







The identification and measurement of potential allergens in wine

FINAL REPORT to:

The Grape and Wine Research and Development Corporation

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Principal Investigators: **Professor Robyn O'Hehir, Ms Creina Stockley**

Research Organisations: **Department of Allergy, Immunology and Respiratory Medicine, The Alfred**
Monash University
The Australian Wine Research Institute

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1. Abstract

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. Similar regulations are pending in the European Union and USA. The objectives of the research project were to establish sensitive assays to detect and measure allergenic proteins from some commonly used processing aids in bottled wine, and to determine whether individuals with a known allergy to these proteins exhibit an allergic reaction on consumption of wine fined with these processing aids.

A double-blind placebo-controlled trial was performed to determine whether allergic reactions followed consumption of Australian commercial wines fined using one or more of the legislation-targeted food proteins. Additionally, allergenicity of a larger panel of these wines was evaluated by blood basophil activation. No anaphylaxis was induced by wine consumption. Three mild clinical reactions to protein-fined wine and two mild reactions to unfined wine occurred, but there was no statistically significant difference in reaction parameters between subject groups or between processing aids. No pattern of basophil activation correlated with wine type, processing aid or subject group. Wines fined with egg-white, isinglass or non-grape-derived tannins present an extremely low risk of anaphylaxis to fish, egg or peanut allergic consumers. Although consumption of milk protein-fined wine did not induce anaphylaxis, there were insufficient subjects to statistically determine whether wines fined with milk proteins present a risk to the rare milk allergic consumers. In summary, the observed lack of anaphylaxis and basophil activation induced by wines made using the legislation-targeted food proteins according to good manufacturing practice suggests negligible residual food allergens in these wines.

Sensitive and wine-specific enzyme-linked immunosorbent assays (ELISA) were developed and established for the proteins casein, ovalbumin and peanut. These ELISA failed to detect residual allergens in the fined wines, where the lower limit of detection was 8 ng/mL, 1 ng/mL and 8 ng/mL, respectively.

Together these findings are consistent with a lack of residual potentially allergenic 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, fish, milk or peanut/tree nut allergic adult consumers.

2. Executive summary

The Grape and Wine Research and Development Corporation (GWRDC) funded a research project entitled *The identification and measurement of potential allergens in wine* (ADF 02/01) in 2002, which has been undertaken by in conjunction with The Australian Wine Research Institute (AWRI). Substantial cash and in kind contribution to this project was also contributed by The Alfred/Monash University and the AWRI. The research project was instigated by changes to the mandatory declarations for labels of all food packaging to include the presence of potential allergens such as egg, fish, milk, nuts and their products in a food. Under Clause 4 of Standard 1.2.3 *Mandatory warning labels and advisory statements and declarations* of the Australian and New Zealand Food Standards Code (ANZFSC), presence of the following winemaking processing aids must now be declared on the label of all wine and wine products in Australia: casein; egg white; evaporated milk; isinglass; lysosyme; milk; and potassium caseinate. The Australian wine industry has been advised by botanists, however, that non-grape-derived tannin, such as that derived from chestnuts and oak, are derived primarily from the bark and galls of the trees, which are physically and pharmacologically distinct from the potentially allergenic nuts. Similar labelling legislation is pending in Canada, the European Union and the USA, which are the three primary export markets for Australian wine (Johan Bruwer, personal communication, September 2004).

Clause 4 of Standard 1.2.3 does not require labelling where these processing aids are in the winemaking process and cannot be detected in the final wine. While there are no assays commercially available that are sufficiently sensitive and specific to detect residual processing aid in the final wine, winemakers have a duty of care to consumers, in particular the 1% who are allergic to egg, fish, milk or nuts. There are, however, no reported cases of allergy definitively attributed to wine fined with casein, egg white, isinglass or milk recorded in the medical and scientific literature, nor do colleagues of Professor Robyn O'Hehir, Director of the Department of Allergy, Immunology and Respiratory Medicine at The Alfred, know of any adverse reactions following wine ingestion due to these processing aids. If a winery chose not to determine whether there was residual egg, fish, milk or nut in a final wine, and also chose not to label that there may be an allergen present in the wine, and a consumer with a known allergy to either eggs, fish, milk or nuts, had a severe allergic reaction to the wine, the current good image and reputation of the Australian wine industry could be immeasurably tarnished by this lack of duty of care to the consumer. Also tarnished would be the perception of quality and purity associated with that winery and Australian wine per se. It is becoming increasingly obvious that the consumer wishes to be better informed about potential risks to their health and safety, and that they conscientiously consult the product label.

Consequently, the objectives of the GWRDC-funded research project were two-fold. The first objective was to establish sensitive, specific and reliable assays to detect and measure any residual allergenic proteins from the processing aids casein, egg white, isinglass and milk in the final bottled wine. The second objective, irrespective of whether residual processing aids could be detected and measured in the final bottled wine, was to determine whether individuals with a known allergy to eggs, fish, milk and/or nuts exhibit an allergic reaction on consumption of wine known to have been fined with either casein, egg white, isinglass and/or milk, or to which non-grape-derived tannins have been added. These two objectives should enable the Australian wine industry to in order to determine the degree of

risk on which to base a decision regarding labelling and a possible application for exemption for wine from FSANZ and the European Commission.

A panel of 113 Australian wines was collected and coded by the AWBC and AWRI for blind analysis by The Alfred/Monash University investigators, and included at least 20 wines that had been fined with: egg white or had whole egg added; casein or potassium caseinate; isinglass; milk; or had non-grape derived tannin added. Four control non-fined wines were also collected. Clinical challenge of 26 food-allergic and 11 non-food allergic subjects using 26 of the above wines and three control unfined wines revealed no episode of anaphylaxis and no symptom or sign of adverse reaction consistent with fining agent for the relevant allergic subject group. These findings were supported by the results of *in vitro* basophil activation assays performed using blood samples from the above subjects tested against a larger panel of 100 wines fined with the same panel of agents. Only nine of the 100 fined wines tested gave basophil activation >10% with a ratio above the reference range upper limit of 2.85 for some subjects (data summarised in Table 4.4), but no pattern could be identified with respect to wine variety, fining agent or geographic indication, or to subject group.

Sensitive enzyme linked immunosorbent assays (ELISA) have also been developed to detect residual fining agents in wine. An ovalbumin (egg)-specific ELISA was established using commercial monoclonal and polyclonal antibodies and used to screen the above panel of 100 wines. Apart from the two wines to which whole eggs had been added rather than being fined with egg white proteins, the concentration of ovalbumin was below the detection limit of the assay (1 ng ovalbumin/L). A casein-specific ELISA and a peanut-specific ELISA also generated in-house failed to detect residual allergens in the fined wines (< 8 ng/ml α-casein; < 8 ng/ml peanut).

Only a limited number of milk allergic (one) and egg allergic (five) subjects could be recruited for this study due to the paucity of these allergies in adults, and the minor symptoms noted by the one milk allergic subject after two blinded encounters with wine fined with milk proteins suggested that milk-fined wines should be further investigated. A follow-up study was therefore performed using serum from children with milk and egg allergies in a stripped basophil activation assay. The stripped basophil assay was established and validated using control milk and egg white extracts. Wines fined with egg did not induce a statistically significant increase in basophil activation when cells were resensitised with egg allergic sera compared with control sera, but milk fined wines caused an increased basophil activation when cells were resensitised with milk allergic sera compared with controls.

Together these findings are consistent with a lack of residual potentially allergenic processing aids in final bottled wine manufactured in Australia at a concentration that could cause an adverse reaction in fish, peanut/tree nut or egg allergic adult subjects. Wines to which whole eggs are added cannot be included in this conclusion; the two such wines in this survey panel contained detectable ovalbumin and were not tested in clinical challenge. The rarity of IgE-mediated milk allergy in adults prevented a statistical analysis for clinical challenge and direct basophil activation with milk-fined white wines, but this rarity makes potential allergic reactions to these food proteins in wine rather a theoretical problem. Nevertheless, the results of the follow-up study of milk-fined wines using a stripped basophil assay suggest that the potential of an allergic reaction in a severely milk allergic child or adult following ingestion of a milk-fined wine cannot be excluded.

3. Background

3.1 Introduction

Wine sold in Australia must be labeled according to the provisions of the Australia New Zealand Food Standards Code (ANZFSC). Prior to December 2002, specific labelling requirements were for the presence of the added preservatives (for example, sulfur dioxide and sorbic acid), added antioxidants (for example, ascorbic and erythorbic acids), and colouring materials (for example, caramel). Labelling for sulfur dioxide in particular, is related to its potential to cause an adverse reaction in sulfite-sensitive asthmatic individuals (Vally and Thompson 2001). The Australian wine industry has always acknowledged and accepted these health and safety labelling provisions, and indeed has worked with Food Standards Australia New Zealand (FSANZ) to inform consumers of the potential risks of wine consumption as exemplified by the support and subsequent adoption of standard drinks labelling in 1995.

3.2 Changes to the labelling provisions

Egg, fish, milk and nuts and their products contain potential food allergens and have been associated with allergic reactions in approximately 7.5% of children up to five years and 1% of adults (Chandra 1977). This percentage is, however, growing. In 1995, the Codex Alimentarius Commission established a list of eight key allergenic foods and Japan introduced allergen labelling for food in 2002. Australia is the first country, however, to introduce allergen labelling for wine. From December 2002, there has been an additional requirement that if wine is made with the use of egg, fish, milk and nuts or their products, this must be included on the wine label, for example, according to the ANZFSC, Standard 1.2.3 *Mandatory warning labels and advisory statements and declarations*:

Clause 4

(1) The presence in a food of any of the substances listed in the Table to this clause, must be declared in accordance with subclause (2), when present as—

(a) an ingredient; or
(b) an ingredient of a compound ingredient; or
(c) a food additive or component of a food additive; or
(d) a processing aid or component of a processing aid.

(2) Any substance required to be declared by subclause (1) must be—

(a) declared on the label on a package of the food;…

Table to clause 4

…
Egg and egg products
Fish and fish products
Milk and milk products
Nuts and sesame seeds and their products
…
Added sulphites in concentration of 10 mg/kg or more

3.3 Implications for the Australian wine industry

The following legally permitted processing aids have been captured by Clause 4 of Standard 1.2.3: casein; egg white; evaporated milk; isinglass; milk; potassium caseinate; and non-grape derived tannins, such as that derived from chestnuts and oak galls. The Australian wine industry has been advised by botanists, however, that these latter products are derived primarily from the bark and galls of the trees, which are physically and pharmacologically distinct from the potentially allergenic nuts.

There have been no exemptions for inclusion of this labelling information granted by FSANZ to date. Standard 1.2.3 does not require labelling where these processing aids are used in the manufacturing process and cannot be detected in the final wine. It is the duty of the manufacturer, however, to determine whether residual of the processing aid is present in the final wine, and accordingly requires declaration under Clause 4 of Standard 1.2.3. Manufacturers may voluntarily provide this information if there is any uncertainty. At present, however, commercially available assays may not be sufficiently sensitive to detect residual processing aid in the final wine at a concentration that potentially could provoke an allergic reaction.

While there have been no previous proteinaceous allergic reactions to wine recorded in the literature, the industry has a duty of care to the consumer and should demonstrate due diligence. This is the premise of the Food Safety Standards component (Chapter 3) of the ANZFSC. Due diligence acts as a balance to, or protection against, the principle of strict liability, which forms the tenet of consumer law, that is, the defendant is guilty whether or not he intended to commit the offence. The defendant can be acquitted, however, if he is able to demonstrate that he has taken all reasonable precautions and exercised all due diligence to avoid the commission of the offence (Law 2000). For example, 'all reasonable precautions' means that a system of controls was in place and 'all due diligence' means that it can be demonstrated that the system worked. The test of reasonableness is related to the size and nature of the business and also the risk, which the precautions are designed to avoid. Applied to food allergenicity for the Australian wine industry, 'all due diligence' means knowing confidently that the wine product contains or does not contain allergens and specifically that it does or does not contain residuals of the aforementioned processing aids. It also means knowing the degree of risk to the consumer of not labelling following the use of these potentially allergenic processing aids. The most severe reaction to an allergen is anaphylactic shock, which may result in death. However, it is difficult to proscribe, definitively, a lower limit for the concentration below which the risk of an allergic reaction is minimal, due to the numerous factors involved, such as age, gender, genetic constitution, dietary habits, the allergenicity of dietary components and as yet largely unidentified environmental factors (Sampson 1996, Dean 1997, British Nutrition Foundation Task Force 2002). The prevalence of incontrovertible food allergy in the adult population is 0.3% but ranges from 0.1–1.0% (Chandra 1997).

If the Australian wine industry chose not to determine whether there was residual egg, fish, milk or nut in a final wine, and also chose not to label that there may be an allergen present in the wine, and a consumer with a known allergy to either eggs, fish, milk or nuts, had a severe allergic reaction to the wine, the current good image and reputation of the Australian wine industry could be immeasurably tarnished by this lack of duty of care to the consumer. Also tarnished would be the perception of quality and purity associated with Australian wine. It is becoming increasingly obvious that the consumer wishes to be better

informed about potential risks to their health and safety, and that they conscientiously consult the product label.

Subsequent to the FSANZ legislation, the European Commission has also legislated for foods, including alcoholic beverages, to list all potential allergens on the label, but this may only come into force from approximately 1 December 2007 for alcoholic beverages. On 12 December 2001, the European Commission confirmed that if a listed allergen has been used during production it must be listed on the label, irrespective of the level, if any, present in the finished product. The EU legislation (COD/1997/0027) reflects Article 4.2.1.4 of the Codex Alimentarius standard on food labelling. For example, *Document 501PC0433 Proposal for a Directive of the European Parliament and of the Council amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs,* states:

> "(9) in order to provide all consumers with better information and to protect the health of certain consumers, it should be made obligatory to include, in the list of ingredients all ingredients present in the foodstuff and, in the case of ingredients known to be allergenic, to declare them by their specific name in all cases, including in alcoholic drinks, allowing no possibility…in the case of additives, any exemption from inclusion in the list of ingredients; and
>
> (21) Lastly, in order to achieve the objective of a high level of health protection by improved information on the composition of foodstuffs, it would be inconceivable to exclude alcoholic drinks from the obligation to indicate the presence of an ingredient that is recognized to be an allergen…
>
> Ingredients referred to…
> Egg and egg products thereof
> Fish and fish products thereof
> Peanuts and products thereof
> Milk and dairy products thereof…
> Nuts and nut products thereof…
> Sulphite at concentrations of at least 10 mg/kg"

All Australian wine exported to the European Union, approximately 60.95% of total Australian wine exports, will be have to comply with this Directive unless there is sufficiently strong evidence to suggest otherwise.

Australian wine exports to the USA and Canada will also have to comply with allergen labelling legislation, as the USA will introduce similar allergen labelling legislation for all foods including alcoholic beverages from 1 January 2006, but Canada will only introduce labelling legislation for gluten and sulfur dioxide for alcoholic beverages .

3.4 What is an adverse food reaction?

An 'adverse food reaction' is a generic term referring to any untoward reaction following the ingestion of a food or beverage which may be immunologically (food allergy) or non-immunologically (food intolerance) mediated (Bruijnzeel-Koomen et al. 1995).

Essentially, allergy is 'an inappropriate and exaggerated immune response' where a normally harmless substance is perceived as a threat by the body's immunological defences. Food allergy is an immunologically mediated hypersensitivity after the ingestion of a particular food, usually involving the production of specific immunoglobulin E antibodies (IgE) by B lymphocytes (white blood cells) in response to a food protein.

Within the body, IgE binds to mast cells, which release molecules such as histamine that causes an inflammatory reaction. Mast cells are scattered just below the surface of the skin and below the mucosal surfaces of the eyes, nose, mouth, respiratory tract and intestine (Costa et al. 1997).

The most commonly involved foods in children under five years old are egg, milk, peanut and other nuts, soya, fish and wheat, which account for around 90% of episodes. In older children and adults, peanut and other nuts, fish and shellfish account for most reactions. Allergies to egg, milk, soya and wheat proteins usually resolve by three or four years of age. The prevalence of true food allergy is, therefore, quoted as up to 7.5% in infancy, falling rapidly to 1 to 2% by age three and is approximately 0.1 to 1% in adults (Chandra 1997). Patients are typically atopic with additional sensitivity to one or more common aeroallergens, such as house dust mite and/or pollen (Bishop et al. 1990, Høst and Halken 1990, Hill et al. 1994, Jansen et al. 1994, Sampson 1996, Tariq et al. 1996).

True food allergy usually has a rapid onset following ingestion of the food protein with symptoms commencing within minutes and up to two hours post ingestion (Hourihane et al. 1997a, 1997b). These include oral and perioral itching and burning, gastrointestinal symptoms of nausea, vomiting, diarrhoea and severe colicky pains, respiratory symptoms of wheeze and asthma, skin reactions of urticaria and angioedema, and cardiovascular reactions of dizziness and occasionally fatal anaphylaxis (Sampson 1988, Metcalf et al. 1996).

Food intolerance is the result of non-immunological mechanisms with an abnormal physiological response to an ingested food or food component. Such responses may include pharmacologic, toxic (food poisoning), psychological (food aversion) or metabolic reactions (lactase deficiency or favism) (Bruijnzeel-Koomen et al. 1995). Food intolerance is much more common than true food allergy and is more commonly associated with reactions to multiple foods (Parker et al. 1990). Pharmacologic responses may result in drug-like side-effects in sensitive individuals which typically commence from one to two hours after food ingestion, but may commence at up to 48 hours (Parker et al. 1990, Parker et al. 1993). These include responses to low molecular weight chemicals that are either added to food as flavour enhancers or preservatives (such as monosodium glutamate [MSG], metabisulphite and colourants) or occur naturally within the food (such as salicylates, biogenic amines including histamine and tyramine, MSG and glutamates). Pharmacologic food intolerance is estimated to occur at a frequency of 20% of the population with a female preponderance, but no definitive laboratory tests are available for simple diagnosis. Reactions typically occur at any age, most commonly in nonatopic individuals, and episodes may be triggered by many different foods.

Symptoms of food intolerance are usually non-specific and may involve one or more body systems (Parker et al. 1990, Parker et al. 1993):

1. neurological system, including malaise, headache, lethargy and myalgia;
2. skin, including urticaria, angioedema and/or eczema;
3. gastrointestinal system, including nausea, vomiting, flatulence, diarrhoea, colicky abdominal pain, and aphthous ulcers;
4. respiratory system, including rhinitis, pharyngitis and asthma; and
5. anaphylactoid reactions.

3.5 Food allergens

Foods contain lipids, proteins and carbohydrates. Food allergens are usually water-soluble glycoproteins of molecular weight from 10,000 to 60,000 daltons (Astwood et al. 1997). Water-soluble food proteins are albumins and saline-soluble food proteins are globulins. No identifiable biochemical or immunochemical features have been identified that are unique to all food allergens. Food allergens tend to be quite resistant to food processing and preparation procedures including proteolysis, digestion, and heat and acid treatment. Allergens in fresh fruit and vegetables may be inactivated, however, by cooking.

Sensitisation to food allergens requires a genetic predisposition combined with environmental exposure. The minimum sensitising level of exposure is not known but, once sensitised, individuals may react to microgram to milligram quantities of allergen with reports of highly sensitive peanut allergic subjects experiencing anaphylaxis on exposure to aerosol particles distributed by opening a packet of peanuts on an aeroplane (Sicherer 1999). Typically food allergic individuals react to only one or two different foods.

3.5.1 Egg allergens

Egg allergy is more common in younger children but may occasionally persist into adulthood. Egg white (albumin) is more allergenic than egg yolk. Egg white contains predominantly ovalbumin with additional ovotransferrin, ovomucoid, ovomucin and lysozyme (Powrie and Nakai 1985). The ovomucoid allergen Gal d 1 of MW 28kD has been identified as the major egg allergen (Bernhisel-Broadbent et al. 1994). Ovalbumin (Gal d 2) of MW 43-45 kD has also been identified as a major egg allergen. Other characterised lesser allergens in egg include ovotransferrin (Gal d 3) MW 77 kD, lysozyme (Gal d 4) MW 14.3 kD, and the minor allergens apovitellin, ovomucin and phosvitin (Bleumink and Young 1969, Bleumink and Young 1971, Bernhiesel-Broadbent et al. 1994, Hoffman 1983, Anet et al. 1985, Langeland 1982, Langeland and Harbitz 1983). The carbohydrate portion of egg glycoproteins has not been shown to bind specific IgE. There may be cross reactivity between hen eggs and those from other birds (Lessof 2002).

3.5.2 Fish allergens

Fish allergy is important in both children and adults, and once present tends to persist throughout life. Parvalbumins (including Gad c 1) MW 12 kD have been identified as important muscle allergens in fish (Elsayed and Bennich 1975, Elsayed and Apold 1983, Elsayed et al. 1991); the carbohydrate moiety of parvalbumins, however, has no demonstrable allergenicity. There is cross-reactivity between the parvalbumins of different fish species (Hansen et al. 1997, Bugajska-Schretter et al. 1998, Lessof 2002). Fish collagen obtained from muscle has also been reported as being allergenic and there may also be cross-reactivity between the collagen of different fish species but not with animal collagen (Hamada et al. 2001). The wine fining agent, isinglass, is a fish collagen extracted from swim bladders (especially sturgeon, cod and hake), cleansed and either desiccated or dried, and is not normally ingested. The possible importance of swim bladder collagen as an allergen is unclear.

3.5.3 Milk allergens

Cow's milk allergy is extremely uncommon beyond early childhood. Caseins form 80% of milk proteins with whey proteins comprising the residual 20% of total protein (Swaisgood 1985). Milk allergic patients usually have specific IgE to more than one form of milk

protein (Baldo 1984). Casein and beta-lactoglobulins are the major allergens in cow's milk (Goldman et al. 1963a, Goldman et al. 1963b). There may be cross reactivity between cow's milk and that from other animals (Lessof 2002).

3.5.4 Nut allergens

There are two types of nut allergies - peanut, which is a legume, and tree nut. The onset of peanut allergy is usually in early childhood and generally persists in adulthood. Allergy to peanut is the most frequent cause of food-associated anaphylaxis in North America and Europe (Committee on toxicity of chemicals in food, consumer products and the environment 1998). Peanut kernels contain 25 to 30% protein (Arthur 1953), where α-arachin, conarachin, peanut agglutinin, the vicilin Ara h 1 and the conglutin Ara h 2 have been identified as the more important allergens in peanuts (Barnett et al. 1983, Barnett and Howden 1986, Burks et al. 1991, Burks et al 1992, Burks et al 1994). Individuals allergic to peanut may exhibit cross reactivity with various tree nuts but rarely with other legumes (Loza and Brostoff 1995, Lessof 2002).

Tree nuts implicated in allergic reactions include almond, Brazil nut, cashew, chestnuts, walnut, hazelnut, macadamia, pecan, pine nut, pistachio, filbert and hickory. The onset of tree nut allergy is also usually in early childhood and generally persists in adulthood (Sicherer and Sampson 2000).

3.6 Potential allergens in wine

The potential allergenic wine processing aids are casein, egg white, evaporated milk, isinglass, milk and potassium caseinate. While egg white is generally used to remove tannins from red wine, the other permitted processing aids are generally used to remove phenolic and tannin compounds from white wine. Another potential allergen used in winemaking is tannin if it is produced from tree nuts such as chestnuts or oak galls.

Because wines differ in their composition, there is no set recommendation on the amount of processing aid to be employed. From the winemaker's perspective it is important that little of the protein remains in the wine after the fining/clarification, as the presence of relatively large amounts of residual fining agent will lead to visible protein precipitates and necessitate further remedial processes. Therefore, most fining processes are based on laboratory trials on individual batches of wine.

While the trials will determine the amount of processing aid to be used for individual batches of wine, the general range of additions is as follows:

- casein: 10–50 g/hL for white wine;
- isinglass: 1–2.5 g/hL for white wine;
- skim milk: 0.5% (w/v) for white wine;
- egg white: 5–15 g/hL for red wine; and
- tannins: 3–10 g/hL for white wine (Ribéreau-Gayon et al. 1998).

The final concentration of these proteinaceous processing aids in the wine is likely to be significantly lower due to the removal of precipitates through the clarification process.

There is no published literature available on the concentration of these proteinaceous processing aids in the finished wine, nor are there published assays for measurement of their concentration in wine. A recent publication investigating the use of wheat gluten as a fining agent of red wines, however, describes non-detectable levels (less than 2.5 mg/L) of wheat gluten by immunodetection post fining (Marchal et al. 2002).

There are, however, commercially available assays to measure the concentration of proteinaceous fining agents in foods: ELISA and PCR. ELISA is an enzyme linked immunosorbent assay, where a specific antibody recognizes the target protein, for example, the peanut allergen Ara h 1 protein, and a colour reaction makes its presence visible. PCR or polymerase chain reaction is the amplification of DNA-target molecules, for example, a specific sequence from the peanut DNA coding for the Ara h 1 protein. The amplification is performed using an enzyme that copies the target sequence so often that it can be visualized by colour staining. The PCR assay is only an indirect test, which detects whether DNA from a specific organism is present and, therefore, can only be used to determine whether a particular product may have been used. It says nothing about whether the allergen exists in the wine. In addition, although most of the PCR assays amplify very small fragments, there is no guarantee that the DNA found in wine will actually have fragment sizes sufficient for amplification. Unfortunately the lower level of sensitivity of commercial ELISA assays is at the mg/L level, which is approximately 100 to 1000-fold higher than the likely level of processing aid residue in wine when GMP is adopted. In addition, The Australian Wine Research Institute has only one recorded incident each of an Australian winery over-fining with casein and isinglass in the past 10 years.

3.7 Reported adverse reactions to wine

The 13-year database of consumer/general public enquiries to the Health Regulatory Information Manager at The Australian Wine Research Institute attributes only one reported allergic reaction to the use of these fining agents in winemaking; approximately 85 health-related enquiries are received each year. Similarly, The Alfred Hospital allergy specialists do not know of any allergic reactions to these fining agents following wine ingestion (personal communication). A comprehensive literature review identifies only a few case reports of severe adverse reactions to wine ingestion largely anecdotally attributed to biogenic amines, salicylates or sulfites (Clayton and Busse 1980, Littlewood et al. 1988, Alibrandi et al. 1990), but the question of a reaction due to fining agents is not specifically considered. Other adverse reactions to wine have been anecdotally attributed to an alcoholic fermentation wine yeast, *Saccharomyces cerevisiae*, which is taxonomically related to the *S. cerevisiae* yeast strains used in bread making and brewing. One study analysed ultrafiltered samples of beer, aged red wine, young white wine, sparkling wine and extracts of fresh bread and dried rye bread by skin prick test, radioallergosorbent test inhibition, SDS PAGE and immunoblotting to identify *S. cerevisiae* (bakers yeast) allergens (Kortekangas-Savolainen et al. 1994). The results suggest that IgE-reactive *S. cerevisiae* yeast allergens are not present in significant concentration in breads, beer or wine.

3.8 Overall aim of research project

As a consequence of the lack of data available on the residual of processing aids in wine and the inability to accurately and sensitively measure the residual at present, a research project commenced on 1 July 2002, in order to determine the degree of risk on which to base a decision regarding labelling and a possible application for exemption for wine from FSANZ and the European Commission.

The research project was undertaken at the Department of Allergy, Immunology and Respiratory Medicine at The Alfred and Monash University in conjunction with The Australian Wine Research Institute.

4. Study A

4.1 Specific Project Aims and Performance Targets

The aims of the project are as follows:

1) to establish sensitive and reliable assays to detect and measure allergenic proteins from the processing aids casein, egg white, isinglass and milk in final bottled wine;

2) to determine if there is any detectable residual allergenic proteins from the processing aids casein, egg white, isinglass and milk in final bottled wine, that has been produced with the aforementioned processing aids; and

3) to determine whether individuals with a known allergy to eggs, fish, milk, and nuts exhibit an allergic reaction on consumption of wine that has been fined with the aforementioned processing aids.

Section I

Outputs (aim)	Performance Targets
1. Wine sample collection to survey whether there are detectable allergenic proteins in wine	1. 100 wine samples collected for subsequent analysis of basophil activation and enzyme immunoassay by September 2002 (survey)
2. Basophil activation assays specific to egg, fish, milk and peanut/tree nut allergens related to the processing aids casein, egg white, isinglass, milk and non-grape derived tannins	2. Basophil activation assays developed specific to egg, fish, milk and peanut/tree nut allergens and 100 survey wines analysed with these assays to determine whether they contain detectable allergenic proteins by March 2003 (publication)
3. Enzyme immunoassays specific to egg, fish, milk and peanut/tree nut allergens related to the processing aids casein, egg white, isinglass, milk and non-grape derived tannins	3. Polyclonal and monoclonal antibodies developed to each of ovomucoid, casein, β-lactoglobulin and isinglass, and the commercial monoclonal and polyclonal antibodies for ovalbumin purchased; 4. Establish and optimise high sensitivity ELISA; and 5. Analyse 100 survey wines using these assays to determine whether they contain detectable allergenic proteins by March 2004 (publication)
4. Provisional patents for the enzyme immunoassays specific to egg, fish, milk and peanut/tree nut allergens	6. Prepare provisional patents for the enzyme immunoassays that have been developed in output 3) by June 2004

Section II

Outputs	Performance Targets
1. Information on whether patients with a confirmed allergy to eggs, fish, milk or peanuts/tree nuts have a clinically elicitable allergy to wine fined with casein, egg white, isinglass, milk and non-grape derived tannins	1. Forty subjects at risk of reaction to wine containing food allergens recruited, clinically characterised and challenged with wines fined with casein, egg white, isinglass, milk and non-grape derived tannins by December 2003
2. Information on whether patients with a confirmed allergy to eggs, fish, milk or peanuts/tree nuts have a clinically elicitable allergy to wine which have no detectable residue of processing aids	2. Subjects giving a positive challenge in output 1), will be further challenged with wines which have no detectable residue of processing aids by June 2004
3. Peer-reviewed manuscript published	3. Preparation of a peer-reviewed manuscript to be published in an international peer-reviewed *Allergy and Immunology Journal* by June 2004
4. Industry-published manuscript	4. Preparation of a manuscript to be published in the Australian and New Zealand Grapegrower and Winemaker by June 2004
5. Submission to FSANZ	5. Preparation of a submission to FSANZ dependent on the results of outputs 1) and 2) as to whether wine and wine products should be labelled for potential allergens by 30 June 2004

4.2 Materials and Methods

4.2.1 Wines

4.2.1.1 Wine sample collection

A panel of 113 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 at least 20 wines from each of the following categories:

1. egg white fined or whole egg added (red wine);
2. non-grape tannin added (red wine);
3. milk fined (white wine);
4. isinglass fined (white wine); and
5. casein fined (white wine).

Of the 109 fined wines collected for analysis, some had been fined with more than one of the above fining agents, such that the actual number of wines processed with each agent was:

1. egg white fined or whole egg added	22
2. non-grape tannin added	25
3. milk fined	34
4. isinglass fined	20
5. casein fined	21

Wines were tested in groups according to the primary category above, but in data analysis, any additional processing aids used for a wine were taken into account.

In addition to the fined wines, two red and two white control wines were also selected on the basis of preparation in the absence of the above processing aids.

A description of the wines used in this study is shown in Appendix 7.5.6.

4.2.1.2 Wine pre-treatment

Preliminary tests showed that wine samples inhibited ELISA reactions and were cytotoxic at the concentrations to be used for *in vitro* assay. After comparison of different procedures for removing toxic effects of the wines, the following protocols were adopted for pre-treatment of wines before *in vitro* analysis. White wines were dialysed (3.5 kDa cut-off) in SnakeSkin® pleated dialysis tubing (Pierce, USA) against 5 L of phosphate buffered saline (PBS), pH 7.4 for 24 h at room temperature, with three changes of PBS. Red wines were diluted 1:4 in cold ethanol and incubated overnight to precipitate the proteins in the wine. After centrifugation at 6238 x g for 10 min at 4^{o}C, the protein pellet was resuspended in PBS to the original volume of wine. Pre-treated wines were aliquotted into 1 mL Eppendorf tubes and freeze-stored at -20ºC until use. Once thawed, the remaining sample was discarded.

Wine samples were found to be cytotoxic and unsuitable for *in vitro* assay, likely due to alcohol content and low pH value and, therefore, wines were de-alcoholised prior to assay. The method of dialysis against PBS was found to overcome this problem for white wines.

A small cut-off pore size of 3.5 kDa for the dialysis tubing was used to prevent loss of allergenic proteins. For red wines, this method was unsuitable because a fine sediment would form during assay. Proteins were thus precipitated from red wines and redissolved in PBS eliminating the sediment problem.

4.2.2 Analytical methods

4.2.2.1 Basophil activation assay for determination of allergenic wine processing aids in final bottled wines

4.2.2.1.1 *In vitro* challenge of basophils with wine samples

The basophil activation test using whole blood samples was modified from that described by Sanz et al. (2001). Preliminary experiments were performed to optimise antibody concentrations and incubation times, and the following protocol was adopted. Aliquots of heparinised blood, 100 µL, were incubated with 20 µL of stimulation buffer containing IL-3 (20 µL/10 mL; R & D Systems, USA) and heparin (200 µL/10 mL; David Bull Laboratories, Australia) for 10 min at 37°C. Pre-treated wine samples or allergen solutions, 100 µL, were added and the cells further incubated at 37°C for 20 min. Positive control cell samples were stimulated with 100 µL rabbit anti-human IgE antibody (DAKO Corporation, USA; diluted 1:1000 in stimulation buffer containing IL-3 and heparin) or fMLP (Sigma-Aldrich Co., USA; diluted 1:200 in stimulation buffer containing IL-3 and heparin); negative controls included cells alone (assay control) or cells incubated with stimulation buffer alone. Activation of basophils was stopped by incubating cells on ice for 5 min.

4.2.2.1.2 Fluorescent labelling of cells

Following allergen challenge, cells were incubated with normal goat serum (10 µL/ tube; Sigma-Aldrich, USA) on ice for 10 min to reduce non-specific binding of labelled antibodies. Cells were stained with PE-conjugated mouse anti-human CD63 (Caltag Laboratories, USA) and FITC-conjugated goat anti-human IgE (Caltag Laboratories, USA) at the previously optimised antibody dilutions and subsequently incubated on ice for 20 min in the dark. B cells and monocytes present in the cell suspension were detected by staining cells with APC-conjugated mouse anti-human CD19 (BD Pharmingen, USA) and APC-conjugated mouse anti-human CD14 (BD Pharmingen, USA) respectively, at the previously optimised antibody dilutions. Controls for antibody isotype were also included by staining cells with the relevant isotype control antibodies (BD Pharmingen, USA). Red blood cells were lysed by incubation with red cell lysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.806 mM EDTA; 2 mL/tube) for 15 min at room temperature. Cells were pelleted by centrifugation at 250 x g for 5 min (4°C) and washed once with wash buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 0.27 mM EDTA; 3 mL/tube) followed by centrifugation as described above. Cell pellets were resuspended in 150 µL wash buffer per tube and 7-amino-actinomycin D (7AAD; Sigma-Aldrich Co., USA) was added to the cells to exclude non-viable cells. Flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, USA) and Cell Quest software (Becton Dickinson, USA). Approximately 300,000 total events were collected per test to obtain sufficient numbers of basophils for analysis. The gating of $CD63^+$ cells was based on the isotype control staining and on the discrimination between the negative control (no antigen control) and positive control staining (fMLP and anti-IgE stimulation). To permit comparison between different assays, a basophil activation ratio was calculated as the proportion of CD63-positive high IgE staining cells for cells incubated with the test sample divided by the proportion for cells with stimulation buffer alone.

4.2.2.2 ELISA for detection of residual processing aids in wine

4.2.2.2.1 Generation of monoclonal antibodies

As monoclonal antibodies were only available commercially for ovalbumin, this project sought to prepare mouse monoclonal antibodies to ovomucoid, casein, β-lactoglobulin, isinglass and peanut. Polyclonal antibodies to these allergens generated by the immunised mice were also evaluated as reagents for the ELISA.

The procedure outlined below is a well-established and routine protocol for the production of monoclonal antibodies that was successfully established in our laboratory for the generation of monoclonal antibodies to natural rubber latex allergens (Sutherland *et al.* 2002). Approval from the AMREP Animal Ethics Committee was obtained (AEC approval number MU999).

(i) Immunisation procedure

On day 0, a saphenous vein bleed of 100 µL was obtained from BALB/c mice (age 4-6 weeks) for pre-immune serum. On day 1 mice were injected intraperitoneally with allergen solution (either ovomucoid, casein, β-lactoglobulin, isinglass or peanut; 10-30 µg/100 µL PBS) mixed with 100 µL of Freund's Complete Adjuvant (Sigma-Aldrich Co., USA). This allergen immunisation procedure was repeated at day 14 using 100 µL of Freund's Incomplete Adjuvant (Sigma-Aldrich Co., USA). At day 24, saphenous vein bleeds were collected and the serum screened for reactivity with the appropriate allergen (refer below for screening protocol). Mice were again immunised with a mixture of 100 µL of allergen and 100 µL of Freund's Incomplete Adjuvant on day 35. Blood was collected from mice on day 45 and screened again to select the mouse with the highest antibody titre. This mouse was then immunised again on day 56 with 100 µL of allergen solution without adjuvant. The mouse was killed by CO_2 asphyxiation on day 60 and the spleen removed aseptically.

(ii) Antibody screening protocol

Serum antibody screening assays were performed by ELISA against the relevant protein. A Costar® 96-well flat-bottom EIA/RIA plate (Corning, USA) was coated with 50 µL of allergen solution (2 µg/mL in 0.1 M bicarbonate buffer) and incubated overnight at 4°C. The plate was washed four times in 0.05% PBS-Tween and then blocked with 200 µL of 0.1% gelatin in PBS (PBS-gelatin) at 37°C for 1 h. The wash step was repeated 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 HRP-conjugated sheep anti-mouse Ig antibody (Silenus Labs, Australia) diluted 1:5000 in PBS-gelatin added followed by incubation for 1 h at 37°C. Washing was again repeated and antibody binding detected using the substrate o-phenylenediamine (OPD; Sigma-Aldrich Co., USA). OPD, 5 mg, dissolved in 12.5 mL of 0.05 M phosphate citrate buffer with perborate (Sigma-Aldrich Co., USA), was added (50 µL/well) and incubated for 10 min in the dark at 37°C. The reaction was stopped by adding 50 µL/well 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, USA). Control wells included those incubated with immune mouse serum (positive control) and with pre-immune serum from the immunised mouse (negative control).

(iii) Hybridoma generation

The splenocyte fusion partner, murine myeloma cell line X63-Ag8.653, was prepared 3–4 days prior to the fusion procedure. Cells were thawed rapidly in a 37°C water bath and washed with RPMI medium containing 10% FCS and PSG (CRPMI). The cells were placed in a 50 mL tissue culture flask and incubated at 37°C in 5% CO_2 and monitored for growth. The cell suspension was split into a 250 mL tissue culture flask when necessary to ensure that cells were in exponential growth phase on the day of the fusion.

A single cell suspension from the spleen removed from the immunised mouse on day 60 (refer to immunisation protocol) was prepared using a cell strainer. The cell suspension was washed by centrifugation at 250 x g for 8 min, twice, using RPMI-PSG. At the same time, the myeloma fusion partner was centrifuged to obtain a pellet of similar size to the splenocyte pellet. The splenocytes were mixed with the myeloma fusion partner and centrifuged at 250 x g for 5 min. The supernatant was removed and 1 mL of 50% polyethylene glycol in RPMI (pre-warmed at 37°C) was added to the pellet, dropwise over 1 min. The suspension was stirred for 1 min and then RPMI-PSG (10 mL) was slowly added over a 2 min period. Cells were centrifuged at 250 x g for 5 min and the pellet resuspended in 30 mL RPMI/20%FCS/HAT/OPI/PSG medium and subsequently expanded to 120 mL. The cell suspension was then aliquoted into six 96-well flat bottom plates (200 µL/well).

(iv) Culture, screening and cloning of hybridomas

The hybridoma cells were cultured in RPMI/20%FCS/HAT/OPI/PSG medium in an incubator at 37°C with 7% CO_2. Only fused hybridoma cells survived culturing in this medium. Cultures were 'fed' with a further 100 µL per well of RPMI/20%FCS/HAT/OPI/PSG at days 4 and 7. The first screening assay was carried out on day 10 and repeated on day 14 for the slower-growing colonies. For this, an ELISA plate was coated with 50 µL of allergen solution (2 µg/mL in 0.1 M bicarbonate buffer), and incubated overnight at 4°C. The plates were washed four times in 0.05% PBS-Tween and then blocked with 200 µL of 0.1% gelatin in PBS (PBS-gelatin) at 37°C for 1 h. The wash step was repeated and 50 µL of hybridoma cell culture supernatant was added and incubated at 37°C for 1 h. Washing was repeated and 50 µL of peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich Co., USA) diluted 1:1000 in PBS-gelatin added. After incubation for 1 h at 37°C, washing was again repeated and the substrate, OPD, 5 mg dissolved in 12.5 mL 0.05 M phosphate citrate buffer with perborate, was added (50 µL/well) for 10 min in the dark at 37°C. The reaction was stopped with 50 µL/well of 4 M HCl and colour development was quantified by measurement of optical density (OD) at 490 nm using a microplate reader as described previously.

Hybridoma cell cultures with supernatants which had shown positive specific IgG antibody reactivity by ELISA were removed from the 96-well plates and transferred into 24-well plates containing 200 µL RPMI, 10% FCS, PSG and HT supplement per well. Cell cultures were then observed daily and additional medium added as required. Once cell growth was confluent, cells were split with some cells taken for cloning by limiting dilution or single cell sorting using a flow cytometer. Limiting dilution was performed according to established methods (Goding, 1996). Briefly, 200 µL of cell suspension at 1 x 10^5 cells/mL RPMI with FCS/PSG/HT/OPI were placed in well A1 of a 96-well plate. Doubling dilutions were conducted down column 1 (B1, C1 and so on). Once column 1 was completed, a further 100 µL of medium was added to each well of column 1 and then doubling dilutions of 100 µL performed across the plate from columns 1 to 12. All wells

were topped up with medium containing a non-immune BALB/c mouse splenocyte suspension (100 µL of 0.5-1x10^6/mL) prepared in the same way as the immune mouse splenocytes for a fusion.

The plates were then left for 10–14 days until the most distal wells containing a single colony of cells had grown to a sufficient size to allow screening. If wells were found to be positive, the process was repeated, again using the most distal hybridomas. On the third subcloning, the most distal cells were expanded for large scale supernatant antibody purification.

(v) Isotyping of monoclonal antibodies

Immunoglobulin isotyping was performed by ELISA using a commercial kit (Becton Dickinson, USA) according to the manufacturer's instructions.

(vi) Purification of monoclonal antibodies

Monoclonal antibodies were purified from culture supernatants using affinity chromatography columns; Protein G (Pharmacia, Sweden) or Biosepra© Protein A (Life Technologies, USA) depending on the isotype and according to the manufacturer's instructions.

4.2.2.2.2 ELISA for detection of ovalbumin in wine

A sensitive and specific antigen capture ELISA was established for ovalbumin detection in wine based on the method of Hefle et al. (2001). Following preliminary experiments to optimise antibody concentrations and incubation times the following protocol was adopted. Rabbit anti-ovalbumin antibody (Research Diagnostics, USA) 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 (5 times) and blocked with 0.1% gelatin in PBS (PBS-gelatin) blocking solution (200 µL/well) for 1 h at room temperature. After washing 5 times with 0.05% PBS-Tween, 50 µL of ovalbumin standard solutions (1 ng/mL–2 µg/mL in PBS-gelatin) or the pre-treated wine samples (neat), were added to the wells and incubated at room temperature for 1 h. Plates were washed 5 times with 0.05% PBS-Tween and incubated with mouse anti-chicken ovalbumin monoclonal antibody (1:10,000 at 50 µL/well; Sigma-Aldrich Co., USA) for 1 h at room temperature, followed by horse radish peroxidase (HRP)-labelled sheep anti-mouse Ig antibody (1:1000 at 50 µL/well; Silenus, Australia) for 1 h at room temperature, with washes in between incubations. Antibody binding was detected using the substrate OPD as before [Section 4.2.2.2.1(ii)]. 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 non-specific 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.

4.2.2.2.3 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 antibody (Biodesign International, USA) 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 (5 times) and blocked with 0.1% gelatin in PBS (PBS-gelatin) blocking solution (200 µL/well) for 1 h at room temperature. After washing 5 times with 0.05% PBS-Tween, 50 µL of casein standard solutions (0.008 µg/mL–0.5 µg/mL in PBS-gelatin) or the pre-treated wine samples (neat), were added to the wells and incubated at room temperature for 1 h. Plates were washed 5 times with 0.05% PBS-Tween and incubated with mouse anti-casein monoclonal antibody (1:100 at 50 µL/well; see section 4.2.2.2.1 for preparation of monoclonal antibodies) for 1 h at room temperature, followed by horseradish peroxidase (HRP)-labelled sheep anti-mouse Ig antibody (1:1000 at 50 µL/well; Silenus, Australia) for 1 h at room temperature, with washes in between incubations. Antibody binding was detected using the substrate OPD as described above for ovalbumin.

4.2.2.2.4 Inhibition ELISA for detection of peanut in wine

A sandwich ELISA for the detection of residual peanut in wine was initially established using a monoclonal antibody which was raised against the major peanut allergen, Ara h 1, however, the sensitivity of this assay was insufficient. Consequently, a sensitive and specific inhibition ELISA was established based on the method of de Leon et al. (2003). 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 PBS-Tween (PBS-T) (200 µL/well) for 1 h at room temperature. Serum from a peanut allergic subject (diluted with 1% SMP in PBS-Tween for an OD 490 nm reading of 1.0 for peanut extract) was pre-incubated with de-alcoholised wines neat or peanut extract at a final concentration of 0.008, 0.04, 0.2, 1, 5, 25 and 125 µg/mL at room temperature for 1 h. The inhibition mixtures (including serum with no inhibitor as positive control) were then aliquoted into wells (50 µL/well) and incubated at 37°C for 1 h. Plates were washed with PBS-T and incubated with rabbit polyclonal anti-human IgE antibody (1:1000; 50 µL/well; DAKO) for 1 h at 37°C, followed by HRP-labelled goat anti-rabbit IgG antibody (1:1000; 50 µL/well; Promega) incubated again for 1h at 37°C, with PBS-T washes in between incubations. IgE binding was detected using OPD tablets as described for the ovalbumin ELISA. Percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = 100 - \left[\frac{\text{OD490 of serum with inhibitor}}{\text{OD490 of serum without inhibitor}} \right] \times 100$$

4.2.3 Clinical methods

4.2.3.1 Subject recruitment and clinical characterisation

Subjects were recruited from the Allergy and Asthma Clinics at The Alfred, Melbourne between January 2003 and February 2004. Subjects were initially contacted based on their clinical history of reaction to egg, fish, peanut/tree nuts or milk or as potential non-allergic control subjects, that is, no history of food or wine reactions and no specific IgE to any of the study allergens. The subjects then completed a questionnaire to determine whether they would be eligible to participate in the study (see Appendix 7.5.1).

The study was approved by The Alfred Hospital and Monash University Ethics Committee (project number 124/02) and signed informed consent was obtained from all subjects. Each

food allergic subject described features of anaphylaxis in association with the demonstration of specific IgE to relevant food allergens, either via radioallergosorbent testing (RAST; CAP, Pharmacia) or skin prick testing. Clinical characteristics of the subjects are shown in Appendix 7.5.2.

Thirty-seven subjects were recruited for the trial all of whom successfully completed the study. The study cohort included 11 non-food allergic control subjects, 10 peanut/tree nut allergic subjects, 10 fish allergic subjects, 5 egg allergic subjects, and 1 milk allergic subject (see Appendix 7.5.2). As milk allergy is very rare in adults, only one subject could be identified for this group. The age of the subjects ranged from 18–62 years and there were 21 females and 16 males.

4.2.3.2 Wine challenge protocol

The study was conducted in a double-blind, placebo-controlled manner. Subjects underwent two wine challenges separated by at least 7 days; they received the test wine on one occasion and a control unfined wine on the other. Prior to each visit subjects were asked to withhold any anti-histamine medication for 3 days, short-acting bronchodilator therapy for four hours, and long-acting bronchodilator therapy for 12 h. Each subject abstained from alcohol consumption for at least 3 days prior to the challenge and fasted for at least 8 h beforehand. Subjects were asked to complete a survey to exclude the presence of any factors that might invalidate the interpretation of results or increase the risk of challenge testing to the patient (see Appendix 7.5.3). Subjects then completed a questionnaire examining for the presence of typical features of anaphylaxis, scoring the severity of those symptoms on a visual analogue scale (VAS; see Appendix 7.5.4). A physical examination was performed within which signs of anaphylaxis were sought (see Appendix 7.5.5). Finally, baseline spirometry was performed (including FEV_1 and FVC) using a turbine spirometer (Micromedical, UK) according to ATS criteria for spirometry equipment and methodology (American Thoracic Society, 1994).

Subjects ingested 100 mL of either the test wine or control wine over 10-15 min. Serial assessments of the subjects (visual analogue and physical examination) were then made at 15 min intervals for a total of 2 h. Spirometry was repeated every 30 min during this period. Each individual score was subsequently measured and recorded.

Clinical observations included:

- vital signs (blood pressure, pulse and respiratory rate)
- oropharynx (assessing uvula, tongue, lips)
- chest (auscultation of chest assessing for any wheeze present)
- periphery (assessing for urticaria and angioedema)

Subsequent to the completion of each challenge, subjects completed a daily symptom questionnaire for the next six days, during which time they abstained from all alcoholic beverages.

The diary card assessed the following symptoms:
- asthma;
- urticaria;
- angioedema;
- laryngeal oedema;
- GIT upset; and
- circulatory collapse.

4.2.3.3 Challenge wines

Test wines were selected for each subject on the basis of their preparation with the relevant potential food allergen as a fining agent or, for peanut/tree nut allergic subjects, containing a high quantity of non-grape tannin. Control wines were prepared in the absence of any food allergens. Each wine was opened fresh on the day of challenge or had been stored under vacuum after opening for no more than five days.

4.2.4 Statistical analysis

Statistical analysis was conducted using InStat 2.0 software. Continuous variables were assessed for normality and log-transformed where appropriate. Comparison between groups (wine fining agent or subject group) was performed using Repeated Measures ANOVA. Categorical data were examined by chi-square tests. A p value of <0.05 was considered statistically significant.

4.3 Results and Discussion

4.3.1 Chemical analysis of wine samples

Wines used for the clinical challenge study were tested for total and free sulfur dioxide concentration (Table 4.1). Analysis of Australian wines by the Analytical Service of The Australian Wine Research Institute in 2004, shows that the average concentration of total sulfur dioxide in red and white wines is 50 mg/L and 130 mg/L, respectively, while the range is from 38–60 mg/L for red wine and from 112–143 mg/L for white wine. The concentration of total sulfur dioxide in the clinical challenge wines is generally within these ranges, and below that observed to elicit an adverse reaction in sulfur dioxide-sensitive asthmatics (300 mg/L; Vally and Thompson 2001).

Table 4.1: Concentration of sulfur dioxide (free and total) in wines used in clinical challenge

Wine sample Number	Type	Fining agent	Free sulfur dioxide (mg/L)	Total sulfur dioxide (mg/L)
1	white	Milk	23	112
6	red	Egg white	not detected	27
10	white	Isinglass	21	85
13	red	Egg white		
14	white	Isinglass	18	88
15	white	Isinglass	9	113
16	white	Isinglass	8	96
21	red	Egg white, non-grape derived tannin	9	40
22	white	Isinglass	20	175
24	red	Non-grape derived tannin		
25	red	Non-grape derived tannin	11	56
27	red	Egg white	9	40
28	red	Egg white	7	43
29	red	Egg white		
37	white	Milk	not detected	95
42	white	Isinglass		
61	red	Non-grape derived tannin	17	42
62	red	Non-grape derived tannin	13	126
63	red	Non-grape derived tannin	15	58
66	red	Non-grape derived tannin	4	22
104	red	Egg white	9	57
105	red	Egg white		
107	white	Isinglass	19	177
108	red	Isinglass	19	97
109	white	Isinglass		
120	white	None	18	100
122	red	None	3	41
123	red			

A selection of wines was also analysed for other compounds anecdotally associated with adverse reactions in patients and/or which might interfere in basophil assays and ELISA, both via non-immunological mechanisms (Table 4.2). The concentration of histamine in

wine has generally been observed to be less than 5 mg/L; the average concentration in Australian wine is generally less than 1 mg/L (Costello et al. 1993, Costello et al. 1996, unpublished data). The concentration of salicylic acid, histamine and tyramine in the wines analysed was very low or undetectable; the histamine concentration in the wines was also below that observed to elicit an adverse reaction in histamine-sensitive subjects (Kanny et al. 2001). It has been purported that the primary metabolite of ethanol, acetaldehyde, can release histamine and elicit an adverse reaction (Lowenberg et al. 1981, Shimoda et al. 1996, Zimatkin and Anichtchik 1999), in particular in individuals who have significantly reduced acetaldehyde dehydrogenase activity (Harada and Agarwal 1981). There was, however, no correlation between any of these concentrations and an adverse clinical reaction and/or basophil activation.

Table 4.2: Chemical analysis of selected wine samples

Wine no.	Acetaldehyde mg/L	Salicylic acid mg/L	Histamine mg/L	Tyramine mg/L
8	41	Trace	Not detected	Not detected
20	33	Trace	Not detected	Not detected
22		Trace	Trace	Not detected
26				
29				
37				
40	46	0.2	Not detected	Not detected
47	41	Not detected	Not detected	Not detected
60				
62				
68		Trace	Trace	Not detected
71		Trace	2.3	Trace
88		Trace	Trace	Not detected
90	61	Trace	Trace	Not detected
92		Trace	1.5	Not detected
120				
122				

Histamine and tyramine: limit of detection = 0.2 mg/L
trace = 0.2 – 0.8 mg/L

Salicylic acid: limit of detection = 0.1 mg/L
trace = 0.1 – 0.2 mg/L

4.3.2 Basophil activation assay

4.3.2.1 Optimisation and specificity of basophil activation assay

The ability of wines and fining agent proteins to activate basophils from food-allergic donors was assessed by flow cytometry. The activation of basophils was detected via surface CD63 expression following stimulation of whole blood with wines and fining agent proteins. Figures 4.1–4.3 show typical flow cytometric analyses of basophil activation. The method for analysis is as follows. Cells were first gated on a forward scatter versus side scatter region (Figure 4.1a) later validated to contain basophils by back-gating of high IgE staining cells (Figure 4.1d). Viable cells within this scatter gate were selected via exclusion of the vital dye 7AAD (Figure 4.1b) and basophils identified by high intensity anti-IgE staining (IgE^{hi}; Figure 4.1c). B cells and monocytes may stain weakly with anti-IgE antibody but were shown to be excluded from the gated IgE^{hi} cell population by CD19 and CD14 staining respectively (Figure 4.1f,g). The expression of CD63 by the gated IgE^{hi} cells was analysed (Figure 4.1h).

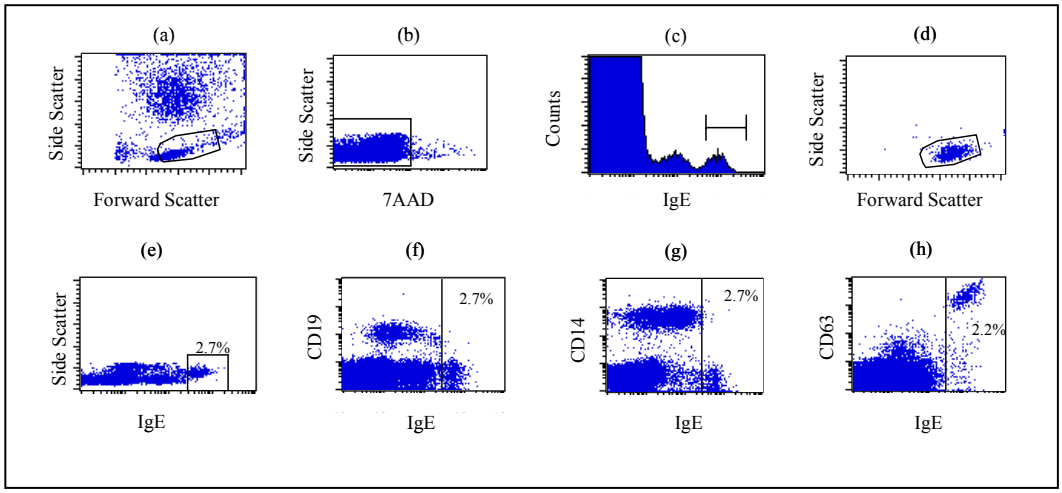


Figure 4.1: Flow cytometry plots showing gating of viable basophils based on forward and side scatter profile (a), 7AAD negativity (b) and high IgE staining (c), with confirmation of scatter gate by back-gating on high IgE staining cells (d). The absence of B cells and monocytes in the high IgE gate is shown by the lack of CD19-positive (f) and CD14-positive (g) cells. Strong staining for CD63 is seen for the majority of IgE^{hi} cells in this sample.

A representative analysis of basophil activation following incubation of whole blood from an egg-allergic patient with the egg allergens ovalbumin and ovomucoid, other wine fining agent proteins (casein, β-lactoglobulin, isinglass and non-grape tannin), roasted peanut, an egg-fined wine and a control unfined wine is shown in Figure 4.2. It can be seen that incubation with 10 µg/mL of ovalbumin and ovomucoid resulted in the activation of 52% and 75% basophils, respectively while incubation with the other fining agent proteins and peanut proteins resulted in 1–6% activation, consistent with specific basophil activation by egg allergens for this egg-allergic subject. Neither of the wine samples induced basophil activation, values being similar to that for the 'no antigen' negative control (stimulation buffer alone; 2% CD63 positive). Positive controls fMLP and anti-IgE demonstrated 60% and 55% activation respectively. fMLP was used to demonstrate IgE-independent CD63 expression and anti-IgE was used to demonstrate the expression of CD63 following cross-

linking of surface IgE on basophils, thereby confirming the viability and functionality of the basophils present in the patient's blood sample.

As a negative control, whole blood from a non-allergic control subject, that is, a subject with no history of food or wine reactions and no specific IgE to any of the study allergens, was incubated with the same concentration of fining agent proteins and wines. Basophil activation was minimal (6–15%; Figure 4.3b), similar to the 'no antigen' control for this sample (14%; Figure 4.3c).

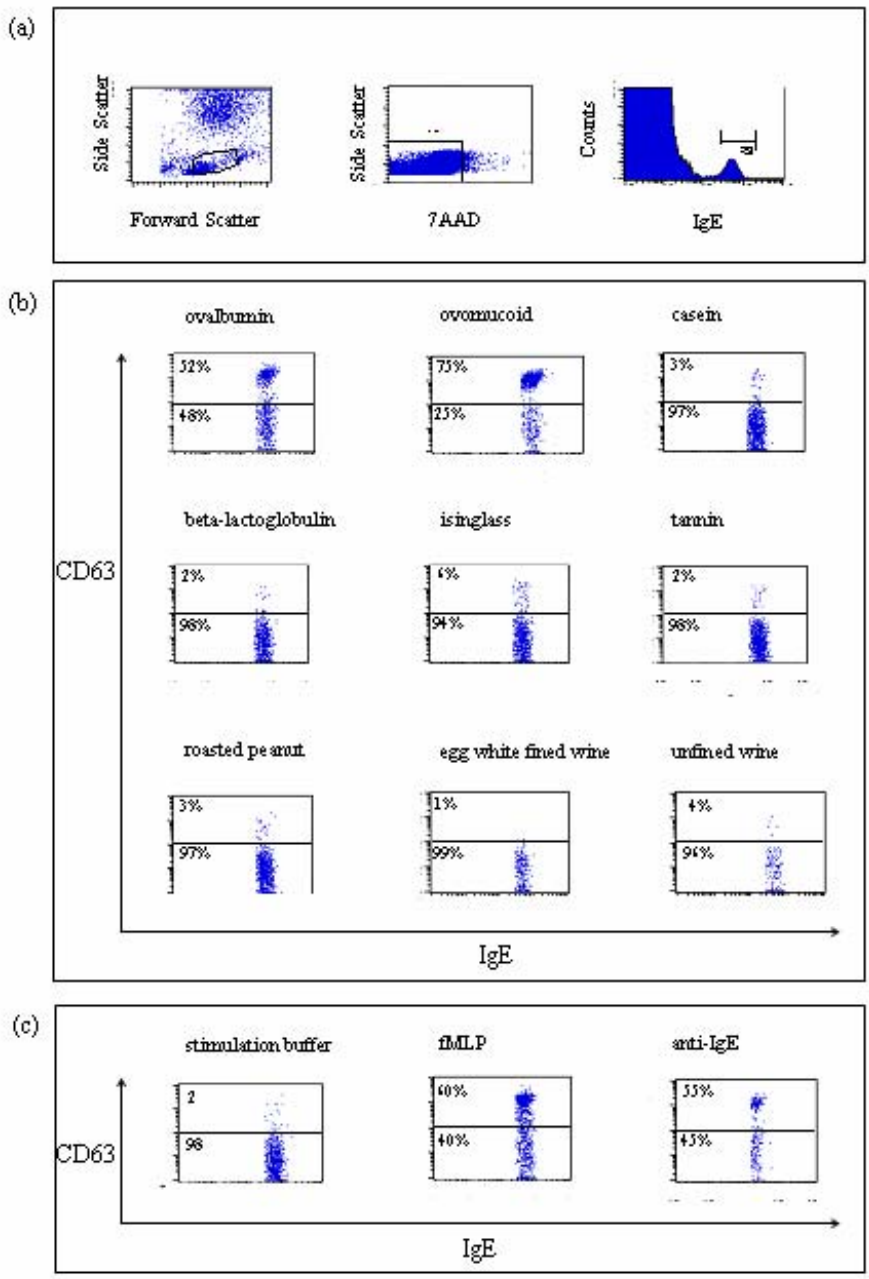


Figure 4.2: Flow cytometric analysis of activation of blood basophils from an egg-allergic patient

(a) Viable basophils are identified by size and granularity (light scatter), viability (exclusion of the vital dye 7AAD) and high anti-IgE staining.
(b) Shows CD63 expression on basophils incubated with the fining agent proteins or roasted peanut extract (at 10 μg/mL), with an egg white-fined wine and an unfined wine.
(c) Shows CD63 expression on basophils without added allergen (i.e. stimulation buffer alone), or with fMLP and anti-IgE as positive controls.

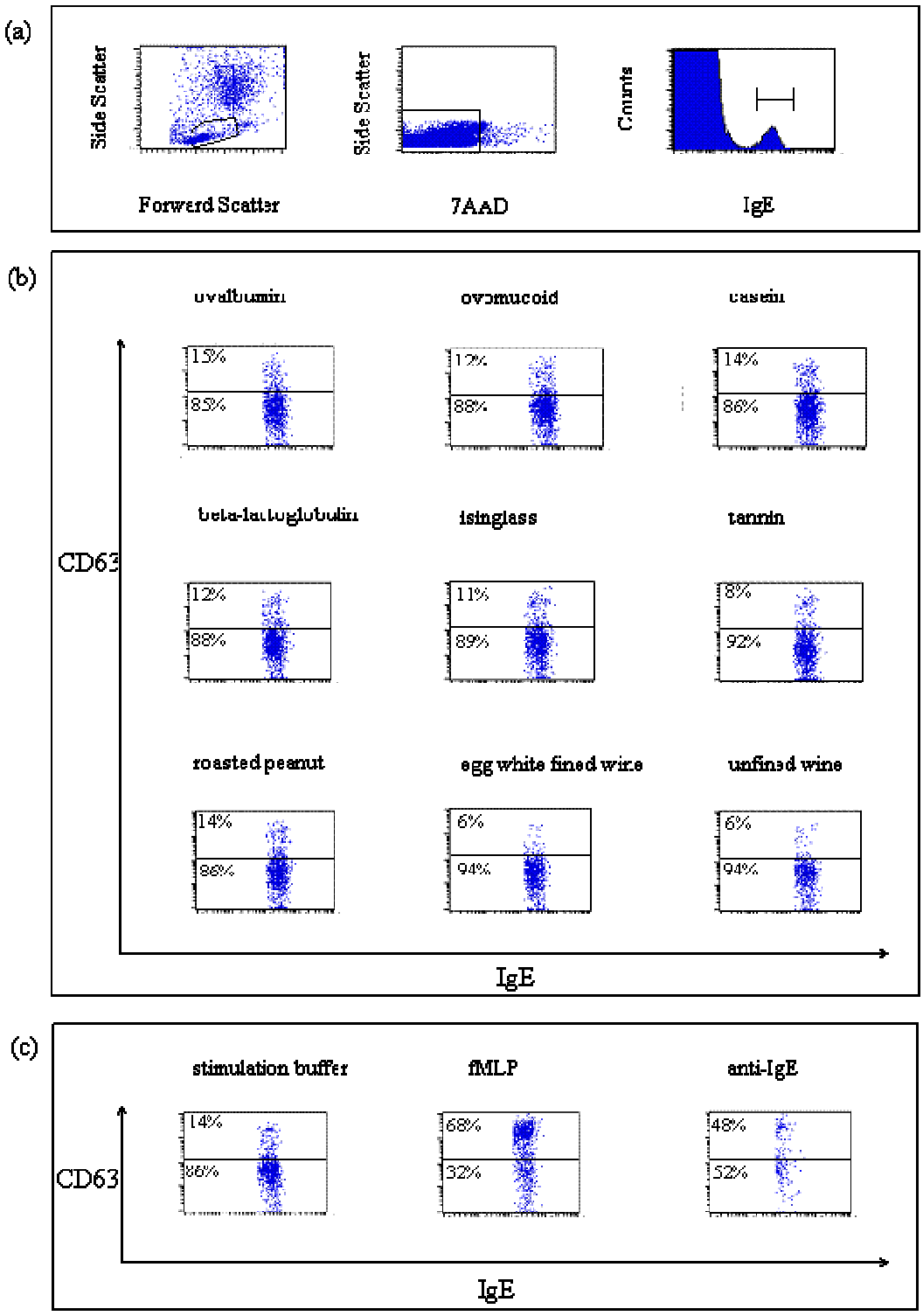


Figure 4.3: Flow cytometric analysis of activation of blood basophils from a non-allergic patient

(a) Viable basophils are identified by size and granularity (light scatter), viability (exclusion of the vital dye 7AAD) and high anti-IgE staining.
(b) Shows CD63 expression on basophils with the fining agent proteins or roasted peanut extract (at 10 μg/mL), with an egg white-fined wine and an unfined wine.
(c) Shows CD63 expression on basophils without added allergen (i.e. stimulation buffer alone), or with fMLP and anti-IgE as positive controls.

In pilot basophil activation assays, a range of concentrations (0.01–10 μg/mL) of the allergen proteins was tested to determine the optimal concentration for activation of basophils from allergic subjects. From these studies, an allergen protein concentration of 10 μg/mL was selected for use in this project. Table 4.3 shows the results for one of these studies in which basophil activation for five peanut allergic subjects was tested to roasted peanut extract at different concentrations.

Table 4.3: Optimisation of allergen concentration for basophil activation assay using roasted peanut extract. Basophil activation ratios (that is, % activated basophils with allergen divided by % activated basophils in stimulation buffer alone) are shown for five peanut-allergic subjects.

	Basophil activation ratio for a different concentration of roasted peanut extract			
Subject	**0.01 μg/mL**	**0.1 μg/mL**	**1 μg/mL**	**10 μg/mL**
A	1.1	1.1	1.4	1.4
B	1.7	1.8	1.9	2.2
C	25.7	28.0	29.3	28.7
D	10.8	11.4	11.3	11.1
E	1.8	2.1	2.2	2.0

4.3.2.2 Analysis of survey wines by basophil activation assay

Blood for basophil activation assays was collected from each subject prior to the wine challenge procedure on one of their two visits. It was not feasible to test the blood of all subjects with all the wines so a chequerboard grid (Figure 4.4) was established to ensure that all wines were tested against the blood of at least one (and usually two subjects) from each allergy group. Representative data for a basophil activation assay of wine samples using the blood of a single allergic donor are shown in Figure 4.5.

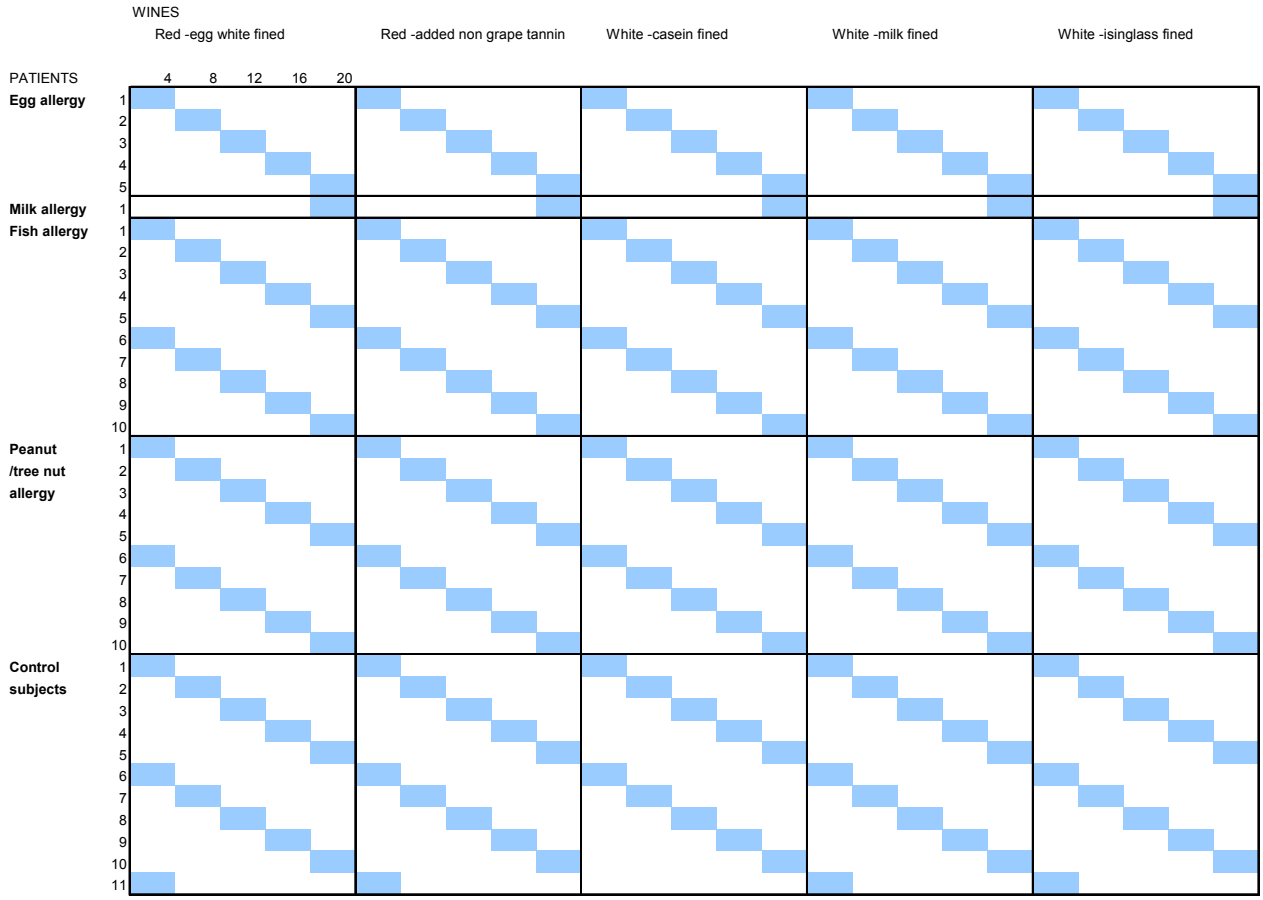


Figure 4.4: Plan for testing survey wines by basophil activation assay

Wines were initially allocated to a fining agent group, then four wines from each of the five groups (shown in blue) were selected as a panel of 20 to be tested in the basophil activation assay for one subject. Wines were distributed such that a different panel of 20 was selected for each subsequent subject in each allergy group (up to five, then repeated) so that the entire wine panel was tested by at least one and usually two subjects in each allergy group. Wines were similarly tested for control subjects.

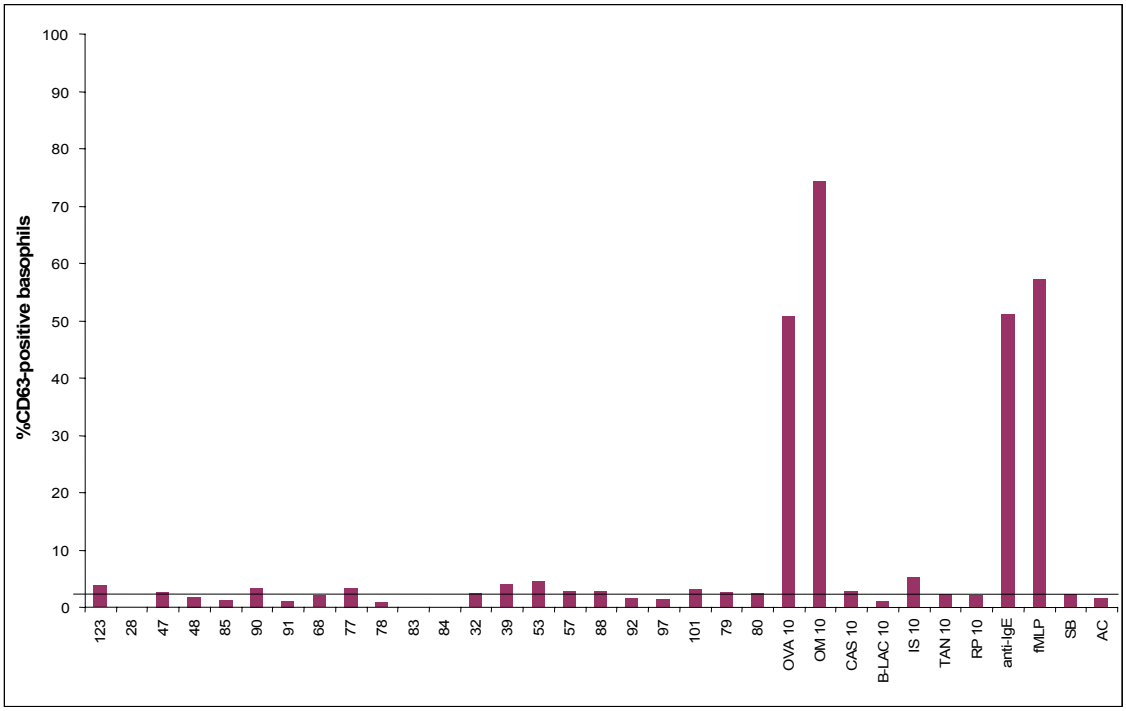


Figure 4.5: Representative data for basophil activation assay of survey wines

Expression of CD63 as a marker of basophil activation for cells from an egg-allergic patient incubated with a panel of test wines (pre-treated as described previously) and fining agents. Results show background expression of CD63 on cells incubated with the panel of wines fined with different processing aids, the fining agents casein (CAS), β-lactoglobulin (B-LAC), isinglass (IS) and non-grape tannins (TAN), and the control allergen roasted peanut (RP), but good expression of CD63 in positive controls (cells incubated with ovalbumin, ovomucoid, anti-IgE or fMLP).

Key

Numbers: wines (fined and unfined)
OVA 10: ovalbumin at 10 µg/mL
OM 10: ovomucoid at 10 µg/mL
CAS 10: casein at 10 µg/mL
B-LAC 10: β-lactoglobulin at 10 µg/mL
IS 10: isinglass at 10 µg/mL
TAN 10: non grape derived tannin at 10 µg/mL
RP 10: roasted peanut at 10 µg/mL
anti-IgE: anti-IgE (positive control)
fMLP: N-formyl-Met-Leu-Phe (positive control)
SB: stimulation buffer (baseline)
AC: assay control (negative control)

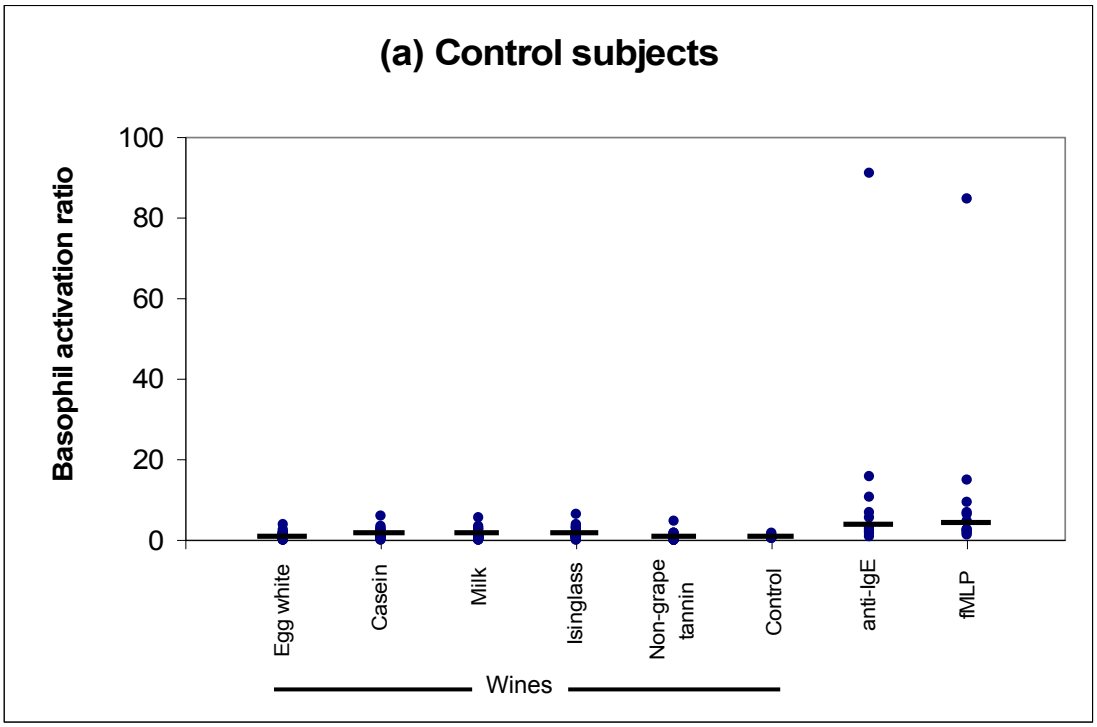
The results for testing blood samples from the 37 study subjects against the complete panel of survey wines as described above are summarised in Figure 4.6a-e and Table 4.4. For all assays, the percentage of activated basophils in positive controls (anti-IgE, fMLP and/or relevant allergen extract) was greater than the positive cut-off value of 15% reported in the literature (Erdmann et al. 2004), validating each assay. Where the percentage of activated basophils for a test sample was not above 10%, the test was considered negative. Since values for cells incubated in stimulation buffer alone differed between subjects, the results are shown as basophil activation ratios to permit comparison between different subjects, that is, % CD63 positive cells incubated with test allergen divided by % CD63 positive cells incubated in stimulation buffer alone. Where a wine was fined with more than one agent, the basophil activation ratio value is shown against all relevant fining agents. Median values for each group of tests are shown as bars.

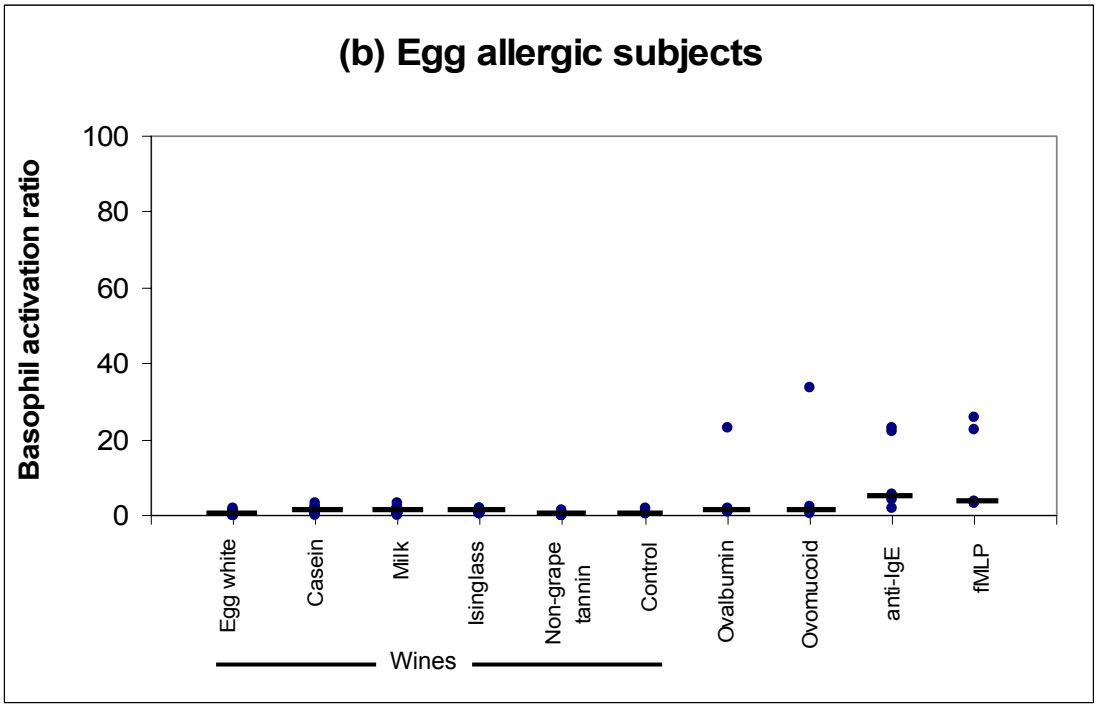
A reference range was established based on the basophil activation ratios for control non-food allergic subjects tested against test and control wines. The upper limit for the 95th percentile of these tests was 2.85. Median values for basophil activation ratios for each group of wines for a particular subject group all lay within this range. When data for basophil activation for blood cells from allergic subjects incubated with the wine samples were analysed, no statistically significant difference in basophil activation ratios between fining agents within a subject group or for a particular fining agent between subject groups was found.

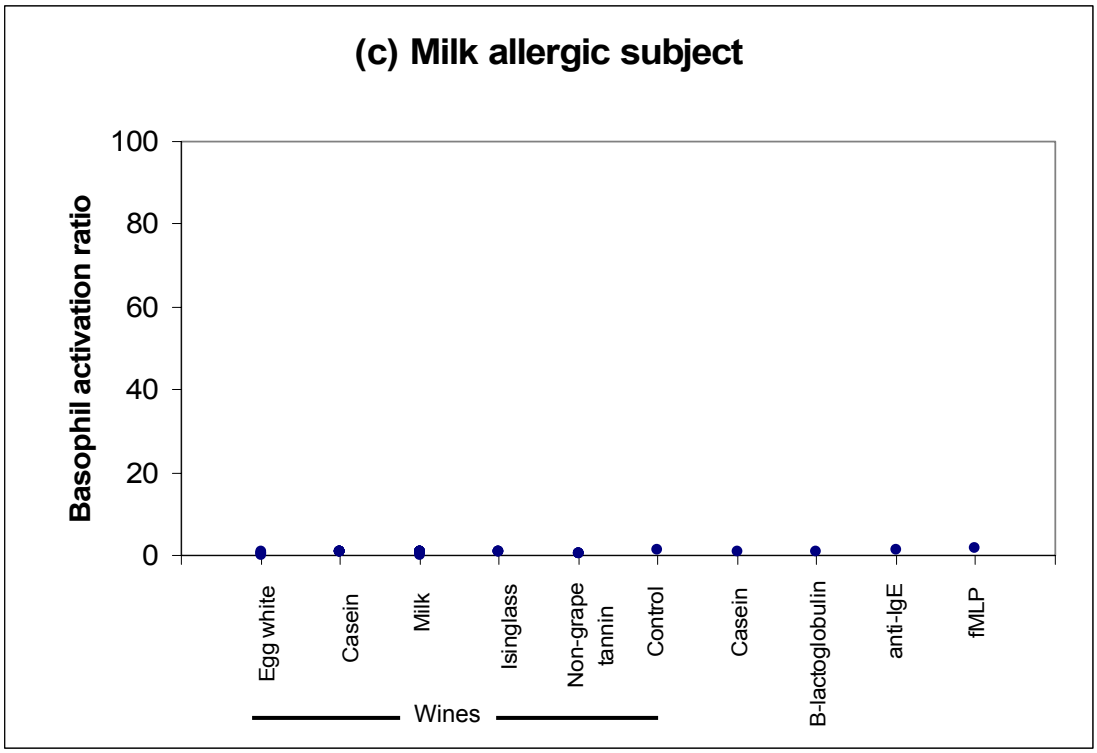
This implies that for the egg allergic subjects, incubation of their blood basophils with wine fined with egg white did not cause a statistically significantly greater activation of the blood basophils than did the control non-fined wine or any other fined wine. This can be also implied for the milk-allergic patient with wine fined with casein and milk, the nut-allergic patients with wine fined with non-grape tannin, and the fish-allergic patients with wine fined with isinglass. Likewise, the egg white fined wines did not cause greater activation of basophils from egg allergic subjects than from any other subject group, and so on for the other wine fining agents.

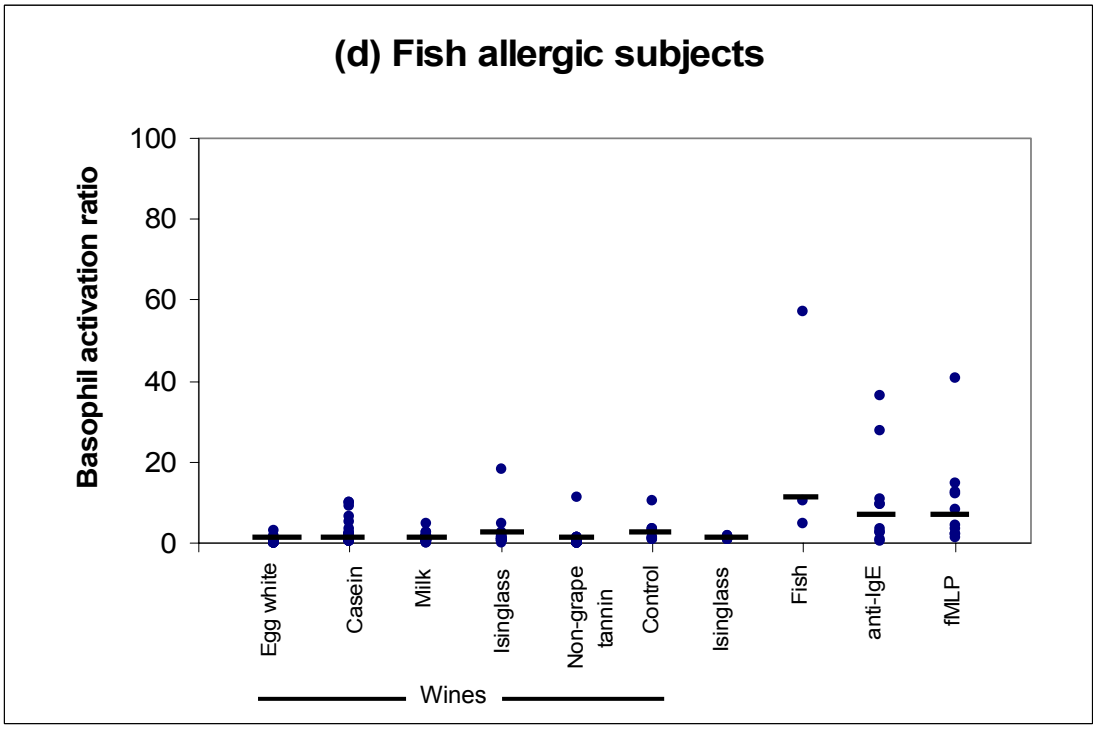
Only nine of the 100 fined wines tested gave basophil activation >10% with a ratio above the reference range upper limit of 2.85 for some subjects (data summarised in Table 4.4), but no pattern could be identified with respect to wine variety, fining agent or geographic indication, or to subject group. One isinglass fined wine (#20) caused weak basophil activation for two fish allergic subjects, but this same wine caused weak activation of basophils from a peanut allergic individual. The two fish allergic subjects showed no clinical adverse effects to another wine fined with isinglass. One of the control unfined wines (#120) caused basophil activation in three fish allergic subjects (two of these reactions were weak), while basophils from one control non-food allergic subject reacted weakly to an isinglass fined wine (#22).

The gold standard in food allergy assessment is the double blind placebo controlled food challenge as we have developed for the clinical arm of this study. Our findings show that the basophil activation assay has correlated well with the clinical challenge results, but its lower specificity than clinical challenge suggests that this assay alone would not be a reliable routine testing procedure.


(a) Control subjects
Basophil activation ratio
100
80
60
40
20
0
Egg white
Casein
Milk
Isinglass
Non-grape tannin
Control
anti-IgE
fMLP
Wines


(b) Egg allergic subjects
Basophil activation ratio
100
80
60
40
20
0
Egg white
Casein
Milk
Isinglass
Non-grape tannin
Control
Ovalbumin
Ovomucoid
anti-IgE
fMLP
Wines


(c) Milk allergic subject
Basophil activation ratio
100
80
60
40
20
0
Egg white
Casein
Milk
Isinglass
Non-grape tannin
Control
Casein
B-lactoglobulin
anti-IgE
fMLP
Wines


(d) Fish allergic subjects
Basophil activation ratio
100
80
60
40
20
0
Egg white
Casein
Milk
Isinglass
Non-grape tannin
Control
Isinglass
Fish
anti-IgE
fMLP
Wines

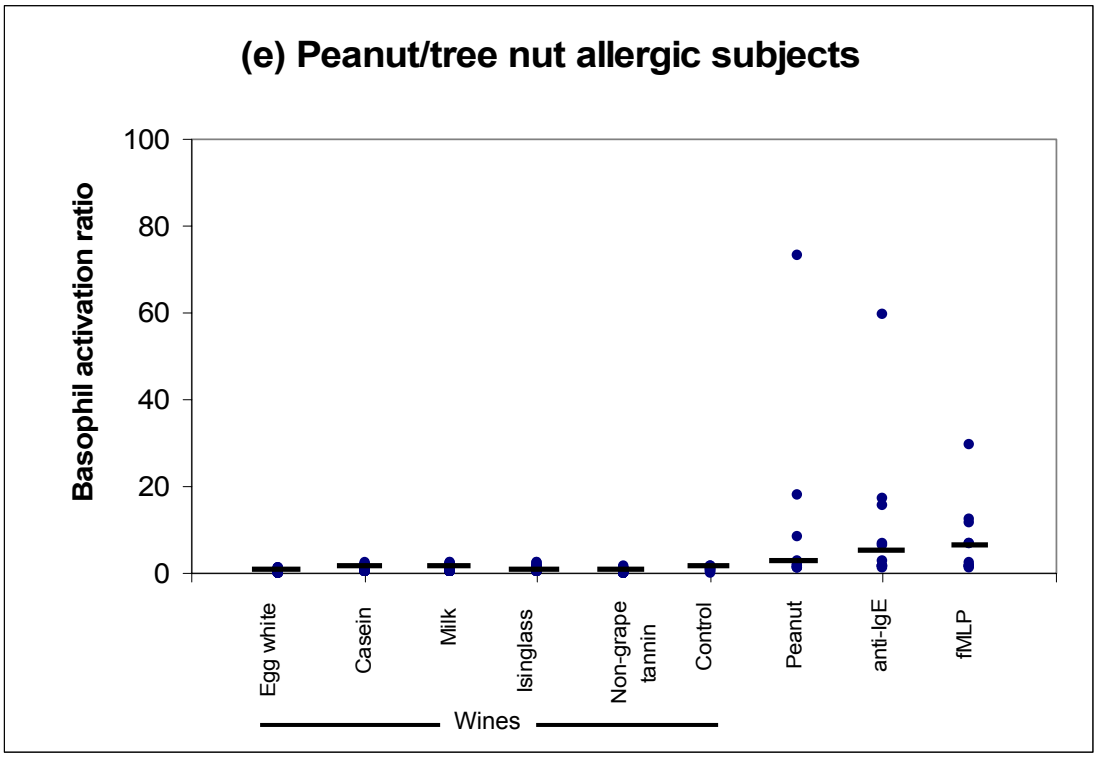


Figure 4.6 Summary of basophil activation ratios for each subject group when blood was stimulated with wines from each of the fining agent groups or with control agents. Bars represent median values.

Table 4.4: Summary of basophil activation assays which showed a ratio of >2.85 (upper limit of 95th percentile for control subject assays vs fined and unfined wine samples). Data not shown for assays where <10% basophils were activated ie test considered negative. A total of 37 assays were performed on control wines and 730 assays on test wines (W); some wines were fined with more than one agent, giving a total of 923 data points.

				No. of assays with basophil activation ratio >2.85						
				Unfined white (#120)	Unfined red (#122)	Fined – egg white	Fined – casein	Fined – milk	Fined – isinglass	Fined – non grape tannin
			Total no. wines	1	1	22	21	34	20	25
			Total no. assays	15	22	152	177	231	144	163
Allergy group	No.subjects in group	No.subjects with ratio >2.85								
Egg	5	1		0	0	0	E5 W #60	E5 W #60	0	0
Peanut	10	1		0	0	0	0	0	P7 W #20	0
Fish	10	6		F6, F8, F10	0	0	F5 W #26; F6 W #8; F10 W #68,88,92	0	F7 W #20; F9 W #20)	F7 W #71
Milk	1	0		0	0	0	0	0	0	0
Control	11	1		0	0	0	0	0	C6 W #22	0

4.3.3 ELISA for detection of residual processing aids in wine

4.3.3.1 Generation of monoclonal antibodies

Hybridomas were successfully generated against all allergens but monoclonal antibodies of $IgG_{1\kappa}$ isotype, suitable for sensitive ELISA, were only obtained for casein and peanut. The hybridoma against ovomucoid failed to maintain antibody production and the antibodies against isinglass and ovomucoid were of IgM isotype, giving high background values in ELISA. Repeat immunisations failed to produce IgG monoclonal antibodies against these two allergens.

4.3.3.2 ELISA for detection of ovalbumin in wine

4.3.3.2.1 Optimisation of ELISA for detection of ovalbumin

A sandwich ELISA for the detection of ovalbumin was established using commercially available monoclonal and polyclonal antibodies. Several aspects of the technical procedure, for example, the concentration of antibody, were varied in order to achieve greatest sensitivity. Performing the assay at room temperature rather than at 37°C reduced non-specific binding of antibodies giving better signal detection at a lower concentration (Figure 4.7). Using the optimised method, the limit of detection was 1 ng/mL ovalbumin.

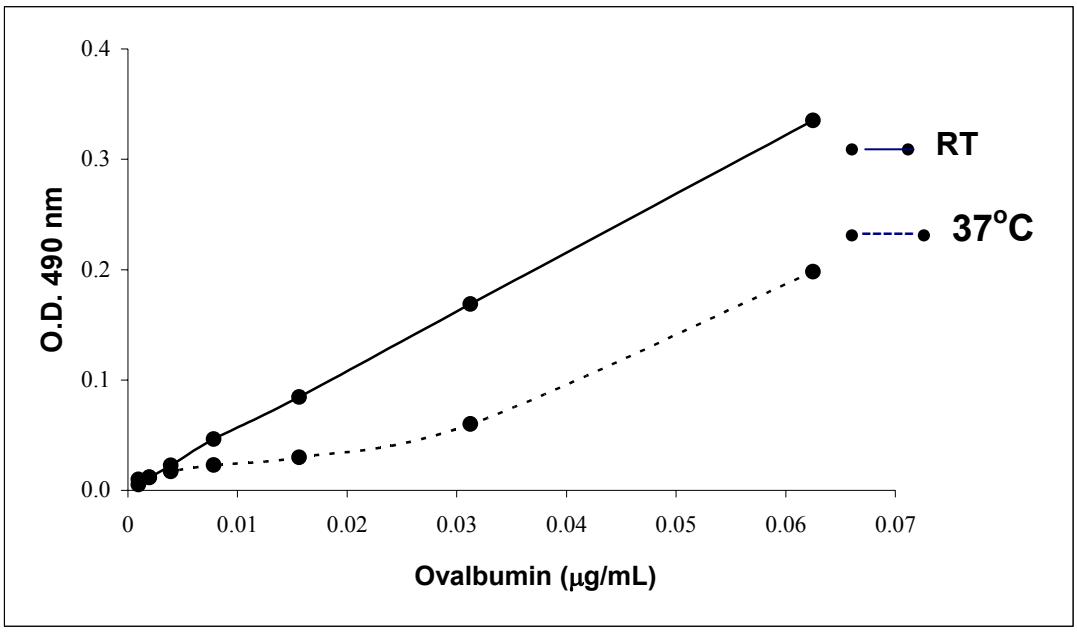


Figure 4.7 The effect of incubation temperature on the sensitivity of the anti-ovalbumin sandwich ELISA

ELISA plates were coated with rabbit anti-ovalbumin antibody (10 μg/mL) and the detection of ovalbumin was assessed after incubations were performed at either room temperature (RT) or 37°C. Assays were performed in triplicate. The mean O.D. of wells containing no antigen was subtracted from the mean O.D. of wells containing antigen.

The ovalbumin ELISA was 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 4.8.

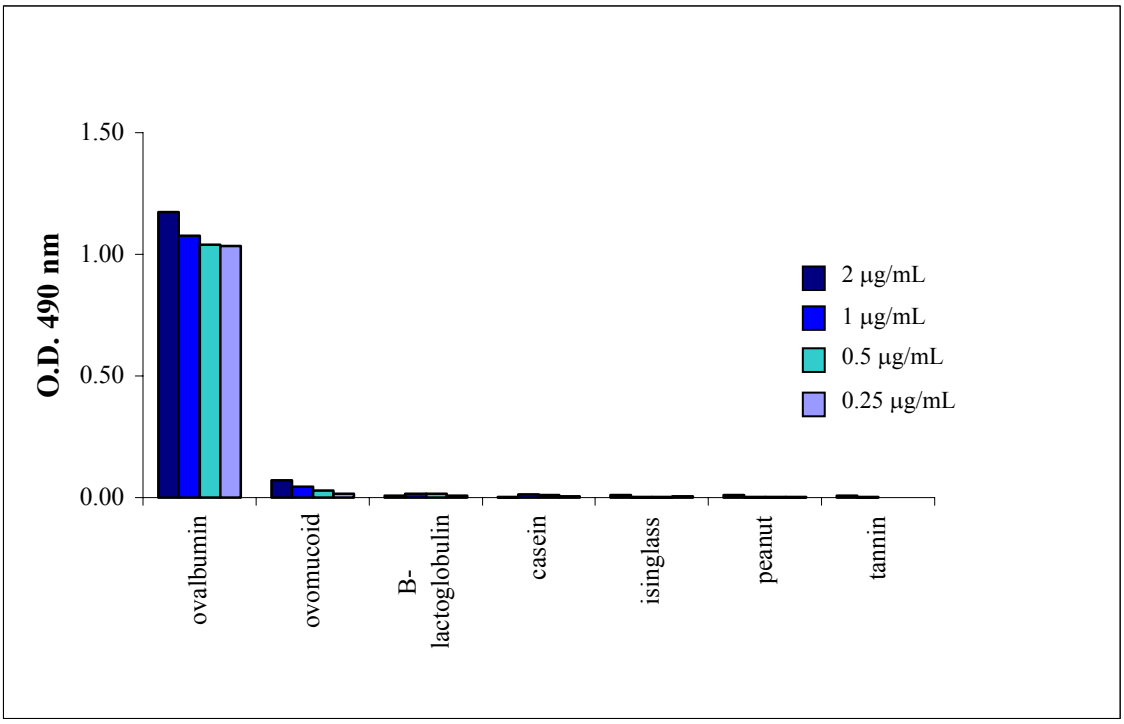


Figure 4.8: Specificity of anti-ovalbumin ELISA

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. Antigen binding was then assessed by binding of anti-ovalbumin monoclonal antibody. The mean O.D. of triplicate wells containing no antigen was subtracted from the mean O.D. of triplicate wells containing antigen.

4.3.3.2.2 Testing of survey wines for detectable ovalbumin

The ovalbumin sandwich ELISA was used to test for ovalbumin in the panel of 102 survey wines (100 fined and 2 unfined as tested in the basophil activation assay). Ovalbumin was undetectable in all wines except two: #98 (0.98 µg/mL) and #99 (0.40 µg/mL). These two wines had not been fined with egg white but rather whole eggs had been added (6 eggs/1000 L). Basophil activation ratios for these wine samples were within the reference range for all of the eight subjects tested including one egg-allergic subject. This subject's basophils, however, were not activated when incubated with control ovalbumin or ovomucoid proteins, suggesting that either the patient was only weakly allergic or reacted to other egg proteins. As egg allergy is predominantly a condition found in children aged less than five years, further testing of basophil activation using a larger panel of sera from children with confirmed egg allergy in a stripped basophil activation assay using adult donor basophils should be considered (see Appendix 7.5.8).

When wines #98 and #99 were analysed without pre-treatment, the concentration of ovalbumin detected was only 0.013 and 0.004 µg/mL, respectively, confirming the importance of pre-treatment for more sensitive detection of ovalbumin in wine.

4.3.3.3 ELISA for detection of casein in wine

4.3.3.3.1 Optimisation of ELISA for detection of casein

A sandwich ELISA for the detection of casein was established using a monoclonal antibody to α-casein developed in our laboratory and a commercially available polyclonal antibody. The commercially available polyclonal sheep antibody was found to have greater sensitivity than polyclonal mouse sera collected during the production of monoclonal antibody (Figure 4.9).

Optimisation studies showed that greatest sensitivity for detection of α-casein was achieved using the polyclonal sheep anti-casein antibody at 0.5 μg/mL as the coating antibody (Figure 4.10) and the monoclonal anti-α-casein antibody at 1:100 dilution as the detecting antibody. Using this optimised protocol, the limit of detection was 8 ng/mL α-casein (Figure 4.11).

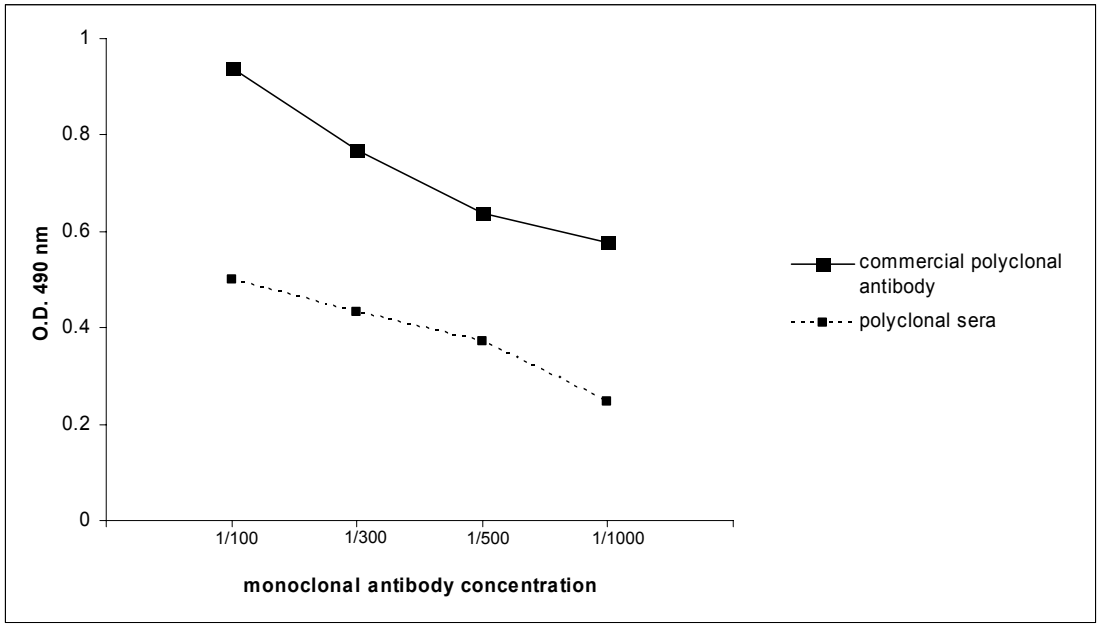


Figure 4.9 Comparison of polyclonal antibodies for the sensitivity of the anti-casein sandwich ELISA and concomitant titration of monoclonal antibody

ELISA plates were coated with either commercial polyclonal sheep antibody to casein (5 μg/mL) or immune mouse sera (1/200). The detection of α-casein was assessed after addition of monoclonal anti-α-casein antibody at a concentration of 1:100. Assays were performed in triplicate. The mean O.D. of wells containing no antigen was subtracted from the mean O.D. of wells containing antigen.

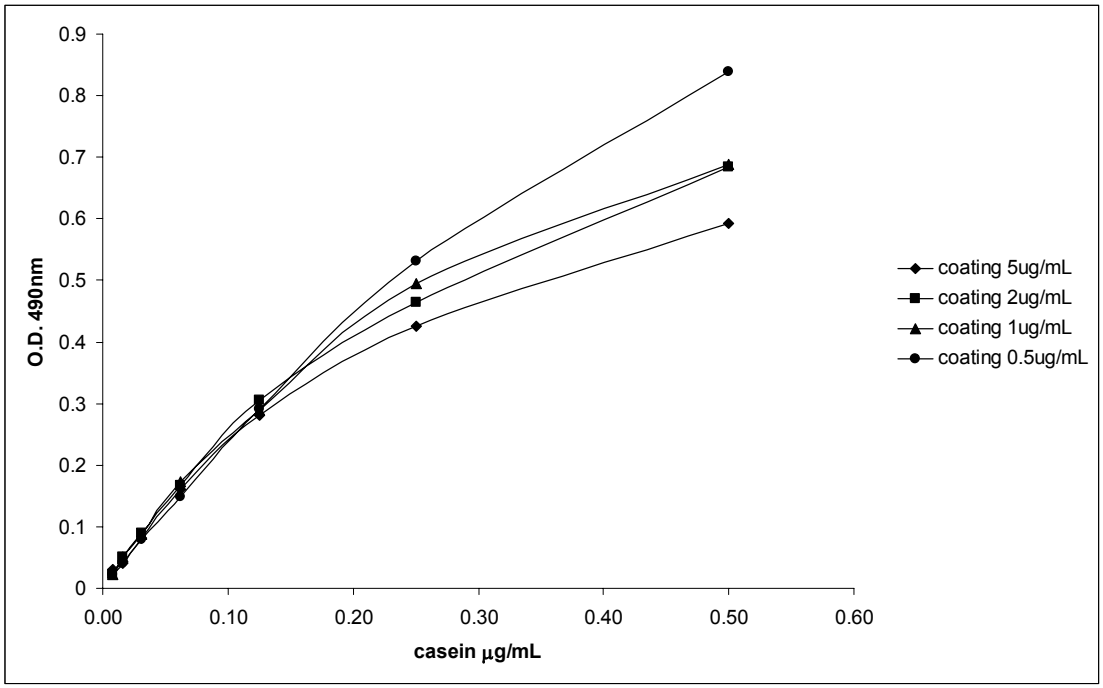


Figure 4.10 Optimisation of the concentration of sheep anti-casein capture antibody
ELISA plates were coated with sheep anti-casein polyclonal antibody at a concentration range of 0.5 to 5 μg/mL. Casein was added in the range of 0.008 to 0.5 μg/mL. The monoclonal antibody to α-casein was used at the optimised dilution of 1:100. Assays were performed in triplicate. The mean O.D. of wells containing no antigen was subtracted from the mean O.D. of wells containing antigen.

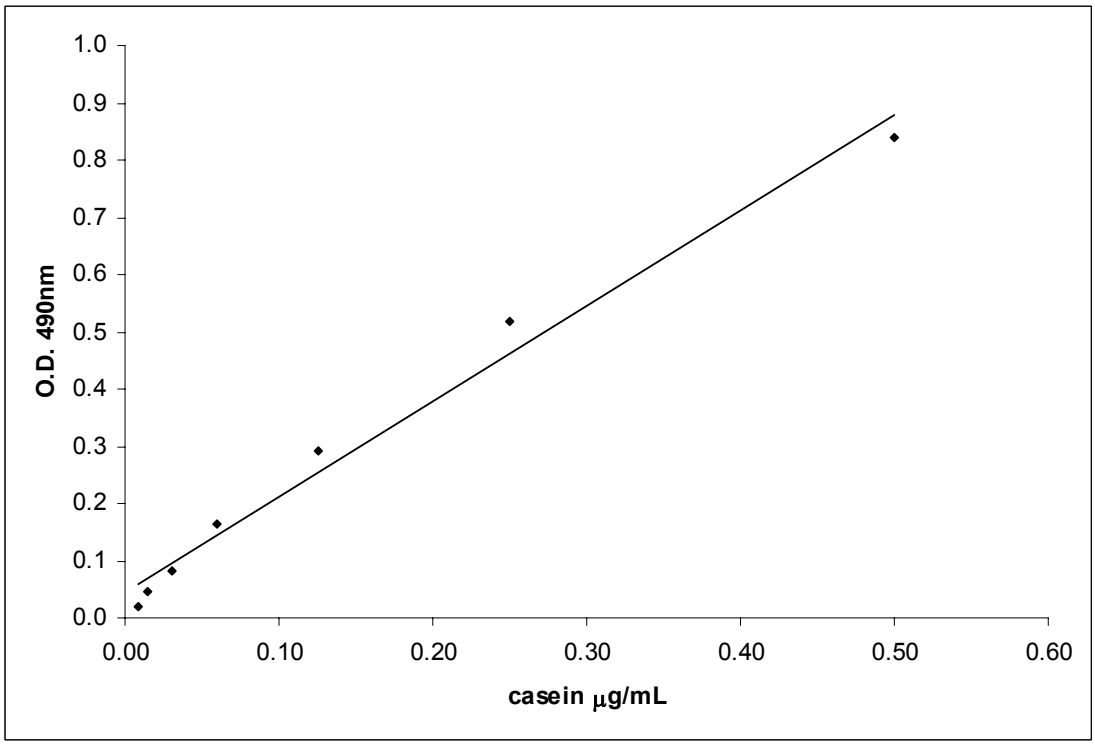


Figure 4.11 A standard curve of the anti-casein sandwich ELISA under optimised conditions
ELISA plates were coated with sheep anti-casein polyclonal antibody at the optimised concentration of 0.5 μg/mL. Casein was added in the range of 0.008 to 0.5 μg/mL. The monoclonal antibody to α-casein was used at the optimised dilution of 1:100. Assays were performed in triplicate. The mean O.D. of wells containing no antigen was subtracted from the mean O.D. of wells containing antigen.

4.3.3.3.2 Testing of survey wines for detectable casein

The casein sandwich ELISA was used to test for α-casein in the panel of 102 survey wines (100 fined and 2 unfined as tested in the basophil activation assay). α-Casein was undetectable (< 8 ng/mL) in all wines.

4.3.3.4 ELISA for detection of peanut in wine

4.3.3.4.1 Optimisation of ELISA for detection of peanut allergens

An inhibition ELISA for the detection of peanut was established using both serum from a peanut allergic patient and an Ara h 1-specific monoclonal antibody. The inhibition assay using peanut allergic serum was found to have greater sensitivity than a sandwich ELISA utilising the monoclonal antibody. The limit of detection for the optimised peanut allergic serum inhibition ELISA was 8 ng/mL peanut.

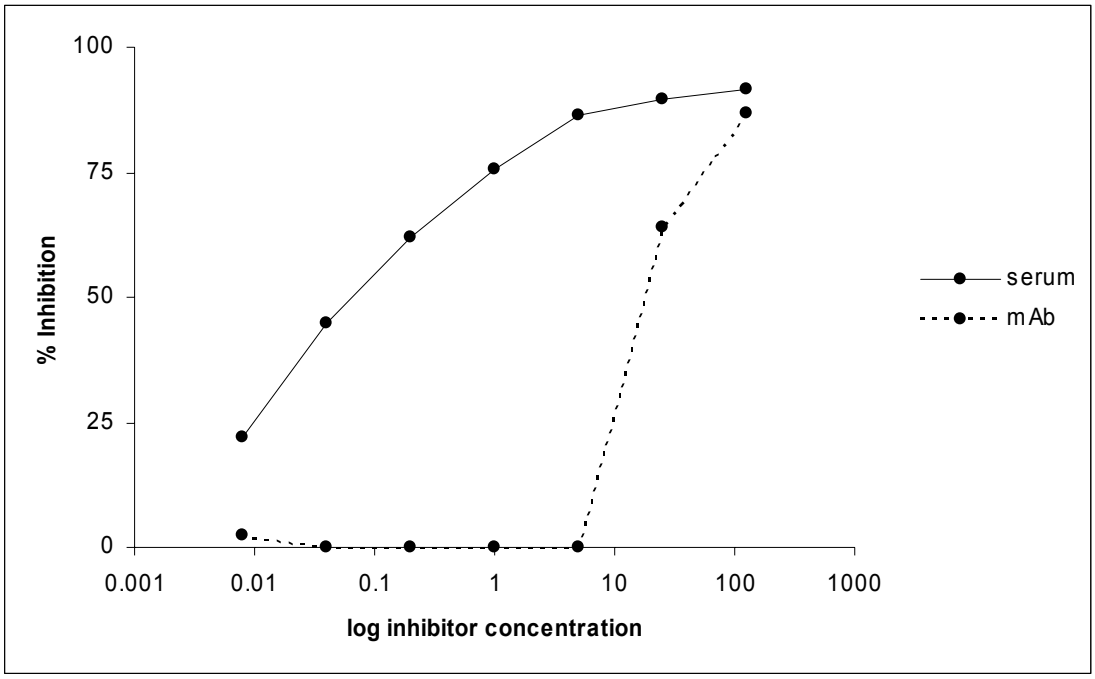


Figure 4.12 Comparison of the sensitivity of peanut-specific IgE with Ara h 1-specific monoclonal antibody using an anti-peanut inhibition ELISA

Peanut allergic serum or the Ara h 1-specific monoclonal antibody was pre-incubated with different concentrations of peanut extract and then added to ELISA plates coated with peanut (1µg/mL). IgE binding to peanut was assessed and expressed as percentage inhibition. Assays were performed in triplicate.

4.3.3.4.2 Testing of survey wines for detectable peanut allergens

The peanut serum IgE inhibition ELISA was used to test for residual peanut in the panel of 102 survey wines (100 fined and 2 unfined as tested in the basophil activation assay). Peanut was undetectable (< 8 ng/mL) in all wines.

4.3.4 Clinical challenge

A total of 76 wine challenges (27 wines including 3 control wines; Table 4.1) were performed on 37 subjects (26 food allergic and 11 non-food allergic). Overall, there was no statistically significant difference in any parameter measured between subject groups or between fining agents. Importantly, no subject experienced anaphylaxis following wine challenge. For four of the 26 food allergic subjects, a symptom (Appendix 7.5.4) or sign (Appendix 7.5.5) was noted during the 2-h observation period following wine consumption (Table 4.5). Two of these events occurred when the subject was challenged with a control wine. No other subject challenged with the control wines showed a symptom or sign of adverse reaction. Only one of the two subjects (E4), who was egg allergic, required treatment with administration of salbutamol and this resulted in complete resolution of the 22% fall in FEV_1. It was notable that this subject also demonstrated an 11% fall in FEV_1 following consumption of an egg-fined wine but FEV_1 normalised spontaneously. Clinically it was considered that this individual had suboptimal asthma management on an ongoing basis with heightened airway reactivity to numerous non-specific stimuli. There was a subsequent adjustment of the maintenance anti-asthma medication.

IgE mediated milk allergy is rarely identified in adult patients but the one confirmed milk allergic subject recruited for this study reported the symptom of a "slight lump in the throat" following ingestion of a milk-fined wine which did not occur with control challenge. A repeated, blinded challenge with the same milk-fined wine resulted in reported itch. No treatment was needed for either active challenge and no signs of allergic reaction were noted. As milk allergy is predominantly a condition found in children aged less than five years, further testing of basophil activation using a larger panel of sera from children with confirmed milk allergy in a stripped basophil activation assay using adult donor basophils should be considered (Appendix 7.5.8).

None of the wines associated with reported symptoms or signs elicited a positive basophil activation assay in any of the same subjects, and except for the unfined wine #120, in any other subject.

No diary card abnormalities were noted in the six-day follow up period for any subject.

Table 4.5: Summary of episodes of symptoms (Appendix 7.5.4) and/or signs (Appendix 7.5.5) following wine (W) consumption in clinical challenge.

Allergy group	No. of subjects in group	No. of subjects with any symptoms or signs following wine consumption	Unfined wine		Fined wine	
			No. of subjects	Subject and wine no. Comments	No. of subjects	Subject and wine no. Comments
Egg	5	1	1	E4 W #122 FEV_1 22% fall, slight chest tightness; fully reversed by salbutamol	1	E4 W #29 FEV_1 11% fall; mild wheeze at time of visit; spontaneous resolution
Peanut	10	1	0		1	P6 W #62 FEV_1 13% fall; spontaneous resolution
Fish	10	1	1	F3 W #120 Mild symptoms of lip numbness; spontaneous resolution	0	
Milk	1	1	0		1	M1 W # 37 Challenge 1: slight lump in throat; spontaneous resolution. Challenge 2: mild itch; spontaneous resolution
Control	11	0				

4.4 Conclusions

Section I

- The survey wine panel (113) was collected comprising control unfined wines and wines fined with the following processing aids: egg white; non-grape tannins; milk; casein; and isinglass.

- A panel of 37 food allergic and non-food allergic subjects was recruited.

- Whole blood basophil activation assays for wine analysis were established and optimised to allow detection of egg white, fish, milk and peanut/tree nut allergenic proteins, related to the processing aids egg white, isinglass, milk, casein and non-grape derived tannins.

- Each of the survey wines was tested by the whole blood basophil activation assay. Overall there was no statistically significant difference in basophil activation ratios between subject groups or between fining agents. 92 of the 102 wines tested gave a basophil activation ratio within the identified reference range. For the 10 wines (9 fined and 1 unfined) which were associated with basophil activation ratios above the reference range for some subjects, no pattern could be identified with respect to wine variety, fining agent or geographic indication, or to subject group.

- An enzyme immunoassay (ELISA) specific to ovalbumin was established and optimised using commercially available polyclonal and monoclonal antibodies. The lower limit of detection of ovalbumin was 1 ng/mL. All survey wines were analysed for ovalbumin, with only two wines giving a detectable concentration. These two wines has not been fined with egg white but had had whole eggs added during production.

- An ELISA specific for casein was established using a monoclonal antibody produced in this project and a commercial polyclonal antibody. The lower limit of detection of casein was 8 ng/mL. All survey wines were analysed and none had detectable casein.

- An inhibition ELISA specific for peanut was established using a peanut-allergic patient serum. The lower limit of detection of peanut was 8 ng/mL. All survey wines were analysed and none had detectable peanut.

Section II

- The panel of 26 food allergic and 11 non-food allergic subjects were challenged with 26 test and 3 control wines in a blinded study. There were no serious adverse events. A review of the five episodes where symptoms and/or signs were noted following wine consumption revealed no consistent pattern with respect to wine type or subject group.

- The lack of any significant clinical adverse response of peanut allergic and fish allergic individuals to double blind placebo controlled challenge of wines fined with non-grape tannins or isinglass suggests a very low risk of allergic reaction attributable to activity from associated food allergenic residual proteins in the Australian wine manufacture process. This conclusion would be strengthened by study of a larger panel of wines and test subjects.

- The low numbers of adult individuals with IgE-mediated milk and/or egg allergy allowed only limited investigation of the potential for clinical challenge with a wine fined with an associated allergenic protein to induce an adverse response. Consideration should be given to further evaluation of the potential for allergic reaction using a stripped basophil activation assay involving sensitisation with serum specific IgE from children with egg and/or milk allergy.

5 Study B - Assessment of potential allergic reactions in children to wine fined with milk and egg products

5.1 Specific Project Aims and Performance Targets

The objectives of the project are as follows:

1) to determine if there is any detectable residual allergenic proteins from the processing aids casein, egg white and milk in the final commercial bottled wine. This will comprise a follow up survey of commercially available Australian wines which have been produced with the aforementioned processing aids;

2) to determine whether serum antibodies from children with a known allergy to eggs and/or milk can elicit *in vitro* basophil activation on incubation with wine fined with egg white and/or milk proteins.

Outputs (aim)	**Performance Targets**
1. Wine sample collection to survey whether there are detectable allergenic milk and egg proteins in wine	1. 40 wine samples to be collected for subsequent enzyme immunoassay (ELISA) and analysis of basophil activation by April 2005 (survey)
2. Stripped basophil activation assays specific to egg and milk allergens related to the processing aids casein, egg white and milk.	2. Stripped basophil activation assays developed with coating of basophils with IgE from allergic children's sera specific to egg and milk allergens by April 2005.
3. Identification of 20 children with confirmed allergy to milk and 20 children with confirmed allergy to egg allergens. (Child may be allergic to both allergens).	3. Up to 40 subjects at risk of reaction to inadvertent encounter with wine containing food allergens recruited, clinically characterised and a serum sample taken during the course of the study with recruitment completed by August 2005.
4. Information as to whether children with a confirmed allergy to eggs and/or milk are at risk of a clinical allergic reaction should they sample wine fined with egg white and/or milk proteins.	4. Subjects identified in output (1), will have their sera tested in a stripped basophil activation assay to determine whether there is indirect ability of 40 survey wines fined with egg white and/or milk proteins to activate *in vitro* sensitised basophils as a surrogate of an allergic reaction. Task completed by September 2005.
5. Information as to whether survey wines contain detectable allergenic egg white and milk proteins by previously developed ELISA.	5. 40 survey wines analysed with previously developed ELISA to determine whether they contain detectable allergenic proteins by December 2005.

6. Peer-reviewed manuscript published.	6. Preparation of a peer-reviewed manuscript to be published in -an international peer-reviewed *Allergy and Immunology Journal* by December 2005.
7. Industry-published manuscript.	7. Preparation of a manuscript to be published in the Australian and New Zealand Grapegrower and Winemaker by December 2005.
8. Submission to FSANZ.	8. Preparation of a submission to FSANZ dependent on the results of outputs 1) and 2) as to whether wine and wine products should be specifically labelled for potential milk and/or egg allergens by December 2005.

5.2 Methods

5.2.1 Serum and blood collection and clinical characterisation

Sera from milk and egg allergic children were obtained from the Royal Children's Hospital (courtesy of Dr. David Hill). Sera were selected based on a clinical history of reaction to milk or egg in association with the demonstration of specific IgE to either milk or egg, via RAST or skin prick testing (Sporik et al. 2000). A total of 20 egg allergic and 20 milk allergic sera were used in this study and their clinical characteristics are summarised in Tables 5.1 and 5.2. Sera were also collected from five non-allergic subjects as controls.

Peripheral blood was obtained from five atopic non-egg allergic and non-milk allergic adult subjects as a source of basophils.

The study was approved by The Alfred Hospital and Monash University Ethics Committee (project number 124/02).

5.2.2 Test wines

Test wines were selected on the basis of their preparation with egg white or milk as a fining agent. Patient sera were tested in the stripped basophil activation assay against a panel of 40 wines: 20 egg white-fined red wines and 20 white wines fined with either milk and/or casein. Each egg allergic serum was tested against four egg white-fined wines and one milk-fined wine as a control. Similarly, each milk allergic serum was tested against four milk-fined wines and one egg white-fined wine as a control. Sera from non-allergic subjects were also tested with milk-fined and egg white-fined wines as a control for specificity. The plan for testing survey wines using the stripped basophil activation assay is summarised in Figure 5.1. Test wines were also analysed using the ovalbumin and casein sandwich ELISA developed in our laboratory as described in Study A Sections 4.2.1.3.2 and 4.2.1.3.3.

Table 5.1: Egg allergic subjects

CODE	Age (when serum was taken)	Sex	Symptoms upon ingestion of egg	Total IgE (kU_A/L)	Egg RAST (kU_A/L)	Egg SPT	Peanut RAST (kU_A/L)
E1	5 yrs	M	urticaria	475	7.7	9 mm	2.38
E2	6 mths	M	unknown	82	14.3	8 mm	1.13
E3	1 yr	F	unknown – avoid	142	22.2	14 mm	2.08
E4	1 yr	M	ND	141	8.48	5 mm	1.54
E5	1 yr	M	unknown	536	39.7	10 mm	51.2
E6	1 yr	M	unknown – avoid	95	31.1	8 mm	7.32
E7	17 mths	M	perioral urticaria	213	11.2	8 mm	8.07
E8	18 mths	M	unknown, DA	672	5.17	11 mm	1.79
E9	6 yrs	M	unknown, AH	373	8.61	5 mm	15.57
E10	16 mths	F	unknown – avoid, DA	69	5.16	12 mm	0.7
E11	15 mths	M	unknown – avoid, FH	95	10.2	7 mm	8.37
E12	21 mths	M	unknown	577	15.2	6 mm	85.4
E13	14 mths	M	ND	825	37.5	10 mm	15.6
E14	13 mths	M	unknown – DA	492	11.22	10 mm	6.89
E15	10 mths	M	unknown	443	14.31	11 mm	0.51
E16	30 mths	F	urticaria	308	8.02	12 mm	0.37
E17	16 mths	F	tolerates small amount of egg	254	5.38	4 mm	>17.5 AEU/ml
E18	9 yrs	F	unknown	583	>17.5	8 mm	<0.18 AEU/ml
E19	31 mths	F	angioedema, rash	189	6.75	8 mm	1.02 AEU/ml
E20	7 yrs	M	tolerated	536	10.64	6 mm	10.13

ND – no data
DA – diagnostic anaphylaxis
AH – anaphylactic hypersensitivity
FH – at risk of food hypersensitivity

Table 5.2: Milk allergic subjects

CODE	Age (when serum was taken)	Sex	Symptoms upon ingestion of milk	Total IgE (kU_A/L)	Milk RAST (kU_A/L)	Milk SPT	Peanut RAST (kU_A/L)
M1	10 mths	F	immediate perioral rash	194	24.8	5 mm	ND
M2	5 yrs	M	hypersensitivity	606	50.2	7 mm	ND
M3	13 mths	M	unknown	165	6.84	3 mm	2.49
M4	13 mths	M	rash, vomiting, cyanosis	328	8.28	10 mm	6.27 AEU/ml
M5	7 mths	F	unknown	15	6.79	6 mm	0.28 AEU/ml
M6	5 yrs	F	ND	1972	>17.5	9 mm	3.57 AEU/ml
M7	7 mths	M	urticaria, vomiting	740	6.29	7 mm	5.01 AEU/ml
M8	12 yrs	M	unknown	864	6.45	8 mm	1.22
M9	10 mths	M	eczema	980	6.63	3 mm	>17.5 AEU/ml
M10	15 mths	M	ND	ND	10.03	6 mm	1.1
M11	3 yrs	F	vomiting, urticaria	77	7.28	0 mm	0.31
M12	4 yrs	M	unknown	>700	8.52	0 mm	6.34
M13	12 mths	F	vomiting	22	6.19	8 mm	<0.18 AEU/ml
M14	8 mths	M	unknown, AHCM	278	8.3	2 mm	5.4
M15	18 mths	M	coughing, rash, vomiting	1592	11.64	9 mm	14.22
M16	11 yrs	F	urticaria	>700	7.01	9 mm	6.58
M17	12 mths	M	unknown, HCM	ND	6.43	5 mm	6.2
M18	6 yrs	F	ND	ND	14.63	8 mm	9.24
M19	14 yrs	M	vomiting, rash	4117	5.08	14 mm	0.32
M20	8 mths	M	unknown	147	5.04	8 mm	5.59 AEU/ml

ND – no data
AHCM – anaphylactic hypersensitivity to cow's milk
HCM – hypersensitivity to cow's milk

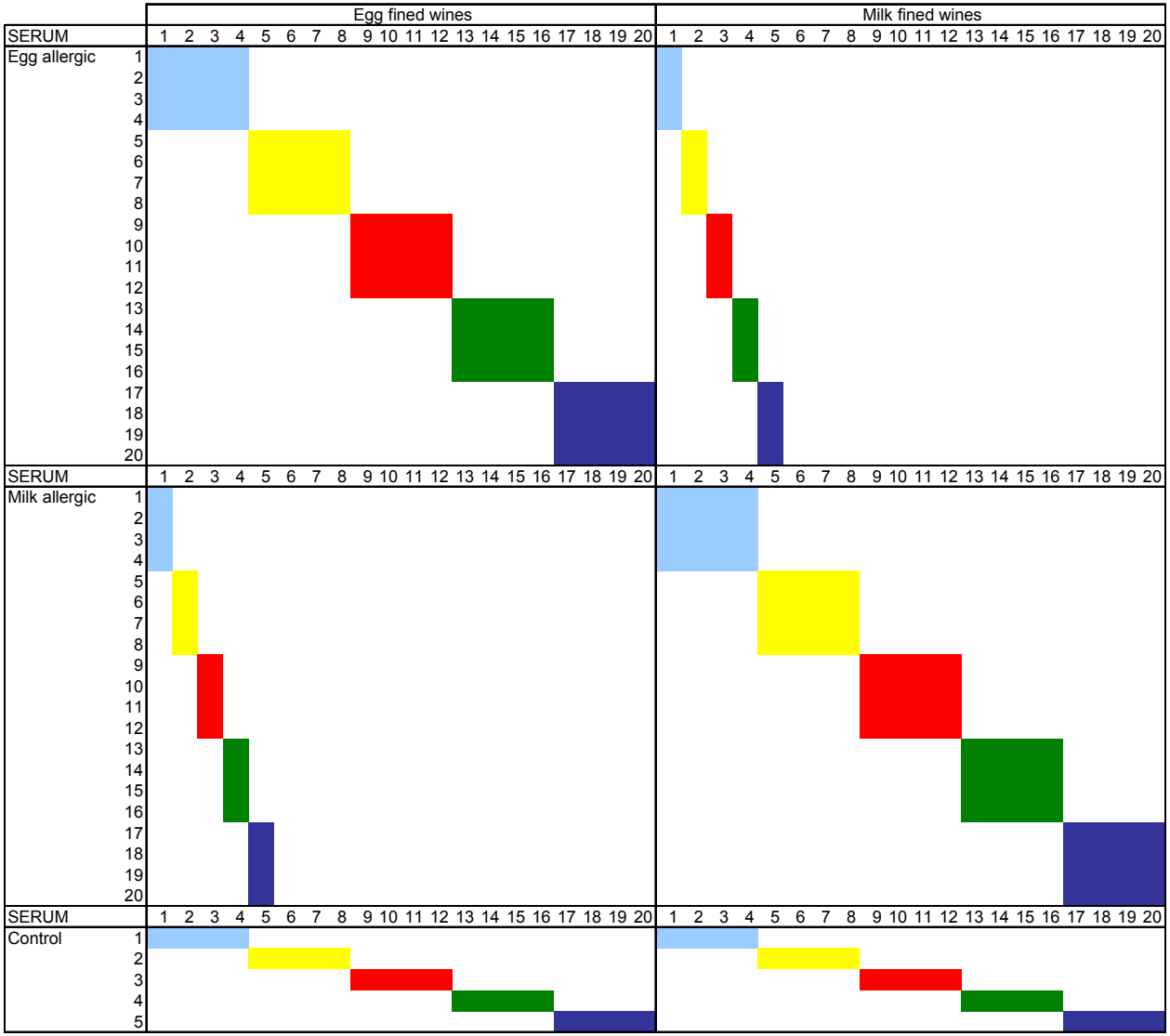


Figure 5.1: Plan for testing survey wines using the stripped basophil activation assay. A total of five basophil donors was used in this study. Basophils from each donor were stripped and resensitised with four egg allergic or milk allergic sera and each serum was tested against four relevant fined wines and one irrelevant fined wine. Basophils were also resensitised with serum from five non-allergic control subjects, with each serum tested against four egg-fined and four milk-fined wines.

5.2.3 Stripping and resensitisation of peripheral blood basophils

This method was adapted from that described by Kleine-Budde *et al.* (2000) and de Leon *et al.* (2005). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood of atopic, non-egg or milk allergic subjects by Ficoll-Paque centrifugation and the cell concentration was adjusted to 5 x 10^6 cells/mL. Surface IgE was removed by incubating cells in an equal volume of lactic acid buffer (13.4 mM lactic acid, 140 mM NaCl, 5 mM KCl, pH 3.9) on ice, for 5 min. The solution was neutralised by adding 2 volumes of HEPES buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, pH 7.4) containing 5% foetal calf serum (FCS; CSL Biosciences, Melbourne, Victoria, Australia) and the cells were pelleted by centrifugation at 286 x g for 5 min. This stripping and neutralisation procedure was repeated once more and cells were washed in HEPES buffer containing 5% FCS. The cells were resensitised by incubation with egg-allergic, milk-allergic or control sera at 37°C for 1 h (200 µL per 2.5 x 10^6 cells), washed once in HEPES buffer containing 5% FCS and resuspended in HEPES buffer containing 1 mM $CaCl_2$, IL-3 (4 pg/mL; R & D Systems, Minneapolis, MN, USA) and 5% FCS to give a final concentration of 5 x 10^6 cells/mL.

Basophil activation and fluorescent labelling of cells was performed as described in Section 4.2.1.2 except that approximately 250,000 events were collected per test sample (500–1000 basophils) for analysis.

5.2.4 Statistical analysis

Statistical analysis was conducted using Excel software. Comparison between groups (subject group) was performed using a Mann Whitney test. A p value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Optimisation of stripped basophil activation assay

The ability of wines and fining agent proteins to activate basophils resensitised with egg allergic sera was assessed by flow cytometry. The activation of basophils was detected via surface CD63 expression following stimulation of resensitised basophils with wines and fining agent proteins. The method for analysis is as outlined in Study A Section 4.3.3.1 except that basophil activation is expressed as the number of $CD63^{hi}$ cells. This was because a clear IgE^{hi} population was not always seen following resensitisation of basophils with sera.

5.3.1.1 Stripped basophil activation assay for egg allergic sera

Figures 5.2 and 5.3 are representative analyses of basophil activation following incubation of IgE-stripped cells with egg white extract and an egg white-fined wine either without (Figure 5.2) or with (Figure 5.3) resensitisation with egg-allergic serum. It can be seen that negligible basophil activation was obtained when IgE-stripped non-resensitised cells were incubated with egg white extract or an egg white-fined wine (Figure 5.2b), levels less than the stimulation buffer alone negative control (Figure 5.2c). The cells, however, were still viable after the stripping procedure as indicated by the high number of activated basophils obtained with the positive controls, anti-IgE and fMLP stimulation. When IgE-stripped basophils were resensitised with egg allergic serum, a higher number of activated basophils was detected following incubation with egg white extract in comparison to the stimulation buffer alone negative control, confirming the presence of egg white-specific IgE antibodies in the egg allergic serum. Negligible basophil activation was obtained with the egg white-fined wine. Positive basophil activation following anti-IgE and fMLP stimulation confirmed that the cells remained viable and functional following stripping and resensitisation with serum.

5.3.1.2 Stripped basophil activation assay for milk allergic serum

Figures 5.4 and 5.5 are representative analyses of basophil activation following incubation of IgE-stripped cells with milk extract or a milk-fined wine either without (Figure 5.4) or with (Figure 5.5) resensitisation with milk allergic serum. Negligible basophil activation was obtained when IgE-stripped cells were incubated with milk extract or a milk-fined wine (Figure 4b), levels similar to the stimulation buffer alone negative control (Figure 5.4c). Donor basophils remained viable after the stripping procedure as indicated by the high number of activated basophils obtained with the anti-IgE and fMLP positive control stimulation. When IgE-stripped basophils were resensitised with milk allergic serum, a higher number of activated basophils was detected following incubation with milk extract (Figure 5.5b) in comparison to the stimulation buffer alone negative control (Figure 5.5c), confirming the presence of milk-specific IgE antibodies in the milk allergic serum. Negligible basophil activation was obtained with the milk-fined wine. The high number of activated basophils obtained following anti-IgE and fMLP stimulation confirmed that the cells remained viable and functional following stripping and resensitisation with serum.

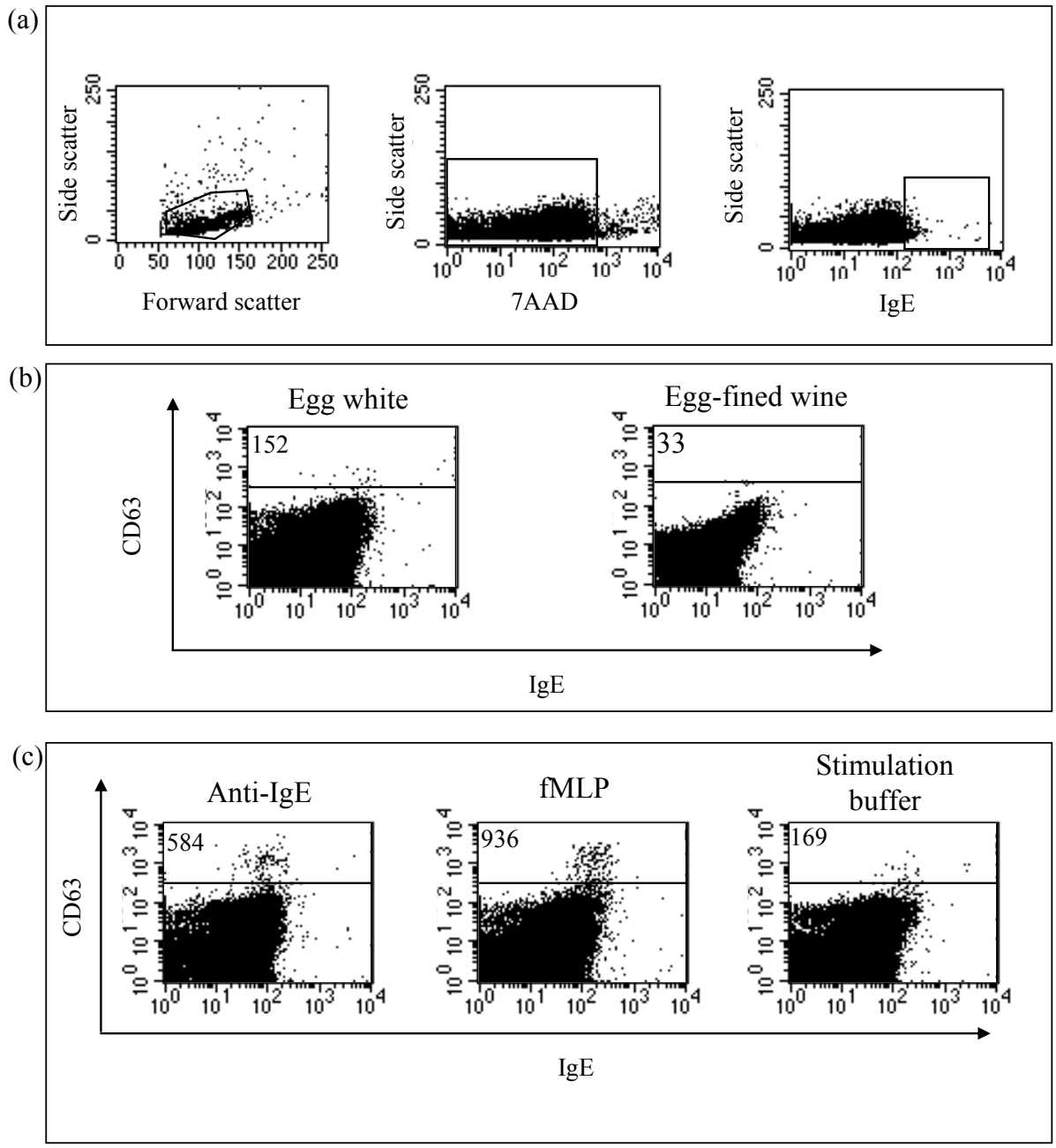


Figure 5.2: Flow cytometric analysis of activation of IgE-stripped basophils without resensitisation

(a) Viable basophils are identified by size and granularity (light scatter),and viability (exclusion of the vital dye 7AAD). Minimal IgE^{hi} staining confirms that basophils are stripped of surface IgE.
(b) CD63 expression on stripped basophils incubated with egg white extract (10 µg/mL) or egg white-fined wine (neat).
(c) CD63 expression on stripped basophils with anti-IgE and fMLP as positive controls, or without added allergen (i.e. stimulation buffer alone)

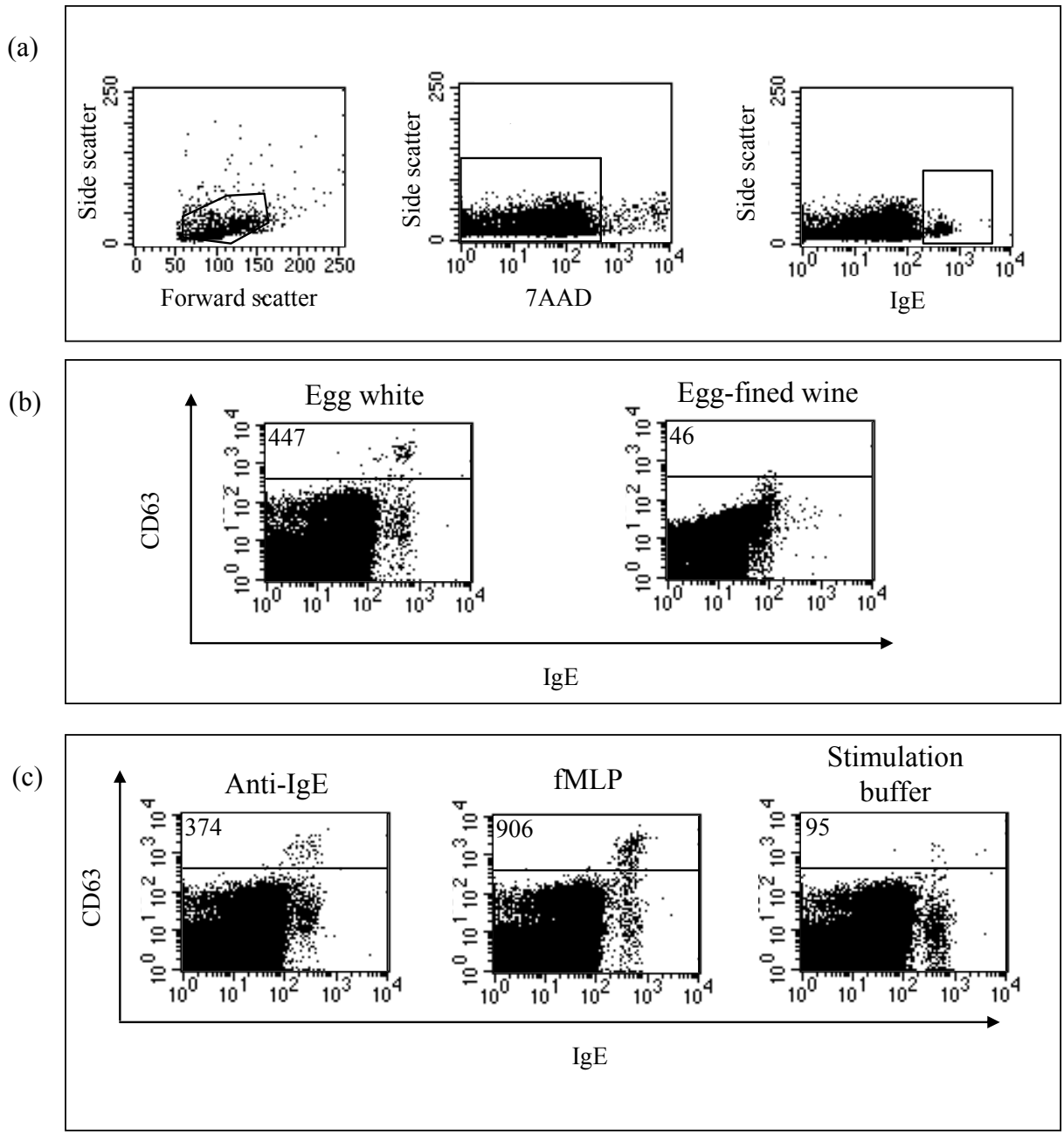


Figure 5.3: Flow cytometric analysis of activation of basophils resensitised with egg allergic serum

(a) Viable basophils are identified by size and granularity (light scatter), viability (exclusion of the vital dye 7AAD). IgE^{hi} staining confirms that basophils have been resensitised with serum IgE.
(b) CD63 expression on stripped-resensitised basophils incubated with egg white extract (10 µg/mL) or egg white-fined wine (neat).
(c) CD63 expression on stripped-resensitised basophils with anti-IgE and fMLP as positive controls, or without added allergen (i.e. stimulation buffer alone).

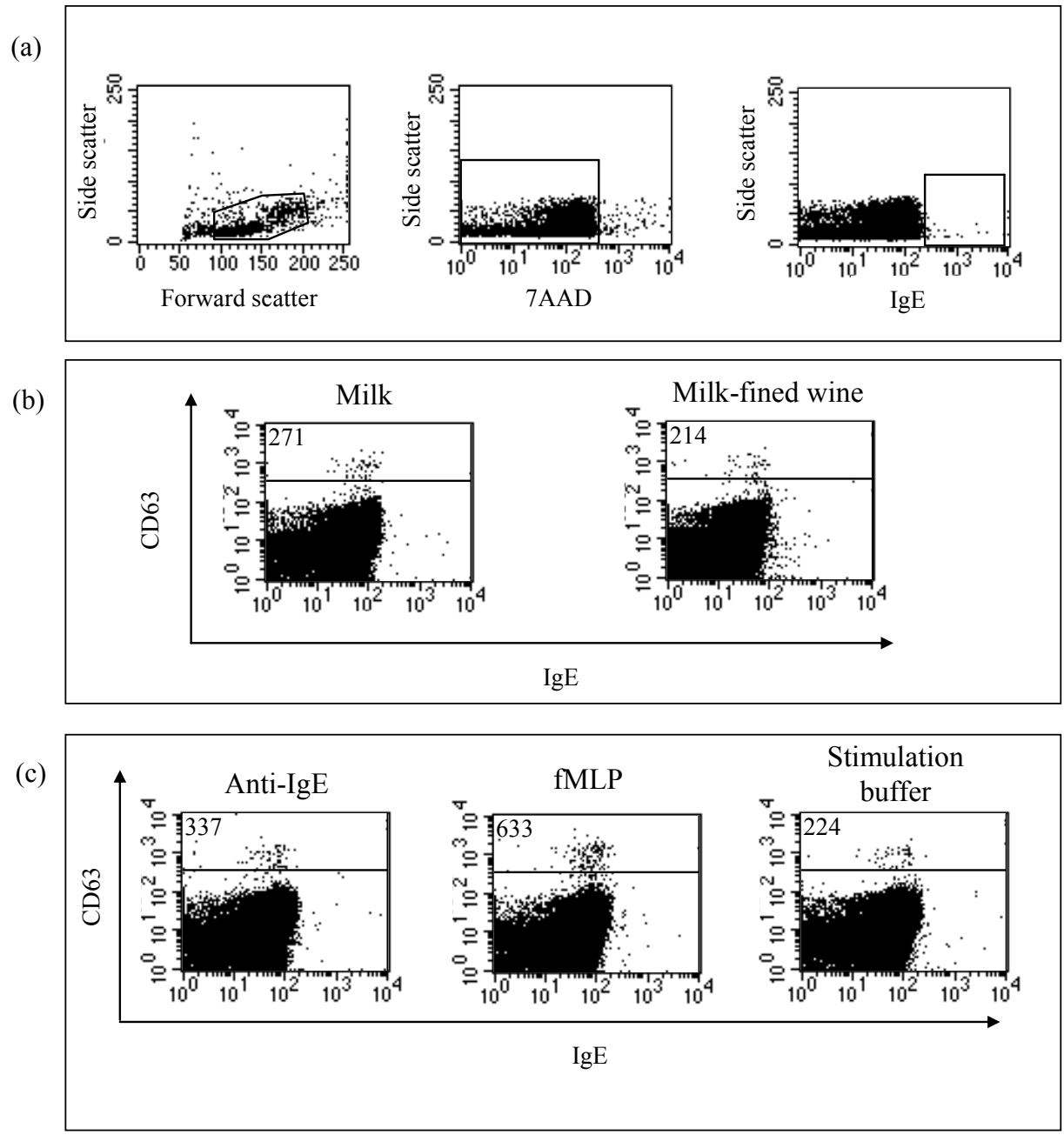


Figure 5.4: Flow cytometric analysis of activation of IgE-stripped basophils without resensitisation

(a) Viable basophils are identified by size and granularity (light scatter), viability (exclusion of the vital dye 7AAD). Minimal IgE^{hi} staining confirms that basophils are stripped of surface IgE.
(b) CD63 expression on stripped basophils incubated with milk extract (10 µg/mL) or milk-fined wine (neat).
(c) CD63 expression on stripped basophils with anti-IgE and fMLP as positive controls, or without added allergen (i.e. stimulation buffer alone).

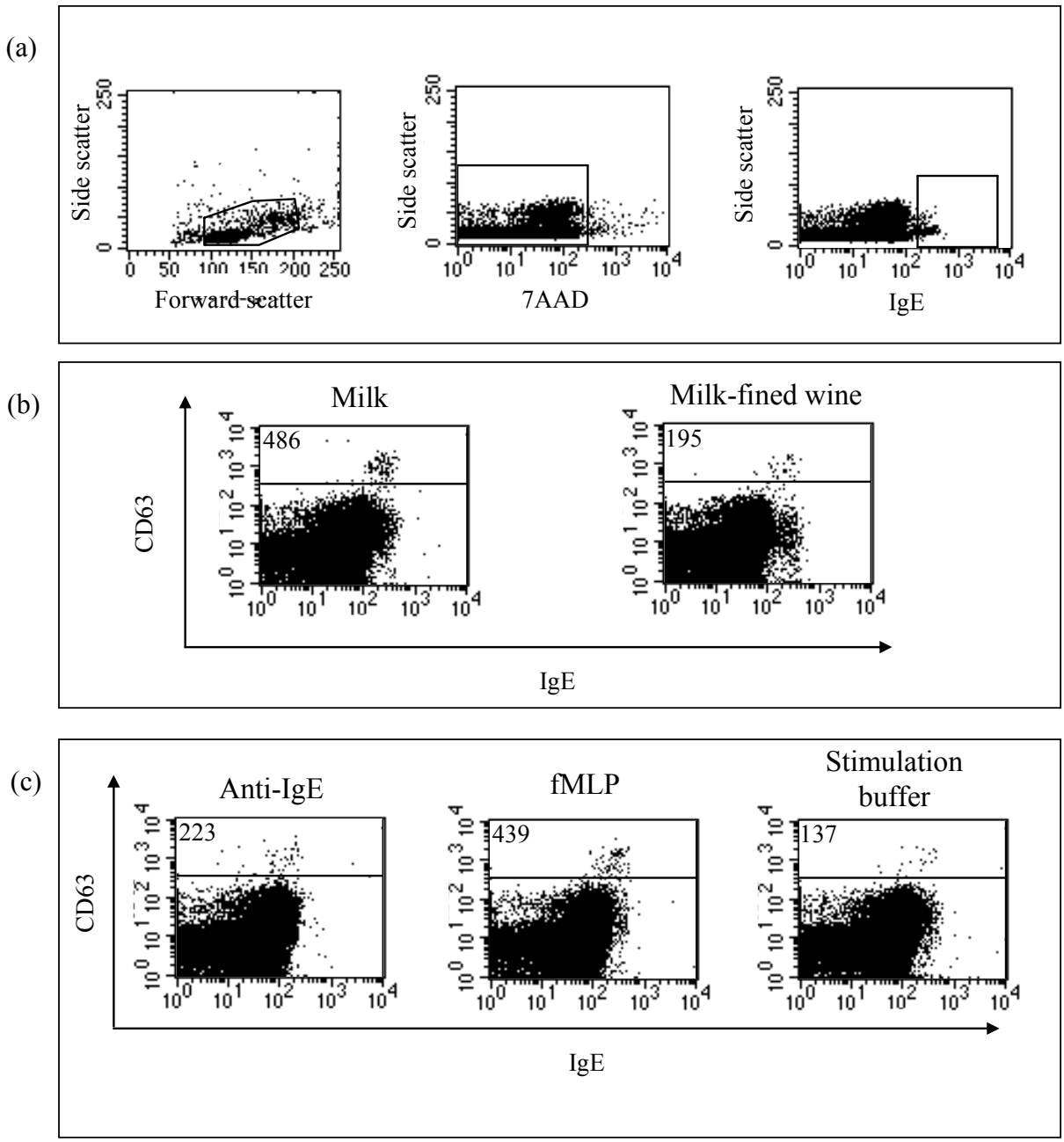


Figure 5.5: Flow cytometric analysis of activation of basophils resensitised with milk allergic serum

(a) Viable basophils are identified by size and granularity (light scatter), viability (exclusion of the vital dye 7AAD). IgE^{hi} staining confirms that basophils have been resensitised with serum IgE.
(b) CD63 expression on stripped-resensitised basophils incubated with milk extract (10 µg/mL) or milk-fined wine (neat).
(c) CD63 expression on stripped-resensitised basophils with anti-IgE and fMLP as positive controls, or without added allergen (i.e. stimulation buffer alone).

5.3.2 Stripped basophil activation assay for egg white fined wines

Donor basophils from atopic, non-egg allergic subjects stripped of surface IgE were resensitised with egg allergic or control sera and subsequently incubated with red wines fined with egg white extract. To permit comparison between subjects, the basophil activation ratio was calculated as the number of CD63-positive cells for cells incubated with the test sample divided by CD63-positive cells with stimulation buffer alone. The results for the 20 egg allergic sera and five control sera are summarised in Figure 5.6. When resensitised cells were stimulated with the positive control egg white extract, the basophil activation ratio was significantly higher when cells were resensitised with the egg allergic sera compared with the control sera ($p<0.05$), confirming the validity of the assay. When resensitised cells were stimulated with the egg white-fined wines, the median basophil activation ratio was slightly higher for the control sera compared with the egg allergic sera ($p<0.05$), but values for both control and egg allergic sera were significantly less than those for the egg allergic sera stimulated with the egg white extract ($p<0.01$ and $p<0.001$, respectively). The viability and function of the resensitised basophils was confirmed for all assays by positive control stimulation with anti-IgE and fMLP.

Three of the four highest basophil activation ratios obtained following incubation of resensitised basophils with egg allergic sera were with the egg white-fined wine R2 (Figure 5.6), and this wine was therefore further analysed in a direct basophil activation assay performed as in Study A. Whole blood was collected from an adult egg allergic patient and subsequently incubated with egg white-fined wine R2. Negligible basophil activation was observed, indicating that the basophil activation obtained with the stripped basophil activation assay was non-egg related.

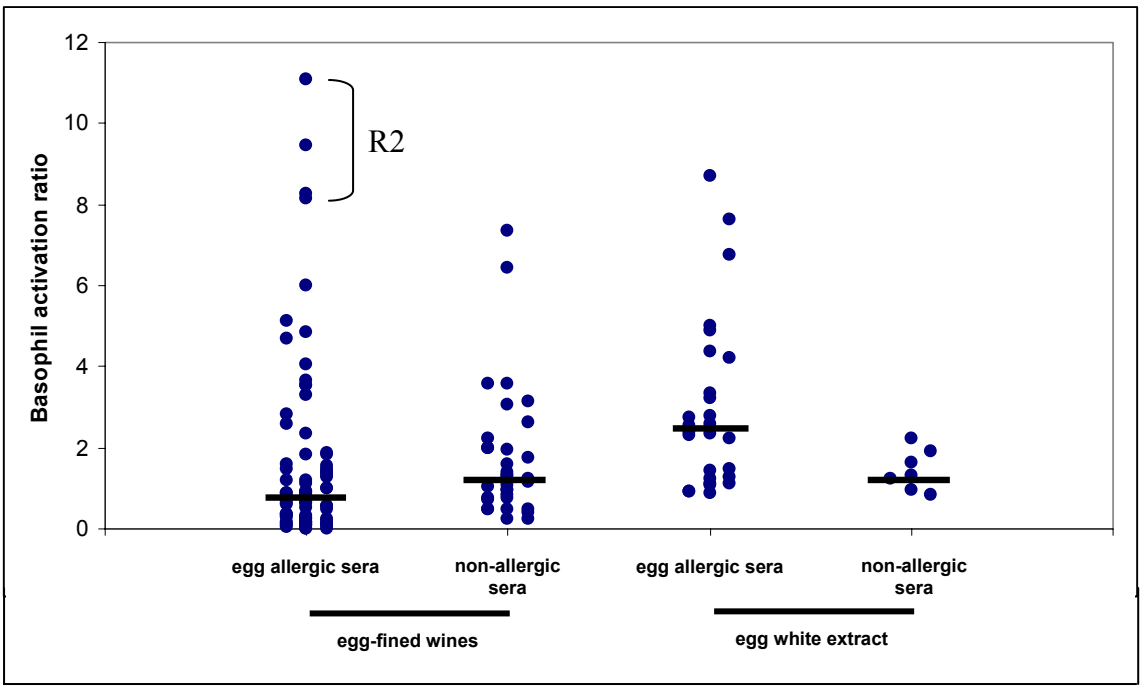


Figure 5.6: IgE stripped and resensitised basophils were stimulated with wine or control egg white allergen extracts. Data are shown as basophil activation ratios with median values indicated by black bars

5.3.3 Stripped basophil activation assay for milk fined wines

Donor basophils from atopic, non-milk allergic subjects stripped of surface IgE were resensitised with milk allergic or control sera and subsequently incubated with white wines fined with milk. As above, the basophil activation ratio was calculated as the number of CD63-positive cells for cells incubated with the test sample divided by CD63-positive cells with stimulation buffer alone. The results for the 20 milk allergic sera and five control sera are summarised in Figure 5.7. When resensitised cells were stimulated with the positive control milk extract, the basophil activation ratio was significantly higher when cells were resensitised with the milk allergic sera compared with the control sera ($p<0.01$), again confirming the validity of the assay. When resensitised cells were stimulated with the milk-fined wines, the basophil activation ratio was also higher for the milk allergic sera compared with the control sera ($p<0.01$). The basophil activation ratios were not significantly different when cells resensitised with milk allergic sera were stimulated with milk-fined wines or with milk extract.

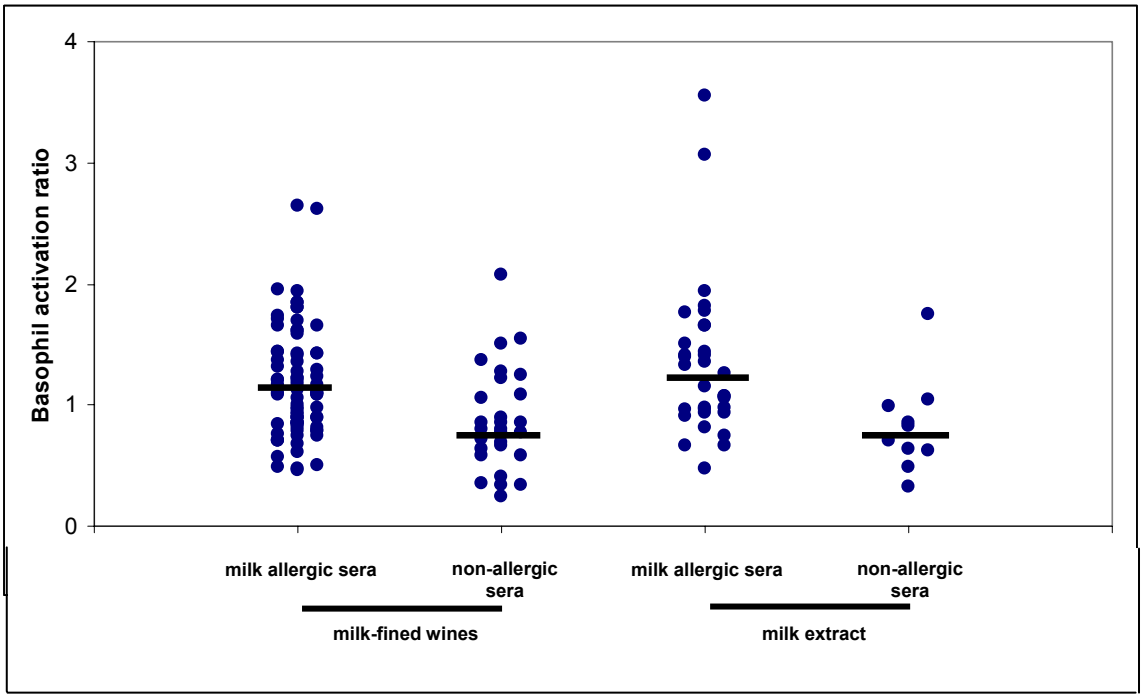


Figure 5.7: IgE stripped and resensitised basophils were stimulated with wine or control milk allergen extracts. Data are shown as basophil activation ratios with median values indicated by black bars.

5.3.4 ELISA for detection of residual allergen in egg white and milk fined survey wines for Study B.

The panel of 40 wines for Study B was tested using the ovalbumin and casein sandwich ELISA developed in our laboratory as described in Study A Sections 4.2.2.2.2 and 4.2.2.2.3. Ovalbumin was undetectable in all egg white-fined wines (limit of detection 1 ng/mL ovalbumin) and casein was undetectable in all milk-fined wines (limit of detection 8 ng/mL α-casein).

An ovomucoid inhibition ELISA was established as described for the peanut inhibition ELISA in Study A Sections 4.2.2.2.4. The egg white-fined wine R2 was further analysed using this method to determine if there was residual ovomucoid

present in this test wine which may be responsible for the higher basophil activation ratios obtained with some egg allergic sera. The results indicated that ovomucoid was undetectable in wine R2 (assay detection limit 1 ng/mL ovomucoid), further confirming the absence of residual egg allergens in this wine.

5.4 Conclusions from Study B

- The survey wine panel (40) was collected comprising wines fined with egg white (20) and milk (20).

- A panel of sera from 20 egg allergic and 20 milk allergic subjects was collected. Sera from 5 non-allergic controls were also collected.

- A stripped basophil activation assay was established and optimised.

- The validity of the stripped basophil activation assays was confirmed by testing with control egg white and milk extracts. Median ratios were significantly higher for the egg allergic sera and milk allergic sera respectively compared with non-allergic sera.

- Each of the survey wines was tested by the stripped basophil activation assay. For the egg white-fined wines, the median ratio was less than that for the control non-allergic sera and median ratios for both egg allergic and non-allergic sera were less than that for the egg white extract stimulated cells. For the milk fined wines, the median ratio was greater than that for the control non-allergic sera and was similar in magnitude to that for the milk extract stimulated cells.

- The egg white-fined wine R2 resulted in three of the four highest basophil activation ratios following incubation of resensitised basophils with egg allergic sera (Figure 5.6). Further analysis of this wine by direct basophil activation assay using blood from an adult egg allergic patient showed negligible basophil activation. This result indicates that the basophil activation obtained with the stripped basophil activation assay was non-egg related.

- The previously established and optimised enzyme immunoassay (ELISA) specific to ovalbumin and casein were used to analyse the survey wines. There was no detectable ovalbumin or casein in any of the survey wines.

- The egg white-fined wine (R2) which gave a high basophil activation ratio with some egg allergic sera was tested additionally for ovomucoid using an inhibition ELISA. Ovomucoid was undetectable.

6. Outcomes and Recommendations

1. Within our subset of the Australian adult population, for patients with known allergies to fish, nuts and egg, there was no significant clinical response (anaphylaxis) to a double-blind placebo-controlled challenge of fined wines that could be attributed to residual food protein processing aids in wine made following good manufacturing practice.

2. The rarity of IgE-mediated milk allergy in adults prevented reliable evaluation of residual milk allergens in wine by direct clinical challenge or *in vitro* basophil activation. However this rarity makes potential allergic reactions to these food proteins in wine rather a theoretical problem.

3. Using the established specific and highly sensitive ELISA techniques (detection limits 1–8 ng/mL), no residual ovalbumin, casein or peanut allergens were detected in the survey Australian wines made following good manufacturing practice. N.B. This does not include wine products containing whole eggs.

4. Although health recommendations advise against children consuming alcoholic beverages, we attempted to evaluate safety of protein fined wines in children with confirmed milk and/or egg allergy using a surrogate *in vitro* assay. The results of the stripped basophil activation assay were difficult to interpret due to the basophils' intrinsic reactivity following *in vitro* manipulation. While testing of egg white-fined wines suggested that in general, residual egg white proteins were not present, stripped basophil activation by the milk-fined wines was similar in magnitude to that of the control milk extract stimulated cells. Clarification by direct clinical challenge or direct basophil activation assay in children could not ethically be pursued.

7. Appendices

7.1 Appendix 1: Communications

7.1.1 Relevant publications

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. (2006) Potential food allergens in wine: double-blind placebo-controlled trial and basophil activation analysis. Nutrition, 22:882–888; 2006.

Stockley, C.S. O'Hehir RE, Rolland, J. (2006) Is allergen labelling necessary for Australian wine? Australian New Zealand Wine Industry Journal, 21(3):17–21.

Stockley C, O'Hehir RE. (2002) *To label or not to label for allergens—the latest question for winemakers.* The Australian and New Zealand Grapegrower and Winemaker, 53-61.

Stockley C.S., Rolland J.M., O'Hehir, R.E. (2004) Allergen labelling—an Australian wine industry case study. Proceedings of the OIV Congress 2004, 4–9 July 2004, Vienna, Austria.

Stockley C.S., Stimson K., Glaspole I., Apostolou E., Rolland J.M., O'Hehir R.E. Double-blind placebo-controlled clinical trial of fined wines in subjects with confirmed sensitivity to eggs, fish, milk or nuts. Proceedings of the 12th Australian Wine Industry Technical Conference, 25–28 July 2004, Melbourne, Victoria.

Stockley C.S., Apostolou E., Glaspole I., Stimson K., O'Hehir R.E., Rolland J.M. An investigation of potential residual allergenic fining proteins in wine. Proceedings of the 12th Australian Wine Industry Technical Conference, 25–28 July 2004, Melbourne, Victoria.

7.1.2 Relevant presentations

Stockley, C., O'Hehir, R. , Rolland, J., Deckert, K., Glaspole, I., Apostolou, E. Double-blind placebo-controlled clinical trial of fined wines in subjects with confirmed sensitivity to eggs, fish, milk or nuts. Organisation de la Vigne et du Vin Subcomission Wine, Nutrition and Health Workshop, Paris, France, March 2006

Apostolou E., Glaspole I., Stimson K., de Leon M., Stockley C., Rolland J. and O'Hehir R. The identification and measurement of potential food allergens in wine. ASCIA Annual Scientific Meeting, Brisbane, 2004

Apostolou E., Glaspole I., Stimson K., de Leon M., Stockley C., Rolland J. and O'Hehir R. The identification and measurement of potential food allergens in wine. Alfred Research Week, Melbourne, 2004

Stockley C.S., Rolland J.M., O'Hehir, R.E. (2004) Allergen labelling—an Australian wine industry case study. OIV Congress 2004, 4–9 July 2004, Vienna, Austria.

7.1.3 Relevant media interviews

24/9/02
Channel 9 News (6.00 pm) – Belinda Byrne (R'OH and CSS)

25/9/02
3AW (Melbourne) – Ernie Sigley (RO'H)
ABC Radio Western Australia and Perth – Liam Barlett (CSS)

26/9 /02
ABC Radio Queensland and ABC Capricornia – David Anderson (CSS)
3AK (Melbourne) – Derryn Hinch (CSS)
ABC Radio Renmark – Susie Hamilton (CSS)

This is in relation to the two articles in *The Weekend Australian* (14/15.9.02) and *The Australian* (25.9.02) on allergen labelling for wine.

7.2 Appendix 2: Intellectual property

Not applicable at this stage.

7.3 Appendix 3: References

Alibrandi B., Parodi A., Varadlo G. Purpura due to ethanol. N Engl J Med, 322:702; 1990.

American Thoracic Society. Standardization of Spirometry, 1994 Update. Am J Respir Crit Care Med, 152:1107-1136; 1995.

Anet J., Black J.F., Baker R.S., Barnett, D., Burley, R.W., Howden, M.E.H. Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteins. Int Arch Allergy Appl Immunol, 77:364–371; 1985.

Arthur Jr J.C., Peanut protein isolation, composition and properties. Adv Protein Chem, 8: 393–414; 1953.

Astwood J.D., Fuchs R.L., Lavik P.B. Food biotechnology and genetic engineering. Metcalf D.D., Sampson H.A., Simon R.A. (eds). Food allergy: adverse reactions to foods and food additives, second edition, Cambridge, Mass, Blackwell Science; 1997.

Baldo B.A. Milk allergies. Aust J Dairy Technol, 39:120–128; 1984.

Barnett D., Baldo B.A. Howden M.E.H. Multiplicity of allergens in peanuts. J. Allergy Clin Immunol, 72:61–68; 1983.

Barnett D., Howden M.E.H. Partial characterisation of an allergenic glycoprotein from peanut (*Arachis Hypogaea* L.). Biophys Acta, 882:97–105; 1986.

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, 93: 1047–1059; 1994.

Bishop J.M., Hill D.K., Hosking C.S. Natural history of cow's milk allergy: clinical outcome. J Pediatr, 116:862–867; 1990.

Bleumink E., Young E. Studies on the atopic allergen in hen's egg. I. Identification of the skin reactive fraction in egg white. Int Arch Allergy Appl Immunol, 35 1–19; 1969.

Bleumink E., Young E. Studies on the atopic allergen in hen's egg. II. Further characterisation of the skin-reactive fraction in egg white: immuno-electrophoretic studies. Int Arch Allergy Appl Immunol, 40: 72–88; 1971.

Brujinzeel-Koomen C., Orolani C., Aaase K., Bindslev-Jensen C., Bjorksten B., Moneret-Vautrin C., Wuthrich B. Adverse reactions to food. Allergy, 50: 623–635; 1995.

Bugajska-Schretter, A., Elfman, L., Fuchs, T., Kapiotis, S., Rumpold, H., Valenta, R, Spitzauer, S. Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive yo periodate treatment and calcium depletion. J Allergy Clin Immunol., 101(pt1): 67–74; 1998.

Burks A.W., Williams L.W. Helm R.M., Connaughton C., Cockrell G., O'Brien T. Identification of a major peanut allergen, *Ara h* I, in patients with atopic dermatitis and positive peanut challenges. J Allergy Clin Immunol, 88: 172–179; 1991.

Burks A.W., Williams L.W. Connaughton C., Cockrell G., O'Brien T., Helm R.M. Identification and characterisation of a second major peanut allergen, *Ara h* II, with use of sera of patients with atopic dermatitis and positive peanut challenge. J Allerg Clin Immunol, 90: 962–969; 1992.

Burks A.W., Cockrell G., Connaughton C., Guin J., Allen W., Helm R.M. Identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. Int Arch Allergy Immunol, 105: 143–149; 1994.

Chandra R.K. Food hypersensitivity and allergic disease: a selective review. Am J Clin Nutr, 66: 526S-529S; 1997.

Clayton D.E., Busse W. Anaphylaxis to wine. Clin Allergy, 10:341; 1980.

Committee on toxicity of chemicals in food, consumer products and the environment. Peanut allergy. Department of Health, London; 1998.

Costa J.J., Weller P.F., Galli S.J. The cells of the allergic response. J Am Med Assoc, 78: 1815–1822; 1997.

Dean T.P. Factors predicting food allergy. Environ Toxicol Pharmacol, 4:85–89; 1997.

de Leon MP., Glaspole IN., Drew AC., Rolland JM., O'Hehir RE., Suphioglu C. Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. Clin Exp Allergy 33:1273-280; 2003.

de Leon MP, Drew AC, Glaspole IN, Suphioglu C, Rolland JM, O'Hehir RE. Functional analysis of cross-reactive IgE antibodies: peanut-specific IgE sensitises basophils to tree nut allergens. Clin Exp Allergy 35:1056-1064; 2005.

Douglas RG, O'Hehir RE Peanut allergy : education, avoidance and adrenaline are the mainstays of management MJA 166 : 63 – 64; 1997.

Elsayed S., Bennich H. The primary structure of allergen M from cod. Scand J Immunol, 4203–208; 1975.

Elsayed S., Apold J. Immunochemical analysis of cod fish allergen M: locations of the immunolglobulin binding sites as demonstrated by native and synthetic peptides. Allergy, 38: 449–459; 1983.

Elsayed S., Apold J., Holen E., Vik H., Florvaag E., Dybendal T. The structural requirements of epitopes with IgE binding capacity demonstrated by three major allergens from fish, egg and tree pollen. Scand J Clin Lab Invest, 204: 17–31; 1991.

Erdmann S.M., Sachs B., Kwiecien R., Moll-Slodowy S., Sauer I., Merk H.F. The basophil activation test in wasp venom allergy: sensitivity, specificity and monitoring specific immunotherapy. Allergy 59:1102-1109; 2004.

Goldman A.S., Anderson D.W., Sellars W.A., Halpern S.R., Saperstein S., Knicker W.T. Milk allergy I. Oral challenge with milk and isolated milk proteins in allergic children. Pediatrics, 32: 425–443; 1963a.

Goldman A.S., Sellars W.A., Halpern S.R., Anderson D.W., Furloe T.E., Johnson C.H. Milk allergy II. Skin testing of allergenic and normal children with purified milk proteins. Pediatrics, 32:572–579; 1963b.

Hamada Y., Nagashima Y., Shiomi K. Identification of collagen as a new fish allergen. Biosci Biotechnol Biochem, 65(2):285–291; 2001.

Hansen, T.K., Bindslev-Jensen, C., Skov, P.S., Poulsen, L.K. Codfish allergy in adults: IgE cross-reactivity among fish species. Am Allergy Asthma Immunol., 78(2): 187–94; 1997.

Harada, S., Agarwal, D.P. Aldehyde dehydrogenase deficiency as cause of facial flushing reaction to alcohol in Japanese. Lancet 2:982; 1981.

Hefle SL, Jeanniton E and Taylor SL. Development of a Sandwich Enzyme-Linked Immunosorbent Assay for the Detection of Egg Residues in Processed Foods. Journal of Food Protection, 64:1812-16; 2001.

Hill D.J., Bannister D.G., Hosking C.S., Kemp A.S. Cow milk allergy within the spectrum of atopic disorders. Clin Exp Allergy, 24 1137–1143; 1994.

Hoffman D.R. Immunochemical identification of the allergens in egg white. J Allergy Clin Immunol, 71: 481–486; 1983.

Høst A., Halken S. A prospective study of cow's milk allergy in Danish infants during the first 3 years of life. Clinical cause in relation to clinical and immunological type of hypersensitivity reaction. Allergy, 45: 587–596; 1990.

Hourihane J.O'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, 100(5): 596–600; 1997a.

Hourihane J.O'B., Kilburn S.A., Dean T.P., Warner J.O. Clinical characteristics of peanut allergy. Clin Exp Allergy, 27: 634–639; 1997b.

Jansen J.J., Kjardinaal A.F., Huijbers G., Vlieg-Boerstra B.J., Martens B.P., Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. J Allergy Clin Immunol, 93: 394–398; 1994.

Kanny, G., Gerbaux, V., Olszewski, A., Fremont, S., Empereur, F., Nabet, F., Cabanis, J.C., Moneret-Vautrin, D.A. No correlation between wine intolerance and histamine content of wine. J Allergy Clin Immunol., 107(2):375-8; 2001.

Kleine Budde I., Aalbers M., Aalberse RC., van der Zee JS., Knol EF. Reactivity to IgE-dependent histamine-releasing factor is due to monomeric IgE. Allergy, 55:653-657; 2000.

Kortekangas-Savolainen O., Savolainene J., Lantto R., Kalimo K. The immediate hypersensitivity to bakery, brewery and wine products in yeast-sensitive atopic dermatitis patients. Clin Exp Allergy, 24:836–42; 1994.

Langeland T. A clinical and immunological study of allergy to hen's egg white studies by crossed radioimmunoelectrophoresis (CRIE). Allergy, 37:521–530; 1982.

Langeland T., Harbitz O. A clinical and immunological study of allergy to hen's egg white. V. Purification and identification of a major allergen (antigen 22) in hen's egg white. Allergy, 38: 131–139; 1983.

Lessof M. Common food allergies. The report of the British Nutrition Foundation Task Force. Adverse reactions to food. Buttris, J. (ed). Blackwell Science, Oxford; 2002.

Littlewood J.T., Gibb C., Glover V., Sandler M., Davies P.T., Rose F.C. Red wine as a cause of migraine. Lancet 1:558–9; 1988,

Loza C., Brostoff J. Peanut allergy. Clin Exp Allergy, 25:493–503; 1995.

Marchal R., Marchal-Delahaut L., Lallement A., Jeandet P. Wheat gluten used as a clarifying agent of red wines. J Agric Food Chem, 50:177–184; 2002.

Metcalf D.D., Sampson H.A., Simon R.A. (eds) Food allergy: adverse reactions to foods and food additives, second edition. Oxford, Blackwell Science; 1996.

Parker S.L., Krondl M., Coleman P. Foods perceived by adults as causing adverse reactions. J Am Diet Assoc. 93(1): 40–44; 1993

Parker S.L., Leznoff A., Sussman G.L., Tarlo S.M., Krondl M. Characteristics of patients with food-related complaints. J Allergy Clin Immunol, 86(4 Pt 1): 503–511; 1990.

Powrie W.D., Nakai S. Characteristics of edible fluids of animal origin: eggs. Fennema, O.R. (ed). Food chemistry, second edition. New York, Marcel Dekker; 1985: 829–855.

Ribereau-Gayon P., Glories Y., Maujean A., Dubourdieu D. Handbook of Enology. Volume 2: The chemistry of wine stabilisation and treatments. Chichester, John Wiley and Sons Ltd; 1998.

Sampson H.A. Fatal food-induced anaphylaxis. Allergy, 53: 125-30; 1998.

Sampson H.A. Food allergy. J Allergy Clin Immunol, 84: 1061–1067; 1989.

Sampson H.A. Epidemiology of food allergy. Pediatr Allergy Immunol, 7: 42–50; 1996.

Sanz ML, Sanchez G, Gamboa PM, Vila L, Uasuf C, Chazot M, Dieguez I, De Weck AL. Allergen-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to Dermatophagoides pteronyssinus and Lolium perenne. Clin Exp Allergy 31:1007-1013; 2001.

Shimoda, T., Kohno, S., Takao, A., Fujiwara, C., Matsuse, H., Sakai, H., Watanabe, T., Hara, K., Asai, S. Investigation of the mechanism of alcohol-induced bronchial asthma. J Allergy Clin Immunol., 97:74-84; 1996.

Sicherer S.H., Furlong T.J., DeSimone J., Sampson H.A. Self-reported allergic reactions to peanut on commercial airlines. J Allergy Clin Immunol 103:186-189; 1999.

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

Sporik R., Hill DJ., Hosking CS. Specificity of allergen skin testing in predicting positive open food challenges to milk, egg and peanut in children. Clin Exp Allergy, 30:1540-1546; 2000.

Sutherland MF, Drew A, Rolland JM, Slater JE, Suphioglu C, O'Hehir RE. Specific monoclonal antibodies and human IgE show Hev b 5 is an abundant allergen in high protein powdered latex gloves. Clin Exp Allergy 32:1-7; 2002

Swaisgood H.E. Characteristics of edible fluids of animal origin: milk. Fennema, O.R. (ed). Food chemistry, second edition. New York, Marcel Dekker; 1985: 791–827.

Tariq S.M, Stevens M., Mathews S., Rodout S., Twiselton R., Hide D.W. Cohort study of peanut and tree nut sensitization by age 4 years. Br Med J, 313: 514–517; 1996.

Vally H., Thompson P.J. Role of sulfite additives in wine induced asthma: single dose and cumulative dose studies. Thorax, 56:763–9; 2001.

Zimatkin, S.M.; Anichtchik, O.V. Alcohol-histamine interactions. Alcohol Alcohol. 34:141–7; 1999.

7.4 Appendix 4: Staff

7.4.1 The Australian Wine Research Institute

- Creina Stockley, Health and Regulatory Information Manager (Principal Investigator)
- David Boehm (Laboratory Technician)

7.4.2 The Alfred and Monash University

- Professor Robyn O'Hehir, Director, Department of Allergy, Immunology and Respiratory Medicine (Principal Investigator)
- Associate Professor Jennifer Rolland (Co-investigator)
- Dr Ian Glaspole FRACP (Clinical fellow)
- Ms Kirsten Stimson (Clinical nurse)
- Ms Efstathia Apostolou (Research assistant)
- Dr Maria de Leon (Research assistant)
- Dr Michael Bailey (Statistician)

7.5 Appendix 5: Data

Appendix 7.5.1 Eligibility criteria for subject selection

Questions to ask wine study candidates:

1. What do you have allergies to?
2. What symptoms do you experience after eating these foods?
3. Have you experienced anaphylaxis before? If so, in what time period?
4. Do you carry around an EpiPen®?
5. Do you have asthma? How is it controlled?
6. Have you ever performed lung function or peak flows before?
7. Is your asthma good at present?
8. Do you drink wine regularly – if not is it to avoid this food?
9. Do you have good health in general?
10. Do you suffer from diabetes, epilepsy, liver disease, kidney disease, stroke, ischemic heart disease, URTI?

Appendix 7.5.2 Subject clinical characteristics

Peanut allergic subjects

Patient no.	Age (yrs)	Sex	Major clinical allergen	Nature of reaction	Symptoms	Total IgE	CAP score					
							Peanut	Hazelnut	Almond	Cashew	Brazil	Nut mix
P1	29	M	peanut	anaphylaxis	laryngeal oedema, hypotension, generalised urticaria, GIT upset	559	6	2	2	2	nd	nd
P2	27	F	peanut	anaphylaxis	laryngeal oedema, facial angioedema, GIT upset	6062	2	nd	nd	0	nd	nd
P3	27	M	peanut	anaphylaxis	asthma, laryngeal oedema, GIT upset	256	2	2	2	2	2	nd
P4	20	F	peanut	anaphylaxis	laryngeal oedema, generalised urticaria, facial angioedema, GIT upset	1658	3	0	0	0	nd	nd
P5	29	M	peanut	anaphylaxis	asthma, laryngeal oedema, generalised urticaria, facial angioedema, GIT upset	nd	2	nd	nd	3	0	0
P6	32	F	peanut	anaphylaxis	asthma, laryngeal oedema, hypotension, generalised urticaria, facial angioedema	688	0	2	1	nd	3	nd
P7	36	M	peanut	anaphylaxis	laryngeal oedema, generalised urticaria, facial angioedema, GIT upset	198	0	nd	nd	nd	nd	2

Patient no.	Age (yrs)	Sex	Major clinical allergen	Nature of reaction	Symptoms	Total IgE	CAP score					
							Peanut	Hazelnut	Almond	Cashew	Brazil	Nut mix
P8	30	F	peanut	anaphylaxis	asthma, laryngeal oedema, generalised urticaria, facial angioedema, GIT upset	186	3	nd	nd	nd	nd	3
P9	34	M	peanut	anaphylaxis	asthma, laryngeal oedema, GIT upset	nd	3	3	nd	nd	nd	3
P10	63	F	peanut	anaphylaxis	laryngeal oedema, generalised urticaria, facial angioedema, GIT upset	168	2	0	0	0	nd	nd

Fish allergic subjects

Patient no.	Age (yrs)	Sex	Major clinical allergen	Nature of reaction	Symptoms	IgE	CAP score		SPT (mm)	
							Seafood mix	Fish	f.fish	c.fish
F1	23	F	fish	anaphylaxis	laryngeal oedema, generalised urticaria, facial angioedema	1198	2	nd	nd	nd
F2	37	M	fish	anaphylaxis	asthma, laryngeal oedema, hypotension, generalised urticaria, facial angioedema, GIT upset	nd	nd	nd	10	nd
F3	50	F	fish	anaphylaxis	laryngeal oedema, facial angioedema	9	nd	0	nd	nd
F4	48	M	fish	anaphylaxis	laryngeal oedema, generalised urticaria, facial angioedema	126	0	nd	10	9
F5	29	F	fish	anaphylaxis	asthma, laryngeal oedema, generalised urticaria, GIT upset	nd	2	nd	nd	nd

Patient no.	Age (yrs)	Sex	Major clinical allergen	Nature of reaction	Symptoms	Total IgE	CAP score		SPT (mm)	
							Seafood mix	Fish	f.fish	c.fish
F6	24	F	fish	anaphylaxis	asthma, laryngeal oedema, hypotension, generalised urticaria, facial angioedema, GIT upset	39	nd	4	0	0
F7	43	M	fish	anaphylaxis	asthma, laryngeal oedema, GIT upset	28	0	0	3	nd
F8	30	M	fish	anaphylaxis	asthma, laryngeal oedema, generalised urticaria, facial angioedema	nd	nd	nd	11	8
F9	?	M	fish	anaphylaxis	laryngeal oedema, generalised urticaria, facial angioedema	129	2	1	nd	nd
F10	27	F	fish	anaphylaxis	asthma, laryngeal oedema, generalised urticaria, facial angioedema	nd	0	1	nd	nd

Egg allergic subjects

Patient no.	Age (yrs)	Sex	Major clinical allergen	Nature of reaction	Symptoms	Total IgE	CAP score	SPT (mm)
							Egg white	Egg white
E1	51	F	egg	angioedema	generalised urticaria, facial angioedema	318	0	4
E2	54	M	egg	anaphylaxis	asthma, laryngeal oedema, generalised urticaria, facial angioedema, GIT upset	1473	nd	7
E3	32	M	egg	anaphylaxis	asthma, laryngeal oedema, facial angioedema, GIT upset	21	2	nd
E4	26	M	egg	laryngeal oedema	laryngeal oedema, GIT upset	1253	3	nd
E5	24	F	egg	laryngeal oedema	laryngeal oedema, GIT upset	9	0	4

Milk allergic subject

Patient no.	Age (yrs)	Sex	Major clinical allergen	Nature of reaction	Symptoms	Total IgE	CAP score	SPT (mm)
							milk	milk
M1	19	M	milk	angioedema, laryngeal oedema	laryngeal oedema, generalised urticaria, facial angioedema	nd	0	15

Appendix 7.5.3 Exclusion criteria for subject selection

Wine allergy study: Data collection sheet – baseline

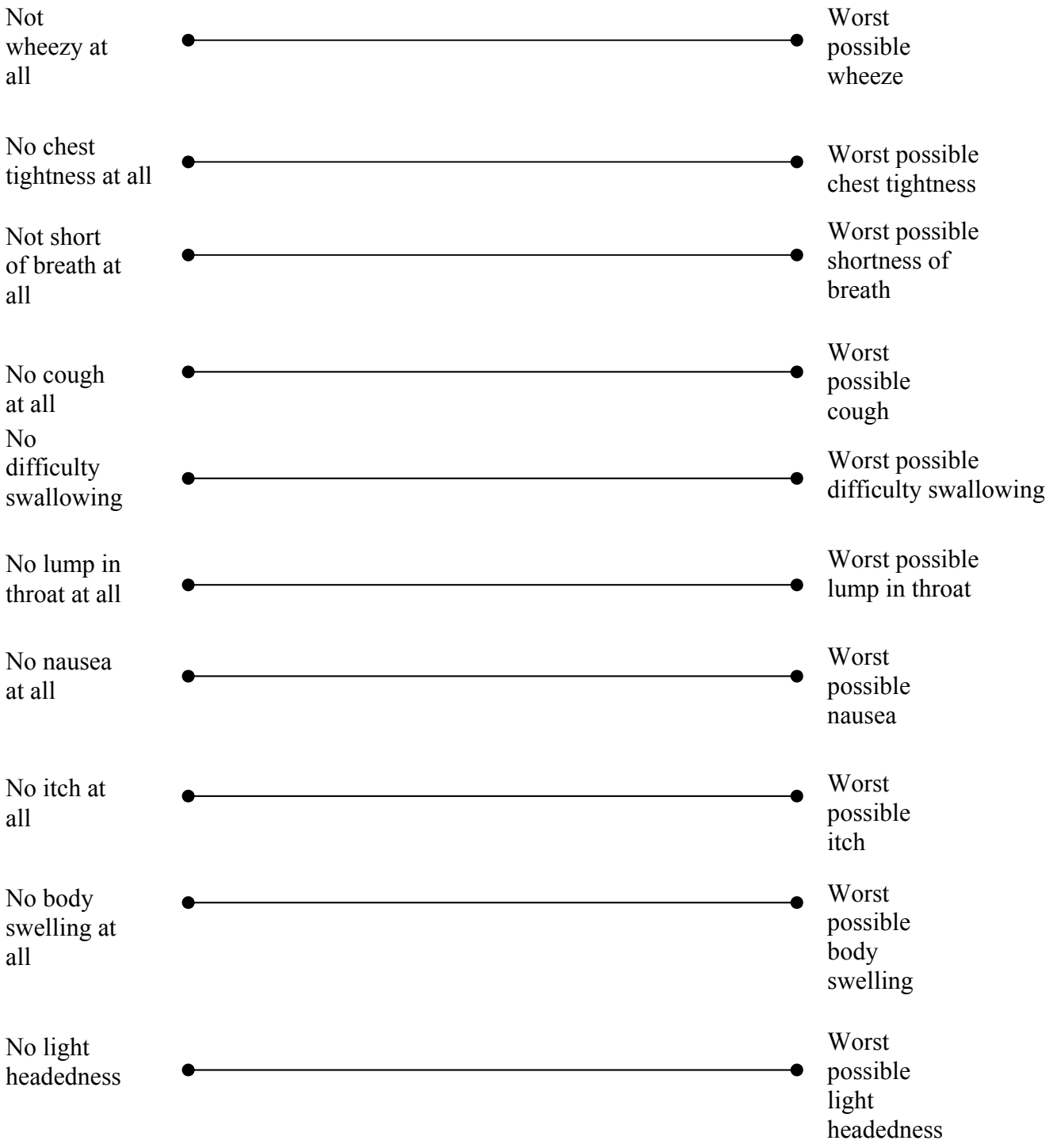
Exclusions (x (cross), if absent)

- asthma
- urticaria
- angioedema
- GIT upset
- URTI
- anti-histamines in last 2 days
- ventolin use in last 4 hours
- seretide/oxis in AM
- alcohol in last 3 days
- ischaemic heart disease
- stroke
- diabetes mellitis
- epilepsy
- renal failure
- liver disease

Appendix 7.5.4 Visual analogue scale (VAS)

Wine allergy study: Data collection sheet – baseline

History



Appendix 7.5.5 Physical Examination

Wine allergy study: Data collection sheet – baseline

Examination findings

Vital signs
BP: Pulse: RR:

oropharynx

uvula:	normal ☐	swollen ☐
tongue	normal ☐	swollen ☐
lips	normal ☐	swollen ☐

chest

wheeze	absent ☐	present ☐

periphery

urticaria	absent ☐	present ☐
angioedema	absent ☐	present ☐

spirometry: attach to sheet

Appendix 7.5.6 Description of survey wines

Wine sample	Processing aid*	Addition (mg/L)	Timing of addition	Variety
1	3	4000	Post cold stabilisation	Chardonnay
2	3	400	Post cold stabilisation	Chardonnay
3	3	5500	Pre cold stabilisation	Chardonnay
4	3	600	Post cold stabilisation	Semillon Chardonnay
5	3	400	Post cold stabilisation	Riesling
6	1	10000		Merlot
7	2	6		Chardonnay
8	2	4		Verdelho
9	2	1		Chardonnay
10	4	2		Chardonnay
11	3 5	10 100		Cabernet Sauvignon
12	5	17		Shiraz
13	1	350		Pinot Noir
14	4	8		Chardonnay
15	4	6		Riesling
16	4	0.8		Chardonnay
17	3	300		Chardonnay
18	2 3	20 400		Riesling
19	2	7.5		Sauvignon Blanc
20	4	5		Chardonnay
21	1 5			Shiraz Cabernet Sauvignon
22	4	0.1		Chardonnay
23	3 4	35 5		Chardonnay
24	5 5 5 5	640 7.5 700 5	At crushing and at pre bottling	Merlot
25	5	11	At crushing and at pre bottling	Merlot
26	2	30	Pre bottling	Chardonnay
27	1	500	Pre bottling	Malbec Merlot Cabernet Sauvignon Cabernet Franc
28	1		Pre cold stabilisation	
29	1		Pre cold stabilisation	
30	5		During	

			fermentation and clarification	
31	3		During fermentation	
32	2 4		Pre clarification and fermentation During cold stabilisation	
33	2		Pre clarification and fermentation	
34	4		During cold stabilisation	
35	3	35	During fermentation prior to racking off	Chardonnay
36	3	25	During fermentation prior to racking off	Chardonnay
37	3	50	During fermentation prior to racking off	Chardonnay
38	3	50	During fermentation prior to racking off	Traminer
39	2 4	5.4 9.5		Chardonnay
40	4	4		Sauvignon Blanc
41	1	0.0665		Cabernet Sauvignon Merlot
42	4	0.5		Sauvignon Blanc
43	4	50	Post heat and cold stabilisation	Riesling
44	3	0.05	Pre settling and bottling	Marsanne
45	3	1.25	Pre settling and bottling	Chardonnay
46	3	0.77	Pre settling and bottling	Chardonnay
47	3	1.15	Pre settling and bottling	Riesling
48	3	2.0	Pre settling and bottling	Marsanne
49	3	0.002	Pre settling and bottling	Chardonnay
50	2			Riesling
51	5			Shiraz

	1			
52	3 2	191 2		Chardonnay
53	3	1		Semillon
54	2	2		Riesling
55	3 2	51 52		Sauvignon Blanc
56	3 2	141 12		Chardonnay
57	3 2	55 18		Chardonnay
58	3 2	2 4		Semillon Sauvignon Blanc
59	2	3		Riesling
60	3 2	423 8		Viognier
61	5 5 5	277 82 31		Merlot
62	5 5 5	7 18 57		Shiraz
63	5 5 5	144 50 6		Merlot Cabernet Sauvignon
64	5 5	24 4		Shiraz Cabernet Sauvignon
65	5 5 5	150 15 35		Merlot
66	5 5 5	217 50 71		Shiraz
67	4	5		Chardonnay
68	2	10		Semillon Chardonnay
69	4	5		Chardonnay
70	4	5		Riesling
71	5	50		Shiraz Cabernet Sauvignon
72	1	3		Merlot
73	5	50		Merlot Cabernet Sauvignon
74	1	0.5		Pinot Noir
75	2 4	50 20		Chardonnay
76	1 3	100 400		Verdelho
77	1 3	50 250		Semillon
78	1 3	300 100		Shiraz
79	1 3	200 50		Merlot Cabernet Sauvignon

80	1	5		Cabernet Sauvignon
81	5	100	On centrifugation	Shiraz
82	5 5	50 250	On centrifugation During time on skins	Cabernet Sauvignon
83	5	40	On centrifugation	Merlot
84	5	50 100	On centrifugation During time on skins	Cabernet Sauvignon
85	2	100	7 days prior to filtration	Chardonnay
86	4	9	5 days prior to filtration	Semillon Sauvignon Blanc
87	1	1	Prior to filtration	Merlot Cabernet Sauvignon
88	2	5	14 days prior to filtration	Semillon Sauvignon Blanc
89	1 4	18 1.25	Prior to filtration	Merlot Cabernet Sauvignon
90	2 3 4	16 366 28	Prior to filtration	Chardonnay
91	3 4	75 119	Prior to filtration	Verdelho
92	2	40	6 weeks pre-bottling	Chardonnay
93	2	25	6 weeks pre-bottling	Semillon
94	3			white
95	1	150	Pre racking	Merlot Cabernet Sauvignon
96	1	350	Pre racking	Shiraz
97	5	150	During time on skins	Grenache
98	1	6eggs/1000L		Marsala
99	1	6eggs/1000L		Marsala
100	1			Merlot
101	5	250 125	On first racking On second racking	Shiraz
102	5	200	During time on skins	Shiraz
103	5	200	During time on skins	Shiraz
104	1	100	Post cold/heat stabilisation, pre filter and	Shiraz

			bottling	
105	1	100	Post cold/heat stabilisation, pre filter and bottling	Shiraz
106	1	100	Post cold/heat stabilisation, pre filter and bottling	Grenache
107	4	3	Post cold/heat stabilisation, pre filter and bottling	Chardonnay
108	4	2	Post cold/heat stabilisation, pre filter and bottling	Grenache Cabernet Franc Petit Verdot
109	4	5	Post 3 month maturation on yeast lees, with 0.5g/L bentonite	Chardonnay
120	—	—		Riesling
121	—	—		Riesling
122	—	—		Grenache
123	—	—		Grenache

***Processing aid key:** **1 = egg white**
2 =casein/potassium caseinate
3 = milk
4 = isinglass
5 = non-grape derived tannin