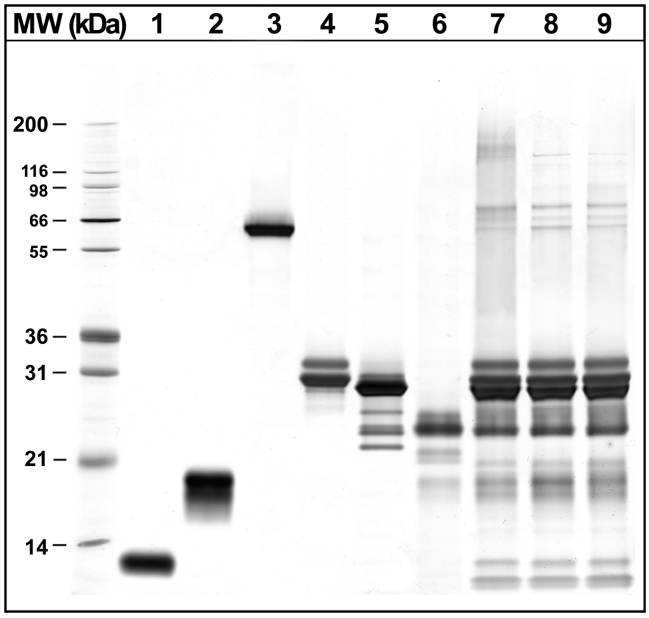

Figure 1. SDS−PAGE silver staining of various milk proteins and casein/caseinate samples. MW = molecular weight; 1 = ALA; 2 = BLG; 3 = bovine serum albumin; 4 = α-casein; 5 = β-casein; 6 = κ-casein; 7 = whole casein; 8 = potassium caseinate 1; 9 = potassium caseinate 2.

However, the oral desensitization indicated a nonblinded oral allergen administration, making this finding untrustworthy.

In conclusion, the most reliable LOAEL was demonstrated by Lam et al. (*6*).

Characterization of Casein and Caseinate Samples by SDS−PAGE. The casein and caseinate samples revealed identical protein compositions in SDS−PAGE analysis as illustrated in **Figure 1**. The predominant proteins were identified as α- and β-caseins. Major protein bands corresponded to κ-casein and BLG, whereas slight protein bands could be assigned to residues of ALA and bovine serum albumin in all three preparations.

Quantitative Indirect ELISA. The indirect ELISA revealed a sigmoidal curve in the range of 0.001−10 mg/L caseinate. No significant differences in antigenicity were found among the three casein derived products as illustrated in **Figure 2**. The LODC was calculated as 0.5 ppb whereas the LOD was calculated as 5 ppb. Intraassay variations were in the range of 0.4−11.5% and

interassay variations between 5.8 and 13.6%, within the range of 0.01−10 mg/L. The sensitivity to whey proteins was determined with a LODC of 0.8 mg/L (BLG) and 2.4 mg/L (ALA), whereas the LOD was identified as 1.8 mg/L (BLG) and 5.2 mg/L (ALA). The assay's sensitivity and accuracy for caseinate in white wine matrices were evaluated by six different wines (**Table 3**). LODCs were determined as 0.2−0.5 mg/L and LODs were 0.2 − 0.6 mg/L, if the 10-fold wine dilution had been considered. Recovery rates were between 68 and 111% and variation coefficients of the recovered caseinates from white wines were between 3.4 and 14.8%, within the steepest curved area of 0.2−2 mg/L. Thus, the lower limit of quantitation (LOQ) was defined as 0.2 mg/L and the upper LOQ as 2 mg/L. This corresponded to values of 2 and 20 mg/L, respectively, if the 10-fold wine dilution had been implemented.

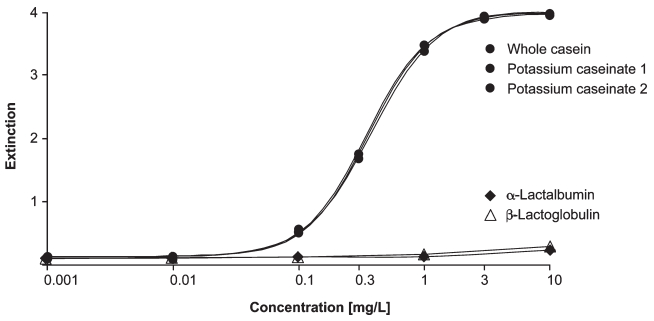

Figure 2. Indirect ELISA curves for α-lactalbumin, β-lactoglobulin, and casein/caseinate samples.

Table 3. LODC, LOD, and Recovery Rates for Caseinate in Various White Wines Investigated by Indirect ELISA

	LODC [mg/L]	LOD [mg/L]	recovery rates [%] between 0.2 and 2 mg/L
Riesling Mosel with bentonite	0.3	0.3	93−102
Riesling Rheinghau with bentonite	0.3	0.3	88−111
Pinot blanc with bentonite	0.3	0.4	102−107
Pinot blanc w/o bentonite	0.5	0.6	68−105
Pinot gris with bentonite	0.2	0.2	87−90
Pinot gris w/o bentonite	0.3	0.3	79−81

Investigations of Wines Fined with Caseinates. A total of 93 wines were investigated: 32 experimental and 61 commercial wines. Two of the 32 experimental wines were found to contain traces of caseinate. Both wines were from the Riesling Rheingau grape variety without the bentonite treatment, and had been fined with 30 g/hL of both possible caseinate preparations. ELISA signals were significantly greater than the LOD, but lower than the LOQ. Thus, caseinate traces were assumed to be greater than 0.2 mg/L, but explicitly lower than 2 mg/L in the undiluted wines. Estimated contents of caseinate traces were in the range of 0.2 mg/L. No traces were found in wines fined with 6 g/hL caseinate without the bentonite treatment or in wines fined with 6 and 30 g/hL caseinate with the bentonite treatment.

Other than casein, the positive findings in two experimental Riesling Rheingau wines may have been triggered as a result of the low but present sensitivity of the assay to whey proteins, particularly BLG. Therefore, both positive Riesling Rheingau wines tested were 150-fold concentrated and investigated by both SDS−PAGE and semidry Western Blot analysis. SDS−PAGE showed two bands that may correspond to α- and β-casein (**Figure 3 A**). No suspecting bands were visible for BLG. This finding was confirmed by semidry Western blot analysis and immunostaining both with antibodies specific to whey proteins and obviously also to caseins and antibodies very specific to caseins (**Figure 3 B**,**C**). Clear immunostainings were obtained within the realm of α- and β-casein for the casein-treated wines, while the staining occurred neither in the untreated control wine nor in the range of ALA and BLG. Thus, whey proteins could be excluded as a reason for positive ELISA results. Due to this analysis, positive ELISA values were triggered by α- and β-casein.

Investigation of commercial wines. Three of 61 commercial wines (4.9%) revealed casein or caseinate traces in the indirect ELISA test: one French Chardonnay, one Italian Pinot gris and one German Bacchus wine. All of these three wines indicated amounts significantly greater than the LOD, but lower than the LOQ. Thus, caseinate traces were assumed to be greater than 0.2 mg/L but explicitly lower than 2 mg/L in the undiluted commercial wines. Estimated contents of caseinate traces were up to 0.4 mg/L.

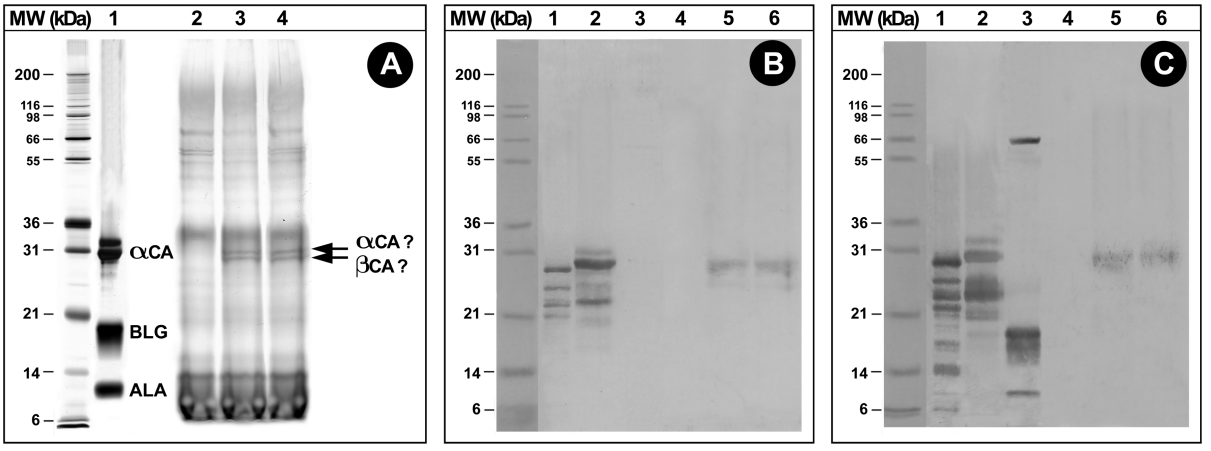

Figure 3. SDS−PAGE and semidry Western blot with immunostaining of various milk proteins and 150-fold concentrated Riesling Rheingau w/o benonite treatment. (**A**) SDS−PAGE with silver staining. (**B**) Immunostaining with anti-bovine casein antibodies. (**C**) Immunostaining with anti-bovine whey antiserum. MW = molecular weight. **A**: 1 = ALA, BLG, α-casein; 2 = Riesling Rheingau w/o bentonite, untreated control, 150-fold concentrated; 3 = Riesling Rheingau w/o bentonite, 30 g/hL potassium caseinate 1, 150-fold concentrated; 4 = Riesling Rheingau w/o bentonite, 30 g/hL potassium caseinate 2, 150-fold concentrated; αca = α-casein; βca = β-casein. **B** and **C**: 1 = α-casein; 2 = β-casein; 3 = ALA, BLG and bovine serum albumin; 4 = Riesling Rheingau w/o bentonite, untreated control, 150-fold concentrated; 5 = Riesling Rheingau w/o bentonite, 30 g/hL potassium caseinate 1, 150-fold concentrated; 6 = Riesling Rheingau w/o bentonite, 30 g/hL potassium caseinate 2, 150-fold concentrated.

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DISCUSSION

This study demonstrated that α- and β-caseins can remain in casein-treated wines and, in some cases, are detected by an indirect ELISA. Whey proteins seem to be of no importance to wine allergy matter. The evaluation of the allergic potential of those residues would demand an establishment of a threshold were no objective allergic reactions occur in the most sensitive individuals. This threshold is commonly defined as a no observed adverse effect level (NOAEL) and could be derived by clinical studies from individuals allergic to milk proteins (*7*).

A comprehensive literature research about the known thresholds for objective allergic reactions present in adults sensitized to bovine milk proteins revealed only three meaningful studies with a total of $n = 22$ investigated individuals: the studies of Lam et al. (*6*), Norgaard et al. (*22*) and Olalde et al. (*23*). Thereof, Lam et al. found the lowest LOAEL of 300 mg of low-fat milk powder which corresponded to approximately 105 mg of milk protein or 90 mg of casein (*6*). The other five studies were not considered to present reliable LOAEL data because of delayed allergic reactions and an unclear placebo usage (*17*), reactions to the lowest administered dosage (*19*, *24*), or nonblinded test conditions (*27*, *28*). Based on the LOAEL of 90 mg of casein, we attempted to estimate a suitable NOAEL for casein. An uncertainty factor of 10 was used to consider interindividual differences as discussed by Taylor et al. (*7*). Implementing an additional uncertainty factor of 10 to extrapolate from LOAEL to NOAEL, the total uncertainty factor of 100 would lead to a NOAEL of 0.9 mg of casein based on the data from the three previously mentioned studies. These findings are in accordance with other authors since the thresholds for allergenic reactions are usually considered in the lower milligram range (*7*, *29*).

To assess a representative value of the daily wine consumption in the adult population, two surveys were analyzed. According to the "Vienna Health and Social Survey" released in 2001 by the Vienna Health Reporting, about 99.2% of the male adult and 99.9% of the female adult Viennese population consume less than 500 mL of wine per day, or 0.8% of the male and 0.1% of the female populations consume more than 500 mL of wine per day. The highest rate was found among the group of 60−74 year old inhabitants, whereof 2.2% of the male and 0.4% of the female adult population consumed more than 500 mL of wine per day. According to the "National Nutrition Survey: Foods Eaten" from 1995 and released by the Australian Bureau of Statistics, 79 mL of wine were consumed daily by the average Australian population, whereas the highest average daily consumption of 312 mL was achieved among the population of 45−64 year old citizens (*8*). Both reports did not collect data about the maximal daily wine consumption, which is the most important value for a threshold estimation. Based on both surveys, a volume of 1 L wine was suggested as a good approximation to such a value. This volume has also been used by Roland et al. (*8*). However, the limited data pool for this volume suggestion must be noticed.

Putting both findings together, the estimated NOAEL and the suggested maximum average daily wine consumption, a NOAEL of 0.9 mg/L wine (= 0.9 mg/L) was estimated as a demand for an analytical system in order to evaluate the risk for allergic reactions, based on current knowledge, after consumption of casein-containing wines. Casein contents in wines were estimated at approximately 0.4 mg/L or lower. Considering the uncertainty of this estimation, casein contents may be in the range or even lower than the estimated NOAEL of 0.9 mg/L. This finding seems to be supported by the survey of Rolland et al., reporting repeatedly mild subjective symptoms after the consumption of a milk-treated wine by a 19 year old male who was the only adult allergic to bovine milk in this survey (*4*). However, it must be noticed that the establishment of a representative NOAEL, based on LOAEL data, is currently limited. Reliable LOAEL data for casein is based merely on 22 individuals, whereof 21 were tested with whole milk protein. Thus, it is not clear whether the observed LOAEL was due to the presence of casein or whey proteins. Moreover, more information is needed regarding the maximal daily wine consumption and also no consensus is current yet about the extent of applicable uncertainty factors. Though this is one of the first efforts to establish a NOAEL for a food allergen based on available clinical data, it must be consequently noticed that this NOAEL of 0.9 mg/L casein is no representation of any common applicable value. The demand for further clinical studies and assessment of any applicable uncertainty factors is greatly appreciated as discussed earlier (*7*, *30*).

This study indicates that the detection of casein in casein treated wines does not necessarily occur. Indeed, it was previously demonstrated that residues of other allergic fining agents and stabilizers, such as isinglass, fish gelatin, egg, or lysozyme, remain in wines in amounts of up to 1 mg/L (*3*, *8*, *9*, *12*, *13*), and are suspected to present, under some circumstances, risks for allergic individuals (*12*). At the same time, significant impacts were found in certain optional processing steps with regard to the amounts of these allergic substances in wines. In this study, the treatment with bentonite and the successive cross-flow and membrane filtration led to no detectable residues of caseins in experimental wines. Successive filtration appears important since Riesling Rheingau with bentonite was found to contain no detectable casein, despite the fact that no bentonite requirement was found (see **Table 1**) and that this same wine w/o bentonite was tested positive. Thus, both Riesling Rheingau with bentonite and Riesling Rheingau w/o bentonite only differ in the final applied cross-flow and membrane filtration steps. The significant impact of the bentonite treatment was confirmed as well by other studies (*12*, *13*). Likewise, the treatment of wines with metatartaric acid was found to have a significant impact in the amount of lysozyme present (*31*). Thus, it seems reasonable to consider various additional applied processing materials, such as bentonite, metatartaric acid, charcoal or diatomite, and certain filtration steps in the risk evaluation. Some of these steps seem to have the potential of lowering the amount of allergic substances and, thus, avoiding the risk for allergic persons and the labeling of wines as legally regulated in several nations.

As mentioned in the Introduction, there are currently four other *in vitro* studies available that deal with the residues of casein in wines. No detectable residues of caseins were found in various wines by Weber et al. (*3*, *5*) and Rolland et al. (*8*), while Lifrani et al. was able to detect casein in 13 of 400 commercial wines (*9*). Positive results achieved by Lifrani et al. seem to be supported by this study, although the accuracy of their sw-ELISA in wine matrices still remains unclear. According to the studies of Weber et al. (*3*, *5*), the higher LODs between 1−3 mg/L obtained by a competitive ELISA seem to be a suitable explanation for their different findings. Rolland et al. applied dialysis and protein precipitation for sample preparations and a sensitive sw-ELISA for the detection of casein residues in wines (*8*). This method seemed not validated by spiking experiments of the wine matrix. Furthermore, the antibodies specific to α- but not to β-casein may have contributed to the negative results. We found dialysis and protein precipitation not suitable for the quantitative analysis of fining agents in wines mostly due to protein adsorption of the dialysis membrane and to the incomplete protein precipitation by hydrophilic organic solvents, such as methanol (results not shown). A 10-fold wine dilution was applied in this study for an acceptable recovery in all tested wine matrices. However, matrix

effects led to a 1000-fold decrease in the assay's sensitivity (from 0.5 μg/L to 0.2−0.5 mg/L). Thus, it is demonstrated that a method validation is essential for an accurate method development.

In conclusion, α- and β-caseins are present in wines and are detectable by indirect ELISA. Estimated amounts were found to be in the range or below an estimated NOAEL of 0.9 mg/L. The NOAEL was estimated by currently available clinical and statistical information, but it was concluded that there is still an uncertainty about this value. Thus, according to this work, allergic reactions due to consumption of casein treated wines cannot fully be excluded. Bentonite, an additional processing material, along with successive cross-flow and membrane filtration, was identified to contribute to a significant decrease of casein residues in wines and other processing materials are suspected to have a similar impact. However, current knowledge does not allow any definite conclusions concerning the decrease or elimination of the allergic potential by the use of these materials.

ABBREVATIONS USED

ALA, α-lactalbumin; B_0, blank value; BLG, β-lactoglobulin; DBPCFC, double-blind placebo-controlled food challenge; ELISA, enzyme-linked immunosorbent assay; LOAEL, lowest-observed adverse effect level; LODC, limit of decision; LOD, limit of detection; LOQ, limit of quantitation; NOAEL, no observed adverse effect level; PBS, phosphate-buffered saline; *s*, standard deviation; SBPCFC, single-blind placebo-controlled food challenge; sw-ELISA, sandwich ELISA.

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