JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Specific and Sensitive Enzyme-Linked Immunosorbent Assays for Analysis of Residual Allergenic Food Proteins in Commercial Bottled Wine Fined with Egg White, Milk, and Nongrape-Derived Tannins

JENNIFER M. ROLLAND,*,† EFFIE APOSTOLOU,†,‡ MARIA P. DE LEON,†,‡ CREINA S. STOCKLEY,§ AND ROBYN E. O'HEHIR†,‡

Department of Immunology, Monash University, and Department of Allergy, Immunology and Respiratory Medicine, The Alfred, Commercial Road, Melbourne, Victoria, Australia 3004, and The Australian Wine Research Institute, Adelaide, Australia

Regulations introduced by the Food Standards Australia New Zealand in December 2002 require all wine and wine product labels in Australia to identify the presence of a processing aid, additive or other ingredient, which is known to be a potential allergen. The objective of this study was to establish sensitive assays to detect and measure allergenic proteins from commonly used processing aids in final bottled wine. Sensitive and specific enzyme-linked immunosorbent assays (ELISA) were developed and established for the proteins casein, ovalbumin, and peanut. Lower limits of detection of these proteins were 8, 1, and 8 ng/mL, respectively. A panel of 153 commercially available bottled Australian wines were tested by these ELISA, and except for two red wines known to contain added whole eggs, residuals of these food allergens were not detected in any wine. These findings are consistent with a lack of residual potentially allergenic egg-, milk-, or nut-derived processing aids in final bottled wine produced in Australia according to good manufacturing practice at a concentration that could cause an adverse reaction in egg, milk, or peanut/tree-nut allergic adult consumers.

KEYWORDS: Wine; allergen; ELISA; processing aid; food allergy; ovalbumin; casein; peanut

INTRODUCTION

Food-related allergies affect 1–2% of the adult population, but the prevalence is higher in young children (6–8%) (*1*). The inclusion of potential food allergens in ingredient information on food packaging has been inconsistent, increasing the risk of exposure and adverse reaction in food allergic consumers. In 1993, in order to facilitate the development of international labeling regulations on food allergens, the FAO/WHO Codex Alimentarius Commission (Codex) developed a list of the eight ingredients that cause 90% of food-related allergic reactions. The list comprises the following: cereals and their products (wheat, rye, barley, oats, and spelt and their hybridized strains); crustacea and their products; eggs and egg products; fish and fish products; peanuts and soybeans and their products; milk and milk products; tree nuts and sesame seeds and their products; and added sulfites in concentrations of 10 mg/kg or more. Codex subsequently recommended government legislation for mandatory labeling of packaged food containing allergenic foods and their products.

In Australia, labeling regulations for potential allergens in food were introduced in December 2002 by Food Standards Australia New Zealand (FSANZ). These regulations apply also to wine since several potentially allergenic food proteins may be used as fining agents or processing aids in the manufacture of wine. Egg white is generally used to remove tannins from red wine; while milk proteins and the fish swim bladder collagen, isinglass, are used to remove phenolic and tannin compounds from white wine. The new regulations mean that, in addition to labeling for sulfur dioxide, wine and wine product labels must now identify manufacture with the processing aids casein and potassium caseinate, egg white, isinglass, milk, and evaporated milk where there is detectable residual allergenic food proteins. Several allergenic proteins are found in egg white [ovalbumin, ovomucoid, ovotransferrin, and lysozyme (*2–5*)] and milk [casein, β-lactoglobulin, and α-lactalbumin (*6, 7*)], but the predominant allergenic proteins are ovalbumin (*8, 9*) and casein (*6, 7*), respectively. The possible importance of isinglass as an allergen is unclear. Non-grape-derived tannins are also used as processing aids with the potential for cross-

* To whom correspondence should be addressed. Tel: 613 9903 0480. Fax: 613 9903 0038. E-mail: jennifer.rolland@med.monash.edu.au.

† Department of Immunology, Monash University.

‡ Department of Allergy, Immunology and Respiratory Medicine, The Alfred.

§ The Australian Wine Research Institute.

10.1021/jf073330c CCC: $40.75
Published on Web 12/29/2007

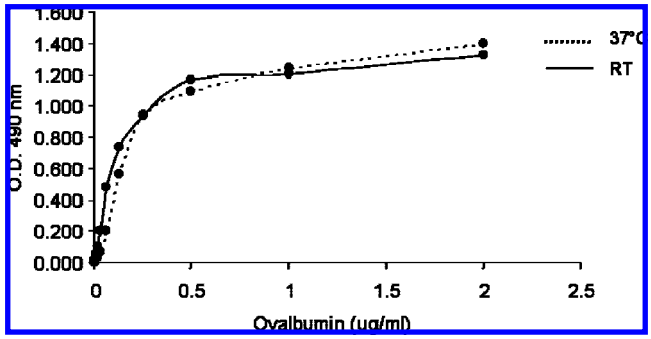

Figure 1. Effect of incubation temperature on the sensitivity of the anti-ovalbumin sandwich ELISA. ELISA plates were coated with rabbit anti-ovalbumin antibody (10 μg/mL), and the detection of ovalbumin in standard solutions was assessed after incubations were performed at either room temperature (RT) or 37 °C. Assays were performed in triplicate. The mean OD of wells containing no antigen was subtracted from the mean OD of wells containing antigen.

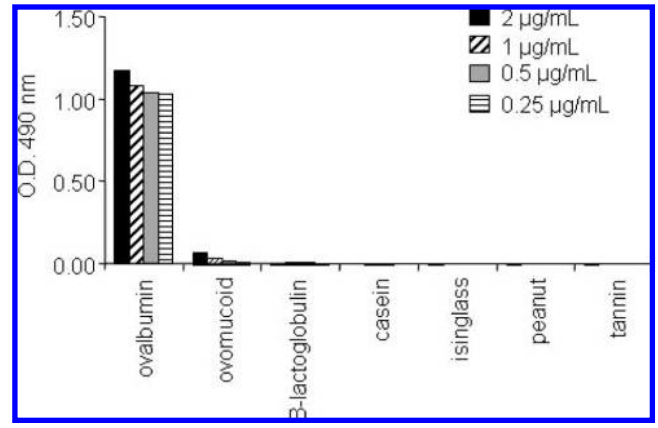

Figure 2. Specificity of anti-ovalbumin sandwich ELISA. ELISA plates were coated with rabbit anti-ovalbumin antibody and incubated with different concentrations of ovalbumin, ovomucoid, β-lactoglobulin, casein, isinglass, peanut, and non-grape tannin protein extracts. Binding of anti-ovalbumin monoclonal antibody was then assessed. The mean OD of triplicate wells containing no antigen was subtracted from the mean OD of triplicate wells containing antigen.

reactivity with tree nut and peanut allergens (*10*). However, labeling is not required where these processing aids are used in the manufacturing process but cannot be detected in the final wine.

If processing aids were used and removed according to good manufacturing practice, there should be negligible residual protein in the final product. We reported previously a double-blind, placebo-controlled clinical trial showing that wine made using egg white, isinglass, or non-grape-derived tannins present an extremely low risk of anaphylaxis to egg-, fish-, and peanut-allergic consumers, respectively (*11*). These results were consistent with negligible residual proteinaceous processing aid in the wines analyzed. However, in that study the paucity of milk allergic adults meant that firm conclusions on the presence of residual casein in wine could not be made. For wider application in the wine industry prior to labeling, suitable laboratory assays for allergenic processing aids in wine with clinically relevant sensitivity are required.

Testing of foods for allergenic proteins is usually done using enzyme-linked immunosorbent assays (ELISA), but while commercial ELISA for measuring the concentration of such proteins in food are available, detection limits are typically milligrams per liter (*12, 13*), which is generally 100-fold higher than the likely level of residual processing aid in wine (nanograms to micrograms per liter). After addition of 1–50 mg/L processing aid to the wine following fermentation, the wine is settled, decanted, and filtered prior to bottling (*14*). Although it has been suggested that commercial ELISA for ovalbumin, casein, and peanut with a detection limit of 1 mg/L have a high negative predictive value for allergic reaction (*12, 15, 16*), the threshold value for inducing an adverse reaction in an allergic individual is likely to be lower and will also be dependent on other factors including an individual's sensitivity as well as properties of the particular allergen (*17*). Weber et al. reported difficulties in performing reliable analyses of residual proteins in wines and evaluated an in-house competitive ELISA (*18*). This assay detected a low concentration (~0.01 mg/L) of egg proteins in four simulated German commercial wines, highlighting the need for further investigation of a wider panel of commercial wines for food safety.

We report here the development of specific and highly sensitive ELISA for detection of clinically important food allergens and the application of these assays to the analysis of residual proteinaceous processing aids in a panel of commercially available bottled Australian wines.

MATERIALS AND METHODS

Wine Samples. A panel of 153 commercially available bottled Australian wines was collected and coded by staff at The Australian Wine Research Institute and the Australian Wine and Brandy Corporation to ensure blind analysis by The Alfred/Monash University investigators. The panel included 149 wines manufactured using one or more proteinaceous processing aids and comprised 40 egg white-fined and 2 whole egg-added red wines, 54 milk-fined white wines, 21 casein-fined white wines, 25 non-grape tannin-added red wines, and 23 isinglass-fined white wines. Some wines were fined with more than one processing aid. In addition, two red and two white control wines were selected on the basis of manufacture in the absence of the above processing aids. While the amount of fining agent added, and the timing of additions, differed between the wines which had differing concentrations of phenolic and tannin compounds, the general range of amounts was 10−50 g/hL casein for white wine, 0.5% (w/v) skim milk for white wine, 1−2.5 g/hL isinglass for white wine, 5−15 g/hL egg white for red wine, and 3−10 g/hL tannins for red wine.

Preliminary tests showed that wine samples nonspecifically inhibited ELISA reactions at the concentrations to be used for this study, likely due to alcohol content and low pH value. After comparison of different procedures for removing inhibitory effects of the wines, the following protocols were adopted for pretreatment of wines before *in vitro* analysis. White wines were dialyzed (3.5 kDa cutoff) in SnakeSkin pleated dialysis tubing (Pierce, Rockford, IL) against phosphate-buffered saline (PBS; 150 mM NaCl, 2 mM $NaH_2PO_4 \cdot 2H_2O$, 7.5 mM Na_2HPO_4, pH 7.4) for 24 h at room temperature, with three changes of PBS. This resulted in a small (<10%) increase in volume for some white wines. The dialysis method of pretreatment was not suitable for red wines due to the formation of a sediment. Red wines were diluted 1:4 in cold ethanol and incubated overnight to precipitate the proteins in the wine. After centrifugation at 6238*g* for 10 min at 4 °C, the protein pellet was resuspended in PBS to the original volume of wine. Pretreated wines were stored frozen at −20 °C until use.

Generation of Monoclonal Antibodies. Monoclonal antibodies against the allergenic proteins were required for the development of ELISA. Commercial antibodies against ovalbumin (Sigma, St. Louis, MO) were available, but other antibodies were generated in-house according to established protocols (*19, 20*), with approval from the Alfred Medical Research and Education Precinct Animal Ethics Committee. Briefly, BALB/c mice aged 4–6 weeks were immunized by three intraperitoneal injections of either casein (100 μg/100 μL of PBS; Sigma) or peanut extract (50 μg/100 μL of PBS; extract prepared as described previously (*10*)) on days 1, 14, and 35. For the first injection, allergen solution was mixed with 100 μL of Freund's complete adjuvant (Sigma) and for the second two injections with

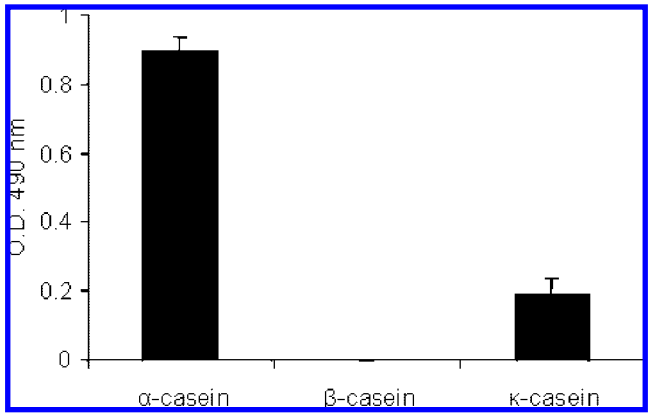

Figure 3. Specificity of anti-casein monoclonal antibody. ELISA plates were coated with α-casein, β-casein, and κ-casein (2 μg/mL) and incubated with anti-casein monoclonal antibody supernatant. Antibody binding was assessed after addition of sheep anti-mouse Ig−HRP (1:1000). Assays were performed in triplicate. The mean OD of wells containing no antigen was subtracted from the mean OD of wells containing antigen.

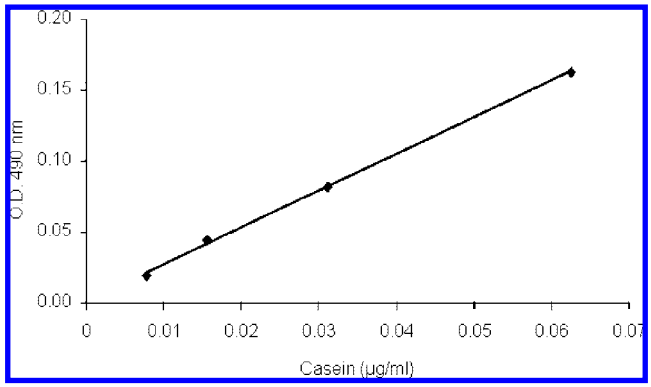

Figure 4. Standard curve for the anti-casein sandwich ELISA under optimized conditions. ELISA plates were coated with sheep anti-casein polyclonal antibody at a concentration of 0.5 μg/mL. Casein was added in the range of 0.008−0.625 μg/mL. The monoclonal antibody to α-casein was used at the optimized dilution of 1:100. Assays were performed in triplicate. The mean OD of wells containing no antigen was subtracted from the mean OD of wells containing antigen.

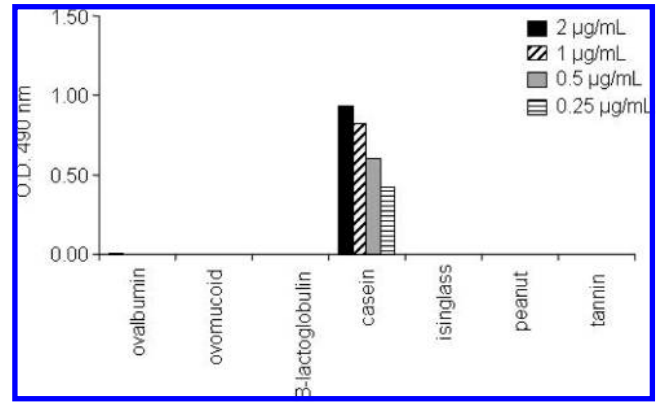

Figure 5. Specificity of anti-casein sandwich ELISA. ELISA plates were coated with sheep anti-casein antibody and incubated with different concentrations of ovalbumin, ovomucoid, β-lactoglobulin, casein, isinglass, peanut, and non-grape tannin protein extracts. Antigen binding was then assessed by binding of anti-casein monoclonal antibody. The mean OD of triplicate wells containing no antigen was subtracted from the mean OD of triplicate wells containing antigen.

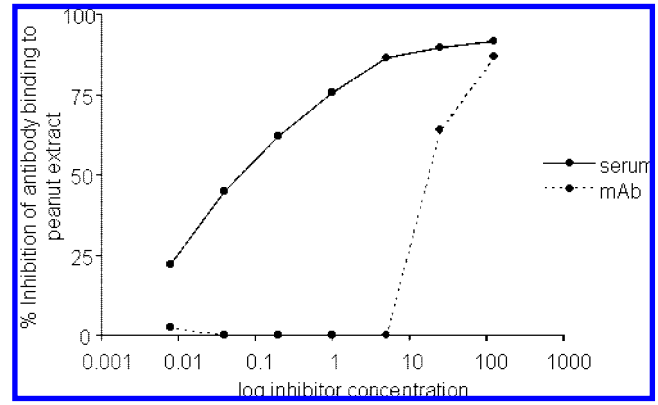

Figure 6. Comparison of the sensitivity of peanut-specific serum IgE with Ara h 1-specific monoclonal antibody using an anti-peanut inhibition ELISA. Peanut allergic donor serum or the Ara h 1-specific monoclonal antibody was preincubated with different concentrations of peanut extract and then added to ELISA plates coated with peanut (1μg/mL). Serum IgE or monoclonal antibody binding to peanut was assessed and expressed as percentage inhibition. Assays were performed in triplicate.

Freund's incomplete adjuvant (Sigma). After screening for specific antibody on day 45 as described below, mice with high antibody titers were reimmunized on day 56 with 100 μL of allergen solution without adjuvant. Mice were killed on day 60 and spleens removed aseptically. Immune mouse splenocytes were fused with cells from the murine myeloma cell line X63-Ag8.653, and hybridomas were screened by ELISA against the relevant allergen (casein or peanut). A Costar 96-well flat-bottom EIA/RIA plate (Corning, Acton, MA) was coated with 50 μL of relevant allergen solution (2 μg/mL in 0.1 M bicarbonate buffer) and incubated overnight at 4 °C. The plate was washed with 0.05% PBS−Tween and then blocked with 200 μL of 0.1% gelatin (Sigma) in PBS (PBS−gelatin) at 37 °C for 1 h. The plate was washed again, and 50 μL of the mouse serum (diluted 1:200 in PBS−gelatin) was added and incubated at 37 °C for 1 h. Washing was repeated, and 50 μL of horseradish peroxidase (HRP) conjugated sheep anti-mouse Ig polyclonal antibody (Silenus, Melbourne, Victoria, Australia) diluted 1:5000 in PBS−gelatin was added followed by incubation for 1 h at 37 °C. Washing was repeated and antibody binding detected using the substrate *o*-phenylenediamine (OPD; 5 mg/10 mL of 0.05 M phosphate citrate buffer with perborate, pH 5.0; Sigma). The reaction was stopped by adding 4 M HCl, and the optical density (OD) of each well was measured at 490 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories, Hercules, CA). Control wells included those incubated with a known immune mouse serum (positive control) and with preimmune serum from the immunized mouse (negative control).

Hybridoma cell cultures with supernatants that showed positive allergen-specific IgG antibody reactivity by ELISA were expanded with some cells taken for subcloning by limiting dilution or single cell sorting using a FACStar Plus or FACSAria flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cells found to produce specific antibody were subcloned three times to achieve monoclonality. Immunoglobulin isotyping of the hybridoma cell culture monoclonal antibodies was subsequently carried out by ELISA (Becton Dickinson) according to the manufacturer's instructions, and large-scale purification of the monoclonal antibodies from culture supernatants was performed using protein G (Pharmacia, Uppsala, Sweden) or Biosepra protein A (Invitrogen Life Technologies, Carlsbad, CA) columns, depending on the immunoglobulin isotype, following the manufacturer's instructions. The specificity of the anti-casein monoclonal antibody was further tested using the ELISA protocol outlined above where wells were coated with 2 μg/mL α-casein, β-casein, and κ-casein extract (50 μL/well; Sigma), diluted in 0.1 M bicarbonate buffer.

ELISA for Detection of Ovalbumin in Wine. A sensitive and specific antigen capture ELISA was established for ovalbumin detection in wine based on a method described previously (*21*). Following preliminary experiments to optimize antibody concentrations and incubation times the following protocol was adopted. Rabbit anti-

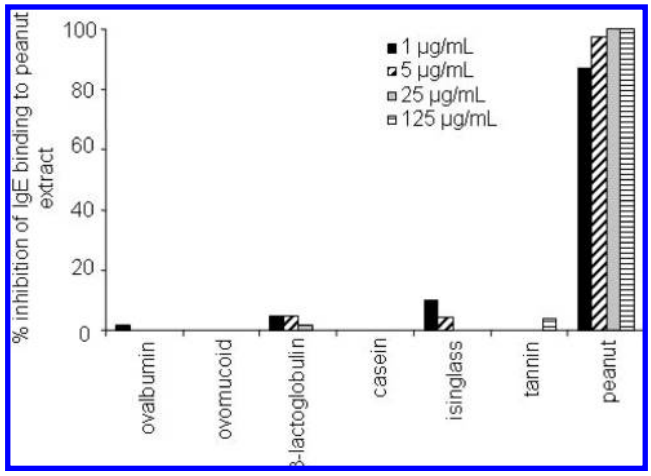

Figure 7. Specificity of anti-peanut inhibition ELISA. Peanut allergic donor serum was preincubated with different concentrations of ovalbumin, ovomucoid, β-lactoglobulin, casein, isinglass, tannin, and peanut extract and then added to ELISA plates coated with peanut (1 μg/mL). Serum IgE binding to peanut was assessed and expressed as percentage inhibition. Assays were performed in triplicate.

ovalbumin polyclonal antibody (IgG fraction purified from rabbit anti-ovalbumin antiserum; Research Diagnostics, Concord, MA) was diluted to a concentration of 10 μg/mL using 0.1 M bicarbonate buffer, pH 9.6, dispensed into Costar 96-well polystyrene plates (50 μL/well), and incubated overnight at 4 °C. Plates were washed with 0.05% PBS−Tween and blocked with PBS−gelatin solution (200 μL/well) for 1 h at room temperature. After being washed with 0.05% PBS−Tween, 50 μL of ovalbumin standard solutions [1 ng/mL to 2 μg/mL ovalbumin (Sigma) in PBS−gelatin], control wine fining agents (0.25–2 μg/ml in PBS-gelatin), or the pretreated wine samples (neat) were added to the wells and incubated at room temperature for 1 h. Plates were washed with 0.05% PBS−Tween and incubated with mouse anti-chicken ovalbumin monoclonal antibody (1:10000 at 50 μL/well; Sigma) for 1 h at room temperature, followed by HRP-labeled sheep anti-mouse Ig polyclonal antibody (1:1000 at 50 μL/well; Silenus) for 1 h at room temperature, with washes in between incubations. Antibody binding was detected using the substrate OPD as outlined above. The testing of wine samples and standards was performed in triplicate, and the mean OD of triplicate negative control wells containing no antigen was subtracted from the OD of wells containing antigen to account for nonspecific binding by detecting antibodies. Ovalbumin concentrations in test wine samples were determined from the standard curve. The assay was performed twice for each wine sample to ensure reproducibility.

ELISA for Detection of Casein in Wine. A sensitive and specific antigen capture ELISA was established for casein detection in wine based on the method described above for ovalbumin. Sheep anti-casein polyclonal antibody (Biodesign International, Saco, ME) was diluted to a concentration of 0.5 μg/mL using 0.1 M bicarbonate buffer, pH 9.6, dispensed into Costar 96-well polystyrene plates (50 μL/well), and incubated overnight at 4 °C. Plates were washed with 0.05% PBS−Tween and blocked with PBS−gelatin solution (200 μL/well) for 1 h at room temperature. After being washed with 0.05% PBS−Tween, 50 μL of casein standard solutions [0.008−0.5 μg/mL casein (Sigma) in PBS−gelatin], control wine fining agents (0.25–2 μg/mL in PBS−gelatin), or the pretreated wine samples (neat) were added to the wells and incubated at room temperature for 1 h. Plates were washed with 0.05% PBS−Tween and incubated with mouse anti-casein monoclonal antibody prepared as described above (1:100 dilution; 50 μL/well) for 1 h at room temperature, followed by HRP-labeled sheep anti-mouse Ig polyclonal antibody (1:1000 dilution; 50 μL/well) for 1 h at room temperature, with washes in between incubations. Antibody binding was detected using the substrate OPD as described above.

Inhibition ELISA for Detection of Peanut-Related Proteins in Wine. A sandwich ELISA for the detection of peanut-related proteins in wine was initially established using an in-house Ara h 1-specific monoclonal antibody, but the sensitivity of this assay was insufficient. Consequently, a sensitive and specific inhibition ELISA was established on the basis of a method which we described previously (*10*), using a peanut-allergic donor serum. The peanut-allergic patient had experienced anaphylaxis on ingestion of peanut and had positive serum specific IgE for almond, cashew, and hazelnut as well as the major peanut allergens, Ara h 1 and Ara h 2. Roasted peanut extract diluted at a concentration of 1 μg/mL in 0.1 M bicarbonate buffer, pH 9.6, was coated onto Costar 96-well polystyrene plates (50 μL/well) and incubated overnight at 4 °C. Plates were washed with 0.05% PBS−Tween (5 times) and blocked with 5% skim milk powder (SMP) in 0.05% PBS−Tween (200 μL/well) for 1 h at room temperature. The peanut-allergic donor serum (diluted with 1% SMP in 0.05% PBS−Tween for an OD 490 nm reading of 1.0 for peanut extract) was preincubated with dealcoholized wines neat, peanut extract, or control wine fining agents at final concentrations of 0.008, 0.04, 0.2, 1, 5, 25, and 125 μg/mL (in PBS−gelatin) at room temperature for 1 h. The inhibition mixtures (including serum with no inhibitor as positive control) were then aliquoted into the peanut antigen-coated wells (50 μL/well) and incubated at 37 °C for 1 h. Plates were washed with 0.05% PBS−Tween and incubated with rabbit polyclonal anti-human IgE antibody (1:1000; 50 μL/well; DAKO, Carpinteria, CA) for 1 h at 37 °C, followed by HRP-labeled goat anti-rabbit IgG polyclonal antibody (1:1000; 50 μL/well; Promega, Madison, WI) incubated again for 1 h at 37 °C, with PBS−Tween washes in between incubations. IgE binding was detected using OPD as described for other ELISA. Percentage inhibition was calculated using the formula:

$$\%\ \text{inhibition} = 100 - \left[\frac{\text{OD}_{490}\ \text{of serum with inhibitor}}{\text{OD}_{490}\ \text{of serum without inhibitor}}\right] \times 100$$

RESULTS AND DISCUSSION

Sensitive ELISA for the detection of ovalbumin, casein, and peanut-related proteins in wine were successfully established in this study and used to evaluate residual processing aids in a panel of Australian wines. Ovalbumin and casein were selected for analysis as these represent the predominant allergenic components in egg white and milk, respectively (*6, 7, 9*). Since there is little information on allergenicity of wood and gall used as a source of non-grape-derived tannins in the wine industry, and in view of the known cross-reactivity between tree nuts and peanuts (*10*) as well as the severe clinical reactions experienced by peanut-allergic subjects on ingestion of peanuts or cross-reacting tree nuts, wines were also probed for peanut-related proteins. The inclusion of isinglass-fined wines in the test panel provided a specificity check for the ELISA.

ELISA for Detection of Ovalbumin in Wine. A sensitive sandwich ELISA for the detection of ovalbumin was established using commercially available monoclonal and polyclonal antibodies. Several aspects of the technical procedure were varied in order to achieve greatest sensitivity. These included the concentration of antibody and incubation time and temperature. Performing the assay at room temperature rather than at 37 °C reduced nonspecific binding of antibodies, giving better signal detection at lower concentrations (**Figure 1**). This was likely due to a higher stringency of the mouse anti-ovalbumin detecting monoclonal antibody at room temperature. Using the optimized method, the limit of detection was 1 ng/mL ovalbumin. The ovalbumin ELISA was also evaluated for specificity by testing different allergen protein solutions including the study wine fining agents. Reactivity was only observed for ovalbumin as shown in **Figure 2**.

The ovalbumin sandwich ELISA was used to test for ovalbumin in the panel of survey wines. Ovalbumin was undetectable in all wines except two red wines, 98 (0.98 μg/mL) and 99 (0.40 μg/mL), which were diluted 1/16 to obtain $\text{OD}_{490\text{nm}}$ values within the linear range of the standard curve. These two wines had not been fined with egg white but had

Table 1. Number of Wines Positive for Allergen Detection

	control unfined wines		test wines: processing aid					
	white	red	egg white fined	whole egg added	casein fined	isinglass fined	milk fined	non-grape tannin fined
ovalbumin ELISA	0/2	0/2	0/40	2/2	0/21	0/23	0/54	0/25
casein ELISA	0/2	0/2	0/40	0/2	0/21	0/23	0/54	0/25
peanut inhibition ELISA	0/2	0/2	0/40	0/2	0/21	0/23	0/54	0/25

whole eggs added during the fining process (6 eggs/1000 L) without subsequent removal. When wines 98 and 99 were analyzed without pretreatment, the concentration of ovalbumin detected was only 0.013 and 0.004 μg/mL, respectively, confirming the importance of pretreatment for more sensitive detection of ovalbumin in wine.

ELISA for Detection of Casein in Wine. A sandwich ELISA for the detection of casein was established using the commercially available sheep anti-casein polyclonal antibody as the capture antibody and the monoclonal antibody to casein developed in our laboratory as the detecting antibody. The casein monoclonal antibody was found to be specific for α-casein, as tested by ELISA, with minimal reactivity to β-casein or κ-casein (**Figure 3**).

Optimization studies showed that greatest sensitivity for detection of casein was achieved using the polyclonal sheep anti-casein antibody as the coating antibody at 0.5 μg/mL and the monoclonal anti-α-casein antibody at 1:100 dilution as the detecting antibody. Using this optimized protocol, the limit of detection of α-casein was 8 ng/mL (**Figure 4**). The casein-specific ELISA was also evaluated for specificity by testing the different study wine fining agents. Reactivity was only observed for casein as shown in **Figure 5**.

The optimized casein sandwich ELISA was subsequently used to test for casein in the panel of survey wines. α-Casein was undetectable (<8 ng/mL) in all wines.

ELISA for Detection of Peanut-Related Proteins in Wine. An inhibition ELISA for the detection of peanut-related proteins in wine was established. Roasted peanut extract was used in this assay because roasted peanuts are commonly consumed, and roasting can enhance the allergenicity of peanut proteins (*22*). Serum from a peanut-allergic patient and an in-house generated Ara h 1-specific monoclonal antibody were compared for use in the antigen incubation mix. The inhibition assay using peanut-allergic donor serum was found to have greater sensitivity than the ELISA utilizing the monoclonal antibody (**Figure 6**). The limit of detection for the optimized peanut-allergic serum inhibition ELISA was 8 ng/mL peanut. The peanut inhibition ELISA was also evaluated for specificity by testing the different study wine fining agents as inhibitors. Inhibition of IgE reactivity to peanut extract was only observed for peanut extract (positive control), as shown in **Figure 7**.

The optimized peanut serum IgE inhibition ELISA was subsequently used to test for residual peanut-related proteins in the panel of survey wines. These were undetectable (<8 ng/mL) in all wines.

Threshold Dose for Food Allergens and Estimated Wine Daily Intake. In this study, 153 survey wines were analyzed for ovalbumin, casein, and peanut-related proteins with only two wines giving a detectable concentration of ovalbumin, and these wines contained added whole egg rather than being fined with egg white (**Table 1**). No wines tested had detectable casein or peanut-related proteins. Duplicate analyses for each wine sample yielded consistent results. The high sensitivities of our ELISA mean that any residual fining agents if present would be at levels (nanograms per milliliter) unlikely to be clinically relevant, but the precise threshold for adverse reactions among food-allergic individuals has yet to be established conclusively (*16, 17, 23*). The threshold dose for allergens varies among individuals and also among sources of the same allergen. There is accumulating evidence to suggest that the majority of food-allergic individuals can tolerate small amounts of allergenic protein [milligram levels (*13*)]. In a challenge study to determine a peanut protein threshold in sensitive individuals, the lowest dose to elicit a mild, nonthreatening adverse reaction was 2 mg, although 50% of subjects could tolerate up to 50 mg (*24*). In another challenge study to determine an egg and milk protein threshold in sensitive individuals, some subjects (11% and 25%, respectively) reacted to doses of 100 mg, but the majority of sensitive individuals could tolerate this dose (*17, 25*). Thus, the lowest observed adverse effect level (LOAEL) for egg, milk, and peanut is commonly in the range of 1−2 mg of a food, which represents approximately 100−200 μg of protein. These minimal levels characterize about 1% of people who suffer from allergies to egg, milk, or peanut, where approximately less than 65 mg characterizes 16% and 18% of patients allergic to egg or peanut while less than 30 mg of milk proteins characterizes 5% of those allergic to milk (*13*).

The national nutrition survey of foods eaten by Australians (Australian Bureau of Statistics 1999) shows that the average consumption of wine for the entire population, taking into account those persons who do not drink wine, is 79 g/day. The subpopulation of wine consumers with the greatest intake are 45−64-year-old males with a consumption rate of 312 g/day. For risk assessment purposes, it is conventional to conduct the evaluation for the "highest" sector of consumers rather than for the median sector. Since there is insufficient information available in the Australian Bureau of Statistics (1999) data source to statistically calculate the consumption rate for the highest sector consumers, an assumption is made that the highest sector consumption could be represented by a 3-fold increase over the median for 45−64-year-old males (i.e., a consumption rate of 936 g/d). Assuming a specific gravity of 1 for wine, the highest sector consumption rate is therefore approximately 1 L per person per day, i.e., ∼10 Australian standard drinks.

In our ELISA, the detection limit for α-casein and peanut was calculated to be 8 ng/mL. At this level, the daily intake of α-casein or peanut-related protein from wine for a consumer who drinks approximately 1 L of wine per day would be 7.488 μg, considerably less than the threshold doses for adverse reactions in allergic individuals. The National Health and Medical Research Council's Australian Alcohol Guidelines: Health Risks and Benefits (2001) low risk guideline is that men should not exceed four standard drinks per day and women should not exceed two standard drinks. Therefore, at low risk alcohol consumption levels, a consumer who drinks 2–4 Australian standard drinks of wine containing 8 ng/mL α-casein or peanut-related proteins per day has a daily intake of 1.5–3 μg of α-casein or peanut-related proteins. This is 1000000 less than that tolerated by 75% of 117 milk allergic subjects in the challenge study undertaken by Sicherer et al. (*25*). In comparison

with α-casein and peanut-related proteins, the calculated daily intake of ovalbumin from wine would be much less given the lower detection limit of 1 ng/mL in our sandwich ELISA.

Conclusions. In conclusion, we have established specific ELISA for ovalbumin, α-casein, and peanut proteins suitable for testing wine samples with allergenic protein detection limits well below threshold levels for eliciting adverse reactions. When these ELISA were used to analyze a large panel of commercially available bottled wines manufactured in Australia, our findings were consistent with a lack of residual potentially allergenic processing aids at a concentration that could cause an adverse reaction in egg, milk, or peanut/tree nut allergic adult subjects. Wines to which whole eggs are added cannot be included in this conclusion as two such wines in this survey panel appropriately contained detectable ovalbumin.

ABBREVIATIONS

ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; SMP, skim milk powder; OD, optical density.

LITERATURE CITED

(1) Sicherer, S. H.; Sampson, H. A. Peanut and tree nut allergy. *Curr. Opin. Pediatr.* **2000**, *12*, 567–573.

(2) Bernhisel-Broadbent, J.; Dintzis, H. M.; Dintzis, R. Z.; Sampson, H. A. Allergenicity and antigenicity of chicken egg ovomucoid (Gal d III) compared with ovalbumin (Gal d I) in children with egg allergy and in mice. *J. Allergy Clin. Immunol.* **1994**, *93*, 1047–1059.

(3) Hefle, S. L.; Nordlee, J. A.; Taylor, S. L. Allergenic foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36* (Suppl.), S69–S89.

(4) Hoffman, D. R. Immunochemical identification of the allergens in egg white. *J. Allergy Clin. Immunol.* **1983**, *71*, 481–486.

(5) Mine, Y.; Zhang, J. W. Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *J. Agric. Food Chem.* **2002**, *50*, 2679–2683.

(6) Wal, J. M. Cow's milk allergens. *Allergy* **1998**, *53*, 1013–1022.

(7) Wal, J. M. Cow's milk proteins/allergens. *Ann. Allergy Asthma Immunol.* **2002**, *89*, 3–10.

(8) Powrie, W. D.; Nakai, S. Characteristics of edible fluids of animal origin: eggs. In *Food chemistry*, 2nd ed.; Fennema, O. R., Ed.; Marcel Dekker: New York, 1985; pp 829–855.

(9) Rupa, P.; Mine, Y. Immunological comparison of native and recombinant egg allergen, ovalbumin, expressed in *Escherichia coli*. *Biotechnol. Lett.* **2003**, *25*, 1917–1924.

(10) de Leon, M. P.; Glaspole, I. N.; Drew, A. C.; Rolland, J. M.; O'Hehir, R. E.; Suphioglu, C. Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. *Clin. Exp. Allergy* **2003**, *33*, 1273–1280.

(11) Rolland, J. M.; Apostolou, E.; Deckert, K.; de Leon, M. P.; Douglass, J. A.; Glaspole, I. N.; Bailey, M.; Stockley, C. S.; O'Hehir, R. E. Potential food allergens in wine: Double-blind, placebo-controlled trial and basophil activation analysis. *Nutrition* **2006**, *22*, 882–888.

(12) Morisset, M.; Moneret-Vautrin, D. A.; Kanny, G.; Guenard, L.; Beaudouin, E.; Flabbee, J.; Hatahet, R. Thresholds of clinical reactivity to milk, egg, peanut and sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or single-blind placebo-controlled oral challenges. *Clin. Exp. Allergy* **2003**, *33*, 1046–1051.

(13) Moneret-Vautrin, D. A.; Kanny, G. Update on threshold doses of food allergens: implications for patients and the food industry. *Curr. Opin. Allergy Clin. Immunol.* **2004**, *4*, 215–219.

(14) Ribereau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D. *Handbook of Enology, Vol. 2: The chemistry of wine stabilisation and treatments*, 1st ed.; John Wiley and Sons: Chichester, 2000.

(15) Bindslev-Jensen, C.; Briggs, D.; Osterballe, M. Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy* **2002**, *57*, 741–746.

(16) Taylor, S. L.; Hefle, S. L.; Bindslev-Jensen, C.; Bock, S. A.; Burks, A. W., Jr.; Christie, L.; Hill, D. J.; Host, A.; Hourihane, J. O.; Lack, G.; Metcalfe, D. D.; Moneret-Vautrin, D. A.; Vadas, P. A.; Rance, F.; Skrypec, D. J.; Trautman, T. A.; Yman, I. M.; Zeiger, R. S. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *J. Allergy Clin. Immunol.* **2002**, *109*, 24–30.

(17) Hourihane, J. O.; Knulst, A. C. Thresholds of allergenic proteins in foods. *Toxicol. Appl. Pharmacol.* **2005**, *207*, 152–156.

(18) Weber, P.; Steinhart, H.; Paschke, A. Investigation of the allergenic potential of wines fined with various proteinogenic fining agents by ELISA. *J. Agric. Food Chem.* **2007**, *55*, 3127–3133.

(19) Goding, J. *Monoclonal antibodies: principles and practice*, 3rd ed.; Academic Press: London, 1996.

(20) Sutherland, M. F.; Drew, A.; Rolland, J. M.; Slater, J. E.; Suphioglu, C.; O'Hehir, R. E. Specific monoclonal antibodies and human immunoglobulin E show that Hev b 5 is an abundant allergen in high protein powdered latex gloves. *Clin. Exp. Allergy* **2002**, *32*, 583–589.

(21) Hefle, S. L.; Jeanniton, E.; Taylor, S. L. Development of a sandwich enzyme-linked immunosorbent assay for the detection of egg residues in processed foods. *J. Food Prot.* **2001**, *64*, 1812–1816.

(22) Maleki, S. J.; Chung, S. Y.; Champagne, E. T.; Raufman, J. P. The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* **2000**, *106*, 763–768.

(23) Hefle, S. L.; Taylor, S. L. How much food is too much? Threshold doses for allergenic foods. *Curr. Allergy Asthma Rep.* **2002**, *2*, 63–66.

(24) Hourihane, J. B.; Kilburn, S. A.; Nordlee, J. A.; Hefle, S. L.; Taylor, S. L.; Warner, J. O. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *J. Allergy Clin. Immunol.* **1997**, *100*, 596–600.

(25) Sicherer, S. H.; Morrow, E. H.; Sampson, H. A. Dose-response in double-blind, placebo-controlled oral food challenges in children with atopic dermatitis. *J. Allergy Clin. Immunol.* **2000**, *105*, 582–586.

Received for review November 14, 2007. Accepted November 26, 2007. This work was funded by the Grape and Wine Research and Development Corporation of Australia, the National Health and Medical Research Council of Australia, and Alfred Research Trusts.

JF073330C