Ascertaining risk of an allergic reaction from consuming wine in Australia

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Declaration

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Creina S. Stockley

Summary

The first recorded risks to food safety date back to the Roman Empire and examples include the preservation of foods and adulteration of wine with the poisonous sweetener, lead acetate, which had fatal consequences for consumers. The latest recorded risks to food safety include allergies to food and food ingredients, which are increasing in prevalence and with life threatening consequences. Allergens can occur unintentionally in foods such as wine, being ingredients not intended to remain in the final product. Such examples are the proteinaceous processing aids used in the clarification of wine, which are derived from egg, fish, milk or nuts, although a review of the published literature revealed no adverse reactions that had been attributed to them. As the basis of food allergy management is the complete avoidance of all foods that could contain the causative allergen, this has resulted in a reduction in choice of potentially safe foods for allergic consumers such as wine, until studies had been undertaken to ascertain their allergic potential. This body of research comprised four inter-related studies undertaken to ascertain the risk of an allergic reaction occurring in sensitive adult individuals from the consumption of protein-fined wine in Australia.

A series of four studies were the first undertaken to ascertain the risk of an allergic reaction in sensitive individuals from Australian wine fined with egg, fish or milk and products derived thereof, and/or to which nut-derived non-grape tannins were added. The four studies comprised: the development of sensitive and specific ELISAs for the candidate allergens in wine; the analysis of a diverse panel of 113 wines, 109 of which were produced with these proteinaceous processing aids; the development of an alternative *in vitro* assay (BAT) to predict the potential allergenicity of protein fined-

wines; and a food challenge of protein-fined and un-fined wines in 37 individuals, 26 of whom were food-allergic.

Sensitive and specific ELISAs were developed for the most abundant potent egg and milk allergens, and for peanut-derived allergens in wine. The level of detection was between 1-8 µg/L and is among the lowest for such assays. When the ELISA were applied to the panel of wines, no residual egg, milk or peanut-related protein was detectable in the protein-fined wines. Residual egg was only detected in two wines to which whole eggs had been added, and these wines were labelled as containing egg. In the food challenge with protein-fined and unfined wines, no clinically significant life threatening adverse reactions were elicited by the wines in the 37 individuals. The subsequently developed BAT was, however, insufficient sensitive to be an alternative to the ELISA or BAT but may be considered as an adjunctive tool to predict potential allergenicity.

This body of work therefore has ascertained that in this food-allergic population of adults, Australian wine fined with egg, fish or milk or to which non-grape, nut-derived tannin has been added and made according to good manufacturing practice, poses a low risk of allergic reaction attributable to allergenic residual proteins in wine.

Chapter 1 Wine and risk

Overview

Foods including wines[1] can induce many different allergic and immune responses. A primary public health concern exists in relation to potential risks to sensitized individuals who have produced immunoglobulin E (IgE) antibodies to proteins in food, which are then implicated in immediate adverse reactions on subsequent exposure to that food (Hefle et al., 1996). Commonly allergenic foods affecting adults that may be present in wine include egg, fish, milk and nuts and ingredients derived from them. Associated adverse reactions can range from mild objective and subjective reactions, to life threatening and fatal anaphylactic reactions (Cianferoni and Spergel, 2009; Cochrane et al., 2012), with an increasing incidence of the latter (Gupta et al., 2011). There is no cure for food allergy at this time in 2014.

In this body of work, and introduced in this chapter, is the recognition of risk to human health of an allergic reaction in susceptible individuals from potentially allergenic proteinaceous processing aids such as the traditional egg, fish, milk and nut-derived fining agents used in the production of wine. Identification, quantification and ascertainment of risk to potentially vulnerable populations is examined. This risk from wine has not been previously researched in Australia. The program of research that is presented here to ascertain this risk has both national and international relevance.

[1] Wine is regulated as a food and, therefore, for the purposes of this thesis, food includes wine, and wine is substituted within all references to food herein, although it is noted that in other contexts this characterisation would not hold or may be challenged.

1.1 Introduction

Risks exist in all facets of life. Potential sources of risk need to be ascertained, assessed and managed. Moreover, standards need to be set for acceptable or tolerable risks. A key public health area where risk manifests is in relation to food and food products such as wine. Food borne illnesses and diseases represent an important public health problem, significantly affecting people's health and with substantial economic consequences (WHO, 2002). A food borne illness is defined by the World Health Organization (WHO) as being caused by ingestion of a food containing a biological or chemical agent, or physical hazard (Schmidt and Gervelmeyer, 2003). One of the 10 great public health achievements for the twentieth century that have been documented by the US Centers for Disease Control and Prevention[2], is the control of food borne illnesses by sanitation, refrigeration, pasteurization and pesticide application. It is expected that this problem will increase in the 21st century as global changes including population growth, poverty, international trade in food and animal feed, continue to influence the safety of food and food products. In addition, the safety and security of the food supply is increasingly threatened by deliberate adulteration and contamination (WHO, 2002).

Wine, like many other ingested food products, has been consumed for thousands of years. At different times in history it has been considered to be a staple part of a daily diet and crucial calorie intake (Babor, 1986). Like many other ingested food products it is also subject to the full range of potential risks to human health and safety, and has become one of the most controlled of all foods and beverages with respect to the use of additives and processing aids. The number of additives approved for use in winemaking

[2] http://www.cdc.gov/about/history/tengpha.htm

has reduced considerably in the past 50 years (Brooke-Taylor et al., 2003), a trend that was in effect well before recent public activity seeking to reduce additives in foods (Stockley et al. 2004).

Current wine regulations are concerned with health and consumer protection, as well as affording protection against fraud. In the Australian and New Zealand governments' current Food Standards Code, to which the Australian and New Zealand wine industries must abide, the addition of an additive or processing aid to wine must follow four principles: it must not compromise health; it must have a beneficial purpose that cannot be achieved by a mechanical process; it should be efficient; and, in the case of a processing aid, it must leave minimal or no residue in the wine (Brooke-Taylor et al., 2003).

1.2 What is a public health risk

Food is a basic prerequisite for human survival and also for economic and social welfare. Ensuring food safety and security and reducing associated risks is a basic tenant of public health. In the two centuries since the industrial revolution the processes by which we obtain resources from our environment, including food, have been substantially modified (Cochran, 1905). Food supply and distribution systems have evolved from relatively simple agrarian systems to complex industrialised systems (Morrison et al., 2010). Food supply and distribution systems that were local or home-based, with minimum processing and production, large loss from spoilage and limited opportunities for adulteration, have been replaced by regional, national and global systems. The latter are both diverse and flexible, and based on large-scale and multi-step processing and production.

Food is also a major source of chemicals (Kroes et al., 2000; Dorne and Fink-Gremmels, 2013). By definition, food contains chemicals apart from added chemicals. A variety of desirable and undesirable chemicals may enter our food supply by means of intentional or unintentional addition, including adulteration, at different stages of the food chain. These chemicals can include food additives and processing aids, pesticide residues, environmental contaminants, pathogenic and spoilage microbials, mycotoxins and micronutrients. Packaging and other food contact material are also a potential source of chemicals in food products and beverages, including wine. Monitoring consumer exposure to chemicals has become an integral part of ensuring the safety of the food supply. Documented health and safety issues associated with the ingestion of chemicals from foods have varied over time and from country to country, as have government regulations and responses to these issues (Keener et al., 2013).

Accordingly, as our food supply chains become increasingly complex and globalised[3] (Bonnano, 1994; Friedland, 1994; Pingali, 2006; Popkin, 2006) the need for ascertainment of risks to health and safety, and consequent control of these risks, will increase in importance for both consumers and governments (Keener et al., 2013). Risks to health and safety for wine extend beyond local population groups to global populations, and are the remit and responsibility of both highly regulated and less regulated governments (Keener et al., 2013).

[3] Of the wine produced in Australia, 60% was exported in 2013 to 122 countries compared to only 28% in 1993;http://www.wineaustralia.com/en/Winefacts%20Landing/Australian%20Wine%20Export%20Approvals/Wine%20Export%20Approvals%20Report.aspx

1.3 Health and safety risks for foods

There are different types of health and safety risks for food. Such risks emanate from aspects of food production through to accidental and deliberate adulteration of the final food product. Of specific relevance to consumers is the use of additives which can have adverse effects on human health. These additives may contain chemical, microbial or physical contaminants which can accidently adulterate foods. An important aspect of the use of an additive, therefore, is its purity or quality. There are specifications and regulations concerning the purity and composition of additives in government regulations, as well as in the Codex Alimentarius[4] (see Chapter 2, Section 2.5.5).

1.3.1 Adulteration

Deliberate adulteration of a food occurs when an additive is knowingly used which compromises human health and safety (Knechtges, 2011). The adulteration of food products has been a major challenge, and the protection of the consumer has occupied the attention of governments from ancient times (Dorne et al., 2010). In keeping with a globalised food supply, food surveillance systems have also been globalised with a dual remit to detect, control and prevent food borne illness and disease outbreaks, as well as to inform longer term issues. These longer term issues include: (1) estimating the burden of food borne illnesses and diseases and monitoring trends; (2) identifying priorities and developing policy for the control and prevention of food borne illnesses and diseases; (3) detecting, controlling, and preventing food borne illness and disease outbreaks; (4) evaluating food borne illness and disease prevention and control

[4] Codex Alimentarius [internet]. International Food Standards. Rome: World Health Organization & Food and Agriculture organisation of the United Nations; 2013. Available from: http://www.codexalimentarius.org/

strategies; and, equally importantly, (5) identifying emerging food safety issues (WHO 2002; Bishop and Tritscher, 2012).

Adulteration has recently re-emerged as a preventable food safety risk and hence a major public health concern. A food is considered adulterated if it contains poisonous or other chemicals, foreign materials, and/or is decomposing, which adversely affect the health of humans. It is also considered adulterated if it contains a non-approved additive such as a colourant or flavourant, or a specified ingredient has been substituted with a non-specified one, or if it contains any substance that increases its bulk or weight (Tsimidou and Boskou, 2003).

Common cases of adulteration can involve basic foods such as dairy products, cereals, flour, fish, meat, oils, fruit juices, flavourings, honey, chocolate and coffee as well as alcoholic beverages such as wine (Tsimidou and Boskou, 2003). Deliberate adulteration can involve false presentation in colour, texture and weight, and/or efforts to increase profit (Jackson, 2009). For example, from the Roman Empire until the 17th century, wine was often adulterated with lead-infused grape juice to sweeten and preserve it. Similarly, the established acceptable daily intake of lead calculated by the WHO in 1999 was 243 µg/day [5], and consumers of such adulterated wine (containing approximately 20 mg/L of lead) experienced debilitating neurological and physical symptoms leading to death (Eisinger 1982). Vinegar was often observed to be adulterated with sulphuric acid, green vegetables with copper, oils with oil of turpentine, boiled lollies with arsenic and lead, chocolate with ferric oxide and red pepper with mercury sulphide (Jackson et al., 2009). In the Spanish toxic oil syndrome

[5] http://apps.who.int/food-additives-contaminants-jecfa-database/chemical.aspx?chemID=3511

of 1981, Spanish producers presented industrial grade refined rapeseed oil as food grade olive oil (Rangan and Barcloux, 2009) which caused 600 deaths and 25,000 injuries (Posada de la Paz et al., 2001; Gelpi et al., 2002). This 'toxic oil syndrome', as it has been coined, highlighted the need to strengthen food safety regulations and their enforcement. Subsequently, the Spanish government initiated the involvement of the WHO.

A more recent example of adulteration with adverse health effects is diethylene glycol. The physicochemical properties of this colourless, practically odourless and hygroscopic liquid with a sweetish taste make it an excellent counterfeit for pharmaceutical grade glycerine or propylene glycol. This poisonous chemical has caused deaths in different countries when used in pharmaceutical and other ingested products including toothpaste (Cantarell et al., 1987; Wax, 1995; Schier et al., 2009; Schep et al., 2009). In 1985, up to 1 g/L of diethylene glycol was found to have been illegally added to Austrian and German wines to improve sweetness in late harvest style wine, but with no reports of significant adverse health effects (van der Linden-Cremers and Sangster, 1985).

More recently, the chemical melamine, which is poisonous in high concentrations, was added to wheat gluten as a thickener to give the false appearance of higher protein in products, so that inexpensive ingredients could pass for more expensive, concentrated proteins (Food Safety and Inspection Service 2009). Adulterated wheat gluten sourced from China was used in animal food products in the USA which caused kidney failure in certain animals, and the adulterated products were duly recalled. The U.S. Food and Drug Administration (FDA) subsequently banned all vegetable proteins imported from

China intended for human or animal consumption. In 2008 in China, however, melamine mixed with cyanuric acid was added to baby formula resulting in six infant children deaths and another 294,000 adversely affected (Knechtges, 2011).

Advanced analytical methods of adulteration and detection have evolved. There has been a concomitant shift in focus from food adulteration to food safety, and to an approach which emphasises not only evaluation of the food, but also an evaluation of individual components such as additives and processing aids. An additive is a chemical that remains in the food product and has a technological function. Additives can be classified according to function, such as acidificants, deacidificants, anti-oxidants, colourants, flavourants, preservatives, stabilisers and sweeteners.

In contrast to additives, processing aids do not remain in the final product, although they can be present as residues in the final product, and include clarifiers, fermentation aids and stabilisers. In addition to remaining in the product, they can also contain contaminants or residues of their constituents or their production processes, which can be transferred into the food. Both additives and processing aids are used in the production of wine and may have inherent risks for consumers.

1.3.2 Contamination

Unlike additives and processing aids, contaminants are chemicals or microorganisms that are not intentionally added to food but may enter the food accidentally during growth, cultivation, transport or preparation. Contaminated food is now one of the most widespread public health problems in the contemporary world (WHO 2008). It is the cause of substantial morbidity and mortality (Hanson et al., 2012). Globally, millions of

consumers are affected by contaminated food and food borne illnesses and diseases (FAO/WHO, 2002)[6]. In 2004, the U.S. Centers for Disease Control (CDC), for example, estimated that food borne diseases resulted in approximately 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths in the USA each year. This rate has not declined for the past seven years[7]. Food poisoning is a significant risk for vulnerable population groups such as infants, children, pregnant women, the elderly, and immune-compromised individuals; the latter two groups are increasing in industrialised nations (Rooney et al., 2004). Diarrheal diseases alone, of which a considerable proportion are food borne related, kill approximately 1.5 million children worldwide every year (WHO 2008, Hansen et a. 2011). Although most of these diarrheal deaths occur in poor countries, food borne diseases are neither limited to developing countries nor to children[8].

Environmental contaminants in a food include fertilisers, pesticides, veterinary chemicals and air, soil and water pollutants, transferred to the food by deposition on leaves or plants or from uptake by roots. Microbial contaminants may result from both pathogenic and spoilage microorganisms associated with the raw materials, production, storage or packaging. They may accumulate during food storage, form in the food though the integration of chemical components or may be concentrated from natural components of the food (Rooney et al., 2004).

[6] http://www.fao.org/docrep/meeting/004/ab524e.htm
[7] http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html
[8] http://www.ifpri.org/sites/default/files/publications/focus10.pdf

1.4 Allergic health risks from food

In addition to risks associated with adulteration of food, there are also risks associated with the inherent qualities of food products. Proteins contained in a food can cause adverse health effects such as allergies or intolerances. Similarly, proteins in additives or processing aids used in food production can also cause allergies or intolerances (Atkins, 2008). Worldwide, prevalence of allergic diseases has continued to increase in the industrialized world for more than 50 years, particularly over the past two decades, and sensitization rates to one or more common allergens among school children are currently approaching 40 to 50% (Parwanker et al., 2011). The prevalence of allergic diseases worldwide from drugs, food and insects is rising dramatically in both developed and developing countries (Sampson, 1988; Metcalf et al., 1996; Taylor and Hefle, 2001; Boyce et al., 2010).

The prevalence of allergy has increased to such an extent that it is now regarded as a major healthcare problem, and in relation to food allergy a major public health and safety risk, markedly affecting quality of life, and negatively impacting the socio-economic welfare of society (Cianferoni and Spergel, 2009; Lieberman and Sicherer, 2010; Burks et al., 2012; Australasian Society of Clinical Immunology and Allergy, 2014). In the EU, acute and life threatening allergic reactions such as anaphylaxis account for a high proportion of hospital admissions for allergic reactions to foods (Worms et al., 2010), while the rate increased between 1993 and 2004 by five-fold in Australia (Poulos et al., 2007).

World-wide, food-related allergies affect 3-4% of the adult population, but prevalence is higher in children (5-8%). Nationally in Australia, food allergy is estimated to currently

affect 1-2% of adults and 4-8% of children under five years of age (SA Department of Health, 2010), and data indicates that up to 10% of children less than one year of age will develop food allergy (Osborne et al., 2011). Allergies to eggs or milk, soya and wheat proteins usually resolve by three or four years of age (Crespo and Rodriguez, 2003; Sicherer and Sampson, 2006). The most common food allergies among adults include shellfish, peanuts, tree nuts and fish (Sicherer et al., 2004; Rona et al., 2007; Boyce et al., 2010), and among children are cow's milk, eggs, peanuts, soy, wheat, tree nuts and fish (Høst and Halken, 1990; Sicherer, 2003; Sicherer and Sampson, 2006; Hare and Fasco, 2008; Blom et al., 2013). Individuals 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; Fernández-Rivas et al., 2008; Hofmann and Burks, 2008; Patelis et al., 2012; Patelis et al., 2014). The most common food allergens affecting adults that may be present in wine are egg, fish and milk.

Food allergies are distinguished from food intolerances. Food intolerances are a non-allergic abnormal physiological or pharmacological response to an ingested food or food component (Johansson et al., 2004). Contemporary nomenclature for food intolerance is "non-allergic food hypersensitivity" (Johansson et al., 2004). Food intolerances occur more frequently than true food allergies, affect approximately 20% of the population, occur more frequently in females than males (Parker et al., 1990; Parker et al., 1993; Bruijnzeel-Koomen et al., 1995; Schäfer et al., 2001; Wigand et al., 2012), and are often associated with reactions to multiple foods (Parker et al., 1990). Symptoms of food intolerance generally occur more than two hours after ingestion of the problematic food (Bruijnzeel-Koomen et al., 1995), are usually non-specific, may

involve one or more body systems (Parker et al., 1990; Parker et al., 1993), typically occur at any age, and are found most commonly in nonatopic individuals. No definitive laboratory tests are available to diagnose food intolerances (Vanderhoof, 1998). Common elicitors of food intolerances include low molecular weight chemical ingredients including preservatives such as sulfur dioxide, flavourants and colourants. They also include components that occur naturally within the food such as salicylates, biogenic amines including histamine and tyramine and glutamates; these are all natural components of wine.

In contrast to food intolerances[9], a food allergy is an immunologically mediated hypersensitivity after the ingestion of a specific food, usually involving the production of specific immunoglobulin E antibodies (IgE) by B lymphocytes in response to a food protein (Hare and Fasno, 2008). A true food allergy usually has a rapid onset following ingestion of the food protein with symptoms commencing within three minutes and up to two hours post ingestion (Hourihane et al., 1997a; Hourihane et al., 1997b; Bindslev-Jensen et al., 2004). The most severe and life threatening symptom is anaphylactic shock (anaphylaxis) which, if not treated immediately, can result in death within 15 minutes.

It is difficult to definitively proscribe a lower limit for the concentration of potential allergenic substances below which the risk of an allergic reaction is minimal. This is because numerous factors may be involved, such as age, gender, genetic constitution, dietary habits, the allergenicity of dietary components and extraneous environmental factors (Sampson, 1996; Dean, 1997; British Nutrition Foundation Task Force, 2002).

[9] Further described in Chapter 3

Prevention of an allergic food reaction is best achieved by complete avoidance of the food in question. In the case of foods produced with potentially allergic proteinaceous food additives or processing aids such as wine, which are not identifiable either in the food product or on its label, such avoidance strategies may be difficult to accomplish.

1.5 Allergic health risks from wine

In addition to chemical and microbiological additives, wine can also be produced with food and food-derived proteinaceous processing aids such as egg, fish, milk and nuts. These processing aids are described further in Chapter 3. Processing aids are not intended to remain in the final food product[10]. They are nonetheless a potential source of contaminants where the presence of these food proteins in wine may potentially provide a threat to the health and safety of food-allergic individuals.

Until 2002, when allergen labelling was introduced into Australia, the allergenic potential of wine as a health and safety risk to sensitive individuals had not been considered by the wine industry, researchers, Australian consumers or government. Despite the potential for an allergic reaction from egg, fish milk or nuts to be fatal (World Allergy Organization, 2011), no investigation had been undertaken of the potential risk to sensitive individuals of an allergic reaction from consuming wine produced with these foods. Thus it was not known whether wine produced with food and food-derived proteinaceous processing aids such as egg, fish, milk and nuts could cause an allergic reaction in sensitive individuals. In other words, it was not known whether wine made with these food proteins posed a risk to human health and safety.

[10] http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=101.100

This thesis therefore investigated the risk to the health and safety of egg, fish, milk and nut-allergic individuals from consuming Australian wine produced with egg, fish, milk and nuts or products derived thereof. To understand the extent and nature of the potential risk, several fundamental research steps were required to be undertaken. These included the development of assays to determine the presence of these processing aids in wine and to provide an assessment of whether allergenic proteins have been removed through food processing. What was further required was an in vivo assessment of the actual risk to individuals exposed to potential allergens and to do this required studies that involved a food challenge of food-allergic individuals with protein-fined wine to see whether these wines could actually elicit an allergic reaction.

This program of research is predicated on the notion and understanding of the concept of risk. The following chapter details and discusses the mechanisms and framework for ascertaining risk. It further indentifies how the scientific research that was undertaken into the risk of an allergic reaction from wine can inform public health policy and thereby protect the health and safety of food-allergic individuals.

Chapter 2. Risk analysis - approaches for ascertaining risk and applying it to public health safety

Overview

To ascertain the safety of foods such as wine, the risks to health have to first be established. To establish risk there is a range of evidence-based assessment models and tools that can be employed by national regulatory bodies to ascertain risk of an allergic reaction from wine. The principles that form the basis of a scientific risk assessment and the steps for undertaking a risk assessment are examined in this chapter. Once mechanisms for ascertaining risk have been identified, they can be applied in appropriate studies to quantify said risk. Subsequent research findings can inform public health food safety policy, legislation and regulations including that for wine.

2.1 Introduction

There is a community expectation that foods such as wine will be safe. Both government and food industry have a duty of care to the consumer that the food source and supply is safe. The safety of food, however, is dependent on many factors, some of which can be controlled through government public health policies, legislation and regulations. Much of the shared responsibility for food safety lies with the agricultural sector and the processed food industry such as the wine industry to ensure that reliable practices and procedures are in place to produce consistently safe primary produce and processed foods.

Public health policy, in the form of legislation, regulation and guidelines also plays a crucial role in food safety. The formulation of public health policy is complex and depends on a combination of often competing economic, social and political factors in

addition to science (Spasoff 1999; Marston and Watts, 2003; Choi et al., 2005). Consequently there may be a disconnect, or gap, between the evidence or results of scientific research and the policies that follow (Lomas, 2000). There may also be a gap in the time taken for research results to translate into policy. For example, Sir Richard Doll's scientific evidence linking tobacco smoking to lung cancer was provided to the UK government in 1950 but it was not until 1957 that any legislation was initiated (Walt, 1994).

2.2 Changes to food safety control

Given that adulterated and contaminated foods and food borne diseases comprise a large part of the global mortality burden (Hansen et al. 2011), food safety control is becoming increasingly important in industrialised and developing countries. Traditionally, the approach to food safety control by both the food industry and governments has been technical, ad hoc and primarily reactive. These approaches were based upon utilisation of experience obtained from many years of exposure to various hazards, considering local practices, traditions and technological possibilities. However, such an approach has proved insufficient to ensure public health and safety and minimise risks.

There is now a recognised general or overarching regulatory framework to guide governments' decision making in managing risks to food safety, and associated administering and advisory bodies (WHO, 1995). Additional considerations include consumer concerns, environmental protection and sustainability (Jukes, 2000; Macfarlane, 2002). The Codex Alimentarius Commission of the Food Agriculture Organization (FAO) and World Health Organization (WHO) is recognised by World Trade Organization (WTO) as the international benchmark or reference for the

resolution of disputes concerning food safety (WHO, 1998). Although the Codex Alimentarius Commission does not have a specific standard for wine, wine additives and processing aids are included in its general food standards. In 1993, it first considered food allergy as an important public health issue and allergen labelling as one approach to risk management, In addition it considered that "attempts should be made to define the ingredients and additives involved"[11] (Codex Alimentarius Commission, 1993, Page 9).

Similarly, the European Food Safety Authority (EFSA) provides independent scientific advice and clear communication on existing and emerging risks associated with the food chain to the European Union (EU)[12]. EFSA was requested by the European Commission in 2004 to advise on: 1) The scientific basis supporting the identification of foods, food components and food ingredients which induce food allergies and food intolerance for foodstuffs labelling purposes; and 2) The possibility of determining thresholds or of identifying other elements (including food processing) which would establish that a food component or a food ingredient is no longer susceptible of inducing adverse reactions. In the USA, the Food and Drug Administration is the federal consumer protection agency responsible for the safety and security of the US food supply and administers a Code of Federal Regulations with which wine must comply in conjunction with those of the Alcohol and Tobacco Tax and Trade Bureau (TTB). Correspondingly, food regulations including Standard 4.5.1 Wine production requirements (Australia only) and Standard 1.2.3 Mandatory warning and advisory statements and declarations are currently developed by Food Standards Australia New Zealand (FSANZ) in Australia.

[11] www.codexalimentarius.org/input/download/report/139/al93_22e.pdf
[12] http://www.efsa.europa.eu/en/aboutefsa.htm

There are also general principles developed by international bodies that apply in addition to individual country's legislation and regulations such as the WTO two agreements relating to food regulation: the Sanitary and Phytosanitary Standards Agreement (SPS) and the Technical Barriers to Trade (TBT) Agreement[13]. These Agreements recognise the rights of countries to set "appropriate levels of protection" for the health of their populations, but state that these measures should be based on the science of risk assessment and should not restrict trade in any way, unless required to achieve the appropriate level of protection, taking into account both economic and technical feasibility (Silverglade, 2000; Horton, 2001; Lang and Heasman, 2004). Risk assessment, the scientific part of risk analysis, is promoted by the SPS Agreements requires that any measures applied to protect human, animal and plant health are developed using a scientific and transparent approach.

2.3 Risk analysis approach

To ascertain a food safety risk is to determine it with certainty, through examination or experimentation. This essentially entails the scientific analysis of a given risk. In reality, risks can never be completely eliminated in any human endeavours, but they can be reduced through design, practices and processes. It has been recognised by governments that food law and control systems cannot deliver a completely risk-free food supply (WHO, 2009). Over the past two decades, the food supply chain has been subject to increasingly strict standards and regulations, total quality management (TQM) controls and monitoring procedures. Nonetheless, there has been an increasing number of food safety alerts (for example, BSE, dioxins and bisphenol A contaminations in food) that have contributed to a loss of consumer confidence in the food supply (Vose, 2008).

[13] http://www.fao.org/docrep/003/x7354e/x7354e01.htm

The concept of risk analysis has gained increasing acceptance by governments and is an important component of food control systems, and its principles are gradually being integrated into food safety laws and regulations and public health policy (Rooney et al., 2004). Risk analysis is recognised as the fundamental methodology underlying the development of food safety standards (WHO, 2002).

Risk analysis is now a formalised, scientifically-based approach to addressing food safety issues on which food safety regulations are based (CAC, 2011). When applied, it provides a tool for the identification, assessment, management and communication of risk. In food safety, risk analysis approaches have been applied to the assessment and management of chemical food hazards such as food additives and pesticides, as well as microbiological risks. It is a discipline that is evolving as new food issues emerge[14] and has been extensively adopted by governments, although not consistently interpreted and regulated by different governments. This highlights the need for assessments to be scientifically based.

The basic risk analysis framework is comprised of three inter-related but theoretically separate components: risk assessment, risk management and risk communication as shown in Figure 1 and further described in Sections 2.3.1 to 2.3.7. This risk analysis framework has been incorporated as general principles in national food laws and forms the legal basis of their food safety systems[15].

[14] ftp://ftp.fao.org/docrep/fao/w4982e/w4982e00.pdf
[15] ftp://ftp.fao.org/docrep/fao/009/a0822e/a0822e.pdf

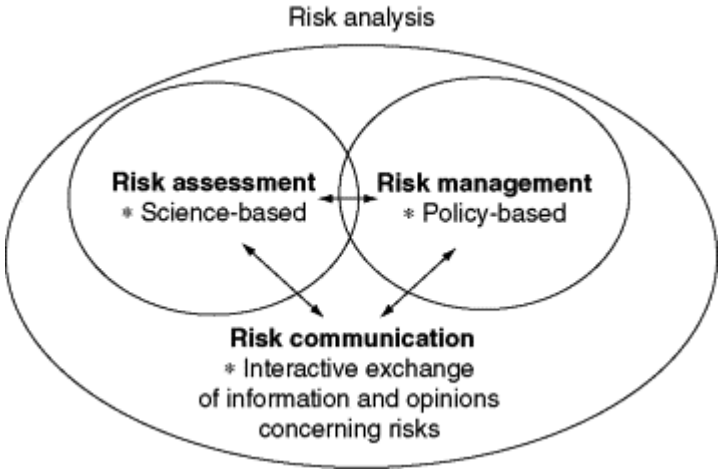

Figure 1 Relationships between the three inter-related components of a risk analysis (WHO, 2010)[16]

The process of risk assessment has been defined by the Codex Alimentarius Commission as a scientifically-based process consisting of hazard identification, exposure assessment, hazard characterisation and risk characterisation. A hazard is considered to be a biological, chemical or physical agent in food with the potential to cause an adverse health effect while a risk is considered to be the function of the probability of the adverse health effect and the severity of that effect, consequential to a hazard in food (CAC, 2003). Simply, risk is the likelihood and severity of a hazard causing death or illness among consumers. Risk assessments focus on estimating the risk (likelihood and severity) that a certain hazard will negatively affect the health of a population or particular population group, by evaluating qualitative and quantitative scientific data.

Risk management is defined as the process of weighing policy alternatives that emerge as a consequence of the result of the risk assessment, as well as selecting and implementing appropriate control options including regulatory measures. Risk

[16] World Health Organization. About Risk Analysis in Food. 2010. Published at: http://www.who.int/foodsafety/micro/riskanalysis/en/.

communication is defined as the interactive exchange of information and opinions concerning risk and risk management among risk assessors, risk managers, consumers and other key stakeholders. Dialogue with the different key stakeholders along the food chain is integral to each component (Fischer et al., 2005).

2.3.1 Risk assessment

Risk assessment is the *scientific component* of a risk analysis and concerns the nature and sources of the hazard, how it affects human health and how it behaves under certain conditions. There are four steps which involve the documentation and analysis of scientific data, the measurement of risk and the identification of factors that influence it. These are: 1. Hazard identification; 2. Exposure and dose-response assessment; 3. Hazard characterisation; and 4. Risk characterisation. Information derived from these four steps is collated to produce the risk estimate. The steps are well defined and developed, and the risk estimation straightforward for chemical hazards.

The evaluation of toxicological data depends on whether the toxic effect of the chemical under examination is considered to have a threshold, that is, a level below which there is no adverse effect. A threshold is assumed to exist for the majority of toxic effects. A key component in the overall risk assessment of chemicals was, therefore, the establishment of advisory standards such as:

- no observable effect levels (NOELs)
- no observed adverse effect levels (NOAELs)
- lowest observable adverse effect levels (LOAELs)
- acceptable daily intakes (ADIs)
- tolerable daily intakes (TDIs) for food additives

- maximum permitted residues of processing aids
- maximum permitted residues levels (MRLs) of pesticides and veterinary chemicals in the final food product, in particular if a chemical is not classified as GRAS (generally recognised as safe).

The estimation of physical hazards is also relatively straightforward, as the characteristics of the physical hazard do not usually change once they have been introduced into the food (Heggum, 2004). The estimation of microbiological hazards however, is complex due to biological diversity and variability (Heggum, 2004).

Consequently, the implementation of hazard analysis critical control points (HACCP) has become a mandatory measure in many countries for food production and food supply. HACCP is a systematic approach to the identification, assessment and control of biological, chemical, and physical hazards in a particular food production process, with the aim of identifying potential food health and safety problems at each critical stage of the production process and establishing measures for their control to prevent their development (Notermans et al., 1994). If the criteria are met at each critical control point of the production process health and safety problems with the food should be minimised.

2.3.2 Hazard identification

Hazard identification is predominantly a qualitative process to identify chemical hazard(s) of concern in a food, and to determine if exposure could cause an adverse health effect in humans, as well as the nature of the health effects. That is, scientific

data are evaluated to establish the possible causal relationship between exposure to a chemical hazard and the occurrence of adverse health effects.

Consideration should be given to the quality and relevance of available scientific data, the characteristics and relevance of experimental routes of exposure such as dermal, inhalation or oral, and the nature and significance of the observed effects as well as the relevance of health effects in experimental animals to effects in humans (Locey, 2005; Nance et al., 2010). Sources of data, which include epidemiological studies, human clinical studies, experimental animal studies, *ex vivo* tests, *in vitro* tests, structure-activity relationships and chemical and physical properties, are as follows:

- epidemiological studies – studies of the distribution and determinants of health effects or health status in human populations;
- human clinical studies – controlled experiments or studies designed to assess particular effects in human of exposure to a chemical;
- experimental animal studies – controlled experiments or studies designed to assess particular effects in animals of exposure to a chemical;
- *in vitro* tests – tests conducted using components of an organism outside of a living organism in an artificial environment, typically in test tube/tissue culture;
- *ex vivo* tests – tests conducted on a tissue outside of a living organism in an artificial environment but with the minimum alteration of natural conditions;
- structure-activity relationships – in the absence of toxicity data, a structure-activity relationship analysis can be used to predict health effects based on understanding of structurally similar chemicals and a specific response in a test system; and

- chemical and physical properties – in the absence of toxicity data, the chemical and physical properties of a chemical can be used to qualitatively predict health effects.

An evaluation should also be undertaken of the likelihood of the effects occurring under certain conditions and environments throughout the food supply chain, with examination of the implications for public health. The relationship is considered causal when one variable has a direct effect or influence on another. Each study should be evaluated with respect to quality, design, interpretation of the data and statistical significance to ensure that conclusions are valid before they can be integrated into the evaluation.

2.3.3 Exposure assessment

The objective of exposure assessment is to obtain a quantitative assessment of actual or anticipated human exposure to a food hazard and is based upon realistic exposure scenarios including the potential extent of food contamination and on accurate dietary data. Susceptible and high-risk population groups should be included in exposure assessments as well as acute, chronic cumulative and/or combined adverse health effects. The ultimate objective of an exposure assessment is the estimation of a hazard in food at the time of its consumption. This requires information about food consumption, and about the concentration and distribution of a particular hazard in food, including:

1. the frequency and level of contamination of the food over time, which are influenced by:

- the characteristics of the hazard;
- the nature of the food;
- the initial contamination of the raw material;
- the level of process controls;
- the methods of processing, packaging distribution and storage of the food; and

2. the amount and patterns of consumption, which relate to socio-economic and cultural backgrounds, ethnicity, seasonality, age differences, geographical or regional differences and consumer preferences and behaviour.

Exposure assessments of food can be generally qualitatively categorised according to the likelihood that the food will/will not be contaminated at its source, and whether or not the level of contamination will increase over time.

2.3.4 Hazard characterisation

The objective of hazard characterisation is to provide a description, either qualitative or quantitative, of the severity and duration of the adverse health effects that may result from ingestion of a hazardous chemical in a food, as well as an estimation of the level of the chemical that causes the adverse health effect (Heggum, 2004). The latter estimation is a dose-response assessment based on the typical dose of the chemical consumed.

2.3.5 Risk characteristation

The final risk characterisation step integrates the previous data collected and collated, and identifies limits and uncertainties in each step of the risk assessment, so a

comprehensive estimate of the potential risk to an exposed population can be confidently provided, including whether it is an acceptable risk (Locey, 2005; Nance et al., 2010). The degree of confidence in the final estimate depends on the variability, uncertainty and assumptions in each previous step. What is an acceptable risk, and how it is expressed is generally derived from a law, regulation or standard, and may be expressed, for example, as a hazard index or in terms of margin of safety.

Assessing the risk from food allergens does not fundamentally differ from assessing the risk from chemicals or microbiological agents in food (Spanjersberg et al., 2007; Madsen et al., 2009; Spanjersberg et al., 2010; Crevel et al. 2014a).

2.3.6 Risk management

Risk management is the public health policy component of the risk analysis and comprises four steps: risk evaluation; risk management options assessment; implementation; and monitoring and review (Figure 2). It has been defined as the decision-making process that entails weighing political, social, economic and engineering information against risk-related information to develop, analyse and compare regulatory options and select the appropriate regulatory response to a potential health or environmental hazard (van Leeuwen and Vermeire, 2007).

The purpose of risk management is the identification of acceptable risk levels and the development and implementation of appropriate control measures including practices, procedures and regulations, within the framework of public health policy (Notermans et al., 1999). This may also include an evaluation and selection of existing control measures, and considers factors contributing to a risk and their quantitative effects, and

a cost-benefit analysis of all options. The FAO/WHO (1997) states that the primary consideration of risk management should be the protection of human health and safety, and that the decisions and control measures associated with management of a risk should be clear. Each participant in the food supply chain has a responsibility to comply with the laws, standards and regulations established by governments.

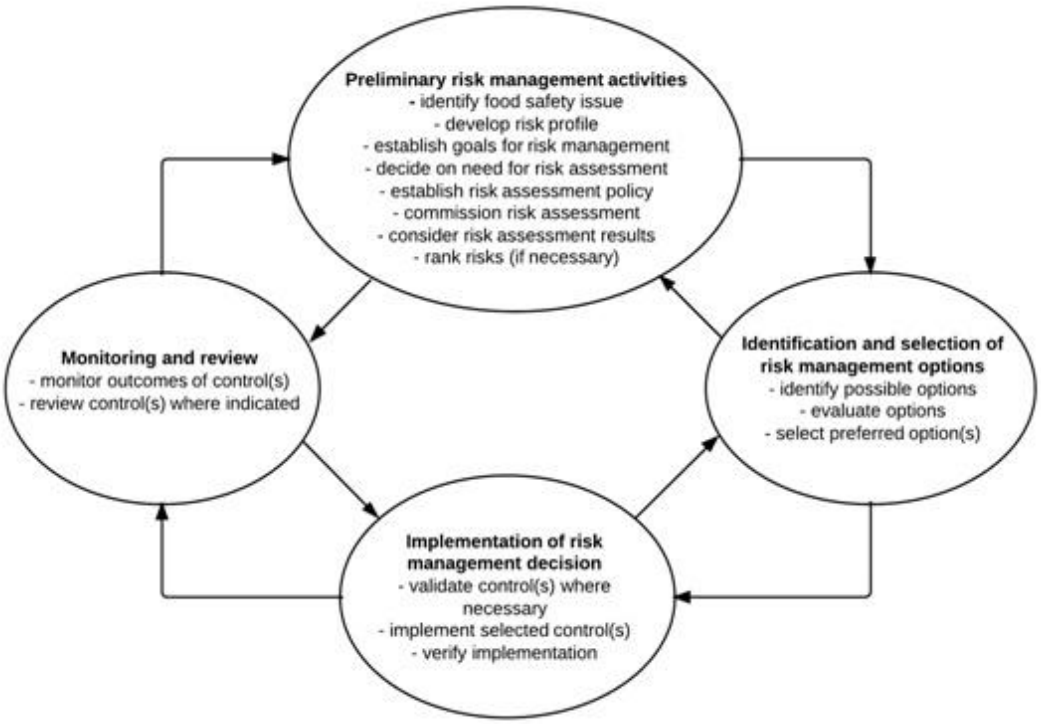

Figure 2 Overview of processes involved in risk management (WHO, 2010)

2.3.7 Risk communication

Risk communication is the third component of risk analysis (see Figure 1) and while it is integral to each component of the risk analysis it is particularly integral to risk management. All stakeholders, including consumers, who may be concerned with the food health and safety risk need to be actively engaged in two-way communication to enable open, transparent, and especially, effective decision making (Heggum, 2011). Two-way communication, for example, involves dialogue between the parties managing

the risk and the public concerned with the risk. Information communicated about the risk should thus be correct and kept current.

In designing a risk communication message for an audience to ensure readily accepted and implemented decisions about managing a risk, the following factors should be considered:

- personal experience with the risk;
- perceived importance of the risk;
- credibility of the communicator; and
- language and format in which the information is presented (Connelly and Knuth, 1998).

2.4 What constitutes tolerable risk?

Food safety has been described the state of acceptable or tolerable risk[17] of illness, disease or injury from the consumption of food (Knechtges, 2011), and as such, public health policy is about defining or establishing and maintaining that state.

Achieving consensus on levels of risk is inherently attractive but difficult to quantitatively establish. Yet, without such a baseline, it would not be possible to set guideline values and standards, given that zero risk is unachievable. The following criteria is a list from the WHO's *Water Quality: Guidelines, Standards and Health*

[17] An acceptable risk represents the level of risk society is prepared to accept without any specific risk management options (Glade et al., 2005, Lee and Jones 2004, Australian Geomechanics Society 2000, IUGS Working Group on Landslides - Committee on Risk Assessment 1997). Lee and Jones (2004) stated, however, that the term acceptable risk is increasingly replaced by tolerable risk.

(2001)[18], that could inform determination of an acceptable risk. A risk is acceptable when:

- it falls below an arbitrary defined probability;
- it falls below some level that is already tolerated;
- it falls below an arbitrary defined attributable fraction of total disease burden in the community;
- the cost of reducing the risk would exceed the costs saved;
- the cost of reducing the risk would exceed the costs saved when the 'costs of suffering' are also factored in;
- the opportunity costs would be better spent on other, more pressing, public health problems;
- public health professionals say it is acceptable;
- the general public say it is acceptable (or more likely, do not say it is not); and
- politicians say it is acceptable (WHO, 2001; p 208).

These listed criteria can be applied to the risk of an adverse reaction from food. Concerning the risk from food allergens, there is growing recognition that consistent risk management approaches with agreed quantitative reference doses based on scientifically robust principles will provide optimal consumer protection (Hattersley et al. 2014). In parallel, stakeholders groups now recognise that zero risk is unrealistic (Madsen et al., 2010; Madsen et al., 2012). They also recognise that any risk management approaches are a shared responsibility across all stakeholders including individuals, clinicians, research scientists, food industry and government regulators. Once a decision has been made on the tolerable frequency of different types of adverse

[18] http://www.who.int/water_sanitation_health/dwq/iwachap10.pdf

allergic reactions, benchmark threshold doses can be determined which meet the appropriate level of protection and can be used by the food industry and regulators (Hattersley et al., 2014). Any public health protection endpoint chosen, such as a threshold dose, needs to be highly protective of the overall population at risk.

2.5 Food risks to human health for which a tolerable risk is currently being established

Common food allergens such as cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products including lactose, nuts, sesame seeds, celery, mustard and sulphites at concentration of 10 mg/kg and above, are relatively recent examples of risks to consumers considered by governments nationally and internationally (Bousquet et al., 1998). Strategies to enable food-allergic individuals to avoid consuming a particular food or food ingredient are needed.

In 2004, EFSA[2] suggested that to establish a tolerable risk for any food allergens, analysis of foods for traces of potential food allergens is necessary. Sensitive test systems were also emerging then and were commercially-available for analysis of certain food allergens but not for wine. However, previous analytical problems identified by EFSA include detection limits outside the range of clinical sensitivity, insufficient specificity due to cross-reaction of allergens and insufficient inter-laboratory reproducibility[18]. Determination of thresholds and/or identification of food processing practices, which would establish whether a food component or a food ingredient is capable of inducing adverse reactions, are also necessary in establishing a

[2] European Food Safety Authority, 2004. Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission relating to the evaluation of allergenic foods for labelling purposes. The EFSA Journal, 32, 1-197. http://www.efsa.europa.eu/en/efsajournal/doc/32.pdf

tolerable risk to consumers. A primary aim of determining a threshold dose is to provide a benchmark that will facilitate assessment of the risk posed by the presence of an allergic food. Any given benchmark threshold dose should actually protect to a greater degrees than the nominal level of protection.

Significant variability in sensitivity exists, however, between allergic individuals. The lowest dose of allergens required to elicit an adverse reaction (LOAEL) generally ranges from µg to g amounts of an allergen (for further detail see Chapter 3, Section 3.5.1). This variability makes it difficult to draw firm conclusions regarding the lowest dose or threshold that would not cause an adverse reaction in the entire population of individuals with a specific type of food allergy. Thus, for these common food allergens - cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products including lactose, nuts, sesame seeds, celery, mustard and sulphites at concentration of 10 mg/kg and above - risk is not simply based on the assessment of no observed adverse effect levels (NOAEL). Therefore, the possibility of specific foods such as wine which potentially contain eggs, fish, milk or nuts, triggering an allergic reaction needs to be evaluated for each candidate food allergen.

The main aim of the development and implementation of a framework for allergen risk management anchored in evidence based threshold doses is to help ensure that food allergic consumers can make safe and informed decisions about what can be safely eaten and drunk. The first stage of any scientific assessment is to identify what is already known about food and wine allergy, and in particular that relating to eggs, fish, milk and nuts. A comprehensive review of the published literature was undertaken and examined for the candidate allergens - egg, fish, milk and nuts - in wine and factors

associated with adverse food reactions (see Chapter 3). Knowledge of derived threshold doses for the candidate allergens in the general food allergic population, and methods for their measurement in wine informed the body of research that was then undertaken which evaluated the likelihood of wine made eggs, fish, milk and nuts or products derived thereof eliciting an allergic reaction in sensitive individuals. Chapters 4 through 7 present investigations undertaken to determine of the amount of residual egg, fish, milk and nuts in Australian wine, whether this could be predicted with an *in vitro* assay, and whether the consumption of wines fined with these processing aids adversely affect egg, fish, milk and nut allergic wine consumers.

Chapter 3 Adverse wine reactions – a literature review

Overview

Adverse food reactions reported in the literature for wine are detailed in this chapter. It then examines what is known about the food allergens and the four candidate egg, fish milk and nuts, as well as what is known about the potential for them to cause an allergic reaction in wine.

3.1 Introduction

Worldwide, allergic reactions to foods are an increasing problem (Sicherer and Sampson, 2007; Branum and Lukacs, 2008; Sicherer, 2011; Burks, 2012). The actual prevalence of food allergy, however, is unknown (Rona et al., 2007; Sicherer and Sampson 2010). Factors such as age, diet, geographical area and genetic predisposition, as well as differing diagnostic procedures make it difficult to precisely estimate the actual prevalence (Sampson, 1996; Dean, 1997; Sicherer, 2002; Sicherer and Sampson 2009).

The Codex Alimentarius Commission (Codex) first considered allergens in 1993[19]. Ten common foods known to cause the most severe adverse reactions and 90% of cases of food-related allergy were subsequently determined in 1995 (Bousquet et al., 1998), and adopted for inclusion on food labels under Section 4.2.1.4 of the Codex *General*

[19] www.**codexalimentarius**.org/input/download/report/139/al93_22e.pdf

Standards for the Labelling of Prepackaged Foods[20] in 1999. These 10 common foods are: cereals containing gluten and their products, namely wheat, rye, barley, oats and spelt and their hybridized strains; crustacea and their products; egg and egg products; fish and fish products; peanuts and peanut products; and soybeans and soybean products; milk and milk products; tree nuts; sesame seeds; and added sulphites in concentration of 10 mg/kg or more [CODEX STAN 1-1985 (Rev. 1-1991)]. Codex recommended that all Member Nations and Associate Members of the Food and Agriculture Organisation (FAO) and World Health Organization (WHO) should accept and adopt this list of 10 common foods in their national food laws and modify their labelling regulations to assure that the potentially allergic ingredients and their products were declared when present in packaged food. Wine is regulated as a packaged food.

In the community, wine is frequently considered responsible for allergic adverse reactions (Wigand et al., 2012). The most commonly reported reactions to wine are cutaneous flushing, itching and nasal congestion (Wigand et al., 2012), which are less suggestive of an immunologically mediated allergy than intolerance to chemical components or ingredients of wine (Parker et al., 1990; Parker et al., 1993). The extent to which wine constitutes a true allergen has not been established although wine could contain up to four of the 10 common foods causing food-related allergies.

Wine can potentially contain residues of egg, fish, milk, nuts and their proteinaceous products. Each of these compounds holds the potential to cause allergic reactions, the most severe of which is anaphylactic shock that may result in death. These residues result from the traditional international standard practice of clarification during the wine

[20] The Codex General Standard for the Labelling of Prepackaged Foods was adopted by the Codex Alimentarius Commission at its 14th Session, 1981 and subsequently revised in 1985 and 1991 by the 16th and 19th Sessions and amended by the 23rd and 24th Sessions, 1999 and 2001.

production process referred to as 'fining'[21]. During the fining process, compounds such as egg white, fish-derived isinglass, and milk and milk proteins (including casein and potassium caseinate) are mixed with the wine, allowed to settle and the clarified wine is transferred or decanted from the deposit. While egg white is generally used to remove unwanted tannins from red wine, the other permitted[22] fining agents are generally used to remove unwanted phenolic and tannin compounds from white wine. These processing agents are expected to precipitate out of the wine during fining and subsequent decanting. Further winemaking practices such as filtration, undertaken after fining, also act to remove any residual processing aids.

As a part of risk communication outlined in Chapter 2 section 2.7.7, which is a component of risk assessment, any wine sold in Australia must be labelled according to the provisions of the Australian New Zealand Food Standards Code (ANZFSC). This includes provisions in Standard 1.2.3 *Mandatory warning labels and advisory statements and declarations*, as well as provisions in Standards A4 *Preservatives* and A7 *Antioxidants.* Prior to 14 December 2002, the provisions specifically stated that the presence in wine of compounds such as added preservatives (including sulfur dioxide and sorbic acid), added antioxidants (including ascorbic and erythorbic acids), and colouring materials such as caramel, which have the potential to cause an adverse reaction in vulnerable wine consumers must be included on the label, and hence subject to these labelling provisions.

[21] Fining is the process of removing undesirable and undissolved microscopic particles from a wine such as protein particles that could cloud the wine and also cause bitterness and astringency (Rankine, 2004).
[22] Permitted according to the provisions in Standard 4.5.1 of the Australian New Zealand Food Standards Code.

Australia was the first country to accept and adopt the list of 10 common foods in their national food law and modify their labelling regulations on 14 December 2002. For wine, this meant that if egg, fish and milk were present in the finished bottled product, their presence must be included on the wine label. However, mechanisms by which to determine the presence of these foods in wine had not been established.

3.2 Literature review of the incidence of adverse food reactions from egg, fish, milk and nuts in wine

Prior to 2002, wine had not been considered as a potentially allergenic food in Australia, even if potentially allergenic egg, fish and milk were permitted as processing aids and tree nut-derived tannin as an additive. Winemakers were uncertain as to whether egg, fish and milk were present in the finished bottled product, were unsure about how to assess their presence and consequently, and were uncertain whether to include their presence on the wine label.

A comprehensive search of electronic databases from 1965[23] to 2005 to inform the development of this program of research was undertaken. The search identified literature on three different topics: 1) adverse food reactions from egg, fish, milk and nuts; 2) adverse reactions and from wine; and 3) assessment of adverse reactions from these proteins in wine. Subsequent searches after 2005 were undertaken to update the initial search throughout the research program. Only English language publications were included. The following key words were used, singly and in combination: allergy; allergen; allergeric; wine; grapes; adverse reaction; hypersensitivity, intolerance; analysis; egg; fish; milk; nut; protein; ovalbumin; ovomucoid; isinglass; and casein, and

[23] Since this area of food allergens began to be research and papers published on case studies

applied in the following databases: EBSCO, EMBASE, MEDLINE (PubMed), MEDLINE (Ovid), Science Direct, and Web of Science.

Numerous studies described general allergic reactions from eggs, fish, milk and nuts with a preponderance related to these reactions in children and a proliferation post 1995 and particularly post 2002. The published literature from 1965 to 2014, however, details relatively few reports of genuine or true allergic reactions following the ingestion of wine, which is discussed as follows. Studies previous to this body of work up to 2005 did not report or investigate allergic reactions from wine associated with the candidate allergens egg, fish, milk or nuts.

3.2.1 The incidence of adverse food reactions from wine

There were 23 studies that reported adverse reactions related to wine and two studies that reported food challenges with egg or milk-fined wine in food allergic individuals (Table 1) These 25 studies in total were divided into three different groups according to the potential allergenic compound in wine that elicited the adverse reaction, which is further discussed below and in Section 3.5.

The first group included 10 studies on IgE-mediated adverse reactions to grapes and grape products such as wine where the allergenic compound was potentially grape protein. The second included 13 studies on adverse reactions to wine but where the allergenic compound was uncertain, and the third included two studies with egg and milk-allergic individuals who did not experience an adverse reaction to egg or milk-fined wine. These studies involved individual case studies, clinical studies and population surveys.

Table 1 Summary of studies on wine-related adverse reactions in the published literature

Reference	Number of subjects	Study type	Food eliciting an allergic reaction	Allergen confirmed
Pastorello et al., 2003	14	Clinical study	11 to grapes 3 to wine	Yes Grape protein
Borghesan et al., 2004	1	Case study	grapes + wine	Yes grape protein
Schäd et al., 2005,	1	Case study	grapes + wine	Yes grape protein
Alcoceba Borràs et al., 2007	3	Case study	2 to grapes 1 to wine	Yes grape protein
Kalogeromitros et al., 2005	11	Clinical study	31.4% to grapes 28.6% to wine	No
Kalogeromitros et al., 2006	61	Prospective study	grapes + grape products	No
Sbornk et al., 2007	1	Case study	Grapes + wine	No
Vassilopoulou et al., 2007	37	Clinical study	37 to grapes 1 to wine	Yes Grape protein
Schäd et al., 2010	1	Case study	grapes + wine	Yes grape protein
Falak et al., 2012	84	Clinical study	grapes	Yes grape protein
Clayton and Busse, 1980	1	Case study	wine	No
Littlewood et al., 1988	9	Clinical study	wine	No
Alibrandi et al., 1990	1	Case study	wine + vinegar	No
Wanke et al., 1993	17	Clinical study	wine	No
Jansen et al., 1994	1	Clinical study	wine	No
Kortekangas-Savolainen et al., 1994	11	Clinical study	wine	No
Kanny et al., 2001	16	Clinical study	Wine	No
Vally et al., 1999	16	Clinical study	wine	No
Vally et al., 2000	138	Clinical study	wine	No
Vally and Thompson, 2001	24	Clinical study	wine	No
Goldberg & Confino-Cohen, 2005	1	Case study	wine	No
Vally et al., 2007	8	Clinical study	wine	No
Wigand et al., 2012	68 reported 2 confirmed	Population survey	wine	No
Kirschner et al., 2009	5 egg-allergic 4 fish-allergic 5 milk-allergic	Clinical study	No reaction to protein-fined wine in egg, fish + milk allergic subjects	
Vassilipoulou et al., 2011	24 53	Clinical study Population survey	47/53 egg, fish + milk allergic subjects did not react to protein-fined wine	

3.2.2 Grape protein-related adverse reactions to wine

Of the 23 studies that reported adverse reactions from wine, 10 studies documented IgE-mediated adverse reactions to grape proteins such as endochitinase 4A and a lipid-transfer protein (LTP) in a very small number of individuals (Pastorello et al., 2003; Borghesan et al., 2004; Kalogeromitros et al 2005; Schäd et al., 2005; Kalogeromitros et al., 2006; Alcoceba Borràs et al., 2007; Sbornik et al., 2007; Vassilopoulou et al., 2007; Schäd et al., 2010; Falak et al., 2012). There are varietal differences in the amount of lipid transfer protein found on grape skins (Wigand et al. 2009), such that sensitive individuals may be able to tolerate certain grapes and wines containing lesser amounts of protein. The regular consumption of wine, however, can lead to the development of oral tolerance to lipid transfer proteins in some sensitive individuals (Schäd et al., 2010). Most of the grape-protein allergic individuals in these studies were also allergic to a variety of fruits from the botanical families *Rosaceae and Cucurbitaceae*, vegetables and pollens that also contain LTP (Garcia Ortiz et al., 1996; Rodriguez and Crespo, 2002; Egger et al., 2006), and reacted adversely to grapes but not always to wine. This lack of reaction to wine raises the question as to what might happen to the grape and other allergenic proteins in the wine production process.

3.2.3 Other adverse reactions to wine

Among the other 13 case reports and studies of adverse reactions to the consumption of wine that were identified in the literature there was uncertainty as to the specific allergen in all cases and they were all anecdotally attributed to acetaldehyde, biogenic amines, salicylates or sulfur dioxide (Clayton and Busse, 1980; Littlewood et al., 1988; Alibrandi et al., 1990; Wanke et al., 1993; Jansen et al., 1994; Kanny et al., 2001; Vally et al., 1999; Vally et al., 2000; Vally and Thompson, 2001; Vally et al., 2007; Goldberg and Confino-Cohen, 2005; Wigand et al., 2012), which are associated with a food

intolerance (Jansen et al., 2003; Maintz et al., 2007). Potential allergens were eliminated but no positive identification of the allergens in the studies.

Other adverse reactions to wine have been anecdotally attributed to the 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* (baker's yeast) allergens (Kortekangas-Savolainen et al., 1994). The results suggested that IgE-reactive *S. cerevisiae* yeast allergens were not present in significant concentrations in breads, beer or wine.

3.2.4 Assessment of adverse reactions from egg, fish and milk proteins in wine

The question of allergic adverse reactions to processing aids in wine had not been considered in the published literature prior to 2005. The publications [24] resulting from the current program of research were the first peer reviewed studies to examine the risk of an allergic reaction from protein-fined wine in sensitive individuals. More recently, however, two studies by Kirschner et al. (2009) and Vassilipoulou et al. (2011), were published which suggest that wines fined with egg or milk and their products do not cause an allergic reaction in sensitive individuals.

The lack of published literature on true allergic reactions following the ingestion of wine does not mean that allergic adverse reactions to wine fined with egg, fish, milk or nuts could and do not occur; rather that they have not been studied or reported, or that

[24] Rolland et al., 2006; Stockley et al., 2008.

the analytical and clinical techniques previously available lacked sufficient sensitivity to detect any possible allergic reactions.

Nonetheless, government and industry still have a duty of care to the consumer to demonstrate due diligence. This is the premise of the Food Safety Standards component (Chapter 3) of the ANZFSC[25]. 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 according to Section 36 of Australian Consumer Law[26]. 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 control 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 circumvent.

Applied to food allergenicity for the Australian wine industry, 'all due diligence' means having an empirical basis upon which to determine and assert confidently whether a wine product contains allergens, and specifically, whether it contains residuals of the aforementioned processing aids. At the time that this program of research was developed and executed, it was not possible to proscribe a definitive lower limit below which the risk of an allergic reaction was negligible and hence acceptable, due to the lack of established appropriate assays and clinical analyses.

[25] http://www.foodstandards.gov.au/code/documents/Commentary_v126.pdf

[26] http://www.consumerlaw.gov.au/content/the_acl/downloads/acl_guide_to_provisions_november_2010.pdf

Therefore, until mechanisms were identified and developed to determine: 1) the presence of egg, fish, milk and nut proteins in wine; and 2) the potential of the proteinacous egg, fish, milk and nut processing aids to elicit an adverse reaction in allergic patients, governments and industry were not able to state with confidence that protein-fined wine will not cause a true food allergy.

3.3 Adverse food reactions

An ‘adverse food reaction’ is a generic term that refers to any abnormal response 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; Ortolani and Vighi, 1995; Sampson, 1999; Sampson, 2004) as shown in Figure 3. As discussed in Chapter 2, food intolerances are all non-immunologically medicated adverse reactions to food with different distinct pathologies. A food allergy may have a life-threatening and/or fatal outcome while food intolerance does not (Jackson, 2003; Johansson et al., 2004). The initial physical manifestations of food allergy and food intolerance can, however, be similar if not identical. Food intolerant consumers can consider themselves to be food-allergic when they are only food intolerant (Young et al., 1994; Ortolani et al., 1999; Woods et al., 2002; Roehr et al., 2004; Wigand et al., 2012), and unnecessarily avoid certain foods.

Accordingly, food allergies need to be distinguished from food intolerances. In a meta-analysis of 51 studies evaluating the prevalence of adverse food reactions, up to 35% of individuals reporting a reaction to a food believed that they had a food allergy, whereas studies confirming food allergy by oral food challenges suggested a much lower

prevalence of approximately 3.5%. Much of this discrepancy is due to a misclassification of adverse reactions to foods that are not allergic in origin, for example, lactose intolerance causing bloating, abdominal pain and diarrhea after consumption of milk products (Rona et al., 2007).

To further illustrate this common misunderstanding, a study of 15,000 UK households found the prevalence of perceived food intolerance was approximately 20% (Young et al., 1994), but a true food allergy was clinically confirmed in only 1.4% to 1.8% of the individuals. Similarly, a study of 4,093 German adults found the prevalence of perceived food intolerance was 34.9%, but a true food allergy was clinically confirmed in only 3.7% of the individuals (Zuberbier et al., 2004). The Young et al. (1994) and Zuberbier et al. (2004) studies highlight that self-reported but clinically unsubstantiated food allergy is relatively common as a reason for unexplained and unexpected 'symptoms'. As there is generally little agreement between self-reported adverse reactions to food(s) known to contain the food allergen of interest and diagnostic tests for food allergies, this suggests that the majority of self-reported adverse reactions are not IgE-mediated (Woods et al., 2002).

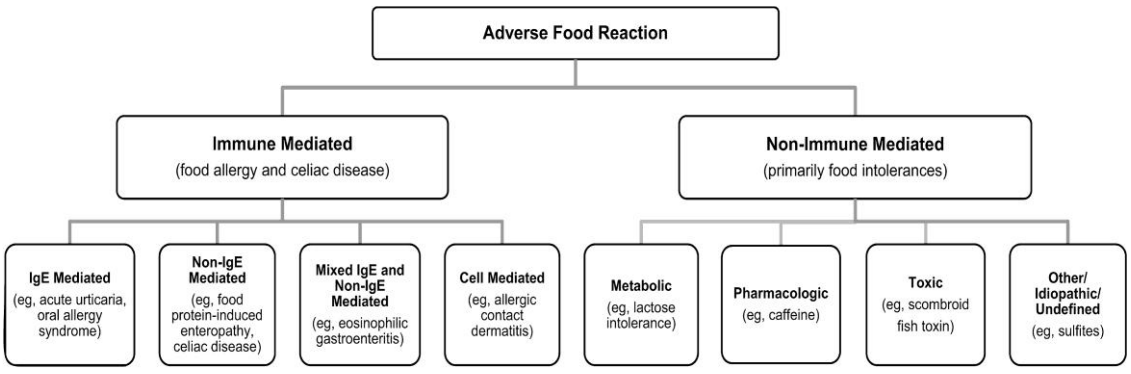

Figure 3 Summary of adverse food reactions (EFSA, 2004; US FDA, 2006).

3.3.1 True food allergy

In contrast to food intolerance, an allergy is essentially 'an inappropriate and exaggerated immune response' where a normally harmless substance is perceived as a threat by the body's immunological defenses. Food allergy is either antibody-mediated or cell-mediated (Johansson et al., 2004). It is an immunologically mediated hypersensitivity that occurs 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 (Hare and Fasno, 2008). Within the body, IgE binds to mast cells which release molecules such as histamine and leukotrienes that cause an inflammatory reaction. As 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, allergic reactions involve multiple body organs and sites (Costa et al., 1997).

An allergic reaction is established in two phases; an initial induction or sensitisation phase to an allergen followed by an elicitation phase of an allergic reaction on subsequent exposure to the same allergen. Sensitisation occurs when a susceptible individual produces IgE antibodies against specific allergenic proteins in a food, and when the individual is again exposed to the same food, the allergenic proteins bind to the IgE on basophil and mast cells activating them to release the inflammatory molecules (Taylor and Hefle, 2001).

True food allergy usually has a rapid onset following ingestion of the food protein with symptoms generally commencing within minutes and up to two hours post ingestion (Hourihane et al., 1997a; Hourihane et al., 1997b; Sampson et al., 2005). The most common symptoms are 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 (Sampson, 1988; Metcalf et al., 1996; Taylor and Hefle, 2001; Boyce et al., 2013). The most life-threatening symptoms are laryngeal edema and anaphylactic shock, that is, hypotension, cardiac arrhythmia and multiple organ failure (Jackson, 2003; Sampson et al., 2005). If these symptoms are not treated immediately, death can occur within 15 minutes.

3.4 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, and accordingly could be resistant to the winemaking fermentation process. Allergens in fresh fruit and vegetables may be inactivated, however, by cooking (Bohle et al., 2006; Bohle, 2007), and several studies have recently suggested that egg allergens may be inactivated by extensive baking and heating (Urisu et al., 1997; des Roches et al., 2006; Konstantinou et al., 2008; Lemon-Mule et al., 2008).

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 et al., 1999). Typically food-allergic individuals react to only one or two different foods.

The most common food allergens affecting adults that may be present in wine are egg, fish and milk. Although nut allergens are not expected to be present in wine as the non-grape derived tannins, such as that derived from chestnuts and oak galls, are derived primarily from the bark and galls of the trees, which are physically and pharmacologically distinct from the potentially allergenic nuts, there is no literature to verify this expectation. Each of these four allergens is described briefly below.

3.4.1 Egg allergens

Egg allergy is more common in younger children but may occasionally persist into adulthood. The estimated prevalence of egg allergy is from approximately 0.07–1.6% of the adult population (Asero et al., 2009a; Asero et al., 2009b; Osterballe et al., 2005). Studies collecting emergency department data have also shown that anaphylaxis to egg in adults is rare (Clark et al., 2004). Egg white (albumin) is more allergenic than egg yolk where allergies to egg yolk are more common in adults (Platts-Mills and Ring, 2005). Egg white contains predominantly ovalbumin (54%) with additional, ovomucoid (11%) (Powrie and Nakai, 1985). Ovalbumin (Gal d 2) of MW 43-45 kD has been identified as a major egg allergen, as well as ovomucoid (Gal d 1) of MW 28kD (Bernhisel-Broadbent et al., 1994). Other characterised lesser allergens in egg include ovotransferrin (12%) (Gal d 3) MW 77 kD, lysozyme (3.5%) (Gal d 4) MW 14.3 kD, and the minor allergens apovitellin, ovomucin and phosvitin (Bleumink and Young, 1969; Bleumink and Young, 1971; Langeland, 1982; Hoffman, 1983; Langeland and

Harbitz, 1983; Anet et al., 1985; Bernhiesel-Broadbent et al., 1994). The carbohydrate portion of egg glycoproteins has not been shown to bind to specific IgE.

3.4.2 Fish allergens

Fish allergy is important in both children and adults, and once present tends to persist throughout life (Kajosaari, 1982; Cresspo, 1995; Eggesbø, et al., 1999; Sicherer et al., 2004; Chiang et al., 2007; Turner et al., 2011). The estimated prevalence of self-reported fish allergy is from approximately 0.2–2.29 % of the adult population, varying between countries and regions (Sharp and Lopata, 2013; Sharp and Lopata, 2014). The allergenic activity resides in the meat of the fish. 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). Only some individuals allergic to fish meat seem also to possess IgE antibodies to fish type I collagen (Sakaguchi et al., 2000). Although fish gelatin obtained from skin and bone has been reported to be allergenic, in a double blinded placebo controlled food challenge none of 30 fish-allergic individuals had an adverse reaction to ingestion of a 3.16 g cumulative dose of commercial grade fish gelatin (Hansen et al., 2004). The wine fining agent, isinglass, is a fish collagen (predominantly type I and type IV) extracted from swim bladders (especially sturgeon, cod and hake), cleansed and either desiccated or dried, and is not normally ingested (Bailey, 1989). Based on the anatomical location

and tissue composition of fish swim bladder, it is unlikely but unknown whether isinglass contains the major allergenic fish protein, parvalbumin. The possible importance of swim bladder collagen as an allergen is unclear.

3.4.3 Milk allergens

Cow's milk allergy is extremely uncommon beyond early childhood. The estimated prevalence of milk allergy is from approximately 0.062−1.4% of the adult population (Asero et al., 2009a; Asero et al., 2009b; Osterballe et al., 2005). Studies collecting emergency department data have also shown that anaphylaxis to milk in adults is rare (Clark et al., 2004). Stöger and Wuthrich (1993) suggested that the main target organs in adult milk-allergic individuals were the skin and the respiratory tract. Gastrointestinal (mild to moderate) and cardiovascular (severe) symptoms were observed less often than in children. Caseins form 80–85% of milk proteins with whey proteins comprising the residual 15–20% of total protein (Swaisgood, 1985; Hermansen et al., 1999). 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). The latter is referred to as whey protein and is less digestible in the intestine, although digested to some small degree. When substantial whey protein is not digested fully in the intestine, some of the intact protein may stimulate a localized intestinal or a systemic immune response.

3.4.4 Nut allergens

There are two types of nut allergies - peanut, which is a legume, and tree nut. The onset of peanut allergy usually occurs in early childhood and generally persists in adulthood. The estimated prevalence of nut allergy is approximately 1% of the adult population,

and again there are differences between countries and regions (Crespo et al., 2006). 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 25–30% 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; Sicherer et al., 1998; Sicherer et al., 2001; 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 also usually occurs in early childhood and generally persists into adulthood (Sicherer and Sampson, 2000).

A variety of tannin sources are used in winemaking, such as chestnut bark and oak galls, that could be potential sources of tree nut allergens, but there is limited information on their potential allergenicity. In view of the reported cross-reactivity between tree nuts and peanuts, and the high potency of peanut allergens, the analysis of wines fined with non-grape derived tannins for ‘peanut-related proteins’ was, therefore, included in the research program.

3.5 Adverse food reactions reported for wine

The review of the literature (Section 3.2) undertaken to identify adverse food reactions from wine, including those related to egg, fish, milk or nuts in wine, found relatively limited literature reporting true allergic reactions following the ingestion of wine.

The consumption of alcoholic beverages such as wine also appears to promote the development of IgE-mediated allergy to some environmental and food allergens in specific genetically predisposed individuals (Linneberg et al., 2001; Linneberg et al., 2003; Alcoceba Borràs et al., 2007; Friedrich et al., 2008). Wine could thus increase the risk of an IgE-mediated allergic reaction to some environmental and food allergens, although this is not been suggested for egg, fish, milk and nut-allergic reactions (Linneberg et al., 2010).

The scarcity of studies also reflects the lack of importance or interest assigned to studying adverse reactions from wine to date. It is also uncertain whether most people are aware that substances derived from these foods are used during winemaking. Some people may ascribe an adverse reaction to other substances including the alcohol content of the wine, or foods ingested at the same time. The concentration of egg, fish or milk derived allergens in the wines was not, however, determined in any case report or study until 2007 (Weber et al., 2007a; Weber et al., 2007b). Thus, there may have been under-reporting of reactions (if any) caused by egg, fish or milk derived allergens in the research literature to date.

3.5.1 Threshold dose

As consuming a food may be harmful to health, ascertaining risk to the consumer is important. There is evidence to suggest that the majority of allergic individuals can tolerate small mg amounts of allergic protein (Hourihane et al., 1997; Moneret-Vautrin et al., 1998; Bindelev-Jensen et al.. 2002; Morriset et al., 2003; Grimshaw et al., 2003; Hourihane and Knulst , 2005; Flinterman et al., 2006; Peeters et al., 2007; Eller et al., 2012), although the threshold dose varies among individuals and also among sources of the same protein/allergen (Hourihane, 2001; Hefle and Taylor 2002; Taylor et al., 2002; Mullins 2003; Brockow and Ring, 2009).

For example, for sulfur dioxide, the threshold dose is usually considered to be 10 mg/L in sensitive individuals, which reflects existing Australian and international legislation (Vally and Thompson, 2001). Foods containing greater than 100 mg/L sulfur dioxide may, however, elicit no reaction in some sulfite-sensitive individuals (Taylor et al., 2002).

Oral food challenge studies are used to diagnose, confirm and characterise food allergy, including determining a threshold dose (Sicherer, 2014)[27]. A threshold dose should be based upon the appearance of objective reactions, although subjective reactions can certainly provide important information and indicate a subsequent objective reaction (Taylor et al., 2003; Moneret-Vautrin, 2004). The challenge procedure involves careful reintroduction of small incremental doses of the relevant food protein under strict medical supervision. In a challenge study to determine a peanut protein threshold in sensitive individuals, the lowest dose to elicit a mild, non-threatening adverse reaction

[27] http://www.uptodate.com/contents/oral-food-challenges-for-diagnosis-and-management-of-food-allergies

was observed to be 2 mg, although 50% of subjects could tolerate up to 50 mg (Hourihane et al., 1997b). In another challenge study to determine an egg and milk protein threshold in sensitive individuals, while 11% of egg-allergic subjects and 25% of milk-allergic subjects adversely reacted to doses of 100 mg, the majority of subjects could tolerate this dose (Sicherer et al., 2000; Hourihane, 2001).

From oral food challenge studies, the lowest observed adverse effect level (LOAEL) for a food protein can be calculated. The LOAEL or threshold for egg, milk and peanut is approximately 1 mg of a food, which represents approximately 100–200 μg of the actual food protein (Moneret-Vautrin and Kanny, 2004). The LOAEL may reflect subjective rather than objective symptoms, as objective symptoms are often only observed at a higher level of food protein (Bernstein et al., 1982; Olalde et al., 1989; Norgaard et al., 1992; Morisset et al., 2003; Moneret-Vautrin and Kanny, 2004; Lam et al., 2008).

Only 16% of egg-allergic subjects and 18% of peanut-allergic subjects appear to have a LOAEL or threshold of less than 65 mg of egg or peanut, and only 5% of milk-allergic subjects appear to have a threshold of less than 30 mg of milk (Moneret-Vautrin and Kanny, 2004).

There is, however, limited information on the LOAEL for fish. For codfish, amounts of less than 3 mg of parvalbumin protein may trigger allergic reactions (Untersmayr et al., 2007), which is less than previously reported (Taylor et al., 2004).

Accordingly, while the precise threshold for adverse reactions among food-allergic individuals has yet to be conclusively established, a ng or µg low level of a food protein is unlikely to be clinically relevant (Table 2). However, the 'gold standard' or definitive test for determining the threshold level for foods and whether a patient is allergic to a particular product is a double-blind, placebo-controlled food challenge (Bock et al., 1988). This is the causal test that needs to be performed to determine, for example, whether wine made with potentially allergenic processing aids can cause an allergic reaction in sensitive individuals.

Table 2 Summary of published threshold values for egg, fish, milk and nuts to elicit an allergic reaction from food challenges (US Food & Drug Administration, 2006)

Food allergy	LOAEL (mg protein)	Number of subjects
Egg	0.13 – 200	281
Fish (variety dependent)	5.00 – 6,000	41
Milk (cow's)	0.60 – 180	299
Nut - hazelnut	1.00 – 1,500	153
Nut - peanut	0.10 – 125	454

Therefore, as long as the amount of residual proteineous fining agent in wine is less than the threshold dose for egg, fish, milk and nut allergy, allergic consumers are unlikely to experience an adverse reaction from consuming potentially allergenic protein-fined wine. The LOAEL also emphasise the necessity of developing and using detection analyses with sensitivity of at least 1 mg/L to ascertain likelihood of an allergic reaction, and hence risk of an allergic for egg, fish, milk or nut-allergic wine consumers.

3.6 The potential for residual egg, fish, milk or nut allergens in wine

As indicated above, the potentially allergenic wine processing aids are egg white, fish-derived isinglass, milk and purified milk proteins (casein and potassium caseinate) and nut-derived tannin. Because wines differ in their composition, there are no set

recommendations on the amount of processing aids to be employed. Most fining processing aids strip aroma, flavour and colour from the wine, and therefore need to be used sparingly. From the winemaker's perspective it is also important that little of the protein remains in the wine after the fining/clarification, as the presence of relatively large amounts of residual fining agents will lead to visible protein precipitates and necessitate further remedial processes. For that reason fining agents are used conservatively. While preliminary small-scale laboratory trials will determine the optimal amount of processing aid to be used for individual batches of wine, the general range of additions is 5–15 mg/L egg white for red wine; 1–2.5 mg/L isinglass for white wine; 0.5% (w/v) skim milk for white wine; 10–50 mg/L casein for white wine; and 3–10 mg/L non-grape derived tannins for white wine (Ribéreau-Gayon et al., 2000). The final concentration of these proteinaceous processing aids in the wine is, however, likely to be lower than the amount added due to the subsequent removal of precipitates through the clarification process, and further clarification and filtration prior to bottling. For example, it is likely that only ng–µg/L of a processing aid would reside in the finished wine. Therefore, any examination of the concentration of egg, fish, milk or nut proteins in wine will require sensitive assays to do so.

3.6.3 Measurement of residual egg, fish, milk or nut allergens in wine

Testing of foods for allergenic proteins is usually done using enzyme-linked immunosorbent assays (ELISA) (Besler et al., 2001). At the time that the present program of research was developed there was no published literature available on the concentration of these processing aids in finished wine, and the only available commercial ELISA were not specific for wine. The lower limit of sensitivity or detection limit of the commercial ELISA was also only at the mg/L level (Morisset et

al., 2003; Moneret-Vautrin and Kanny, 2004). Given that further clarification and filtration processes post protein fining are undertaken to remove the mg/L additions of proteinaceous processing aids to wine to the ng or µg/L level, and that the LOAEL is likely to be 100–200 µg of protein (Hefle and Taylor, 2002; Taylor et al., 2002; Taylor et al., 2004), this level was considered insensitive, and these ELISA unsuitable, to detect residual amounts of allergenic processing aid in finished wine.

3.7 Program of research

The literature prior to 2005 and the development of this program of research contained no reports or studies that addressed whether wine contained potentially allergenic egg, fish, milk or nut proteins that could elicit an allergic reaction in sensitive individuals. There were also no suitable assays available to determine their presence. Hence, a program of research was established to examine the potential for true allergic reactions from Australian wine to occur from eggs, fish, milk and nuts in an adult population. The program involved four separate but inter-related studies.

The first study involved the development of sensitive and specific ELISAs in order to detect and measure residual allergenic proteins from the processing aids egg white, isinglass and milk, and the use of non-grape derived tannin in wine as there were no existing assays which were specific for wine and were sensitive below 1 mg/L of protein in wine. This study involved the development of three new assays and is presented in Chapter 4.

Once established, the three newly developed sensitive and specific assays were applied to a panel of commercially-available bottled Australian wines that had been fined with

one or more of the potentially allergenic processing aids. This study was undertaken in order to accurately determine for the first time the amount of residual fining agent that was present in commercially-available Australian wines. This study is reported in Chapter 5.

A small percentage of egg, fish, milk and nut-allergic individuals, however, have a very low µg/L level threshold for an adverse reaction. A third study was therefore undertaken to determine whether wines could elicit an allergic reaction in susceptible individuals. This study entailed a double blind placebo controlled clinical trial to determine whether egg, fish, milk or nut-allergic individuals experienced an allergic reaction, and were truly allergic to wine fined with egg white, fish, milk, nuts or their proteins. This study is relevant to our understanding of whether the absence of adverse reactions from wine in the published literature means that they do not occur or rather that they may not have been reported and/or studied. This study is reported in Chapter 6.

Finally, as a double blind placebo controlled food challenge is an invasive test that can expose the allergic individual to the risk of a systemic reaction such as anaphylaxis, a fourth study was undertaken to develop a simple and non-invasive predictive basophil activation assay (BAT) specific for egg, fish, milk and nut-fined wine as an alternative to a food challenge. This BAT was then applied to a subset of the panel of commercially-available bottled Australian wines used in Chapter 5 to determine if BAT was an appropriate alternative to the food challenge and ELISA to predict whether protein-fined wines would elicit an allergic reaction in sensitive individuals. This study is reported in Chapter 7.

Therefore, in order to determine whether Australian egg, fish, milk and nut fined wines contained residual food protein, and also whether they could elicit a true allergic IgE-mediated reaction in sensitive individuals, assays were developed which were used to measure the concentration of these food proteins in a panel of Australian fined and unfined wine. Also a double blind placebo controlled food challenge was undertaken, and an alternative non-invasive test was developed and applied to the panel of wines and blood of food challenge individuals, to ascertain whether sensitive individuals adversely reacted to the fined wines.

Declaration – Chapter 4

I declare that all content in the following chapter has been written by me. As the studies conducted for this thesis involved a wider support team, my independent contribution to each aspect of the study is detailed in Appendix 1.

Creina Stockley

Chapter 4 Development of ELISA for detection of residual processing aids in wine

Overview

This chapter presents the findings from the first study in this program of research to determine the risk of an allergic reaction occurring in egg, fish, milk or nut-allergic adults from wine consumption. Risk from potentially allergenic processing aids in wine production had not previously been examined in the researched literature. This chapter describes the development of a sensitive and specific method to detect and measure allergenic proteins from the commonly used egg, fish, milk or nut-derived processing aids in wine and internationally was the first attempt to develop such a method. The results of this study are published in Rolland et al., 2008 and Stockley et al., 2008[28].

4.1 Introduction

In order to be able to accurately assess the potential for true IgE-mediated allergic reactions from Australian wine to occur from eggs, fish, milk and nuts in an adult population, the first step required was the development of assays that could accurately and reliably measure residual egg, fish, milk and nut-derived proteins in wine.

[28] Rolland, J.M., Apostolou, E., de Leon, M.P., Stockley, C.S. O'Hehir RE. (2008). Specific and sensitive enzyme-linked immunosorbent assays for analysis of residual allergenic food proteins in commercial bottled wine fined with egg white, milk, and nongrape-derived tannins. Journal of Agricultural and Food Chemistry, 56(2): 349-354.

Stockley, C.S., de Leon, M.P., Stimson, K., Glaspole, I.N., Apostolou, E,. Rolland, J.M., O'Hehir, R.E. (2008). An investigation of the potential residual allergenic fining proteins in wine and their effect on egg, fish, milk or nut-allergic subjects. Blair, R.; Williams, P.; Pretorius, S. Proceedings of the thirteenth Australian wine industry technical conference; 28 July - 2 August; Adelaide, SA. : p. 380-381.

At the time that this program of research was undertaken there were two alternative types of assays available to measure the concentration of proteins in foods: enzyme-linked immunosorbent assay (ELISA); and polymerase chain reaction (PCR). In an ELISA, a specific antibody recognises the target protein, for example, the peanut allergen Ara h 1 protein, and a colour reaction makes its presence visible. PCR 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 at an exponential rate, making it readily visualised in an electrophoresis gel 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 protein product may have been used. It does not demonstrate whether the allergen is present in the wine. In addition, although most PCR assays amplify very small fragments of target DNA, there is no guarantee that the DNA found in wine will actually have fragment sizes sufficient for amplification.

From the literature reviewed in Chapter 3 and for confirmation, contact with relevant commercial and research facilities in the EU and USA[29], at the time that this program of research was developed, it was identified that there was only one commercially-available enzyme-linked immunosorbent assay (ELISA) specific for egg, milk and peanut allergens in food. This assay was available from the Food Allergy Research and

[29] Commercial companies or research facilities focussing on allergenic proteins in foods included: Laffot Oenologie (France); Molecular Biology & Immunology, Molecular Biology and Immunology, Eurofins Scientific Group (Germany); Enartis, Esseco Group (Italy);Institute of Food Research, Norwich Research Park (UK); Rowett Research Institute (UK); Beverage Alcohol Laboratory of the Alcohol and Tobacco Tax and Trade Bureau (USA); Food Allergy Research and Resource Program, University of Nebraska (USA); and Neogen Corporation (USA).

Resource Program (FARRP) at the University of Nebraska, Lincoln, Nebraska, USA, and it was not established for use in wine. Commercially-available ELISA also had a lower limit of sensitivity or detection limit of mg/L (Morisset et al., 2003; Moneret-Vautrin and Kanny, 2004). Although it has been asserted that commercial ELISA for egg, milk and peanut allergens with a detection limit of 1 mg/L is predictive of inducing an allergic reaction (Bindslev-Jensen et al., 2002; Taylor et al., 2002; Morisset et al., 2003), the threshold value for inducing an adverse reaction in an allergic individual is likely to be lower than this and dependent on other factors including an individual's sensitivity as well as properties of the particular allergen (Hourihane et al., 2005).

Given the limitations of the available assays, there was a need to develop an assay that was specific for the analysis of egg, fish, milk and peanut-related protein in wine, and which was sufficiently sensitive to detect the expected µg-ng/L residual levels in wine.

As described in Chapter 3, while numerous proteins are found in egg white and milk, the predominant allergenic proteins are ovalbumin (constituting 54% of total egg white proteins) (Bernhisel-Broadbent et al., 1994; Powrie, 1985; Rupa, 2003) and casein (constituting 80–85% of total milk proteins) (Hermansen et al., 1999; Wal, 1998; Wal, 2002), respectively. These are also the predominant proteins in egg white, casein, potassium caseinate, milk and skim milk fining agents/processing aids and are, therefore, markers for the presence of any potentially allergenic protein in wine. If ovalbumin and casein are not present above the level of detection of the ELISA assay, as the rates of protein removal by further clarification and filtration processes are expected to be similar for the structurally similar egg proteins and milk proteins (Bragg and Hough, 1961), the other less predominant egg and milk proteins also will be absent.

In terms of allergenicity, ovalbumin and casein are also major egg and milk allergens, respectively (Hoffman, 1983; Holen and Elsayed, 1990; Docena et al., 1996). Therefore, development of a mechanism to ensure the appropriate and rigorous analysis of wines fined with egg white, casein, potassium caseinate, milk and skim milk was warranted, given the importance to consumer health and safety.

Hence, the first step in a program of research was to develop such an assay to measure the concentration of ovalbumin, isinglass-related, casein and peanut-related allergens in finished wine.

4.2 Aim

The aim of this study was to develop sensitive and specific ELISA to detect and measure levels of the clinically relevant food allergens ovalbumin, isinglass-related proteins, casein and peanut-related proteins in wine.

4.3 Method

4.3.1 Generation of food allergen-specific monoclonal antibodies

The first step in the establishment of an ELISA is the procurement or production of specific monoclonal and polyclonal antibodies for use in the assays. As monoclonal antibodies were only available commercially for ovalbumin (Sigma, St. Louis, MO), this study sought to prepare mouse monoclonal antibodies to casein and peanut. Polyclonal antibodies to these allergens generated by the immunised mice were also evaluated as reagents for the ELISA.

The procedure used in this study as outlined below is a well-established and routine protocol for the production of monoclonal antibodies (Goding, 1996; Sutherland et al., 2002). Approval from The Alfred Medical Research and Education Precinct 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 casein 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 immunised again on day 56 with 100 µL of allergen solution without adjuvant. The mouse was euthanised 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, pH 5.0 (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.

The specificity of the anti-casein monoclonal antibody was further tested using the ELISA protocol outlined above where wells were coated with 2 µg/mL α-casein, β-casein and κ-casein extract (50 µL well; Sigma), diluted in 0.1 M bicarbonate buffer.

ELISAs could now be developed following the procurement or production of specific monoclonal (and polyclonal) antibodies for use in the assays.

4.3.2 Generation of ELISA

4.3.2.1 ELISA for detection of ovalbumin in wine

A sensitive and specific antigen capture sandwich ELISA was developed and established for ovalbumin detection in wine based on the method of Hefle et al., (2001). Preliminary experiments were undertaken to optimise antibody concentrations and incubation times and 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),[30] 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 outlined in Section 4.3.1(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 test wine sample to ensure reproducibility.

4.3.2.2 ELISA for detection of isinglass-related proteins in wine

Whilst desirable, it was not possible to develop an assay for isinglass. Antibodies against isinglass were of IgM isotype giving high background values in ELISA. Repeat immunizations failed to produce IgG monoclonal antibodies against isinglass, and generation of antibodies of $IgG_{1\kappa}$ isotype, suitable for sensitive ELISA, was discontinued.

[30] As described in Section 5.3.2 Wine pre-treatment, wine samples non-specifically inhibited ELISA reactions, due to their alcohol content and low pH value, and were pre-treatment before *in vitro* analysis .

4.3.2.3 ELISA for detection of casein in wine

A sensitive and specific antigen capture sandwich ELISA was developed and 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 4.3.1 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.

4.3.2.4 Inhibition ELISA for detection of peanut-related proteins in wine

A sensitive and specific inhibition ELISA was established based on the method of de Leon et al. (2003) using a peanut-allergic donor serum. The peanut-allergic subject has experienced anaphylaxis on ingestion of peanut and had positive serum specific IgE for almond, cashew and hazelnut as well as the major peanut allergens, Ara h 1 and Ara h 2.

Roasted peanut extract diluted at a concentration of 1 µg/mL in 0.1 M bicarbonate buffer pH 9.6, was coated onto Costar® 96-well polystyrene plates (50 µL/well), and incubated overnight at 4°C. Plates were washed with 0.05% PBS-Tween (5 times) and blocked with 5% skim milk powder (SMP) in PBS-Tween (PBS-T) (200 µL/well) for 1 h at room temperature. Serum from the 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:

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

4.4 Results

4.4.1 Generation of monoclonal antibodies

Hybridomas were successfully generated against all allergens and monoclonal antibodies of $IgG_{1\kappa}$ isotype, suitable for sensitive ELISA, were generated for casein and peanut.

4.4.2 Optimisation of ELISAs

4.4.2.1 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 and incubation time, were varied in order to achieve greatest sensitivity. Performing the assay at room temperature rather than at 37^{o}C reduced non-specific binding of antibodies, giving better signal detection at a lower concentration (Figure 4 and Figure 5). This was likely due to astringency of the mouse anti-albumin detecting monoclonal antibody at room temperature. Using the optimised method, the limit of detection was 1 ng/mL ovalbumin.

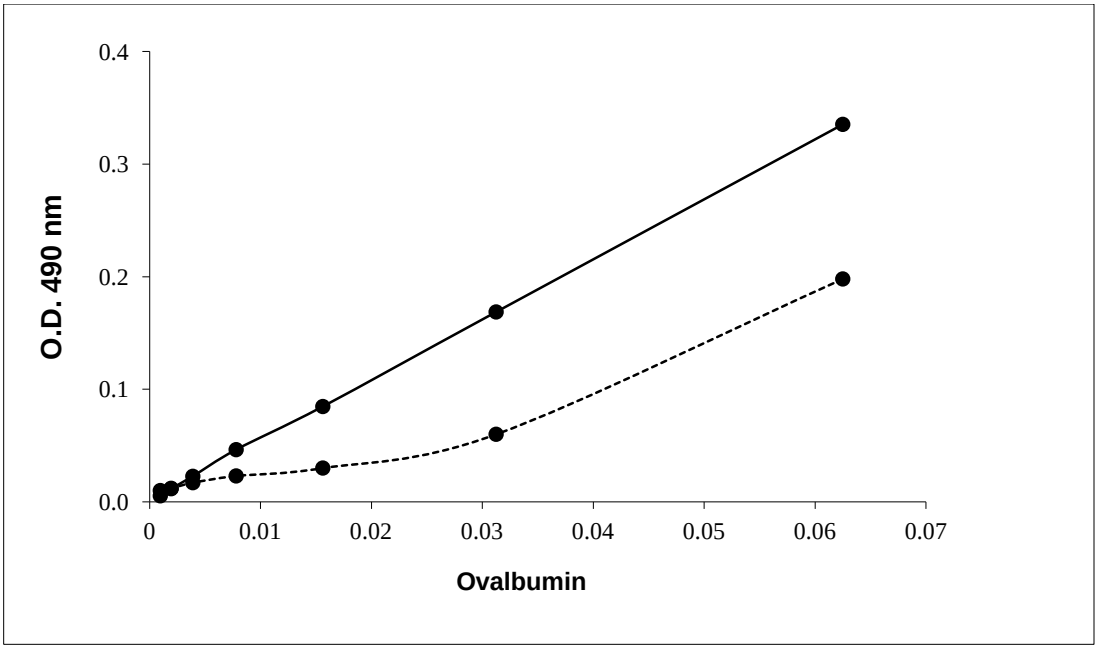

Figure 4 The effect of incubation temperature on sensitivity of the anti-ovalbumin sandwich ELISA

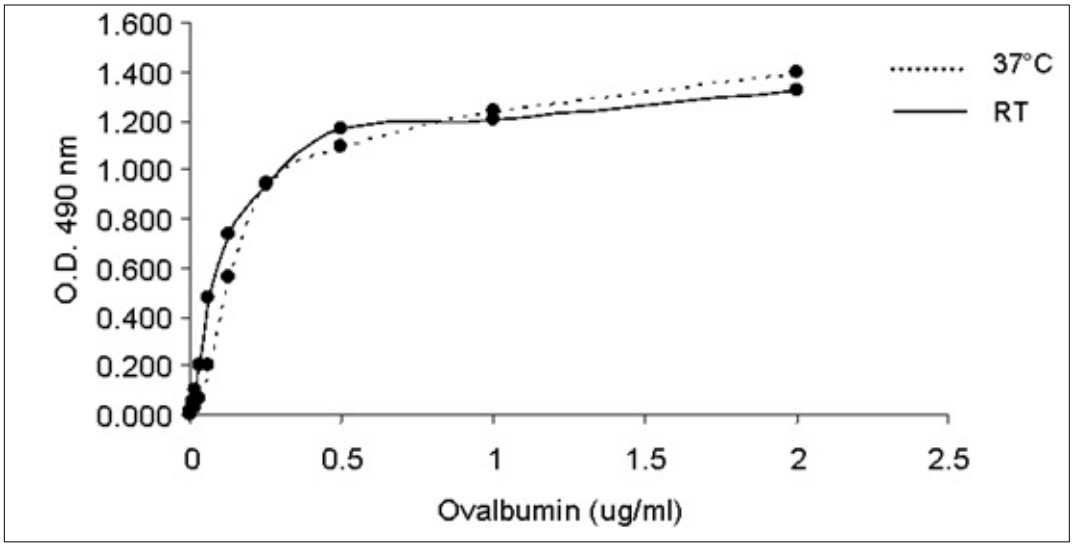

Figure 5 The effect of incubation temperature on sensitivity of the anti-ovalbumin sandwich ELISA

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 also evaluated for specificity by testing different allergen protein solutions including the study wine fining agents egg and milk, as well as the egg protein ovomucoid, milk whey protein β-lactoglobulin, isinglass fining agent and non-grape tannin additive. Reactivity was only observed for ovalbumin as shown in Figure 6, and demonstrates that the assay is specific for ovalbumin and not influenced by the presence of other fining agents if present in wine samples at the concentration range tested.

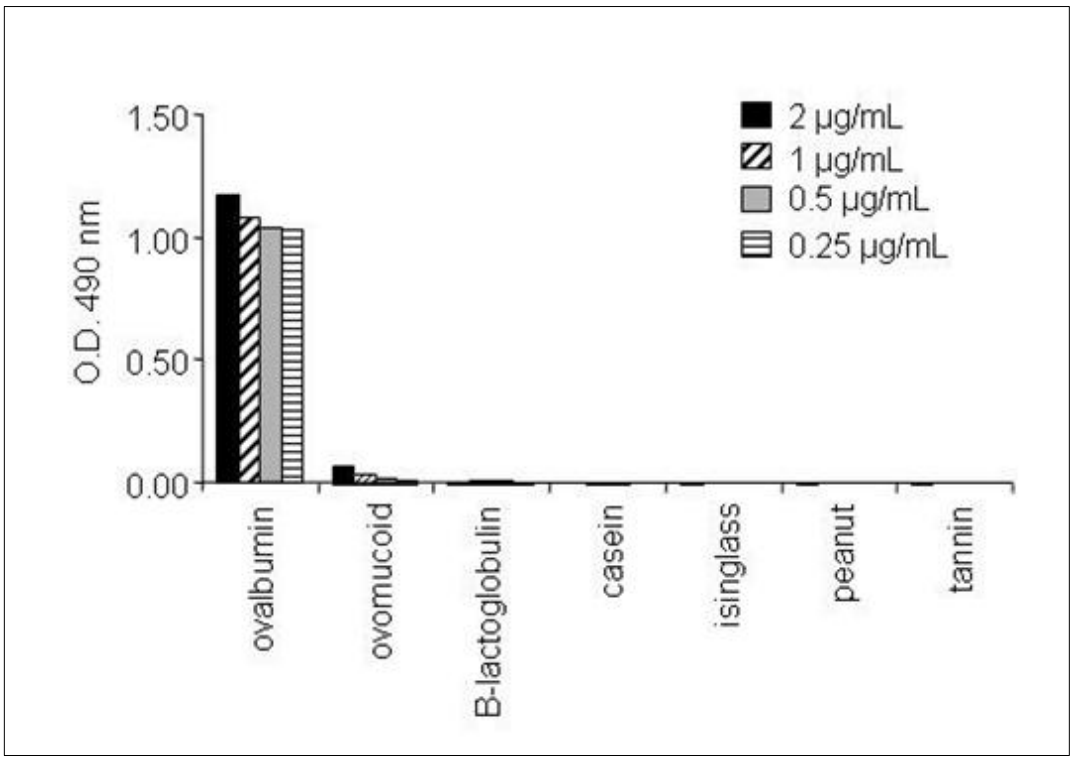

Figure 6 Specificity of anti-ovalbumin ELISA

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.

4.4.2.2 Optimisation of ELISA for detection of casein

A sandwich ELISA for the detection of casein was established using a monoclonal antibody to casein developed inhouse and a commercially-available polyclonal antibody. The specificity of the casein monoclonal antibody was further tested using the ELISA protocol outlined above where wells were coated with 2 μg/mL α-casein, β-casein and κ-casein extract (50 μL/well; Sigma), diluted in 0.1 M bicarbonate buffer. The casein monoclonal antibody was found to be specific for α-casein, as tested by ELISA, with minimal reactivity to β-casein or κ–casein (Figure 7).

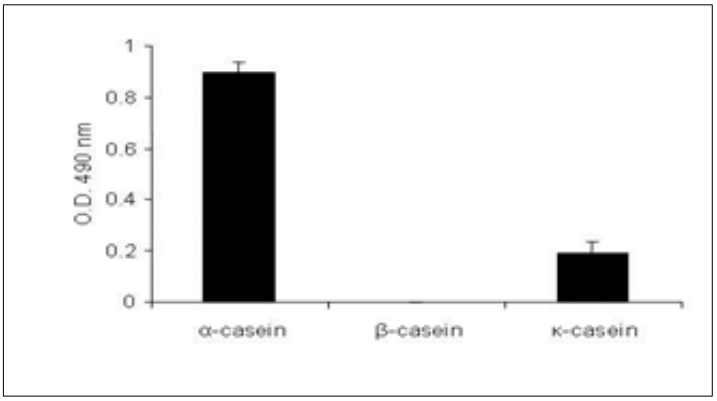

Figure 7 Specificity of anti-casein monoclonal antibody

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

Using commercially-available polyclonal sheep antibody, as the capture antibody, was found to give greater sensitivity than polyclonal mouse sera collected during production of monoclonal antibody (Figure 8).

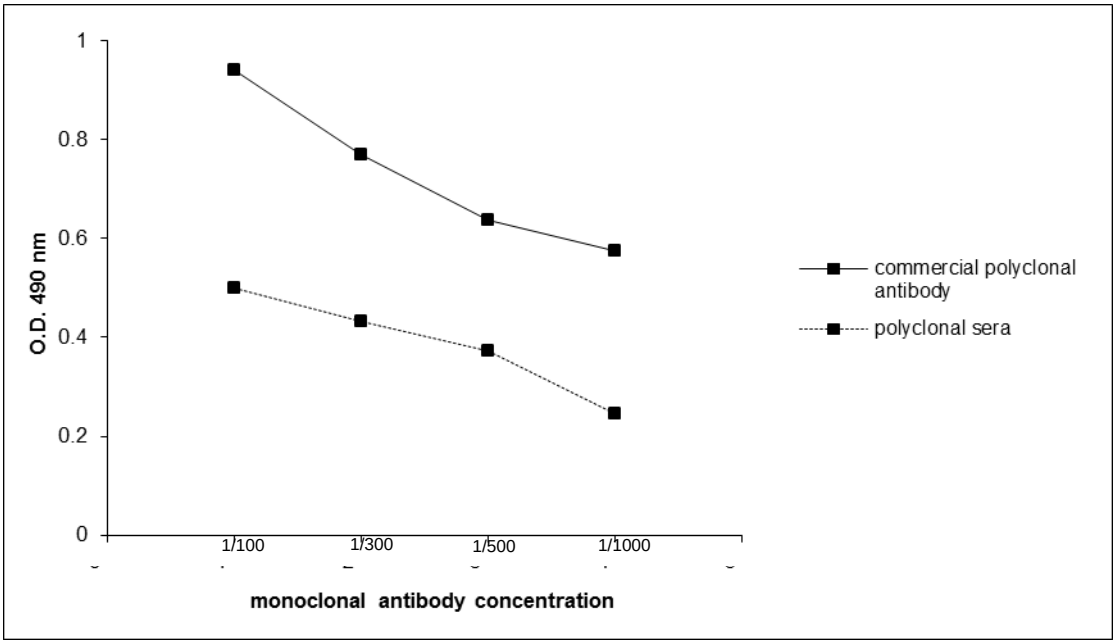

Figure 8 Comparison of polyclonal antibodies for the sensitivity of the anti-casein sandwich ELISA and concomitant titration of monoclonal antibody

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.

Optimisation tests showed that greatest sensitivity for detection of casein was achieved using polyclonal sheep anti-casein antibody at 0.5 µg/mL as the coating antibody (Figure 9) and 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 for α-casein (Figure 10).

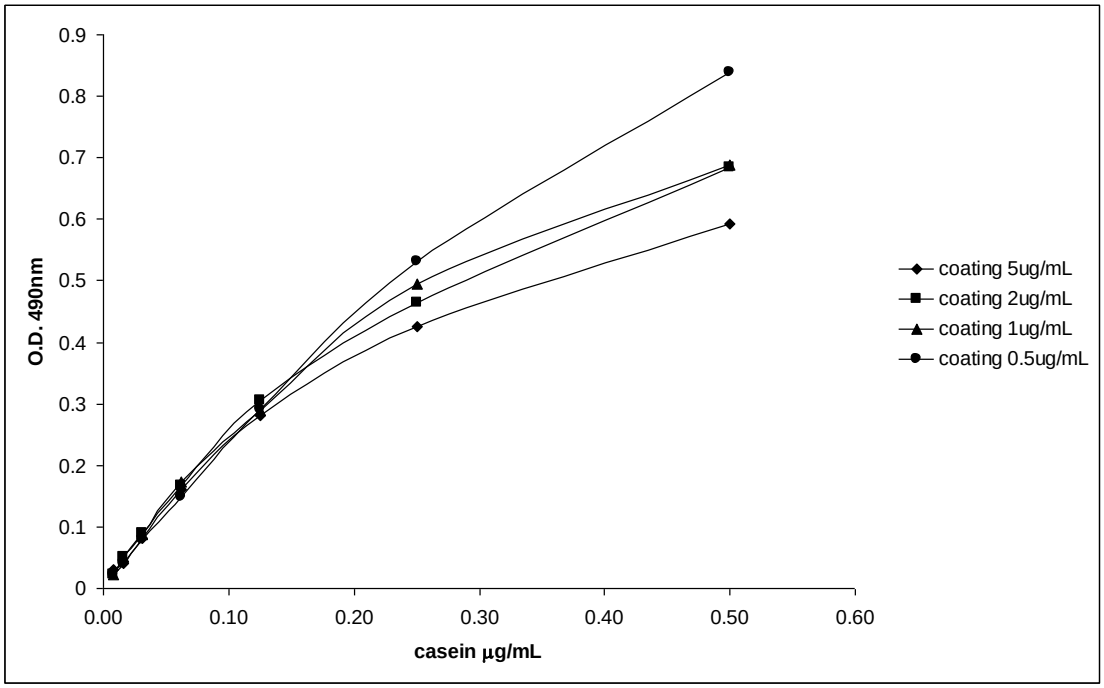

Figure 9 Optimisation of the concentration of sheep anti-casein capture antibody

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.

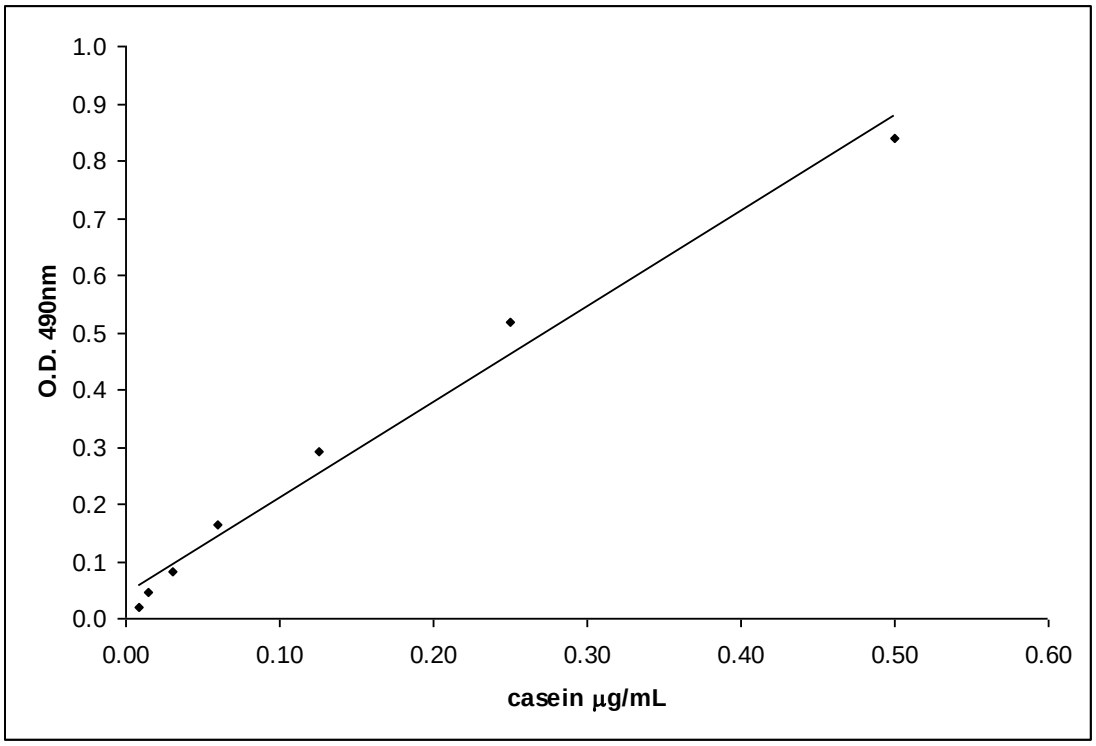

Figure 10 A standard curve of the anti-casein sandwich ELISA under optimised conditions

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.

The casein specific ELISA was also evaluated for specificity by testing the wine fining agents egg and milk, as well as the egg protein ovomucoid, milk whey protein β-lactoglobulin, isinglass fining agent and non-grape tannin additive. Reactivity was observed only for casein as shown in Figure 11, and demonstrates that the assay is specific for casein and not influenced by the presence of other fining agents if present in wine samples at the tested concentration range.

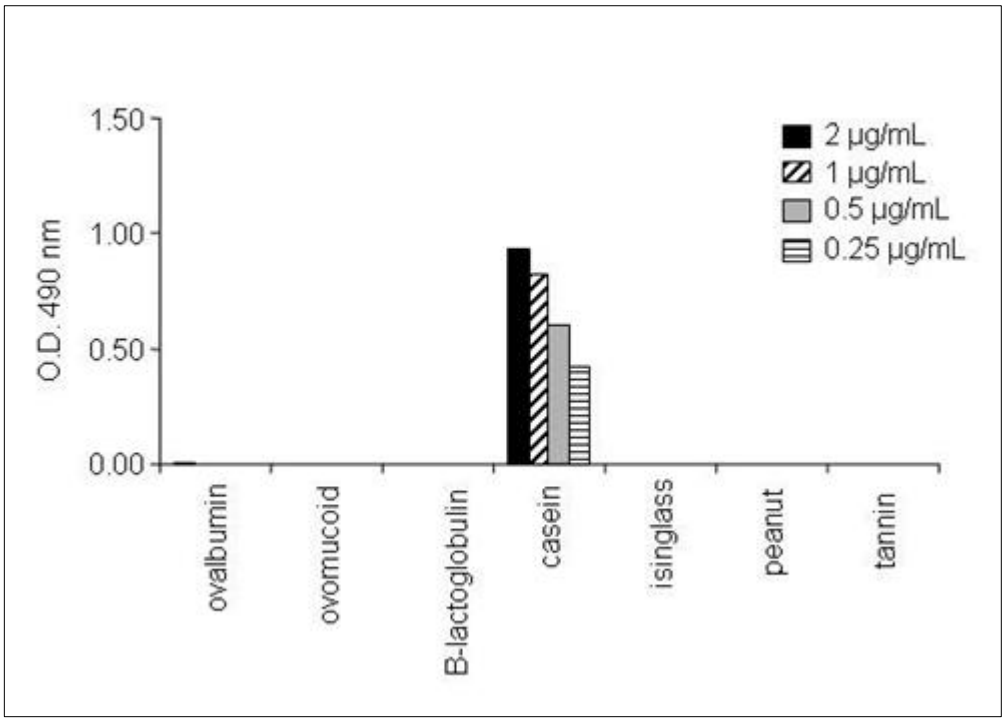

Figure 11 Specificity of anti-casein ELISA

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.

4.4.2.3 Optimisation of ELISA for detection of peanut-related proteins

An inhibition ELISA for the detection of peanut-related proteins was established using both serum from a peanut-allergic patient and an Ara h 1-specific monoclonal antibody. Roasted peanut extract was used in the assay because roasted peanuts are commonly consumed, and roasting can enhance the allergenicity of peanut proteins (Maleki et al., 2000). Serum from a peanut-allergic patient and an inhouse generated Ara h 1-specific monoclonal antibody were compared for use in the antigen incubation mix. The inhibition assay using peanut-allergic donor serum was found to have greater sensitivity than a sandwich ELISA utilising the monoclonal antibody (Figure 12). The limit of detection for the optimised peanut-allergic serum inhibition ELISA was 8 ng/mL (µg/L)

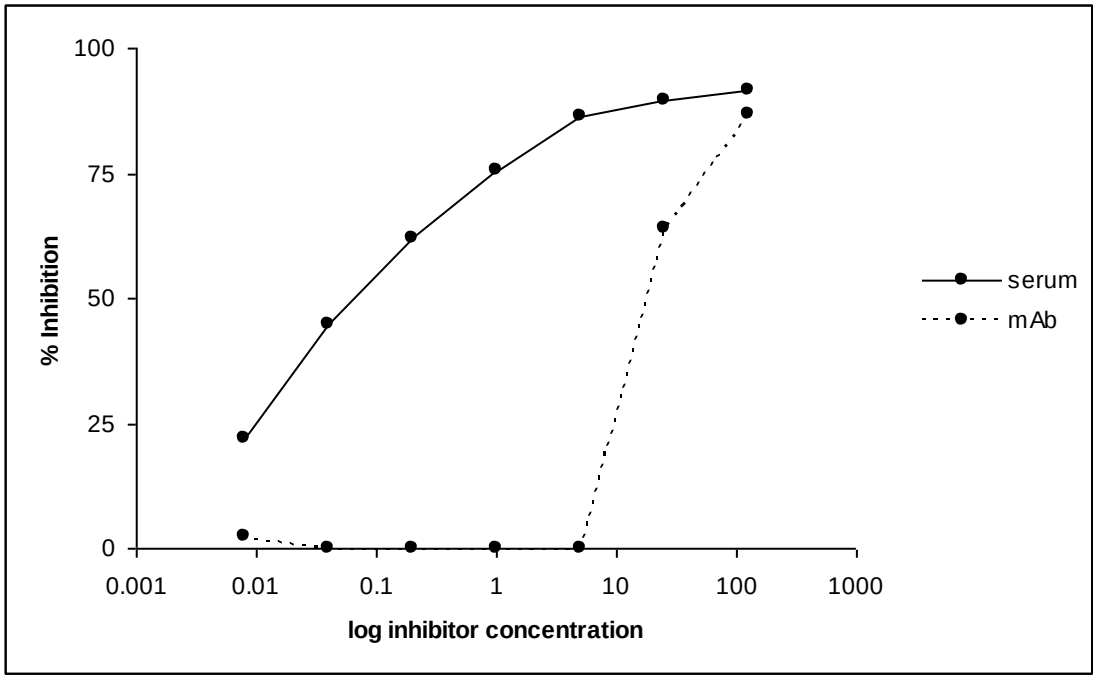

Figure 12 Comparison of the sensitivity of peanut-specific IgE with Ara h 1-specific monoclonal antibody using an anti-peanut inhibition ELISA

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 (1 μg/mL). IgE binding to peanut was assessed and expressed as percentage inhibition. Assays were performed in triplicate.

The peanut inhibition ELISA was also evaluated for specificity by testing the different study wine fining agents egg and milk, as well as the egg protein ovomucoid, milk whey protein β-lactoglobulin, isinglass fining agent and non-grape tannin additive as inhibitors. Inhibition of IgE reactivity of peanut extract was only observed for peanut extract (positive control), as shown in Figure 13, and demonstrates that the assay is specific for peanut and not influenced by the presence of other fining agents if present in wine samples at the tested concentration range.

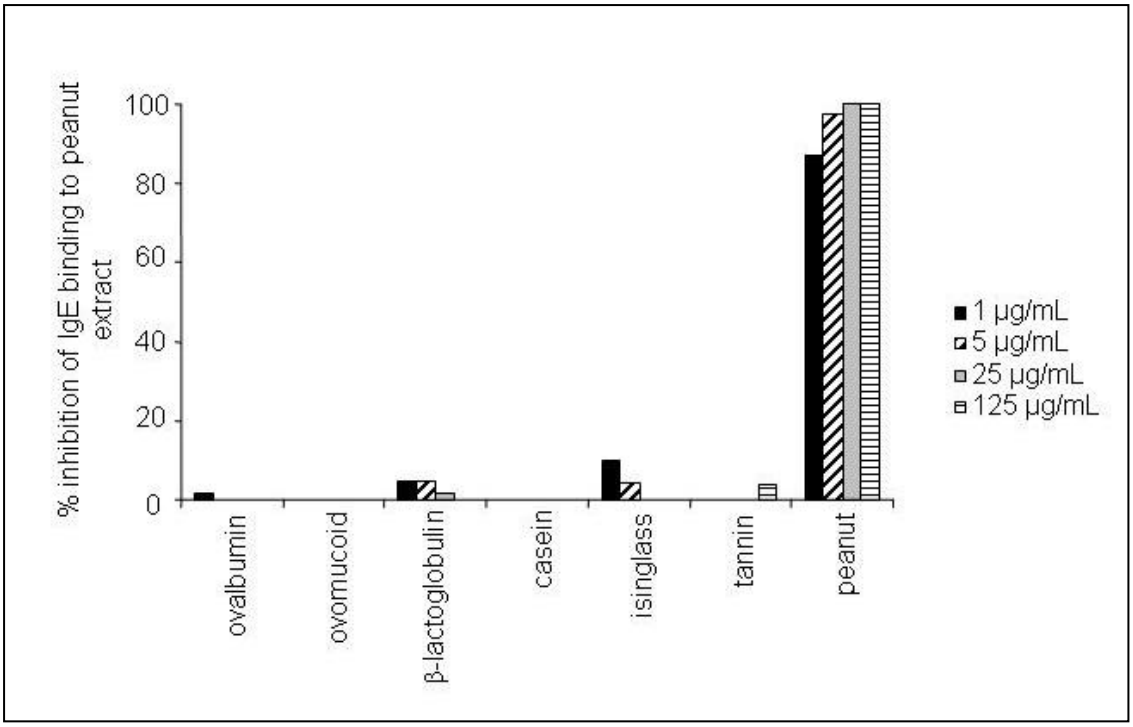

Figure 13 Specificity of anti-peanut inhibition ELISA

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

4.5 Discussion

This study aimed to develop highly sensitive and specific assays to detect and measure allergenic proteins from the commonly used egg, fish, milk or nut-derived processing aids in wine. The result was the successful development of three new and unique assays that were able for the first time to analyse for ovalbumin, casein and peanut-related proteins in wine. No such sensitive or specific assays previously existed commercially or had been previously published in the literature. The predominant allergenic components in egg white and milk were selected for analysis (Wal, 1998; Wal, 2002; Rupa and Milne, 2003) that is, ovalbumin and casein, respectively, and peanut-related proteins were selected as a surrogate for nut-derived proteins.

New and unique ELISAs were successfully developed for ovalbumin, casein and peanut-related proteins.

A sensitive and specific antigen capture ELISA was established and optimised for the detection of ovalbumin in wine, using commercially-available polyclonal and monoclonal antibodies; ovalbumin is the major and predominant protein in egg albumin (54%). The lower level of detection of the ELISA was 1 ng/mL (1 μg/L), approximately 100 or 200-times the calculated lowest observed adverse effect level (LOAEL) or threshold of 100–200 μg/L for eliciting an adverse reaction from food challenge studies as described in Section 3.5.1 of Chapter 3 (Hefle et al., 2002; Taylor et al., 2002; Moneret-Vautrin and Kanny, 2004; Hourihane and Knulst, 2005).

A sensitive and specific antigen capture ELISA was also established for the detection of α-casein in wine, the most abundant of the three casein fractions, using a monoclonal

antibody produced in this project, and a commercial polyclonal antibody; casein is the major and predominant protein in milk (80–85%). The lower level of detection of the ELISA was 8 ng/mL (8 μg/L), approximately 12.5–25-times the calculated LOAEL or threshold of 100–200 μg/L for eliciting an adverse reaction as described in Section 3.5.1 of Chapter 3 (Hefle et al., 2002; Taylor et al., 2002; Moneret-Vautrin and Kanny, 2004; Hourihane and Knulst, 2005). The lower level of detection of the competitive ELISA in this present study were also 25-75-times more sensitive than that obtained for α and β-casein by Weber et al. (2009).

A sensitive and specific inhibition ELISA for peanut-related proteins was also established using a peanut-allergic patient serum and optimised for the detection of peanut-related protein in wine. The lower limit of detection of the ELISA was 8 ng/mL (8 μg/L), approximately 12.5–25-times the calculated LOAEL or threshold of 100–200 μg/L for eliciting an adverse reaction as described in Section 3.5.1 of Chapter 3 (Hefle et al., 2002; Taylor et al., 2002; Moneret-Vautrin and Kanny, 2004; Hourihane et al., 2005).

The high sensitivities of these established ELISAs mean that if any residual fining agent is undetected in wine analysed with these assays it is likely to be present at low μgL levels. These low levels of residual fining agent are also unlikely to elicit an allergic reaction in the majority of egg, milk and peanut-allergic individuals according to the calculated 1–2 mg LOAEL for egg, milk and peanut (Morriset et al., 2003; Moneret-Vautrin and Kanny, 2004). This LOAEL represents approximately 100–200 μg of protein and only 1% of allergic individuals have been observed to have such a low threshold (Morriset et al., 2003; Moneret-Vautrin and Kanny, 2004). This low threshold

also emphasizes the necessity of using assays with a lower limit of detection of at least 1 mg/L.

In the development of these ELISAs, standard procedures for determining the limits of detection, that is, titration of standard samples and curves of best fit, were used. To maximise sensitivity while maintaining specificity of the assays, all methodologies were optimised, such as antigen and antibody concentrations, and temperatures and times for incubations (see Section 4.4.2 Optimisation of ELISAs). The use of secondary and tertiary antibodies was also assessed. Standard curves were generated for each wine testing assay and reproducibility of curves and appropriate controls checked. In each assay, wine samples and standards were tested in triplicate, and each assay was performed twice to ensure reproducibility. Assay specificity was also carefully examined to ensure confidence in the results for a particular allergen.

However, a limitation of this study was that it did not determine and define limits of quantification for ovalbumin, casein or peanut-derived proteins in wine due to budgetary, personnel resource and time limitations, and would therefore form the basis of a future study as discussed in Chapter 8. Validation of the method and hence confirmation of the data from the ELISA was also not undertaken by other analytical techniques such as SDS-PAGE, Western blot and immunostaining that analyse for individual proteins in a protein mixture due to budgetary and time limitations.

Another limitation was that sensitive and specific ELISA for the other potential, but less abundant, egg white and milk proteins in wine, as well as for isinglass proteins in wine, were not developed due to budgetary and time limitations. These include ovomucoid,

ovotransferrin and lysozyme from egg white, and β- and κ-casein and whey proteins, α-lactalbumin and β-lactoglobulin, from milk or present as impurities in casein preparations. While these proteins are also potentially allergenic, they are less potent than ovalbumin and α-casein (Wal, 2001; Besler, et al., 2001) and could still present a risk to sensitive individuals. Development of assays for these less abundant and less potent egg white proteins in wine is therefore recommended to be undertaken in future research. Assays for the less abundant milk proteins in wine and isinglass proteins in wine have been relatively recently published (Weber et al., 2009; Weber et al., 2010).

In conclusion, these newly established ELISAs enabled the detection of low µg/L levels of egg, milk and peanut-related protein in wine, which may be present following fining with proteinaceous processing aids and be clinically relevant. Subsequent to this study, which was published in 2008, other studies have confirmed that such low levels of detection are important for public health and safety to prevent adverse reactions in food-allergic individuals. For example, Weber et al. (2007b) detected residual protein in one of 10 egg-fined wines at a concentration of approximately 200 µg/L, and in another four lysozyme treated wines at a concentration of approximately 100 µg/L. The detection of µg/L levels in their small number (n=64) of experimental wines that were produced following a defined protocol also highlights the need for further investigation of a wider panel of commercially-available wines by sensitive and specific ELISAs for food safety, as there is no single defined protocol for commercial production of wine. Different types and amounts of proteinacous fining agents, for example, can be used in the wine production process. The protein-fined wine can then be clarified by a range of different fining and filtration processes that may remove the protein to a greater or lesser extent which cannot be predicted from the small sample of experimental wines.

In order to undertake the routine analysis of egg and milk-fined wine samples by commercial laboratories and wineries, the ELISA established in this study could be further developed for commercialisation, including undertaking a collaborative inter-laboratory study. Such relatively sensitive and specific ELISA, based on the assays developed here, have since been developed and commercialised for the detection of ovalbumin and α- and β- casein in wine by Restani et al. (2012a) and Uberti et al. (2014), respectively. These ELISA were established according to the reproducibility, repeatability and robustness criteria established by the Organisation International de le Vigne et du Vin Compendium of International Methods of Analysis[31], and subsequently confirmed in a collaborative inter-laboratory study (Restani et al., 2014). Although specific, the assays of Restani et al. (2012a) and Uberti et al. (2014) are less sensitive than the assays developed here, and may be above the allergic reaction threshold in some sensitive individuals. The detection limits of the Restani et al. (2012a) and Uberti et al. (2014) assays are 0.0564 mg/L for ovalbumin and 0.28 mg/L for casein, which are approximately 56 and 35 times higher, respectively, than that of the assays developed in this study.

The next step in this program of research was the analysis of a diverse range of commercially-available Australian wines using the assay developed here, covering a spectrum of brands, producers, varieties and production protocols to determine the presence of egg, milk or nut-derived proteins that they contain with more precision than previously undertaken.

[31] http://www.oiv.int/oiv/info/enplubicationoiv#compendium

Declaration - Chapter 5

I declare that all content in the following chapter has been written by me. As the studies conducted for this thesis involved a wider support team, my independent contribution to each aspect of the study is detailed in Appendix 1.

Creina Stockley

Chapter 5 Measurement of egg, milk and nut allergens in wine

Overview

Having developed sensitive and specific ELISAs to detect and measure levels of the clinically relevant food allergens ovalbumin, casein and peanut-related proteins in wine as detailed in Chapter 4, the next logical study was to apply the newly developed ELISAs to a panel of wines. The wines were unfined or had been fined with one or more of the potentially allergenic processing aids (egg white, isinglass, milk or milk proteins casein or potassium caseinate), or to which non-grape, nut-derived tannins had been added. The research question addressed was whether the newly developed assays could determine the amount of residual fining agents in commercially-available bottled Australian wine.

5.1 Introduction

Wine is frequently anecdotally blamed for eliciting allergic reactions (Wigand et al., 2012), and the literature presented in Chapter 3 revealed that there is often uncertainty as to the source and type of allergen causing the reaction. The review of the published literature also indicated that in assessing allergic reactions to wine, the potentially allergenic proteins from egg, milk and nuts which are used in the wine fining process had not been previously considered. No published literature was found on their concentrations in wine, and there were no available commercial or published assays to enable their analysis in wine, and hence determine the potential for protein-fined wine to compromise consumer health and safety.

As of 2014, only one study has been undertaken to determine the amount of residual fining agent in experimental wines produced with a defined production process. Weber

et al. (2007b) detected a low concentration (approximately 200 μg/L) of egg proteins such as ovalbumin in one of 10 German wines which had been fined with egg white, but at a concentration five-times greater than good manufacturing practice dictates[32], highlighting the need for further investigation of a wider panel of commercially-available bottled wines.

The first study in this program of research established sensitive and specific ELISAs for the predominant allergenic components in egg white and milk in wine, and for peanut-related allergens in wine as described in Chapter 4. The lower limit of detection of these assays was 1, 8 and 8 ng/mL (μg/L) for ovalbumin, casein and peanut-related proteins, respectively.

5.2 Aim

The aim of the present study was to determine if there was any detectable egg white, milk, and peanut-related protein in a broad range of finished commercially-available Australian wines using the assays developed in Chapter 4. Isinglass-fined wines were also included in the present study to provide a specificity check for the ELISA for ovalbumin, casein and peanut-related proteins even though no assay was available to detect their residue.

[32] Organisation International de la Vigne et du Vin (2006) International Oenological Codex. The International Oenological Codex provides the description of the principal chemical, organic or gas products used to make and store wine. The conditions, instructions and limits for their use are set by the International Code of Oenological Practices. http://www.oiv.int/oiv/info/enplubicationoiv#code

5.3 Method

5.3.1 Wine sample collection

A diverse panel of 113 finished and commercially-available bottled Australian wines was collected. Each wine was given a unique identifier numbered from 1 to 113. Of these wines, 109 were protein-fined and 4 were unfined. Included in the fined wine sample were two red wines that had had whole eggs added. The test fined wines included at least 20 wines from each of the following categories: egg white fined or whole egg added (red wine); non-grape, nut-derived tannin added (red wine); milk-fined (white wine); isinglass-fined (white wine); and casein-fined (white wine). Nineteen wines were fined with more than one fining agent. Four wines (two red and two white) were unfined and acted as the control wines, which were selected on the basis of their preparation not involving the above processing aids. Sixteen grape varieties were included in the panel of wines.

Although winemaking processes are highly regulated and standardised[33], some degree of variation in production practices and procedures is permitted under Good Manufacturing Practice (GMP) principles[34]. To accommodate any potential variations in winemaking processes, the selected panel of wines were drawn from well-known brands with high market share in Australia and internationally and covered a spectrum of producers and winemaking processes which were available commercially. On this basis, the findings should apply to the majority wines made according to GMP, including wines that were fined with different amounts of fining agent, by different methods and at different stages of the winemaking process. Only finished, bottled and commercially-

[33] http://www.comlaw.gov.au/Search/Australia%20New%20Zealand%20Food%20Standards

[34] Organisation International de la Vigne et du Vin (2006) International Oenological Codex. The International Oenological Codex provides the description of the principle chemical, organic or gas products used to make and store wine. The conditions, instructions and limits for their use are set by the International Code of Oenological Practices. http://www.oiv.int/oiv/info/enplubicationoiv#code

available protein-fined and unfined wines were used in this study. As the production process had not changed appreciably for some decades, these were all examples of the wines that consumers had been previously exposed to without allergic reactions reported in the published literature. They had all been made according to GMP which meant that they had been further fined and filtered prior to bottling. Unfinished wines may not have undergone further fining and filtration and were not included in the test or control sample.

Of the 109 protein-fined wines collected for analysis, some had been fined with more than one of the above fining agents. Given that the panel included at least 20 wines from each of categories, the actual number of wines processed with each agent was as follows:

1. egg white-fined (including 2 whole egg added) 20
2. isinglass-fined 20
3. milk-fined 34
4. casein-fined 21
5. non-grape, nut-derived tannin added 25

Wines were tested and the data generated analysed in groups according to the primary protein-fining category above.

A description of the 113 wines used in this study is shown in Appendix 2. Post protein fining, all the 113 wines were further clarified and filtered. The type of processing aid and amount used was accessed directly from the winemakers' auditable winery records and recorded for 110% and 92% of the wines, respectively. A description of when they

were used in the winemaking process was recorded for 47% of the wines as provided by the winery.

5.3.2 Wine pre-treatment

As the wine samples non-specifically inhibited ELISA reactions at the concentrations to be used for this study due to their alcohol content and low pH, the following protocols were adopted for pre-treatment before *in vitro* analysis. White wines were dialysed (3.5 kDa cut-off) in SnakeSkin® pleated dialysis tubing (Pierce, Rockford, IL) against phosphate buffered saline (PBS; 150 mM NaCl, 2 mM $NaH_2PO_4.2H_2O$, 7.5 mM Na_2HPO_4, pH 7.4) for 24 h at room temperature, with three changes of PBS. This resulted in a small (<10%) increase in volume for some white wines. The dialysis method of pre-treatment was not suitable for red wines due to the formation of a precipitation or sediment. Red wines were diluted 1:4 in cold ethanol and incubated overnight to precipitate proteins in the wine. After centrifugation at 6238 × 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 -20^{o}C until use. Once thawed, the remaining sample was discarded.

5.3.3 Analytical methods

The panel of 113 wines was analysed for ovalbumin, α-casein and peanut-related proteins using the optimised ELISAs for ovalbumin, α-casein and peanut-related proteins in wine as described in Section 4.3.2 of Chapter 4.

5.4 Results

5.4.1 Analysis of wine panel for detectable ovalbumin

The ovalbumin sandwich ELISA established in Chapter 4 was used to test for residual ovalbumin in the panel of 113 wines (109 fined which included 2 whole egg added and 4 unfined).

Ovalbumin was undetectable in all wines except two red wines (as shown in Table **3**), 98 (0.98 μg/mL) and 99 (0.40 μg/mL), which were diluted 1/16 to obtain OD_{490nm} values within the linear range of the standard curve. These two wines had not been fined with egg white but had whole eggs added during the production process (6 eggs/1000 L), without subsequent removal by filtration. Prior to the dilution pretreatment for wines 98 and 99, the concentration of ovalbumin detected was only 0.013 and 0.004 μg/mL.

5.4.2 Analysis of wine panel for detectable casein

The casein sandwich ELISA established in Chapter 4 was used for the panel of 113 wines (109 fined which included 2 whole egg added and 4 unfined) to analyse for residual α-casein. α-Casein was undetectable (< 8 ng/mL) in all wines (as shown in Table 3).

5.4.3 Analysis of wine panel for detectable peanut-related proteins

The peanut serum IgE inhibition ELISA established in Chapter 4 was used for the panel of 113 survey wines (109 fined which included 2 whole egg added and 4 unfined) to analyse for residual peanut-related protein. Peanut-related proteins were undetectable (< 8 ng/mL) in all wines (as shown in Table 3).

Table 3 Number of panel wines positive for allergen detection

	Control unfined wines		Test wines: processing aid					
	White	Red	Egg white fined	Whole egg added	Isinglass fined	Milk fined	Casein fined	Non-grape tannin fined
Ovalbumin ELISA	0* (n=2)	0 (n=2)	0 (n=20)	2 (n=2)	0 (n=20)	0 (n=34)	0 (n=21)	0 (n=25)
Casein ELISA	0 (n=2)	0 (n=2)	0 (n=20)	0 (n=2)	0 (n=20)	0 (n=34)	0 (n=21)	0 (n=25)
Peanut inhibition ELISA	0 (n=2)	0 (n=2)	0 (n=20)	0 (n=2)	0 (n=20)	0 (n=34)	0 (n=21)	0 (n=25)

*0 refers to no allergen detected in any of the wines

5.5 Discussion

This study examined the presence of ovalbumin, casein and peanut-derived protein in a panel of 113 finished commercially-available bottled Australian wines made according to standard good manufacturing practice. Only two wines had a detectable concentration of ovalbumin and these, unlike the others, contained added whole egg rather than being fined with egg white (Table 3).

Twenty of these wines had been fined with up to 10,000 mg/L (1,000 g/hL) egg white potentially containing 55 mg/hL ovalbumin. No wines had detectable casein or peanut-related proteins, where 34 of these had been fined with up to 5.55 g/L milk potentially containing 1.47 g/L casein. A standard curve was repeated in every assay and duplicate analyses for each wine sample gave consistent results.

The results of this study are consistent with the more recent work of Uberti et al. (2014)[35] who, using similar ELISAs to those used in the present study, analysed 8 experimental (non-commercially-available) wines and 85 commercially-available wines that had been fined with up to 10 g/hL egg white. That study included 12 wines from Australia that had been fined with up to 5.9 g/hL egg white (Uberti et al., 2014). The results of this present study are also consistent with those of Restani et al. (2012a), who analysed 16 experimental and 63 commercially-available wines fined with casein. In the Uberti et al. (2014) and Restani et al. (2102a) studies all experimental wines were further clarified with bentonite[36] post protein fining and the commercially-available wines were filtered post protein fining.

The consistency of results across all three studies, using similar analytical approaches, gives weight to the validity of the approach used in the present study for analysing protein in wine as reported in Chapter 4. Uberti et al. (2014) state that "…ELISA tests previously reported (Rolland et al., 2008) did show good quality characteristics…".

The results of the present study, however, are not consistent with those of Lifrani et al. (2009), where 44 of 400 (11%) commercially-available French wines were observed to contain residual ovalbumin and casein. Of those 44 wines, 37 (84%) were classified as organic and potentially unfiltered post protein fining. The results of this present study are also not consistent with those of Weber et al. (2009), where casein was detected in two of 32 (6%) experimental wines that had not been fined with bentonite post protein

[35] Uberti, F.; Danzi, R., **Stockley, C**., Penas, E.; Ballabio, C.; Di Lorenzo, C.; Tarantino, C., Restani, P. Immunochemical investigations of allergenic residues in experimental and commercially-available wines fined with egg white proteins. Food Chemistry, 159: 343–352; 2014.

[36] Bentonite is a non-allergenic fining agent used for clarification in winemaking permitted according to the provisions in Standard 4.5.1 of the Australian New Zealand Food Standards Code and used according to GMP.

fining, and in three of 61 (5%) commercially-available wines that may not have been benonite fined and/or filtered post protein fining.

All the wines that had been bentonite-fined and/or filtered post protein fining in the present study and in the studies of Uberti et al. (2014), Restani et al. (2012a), Lifrani et al. (2009) and Weber et al. (2009) did not have detectable egg and milk residues. The differences in clarification processes undertaken may explain the anomalies between the studies. Therefore to ensure removal of residual egg and milk protein from wine, it is recommended that bentonite fining and filtration post protein fining are included as per the good manufacturing practices (GMP).

Further studies should also be undertaken to specifically compare fined and filtered wines with fined and unfiltered wines. In addition, to be confident that an analytical method is sufficiently sensitive for wines made according to GMP, analytical methods should be in line with those developed in Chapter 4. This is evident when comparing the ELISAs used by Weber et al. in 2007 and 2009. In the Weber et al. (2007b) study using a competitive ELISA for casein which has a LOD between 1 and 3 mg/L no detectable residues of casein were found in wine, but in the latter 2009 study using an indirect ELISA with a LOD between 0.2 and 0.6 mg/L, residues of casein were detected in five wines.

A limitation of the present study was that all winemaking practices undertaken could not be documented for every wine analysed. This highlights the importance of documentation which would have indicated which winemaking practices potentially had most impact in removing proteinaceous fining agents from wine. Consequently, future

research should include, for each wine, documentation of all the winemaking practices undertaken in its production. For example, a highly controlled laboratory study in which experimental wines spiked with known amounts of the potentially allergenic processing aids were then fined by bentonite and/or filtered should be undertaken to determine the relative efficacies of these different GMP approaches. These winemaking practices and procedures could then be documented in a specific Code of Good Fining Practice for Wines, which if followed should ensure minimal, if any, residual potentially allergenic protein remains in finished wine.

The 113 wines collected for the present study were a sample of a diverse range of commercially-available Australian wines. The 109 wines had been protein-fined with larger as well as smaller amounts of fining agent within the dosage range recommended in GMP, and at different stages of production. In addition, these protein-fined wines were all made according to GMP being further fined and/or filtered prior to the final bottling. The two commercially-available wines that had had whole egg added were labelled as 'egg-marsala'. Further investigation of a wider panel of commercially-available wines fined with different dosages of processing aid by sensitive and specific ELISAs is important for public health and safety. The importance of this is also highlighted by a small study of Weber et al. (2007b) of 64 experimental wines made according to a defined protocol, where 200 µg/L food protein was detected in some but not all wines fined with egg white in excess of the manufacturer's recommended dosage. As the amount detected was equal to the limit of detection of the ELISA, it might be assumed that the other excess fined wines also contained residual food protein in lower amounts that were not detected.

In conclusion, given the diverse range of finished commercially-available Australian wines used in this present study, the findings might be extrapolated to filtered fined and unfined Australian wines made according to GMP with a high degree of confidence.

As reported in Chapter 4, the level of detection of the sensitive ELISAs that were developed for ovalbumin, α-casein and peanut-related proteins in wine was at least 12.5-times the calculated LOAEL or threshold for eliciting an adverse reaction from food challenge studies [see Section 3.5.1 of Chapter 3 (Hefle et al., 2002; Taylor et al., 2002; Moneret-Vautrin and Kanny, 2004; Hourihane et al., 2005)]. Given that none of the fined wines in the sample of 113 commercially-available Australian wines analysed in the present study contained detectable ovalbumin, α-casein and peanut-related proteins, it can be cautiously concluded that wine fined with egg white or milk, or to which nut-derived tannin had been added would not elicit an allergic reaction in food-allergic individuals.

As reported in Chapter 3, however, the precise threshold dose for allergic reactions among food-allergic individuals has yet to be established conclusively and possibly varies among individuals and might also be dependent on the source of the protein/allergen (Helfe et al., 2002; Taylor et al., 2002; Houihane et al., 2005). The literature reviewed in Chapter 3 found no studies that had specifically addressed the issue of whether there is sufficient residual protein in fined wines to elicit an allergic reaction in individuals with known food allergies.

The most appropriate way to determine whether food-allergic individuals suffer from a true food allergy is via a double blind placebo controlled food challenge, which is

considered to be the gold standard for diagnosing food allergy (Bock et al., 1988; Sampson, 1988; Bruijnzeel-Koomen et al., 1995). Therefore, the next step in this program of research was to undertake a double blind placebo controlled food challenge to determine whether food-allergic individuals in the general population suffer from a true food allergy when exposed to wine made with allergenic food proteins.

Declaration - Chapter 6

I declare that all content in the following chapter has been written by me. As the studies conducted for this thesis involved a wider support team, my independent contribution to each aspect of the study is detailed in Appendix 1.

Creina Stockley

Chapter 6 Double blind placebo controlled wine challenge

Overview

The third study in this program of research involved a double blind placebo controlled food challenge. The aim of the food challenge was to determine whether wines which do not contain detectable residual protein could elicit an allergic reaction in susceptible individuals. As the lowest threshold dose for allergens is unknown for these proteins they may be below the detection limit of the ELISA developed in Chapter 4. At the time that this study was undertaken, no similar investigations had been conducted to examine the potential for true allergic reactions from Australian wine to occur from eggs, fish, milk and nuts in an adult population. The results of this study are published in Rolland et al. (2006) and Stockley et al. (2008).[37]

6.1 Introduction

The most rigorous way to determine whether a particular food elicits an allergic reaction in a food-allergic individual is via a double blind placebo controlled food challenge (DBPCC). This is considered to be the gold standard for diagnosing a true food allergy (Bock et al., 1988; Sampson, 1988; Bruijnzeel-Koomen et al., 1995; Bindslev-Jensen et al., 2004). Demonstration of specific sensitizations by a skin prick test or determination of specific IgE is the first step in the confirmation of a true food allergy, as individuals

[37] Rolland, J.M., Apostolou, E., Deckert, K., de Leon, M.P., Douglass, J.A., Glaspole, I.N., Stockley, C.S., O'Hehir, R.E. Potential allergens in wine: double-blind placebo-controlled trial and basophil activation analysis. Nutrition, 22(9):882–8; 2006.

Stockley, C.S. de Leon, M.P. Stimson, K. Glaspole, I.N. Apostolou, E. Rolland, J.M. O'Hehir, R.E. An investigation of the potential residual allergenic fining proteins in wine and their effect on egg, fish, milk or nut-allergic subjects. Blair, R.; Williams, P.; Pretorius, S. Proceedings of the thirteenth Australian wine industry technical conference; 28 July - 2 August 2007; Adelaide, SA. : p. 380-381; 2008.

without sensitizations do not have a food allergy (American Academy of Allergy, Asthma and Immunology, 2003; Sicherer et al., 2014).

6.2 Aim

The aim of this study was 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 treated with processing aids using the assay developed and described in Chapter 4.

6.3 Method

6.3.1 Subject recruitment and clinical characterisation

6.3.1.1 Allergic subjects

Potential allergic subjects were recruited from the patient database of the Allergy and Asthma Clinics at The Alfred Hospital (Melbourne, Victoria) over a 13 month period on the basis of their clinical history of adverse reaction to egg, fish, milk or peanut/tree nuts.

The diagnosis of IgE-mediated food allergy in each food-allergic subject was subsequently confirmed by a clinical allergist, based on a clinical history of anaphylaxis and demonstration of specific IgE to allergens of egg, fish, milk and/or peanut using the ImmunoCAP fluoroenzyme system (CAP-FEIA; Pharmacia Diagnostics, Uppsala, Sweden) or by skin prick testing ($\geq$4 mm diameter wheal). The exception was one fish-allergic patient who had negative tests but a positive oral fish challenge. Clinical characteristics of the food-allergic subjects are shown in Appendix 3.

6.3.1.2 Control subjects

Potential non-allergic control subjects were also recruited from respondents to advertisements at The Alfred Hospital on the basis of no history of food or wine reactions and no specific IgE to any of the study allergens. Confirmation of no IgE-mediated food allergy in each control subject was also determined by demonstration of specific IgE to allergens of egg, fish, milk and/or peanut using the ImmunoCAP fluoroenzyme system (CAP-FEIA; Pharmacia Diagnostics, Uppsala, Sweden) or by skin prick testing ($\geq$4 mm diameter wheal).

6.3.1.3 Eligibility criteria

The potential food-allergic and control subjects completed a screening questionnaire to determine whether they were eligible to participate in the study related to the type, severity and control of their allergies, and whether they were regular wine consumers (Appendix 4). The screening questionnaire also contained strict exclusion criteria such as medical conditions which would confound the results or potentially place the subject at risk of harm (Appendix 5). Subjects (food-allergic and controls) were considered ineligible and were automatically excluded from the study if they had any of the following medical conditions:

- allergies or intolerances related to wine or foods other than the candidate foods
- unstable asthma
- diabetes mellitus
- epilepsy
- liver disease
- kidney disease
- ischaemic heart disease
- ischaemic or haemorrhagic stoke

- upper respiratory tract infection.

Subjects were also excluded if they were not regular wine consumers. Subjects were not excluded if they had previously experienced anaphylaxis to ingestion of a study allergen.

Although the target was to recruit 40 food-allergic subjects, 10 subjects from each food allergy category and 10 controls, only 37 subjects were identified that fulfilled the selection criteria and were successfully recruited to participate to the study, and completed the study.

On each food challenge study day, the recruited food-allergic and control subjects completed a further screening questionnaire related to their recent health behaviours to exclude the presence of any factors that might invalidate the interpretation of results or increase the health and safety risk of challenge testing to the subject. Subjects were excluded from the study day if they answered yes to having any of the following:

- asthma
- urticaria
- angioedema
- an upper respiratory tract infection
- a gastrointestinal tract upset
- used anti-histamines in the last two days
- used ventolin the last four hours; used seretide/oxis medication that morning
- used alcohol in the last three days
- diabetes meliitis

- epilepsy
- liver failure
- renal failure
- ischaemic heart disease
- ischaemic or haemorrhagic stroke

6.3.1.4 Study cohort

The study cohort included 11 non-food-allergic control subjects and 26 food-allergic subjects (five egg-allergic subjects, 10 fish-allergic subjects, one milk-allergic subject and 10-peanut/tree nut-allergic subjects) which is summarized in Table 4. As milk allergy is extremely rare in adults (Sampson, 1999; Osterballe et al., 2005), only one subject could be identified for this group. Subjects ranged in age from 19−63 years old and comprised 16 males and 21 females. All subjects were not naïve wine consumers, and were low risk consumers of wine according to the 2009 National Health and Medical Research Council (NHMRC) definitions of up to two standard drinks per day[38] to ensure that they had been previously exposed to fined wines. The study was approved by The Alfred Hospital and Monash University Ethics Committee (Human Ethics approval number 12402) and signed informed consent was obtained from all subjects.

[38] http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/ds10-alcohol.pdf

Table 4 Summary of selected subjects for wine challenge

Type of subjects	Age of subjects	Gender of subjects
5 egg-allergic	24-54 years	3 male and 2 female
10 fish-allergic	23-50 years	5 male and 5 female
1 milk-allergic	19 years	1 male
10 peanut/tree nut-allergic	20-63 years	5 male and 5 female
11 non-food-allergic (controls)	24-62 years	2 male and 9 female

6.3.2.5 Compliance requirement

Each subject was required to abstain from alcohol for a period of approximately 16 days over the entire food challenge period. This included at least three days before and six days after each of the two food challenge days. Written informed consent was obtained from each subject and subjects were provided with details of potential health and safety risks associated with the food challenge, which was explained when the subjects initially agreed to participate in the study.

Prior to each challenge day, each subject was asked to fast for at least 8 h, withhold any anti-histamine medication for three days, short-acting bronchodilator therapy for 4 h, and long-acting bronchodilator therapy for 12 h.

6.3.1 Food challenge wines

The 27 wines used in the food challenge were a randomly selected subset of the panel of 113 commercially-available wines collected and described in Chapter 4. They comprised 24 fined wines and 3 unfined control wines. These wines had either been fined with one of the candidate proteinaceous processing aids or were unfined control

wines (Table 5). At least two wines were selected from each category of proteinaceous fining agent.

For each of the 37 subjects, an unblinded ombudsman selected two challenge wines: A test fined wine, selected on the basis of whether it had been fined with the relevant potential food allergen or, for peanut/tree nut-allergic subjects, containing a high quantity of non-grape tannin, and a control unfined wine. For example, an egg-allergic subject was matched with an egg-fined wine and with a control unfined wine. The unblinded ombudsman also chose the order in which the two challenge wines were administered such that subjects alternated between the test fined wine and control unfined wine.

Each wine was opened fresh on the day of challenge or had been stored under vacuum after opening for no more than five days to ensure physico-chemical integrity and palatability of the wine.

Table 5 Description of food challenge wines randomly selected by the unblinded ombudsman from the panel of 113 wines collected in Chapter 4

Wine sample number Selected from panel of 113 wines collected in Chapter 4	Fined test or unfined control	Wine type	Fining agent used
6	Fined	red	Egg white
21	Fined	red	Egg white, non-grape derived tannin
27	Fined	red	Egg white
28	Fined	red	Egg white
29	Fined	red	Egg white
104	Fined	red	Egg white
105	Fined	red	Egg white
10	Fined	white	Isinglass
14	Fined	white	Isinglass
15	Fined	white	Isinglass
16	Fined	white	Isinglass
22	Fined	white	Isinglass
42	Fined	white	Isinglass
107	Fined	white	Isinglass
108	Fined	red	Isinglass
109	Fined	white	Isinglass
1	Fined	white	milk
37	Fined	white	Milk
24	Fined	red	Non-grape derived tannin
25	Fined	red	Non-grape derived tannin
61	Fined	red	Non-grape derived tannin
62	Fined	red	Non-grape derived tannin
63	Fined	red	Non-grape derived tannin
66	Fined	red	Non-grape derived tannin
110	Control	white	none
112	Control	red	none
113	Control	red	none

6.3.3 Food challenge study design

The food challenge was a randomized double-blinded study. It comprised two study days separated by a seven day washout period. On the first study day a 100 mL blood sample was taken from each subject by the clinical research nurse and the whole blood was stored between +2 ^{o}C to +6 ^{o}C in a blood bank refrigerator.

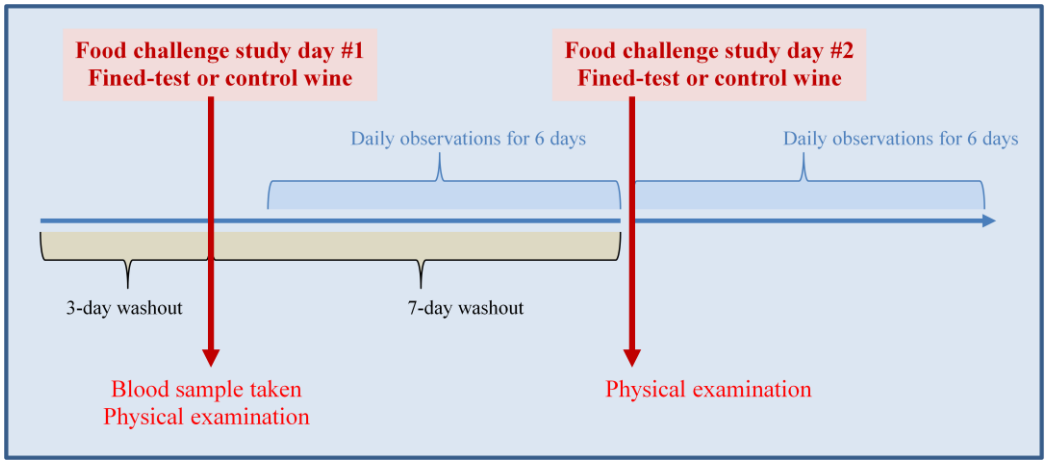

Figure 14 Food challenge study design

6.3.4 Food challenge protocol

A double blind, placebo controlled food challenge with a panel of 27 wines (including three control wines) was conducted in the Allergy and Asthma Clinics at The Alfred Hospital (Melbourne, Victoria). A doctor and a clinical research nurse from the Allergy and Asthma Clinics were in attendance on the food challenges days and administered the food challenge. Subjects underwent two food challenges, one with a test protein-fined wine and one with a control unfined wine separated by a minimum of seven days. They received the test food protein-fined wine on one occasion and the control unfined wine on the other, in random order (Figure 14). Both the subject and the attending doctor and clinical research nurse were blinded as to the test or control status of the wines in the food challenge.

On each challenge day, subjects ingested 100 mL (approximately one Australian standard drink[39]) of either the challenge (fined or unfined control) wine over a 10-15 min period. After the challenge there was an examination period, where serial assessments of the subjects (visual analogue and physical examination) were made at 15

[39] Australia New Zealand Food Standards Code, Clause 3 of Standard 2.7.1 includes the provisions relating to standard drink labelling at: http://www.comlaw.gov.au/Details/F2011C00588

min intervals for a total of 2 h. The subjects completed a questionnaire to assess the presence of typical features of anaphylaxis, scoring the severity of those symptoms on a visual analogue scale (VAS; Appendix 6). A physical examination was performed to identify signs of anaphylaxis (Appendix 7). Clinical observations of the physical examination 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).

Finally, baseline spirometry was performed including forced expiratory volume (FEV_1) and forced vital capacity (FVC) using a turbine spirometer (Micromedical, Chatham, Kent, UK) according to American Thoracic Society criteria for spirometry equipment and methodology (Miller et al. 2005). Spirometry was repeated every 30 min during this 2 h period. Each interval test score was subsequently measured and recorded.

Subsequent to the completion of each food challenge day, subjects completed a daily symptom questionnaire card (diary) for the next six days to record any possible delayed adverse reactions. The daily symptom questionnaire card (diary) assessed the following symptoms:

- asthma
- urticaria
- angioedema
- laryngeal oedema
- gastrointestinal tract upset

- circulatory collapse.

6.3.4 Statistical analysis

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

6.4 Results

A total of 76 food challenges were performed on 37 subjects (26 food-allergic subjects and 11 non-food-allergic controls), with the milk-allergic subject challenged twice with the same wines to confirm the symptoms and physical signs that were observed on initial challenge, thereby totalling 76 and not 74 challenges. All subjects had an FEV_1 >80% predicted in study entry. No subject developed anaphylaxis (laryngeal oedema) following a food challenge, and no adverse effects from the daily symptom questionnaire card were recorded during the six day follow-up by any subject. Overall, there was no statistically significant difference ($p>0.05$) on any parameter measured between subject groups or between fining agents. Three food-allergic subjects, however, experienced mild adverse reactions with wines fined by proteins to which they were allergic. A further two food-allergic subjects experienced mild adverse reactions to unfined control wines. No subject experienced a typical IgE-mediated allergic reaction requiring medication treatment. No control subject experienced any symptom or physical sign of an adverse reaction (Table 6, Table 7, Table 8).

Table 6 Summary of total number of episodes of symptoms (Appendix 6) and/or physical signs of an adverse reaction (Appendix 7) following wine (W) consumption in the food challenge for food-allergic compared to control subjects

Subject	Mild adverse reaction to an unfined control wine	Mild adverse reaction to a fined wine
Food-allergic	2/26	3/26
Control	0/11	0/11

Table 7 Summary of episodes of symptoms and/or physical signs of an adverse reaction following wine (W) consumption in the food challenge

Allergy group	No. of subjects in group	No. of subjects with any symptoms or physical signs of an adverse reaction following wine consumption	Control 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 #112 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
Fish	10	1	1	F3 W #110 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
Peanut	10	1	0	-	1	P6 W #62 FEV_1 13% fall; spontaneous resolution
Control	11	0	0	-	0	-

Table 8 Summary of wine samples eliciting episodes of symptoms and/or physical signs of an adverse reaction following wine (W) consumption in the food challenge

Wine sample number	Fining agent used	Number of adverse reactions in allergic subjects	Allergic subject	Number of adverse reactions in control subjects
29	Egg white	1	E4	0
37	Milk	1	M1	0
62	Non-grape derived tannin	1	P6	0
110	None	1	F3	0
112	None	1	E4	0

Among four (E4, P6, F3 and M1) of the 26 food-allergic subjects, five mild adverse reactions (Appendix 6 or Appendix 7) were observed during the 2-h observation period following wine consumption (Table 7 and Table 8).

Two of these adverse reactions occurred with the same subject (E4) challenged with an unfined control wine. Three of these adverse reactions occurred when a subject (P6, F3 and M1) was challenged with food protein-fined wine. No other subject challenged with the control wines showed symptoms or physical signs of adverse reaction.

One egg-allergic subject (E4) who reacted mildly to an unfined control wine, required treatment with administration of the bronchodilator salbutamol and this resulted in complete resolution of the 22% fall in FEV_1. This subject also showed an 11% fall in FEV_1 following consumption of an egg-fined wine but FEV_1 normalised spontaneously. Clinical assessment suggested that this individual had unstable asthma triggered by the spirometric maneuver, resulting in non-specific airway reactivity combined with suboptimal asthma management. There was a subsequent adjustment of the anti-asthma maintenance medication.

One peanut-allergic subject (P6) experienced a mild asymptomatic decrease in lung function following ingestion of a wine made with a non-grape tannin and one fish-allergic subject (F3) experienced mild lip numbness following ingestion of a control unfined wine. Neither reaction required treatment.

The one milk-allergic subject (M1) reported the symptom of a 'slight lump in the throat" following ingestion of a milk-fined wine which did not occur with the control challenge. This was confirmed by repeat blinded challenge with the same milk-fined wine resulting in a reported mild itch symptom. No treatment was needed for either active challenge and no signs of allergic adverse reaction were noted on either occasion.

The three fined wines (#29, #37 and #62) and two unfined control wines (#110, and #112), which elicited a symptom or physical sign in four food-allergic subjects, were analysed to measure their concentration of sulfur dioxide. As described in Section 3.5 of Chapter 3, sulfur dioxide, a naturally occurring chemical in wine and a permitted additive in winemaking, can cause allergic and food intolerant adverse reactions such as chest tightness and wheeze on auscultation in sensitive subjects. These physical signs were experienced by three of the 26 food-allergic subjects. The concentration of total sulfur dioxide measured in these wines was below that observed to elicit an adverse reaction in sulfur dioxide-sensitive asthmatics (300 mg/L; Vally and Thompson, 2001) (Table 9).

Table 9 Concentration of sulfur dioxide (free and total) in wines that elicited a symptom or physical sign in the food challenge

Wine sample number	Wine type	Fining agent used	Free sulfur dioxide (mg/L)	Total sulfur dioxide (mg/L)
29	red	Egg white	7	43
37	white	Milk	Not detected	95
62	red	Non-grape derived tannin	13	126
110	white	None	18	100
112	red	None	3	41

As the results of the double blind placebo control food challenge were either subjective and hence unmeasurable, or below the threshold values of a clinical response, no statistical analyses were required.

6.5 Discussion

The panel of 26 food-allergic and 11 non-food-allergic subjects were challenged with 27 fined wines, including three control unfined wines in a blinded study. The challenge involved consuming a standard drink (100 mL) ingested after fasting and drunk over a short 10 to 15 minute period. This ensured that absorption of the potentially allergenic protein components and their subsequent distribution from the blood stream into the body's organs and tissues was relatively rapid.

Of the 76 food challenges, no clinically significant adverse reactions were observed. No food-allergic subject experienced symptoms or physical signs of a typical IgE-mediated allergic reaction requiring medical treatment following food challenge, and no food-allergic subject experienced anaphylaxis (laryngeal oedema) following food challenge.

Five mild adverse reactions were, however, observed with five challenge wines, two of which were unfined control wines and three were fined wines. Two of these mild adverse reactions were subjective symptoms which could be independently verified by the attending doctor or clinical nurse, such that subjective symptoms are not used in determining NOAELs and LOAELs for allergenic foods (Taylor et al., 2003). The five episodes where symptoms and/or physical signs were noted following wine consumption revealed no consistent pattern with respect to wine type or food-allergic

subject group. None of the challenge wines contained detectable residues of allergenic protein as assessed by the assay developed in Chapter 4 and tested in Chapter 5.

Although two of the five challenge wines that elicited a mild respiratory function-related adverse reaction in an egg-allergic and a fish-allergic subject were control wines which did not contain any food protein, these wines also did not contain a concentration of sulfur dioxide associated with adverse reactions in sensitive individuals. These five challenge wines may, however, have contained other non-proteinaceous chemical compounds that could elicit an adverse reaction in sensitive individuals. Accordingly, a limitation of this study is that analyses for acetaldehyde, salicylic acid, histamine and tyramine, for example, were not conducted as there was not sufficient sample available for such further analyses.

No control subject experienced a mild or clinically significant adverse reaction to any of the fined or unfined control wines.

The lack of any clinically significant adverse response of peanut or tree-nut-allergic and fish-allergic adult individuals to a double blind placebo controlled challenge of wines fined with non-grape tannins or isinglass suggests that a very low risk exists for allergic reactions attributable to residual allergenic food proteins in the Australian wine manufacturing process.

The low numbers of adult individuals in this study with IgE-mediated egg and milk allergy allowed only limited investigation of the potential for a clinical challenge with a wine fined with an associated allergenic food protein to induce an adverse clinical

response. Nonetheless, the findings were clear and consistent that within this subset of the Australian adult population, for subjects with known allergies to fish or nuts there was no clinically significant adverse response (anaphylaxis or laryngeal oedema) to a double-blind placebo-controlled food challenge of fined wines that could be attributed to residual proteinaceous processing aids in wine made following established manufacturing best practice.

The findings of this study would be strengthened by a further study with a larger panel of fined wines and allergic subjects. In addition, subsequent research could consider recruiting more egg and milk-allergic individuals to increase the number of subjects to enable definitive conclusions to be drawn for these specific subsets of food-allergic individuals of the Australian adult population. A potential limitation of this study is that only non-naïve current wine consumers were included which could be addressed in future studies.

A limitation of the study is that adjunctive diagnostic skin prick tests (Heinzerling et al., 2013) were not undertaken to provide evidence for sensitisation and to confirm the allergencity of the study subjects to the concentrated fining agents (egg white-derived ovalbumin, isinglass and casein), as well as to peanut-related proteins. This was not considered critical in the design of the study as positive food challenges can be observed in subjects with negative SPT responses (Sporik et al., 2000), and was unable to be performed subsequently due to cost and time constraints. Although skin pricks tests themselves do not confirm allergy (Bock et al., 1988), they detect the presence of IgE antibody, and tests would have confirmed that any of the mild adverse reactions observed were not associated with other potential allergens present in wine such as

grape proteins or other egg and milk proteins (Pastorello et al., 2003; Borghesan et al., 2004; Schad et al., 2005; Kalogeromitros et al., 2006; Sbornik et al., 2007, Vassilopoulou et al., 2007). Indeed, another limitation of the study is that the sole milk-allergic individual (who had a SPT of 15 mm) who experienced a mild adverse reaction to milk-fined wine, was only challenged with a milk-fined wine which would contain potentially allergic proteins other than casein, such as the primary whey protein, β-lactoglobulin (Sélo et al., 1999; Lam et al., 2008). Although extremely rare, subjects allergic to both casein and whey proteins display a stronger skin prick test such as 15 mm and IgE reactivity (Lam et al., 2008).

An additional limitation of this study is that the wine challenge only involved the consumption of one standard drink, 100 mL, which may not accurately reflect real-life social situations and may not have been sufficient exposure to generate an adverse clinical response. For example, in 2007 one in five Australians (20.4%) drank at high risk levels at least once a month (Australian Institute of Health and Welfare, 2008). This pattern of drinking is the equivalent to consuming seven or more standard drinks on any one day for males, and consuming five or more standard drinks on any one day for females (National Health and Medical Research Council, 2001). While the moderate amount consumed in this study may not have elicited an allergic reaction in the 37 study subjects, it cannot be concluded that heavier consumption would not induce an adverse clinical response. Kirschner et al. (2009), for example, challenged subjects with a cumulative amount of 200 mL for women and 300 mL for men in four successive incremental amounts at 30 minute intervals. As no allergic reactions were observed with these moderate amounts of alcohol, further studies could challenge subjects with heavier

amounts of wine, such as cumulative amounts of 300 mL and 400 mL or more for women and men, respectively.

Consideration could be given to further evaluation of the potential for allergic reactions using a stripped basophil activation assay involving sensitisation with serum specific IgE from children with egg and/or milk allergy. The rarity of IgE-mediated milk allergy in adults really prevented reliable evaluation of residual allergenic milk proteins in wine by direct challenge or *in vitro* basophil activation (described in Chapter 7). Indeed, there are only six studies, with small numbers of adult milk-allergic subjects recruited, of double blind placebo controlled food challenge considering potential allergic reactions to milk protein (Berstein et al., 1982; Wüthrich et al., 1986; Olalde et al., 1989; Pastrorello et al., 1989; Norgaard et al., 1992; Lam et al., 2008). In the collective 22 milk-allergic adult subjects of these six studies, the lowest dose of milk proteins causing either objective clinical responses or subjective symptoms was approximately 0.10 mg casein, which is greater than the potential amount of residual casein in milk protein-fined wine as described in Chapter 4.

This rarity of milk-allergic adult individuals (Host, 1990; Host, et al., 1997; Lam et al., 2008; Asero et al., 2009a; Asero et al., 2009b) combined with a proposed threshold in excess of the concentration of casein found in fined wine as measured in Chapter 5, suggests that potential allergic reactions to milk proteins in wine are less likely to occur and be observed. This is in comparison with allergic reactions to other food proteins in wine. Therefore, milk-fined wine is less problematic compared to egg, fish fined wine or to which tree-nut derived tannins have been added.

The gold standard in food allergy assessment, especially in research studies, is the double blind placebo controlled food challenge (Boyce et al., 2010; Sampson et al., 2012), where neither the subject nor the clinical nurse/researcher knows whether the test or the control food is being administered in order to prevent both researcher bias and placebo effects. The findings in this study using a double blind placebo controlled food challenge suggest that wines fined with egg white, isinglass, milk or the milk proteins casein and potassium caseinate, or to which non-grape derived tannins have been added, present an extremely low risk of a significant clinical response such as anaphylaxis, to egg, fish, or nut-allergic individuals. Although consumption of milk-fined wine did not induce anaphylaxis, there were insufficient subjects to determine confidently whether wines fined with milk proteins present a risk to the rare milk-allergic adult individuals, which was a limitation of the study as previously stated.

Only mild symptoms were reported by this subject that could not be physically measured or observed, however, subjective symptoms lacking objective adverse reactions after administration of very low doses of an allergen have also been described in some other studies (Hourihane et al., 1997), and thus an emergent allergic reaction to milk-fined wine in this subject cannot be excluded.

However, the subjective milk reaction observed by the milk-allergic adult challenged with milk-fined wine was also at the lowest end of the severity scale and included a small lump in the throat and mild itch. These symptoms disappeared quickly without any treatment. They represent a discomfort and can be graded according to severity (Bousquet et al., 1998, Yanagida et al., 2013) as follows:

1) discomfort – symptoms disappear quickly without treatment

2) discomfort – symptoms disappear more slowly without treatment
3) impedes activity, requiring minor treatment (intervention) to correct
4) seriously impedes activity, requiring significant treatment (intervention) to correct
5) life threatening – may result in death (e.g. anaphylaxis).

This study was designed to test for the most severe symptoms using low dose wine exposure, a single standard drink.

Rare reactions to milk protein exposure have only been observed in challenge studies at the lowest end of this grading scale (1) resulting in discomfort that quickly disappears without treatment. Hence, the risk of an adverse reaction is very low for milk-allergic wine consumers and the level of reaction is very mild, and as such could be considered to be an acceptable or tolerable risk (Knechtges, 2011).

The findings from this study were subsequently supported by those from more recent studies (Kirschner et al., 2009; Vassilopoulou et al., 2011). In a double blind placebo controlled food challenge, Kirschner et al. (2009) observed that none of their 14 egg, fish or milk-allergic individuals experienced either subjective or clinical responses in a double blind placebo controlled food challenge to wine fined with these proteins. Combined, the present study in conjunction with the findings from subsequent studies suggest that wines fined with egg white, fish or milk or their products, or to which tree-nut derived tannins have been added, do not cause an allergic reaction in sensitive adult individuals. However, to more stringently determine the risk of an allergic reaction to egg-fined and milk-fined wine within a population, *in vitro* predictive tests such as

basophil activation assays could be used to increase subject numbers in subsequent studies without the risk of clinically significant adverse reactions. Therefore, the next step in this program of research was to develop and undertake basophil activation assays for egg, fish, milk and non-grape derived tannin in wine to potentially predict whether protein-fined wine would elicit an allergic reaction in sensitive individuals.

Declaration - Chapter 7

I declare that all content in the following chapter has been written by me. As the studies conducted for this thesis involved a wider support team, my independent contribution to each aspect of the study is detailed in Appendix 1.

Creina Stockley

Chapter 7 Basophil activation assay for determination of allergenic wine processing aids in wine

Overview

Food challenges to diagnose or confirm allergic reactions to wines, such as that undertaken in Chapter 6, expose the allergic individual to the risk of a systemic reaction such as anaphylaxis. In line with contemporary trends, exposure to a clinically significant and life-threatening adverse reaction can be avoided by a simple and non-invasive basophil activation assay (BAT) which is a preferable alternative test to a food challenge and where *in vitro* and *in vivo* assays are contradictory, inaccurate or unethical to perform (McGowan and Saini, 2013). This chapter details the development of a predictive BAT and the testing of allergenicity in a selected subset of the 109 commercially-available Australian wines fined with a potentially allergenic food protein described in Chapter 5 as an alternative to a double blind placebo controlled food challenge.

7.1 Introduction

The immediate symptoms of an allergic reaction are caused by an initial systemic histamine release by peripheral blood basophil granulocytes and tissue mast cells when activated by an allergen. A positive skin prick test and/or double blind placebo control food challenge are usually used in the diagnosis and confirmation of allergies as undertaken in the study reported in Chapter 6 for the initial subject assessment for IgE-mediated allergy to eggs, fish, milk and nuts. An adjunct or potential alternative *in vitro* test was subsequently developed to diagnose allergies in individuals, which detects the activation of basophil granulocytes in blood - the basophil activation test or BAT – as it

closely mirrors the *in vivo* situation. That is, BAT can precipitate symptoms but without the risk of a systemic allergic reaction, such as anaphylaxis (Moneret-Vautrin et al., 1999). BAT, or the flow cytometric detection of basophil activation by CD63 expression, has been validated or verified clinically for a variety of IgE-mediated allergens such as inhalants (Paris-Kohler et al., 2000; Erdmann et al., 2003), hymenoptera venoms (Freitag et al., 2001; Erdmann et al., 2004), rubber latex (Sainte-Laudy et al., 1996), neuromuscular blocking agents (Abuaf et al., 1999), beta-lactam antibiotics and clavulanic acid (Sanz et al., 2002), pyrazolones and non-steroidal anti-inflammatory drugs (Rodríguez-Trabado et al., 2008).

The BAT has not been clinically validated, however, for egg, fish, milk or nut allergy from wine. If validated, it could provide an easy non-invasive alternative method to the skin prick test and double blind placebo controlled food challenge, and as an alternative to ELISA, to determine whether food protein-fined wine could elicit an allergic reaction in sensitive individuals. The advantages of BAT in comparison to the other tests are that BAT can be undertaken outside of a hospital clinic, requires only 2–5 mL of whole blood from a sensitive individual and is relatively rapid (2–8 hours) (Lopata, 2008; Khan et al., 2012).

7.2 Aim

The aim of this study was to establish a basophil activation test (BAT) specific for egg, fish, milk-fined wine and wine to which non-grape, nut-derived tannin had been added. The results of the BAT would be validated against the results found in the clinical double blind placebo controlled food challenge reported in Chapter 6. This would determine if BAT was sufficiently sensitive and specific to be an appropriate alternative to predict whether protein-fined wines would elicit an allergic reaction in sensitive individuals.

7.3 Methods

7.3.1 *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). The alcohol content of wine samples was found to be cytotoxic to the basophil cells, and the wines were de-alcoholised as a pretreatment (Table 10). White wines were dialysed (3.5-kDa cutoff) against phosphate buffered saline (PBS), and red wine proteins were precipitated with ethanol and resuspended in PBS to the original volume of wine. Antibody concentrations and incubation times were optimised, 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. The 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. Animal ethics approval was received from the AMREP Animal Ethics Committee (AEC approval number MU999).

Table 10 Effect of different wines, spiked and unspiked on cell viability in the BAT

Sample type	Percent live	Percent dead	pH value
Fortified wine (FW)	95.1	4.9	3.9
FW + house dust mites (HDM)*	93.9	6.1	
FW - dialysed	99.7	0.3	6.8
FW - dialysed +HDM	99.8	0.2	
Red wine (RW)	95.1	4.9	3.4
RW +HDM	97.7	2.3	
RW - dialysed	99.9	0.1	6.8
RW - dialysed +HDM	99.9	0.1	
Dealcoholised wine (DW)	99.9	0.1	3.2
DW + HDM	99.8	0.2	
DW - dialysed	99.8	0.2	6.8
DW - dialysed +HDM	99.8	0.2	
Stimulation buffer (SB)	99.9	0.1	7.4
SB + HDM	99.8	0.2	

* Allergen

7.3.2 Fluorescent labelling of cells

Following the 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 (fluorescein-5-isothiocyanate)-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 isotypes 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 of cells with stimulation buffer alone.

7.3.3 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. Figure 15, Figure 16 and Figure 17 show the flow cytometric analyses of basophil activation. The method for analysis was as follows. Cells were first gated on a forward scatter versus side scatter region (Figure 15a) and later validated to contain basophils by back-gating of high IgE staining cells (Figure 15d). Viable cells within this scatter gate were selected via exclusion of the vital dye 7AAD (Figure 15b) and basophils identified by high intensity anti-IgE staining (IgE^{hi}) (Figure 15c). 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 15f and Figure 15g). The expression of CD63 by the gated IgE^{hi} cells was analysed (Figure 15h).

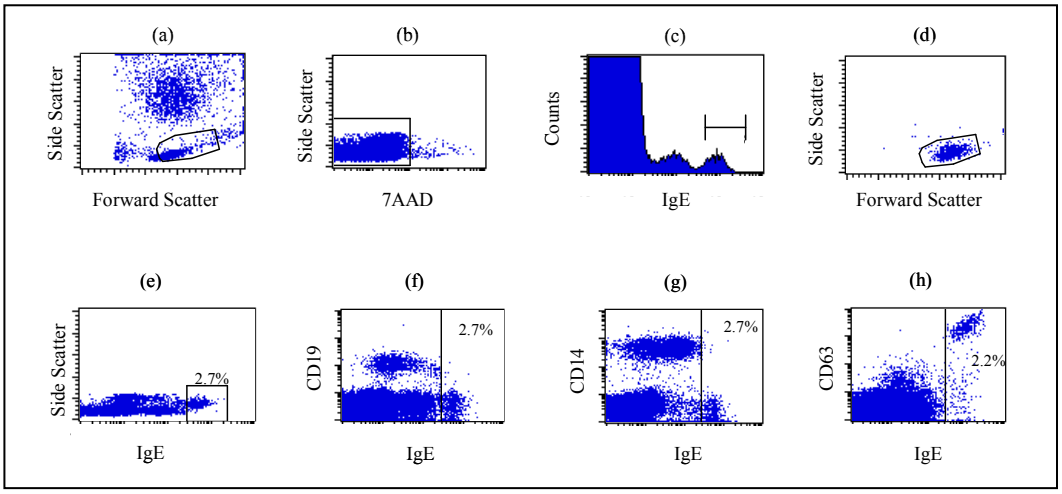

Figure 15 Flow cytometry plots showing gating of viable basophils

Figure 15 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.

Analysis of basophil activation following incubation of whole blood from an representative 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 16. 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 were 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 17b), similar to the 'no antigen' control for this sample (14%) (Figure 17c).

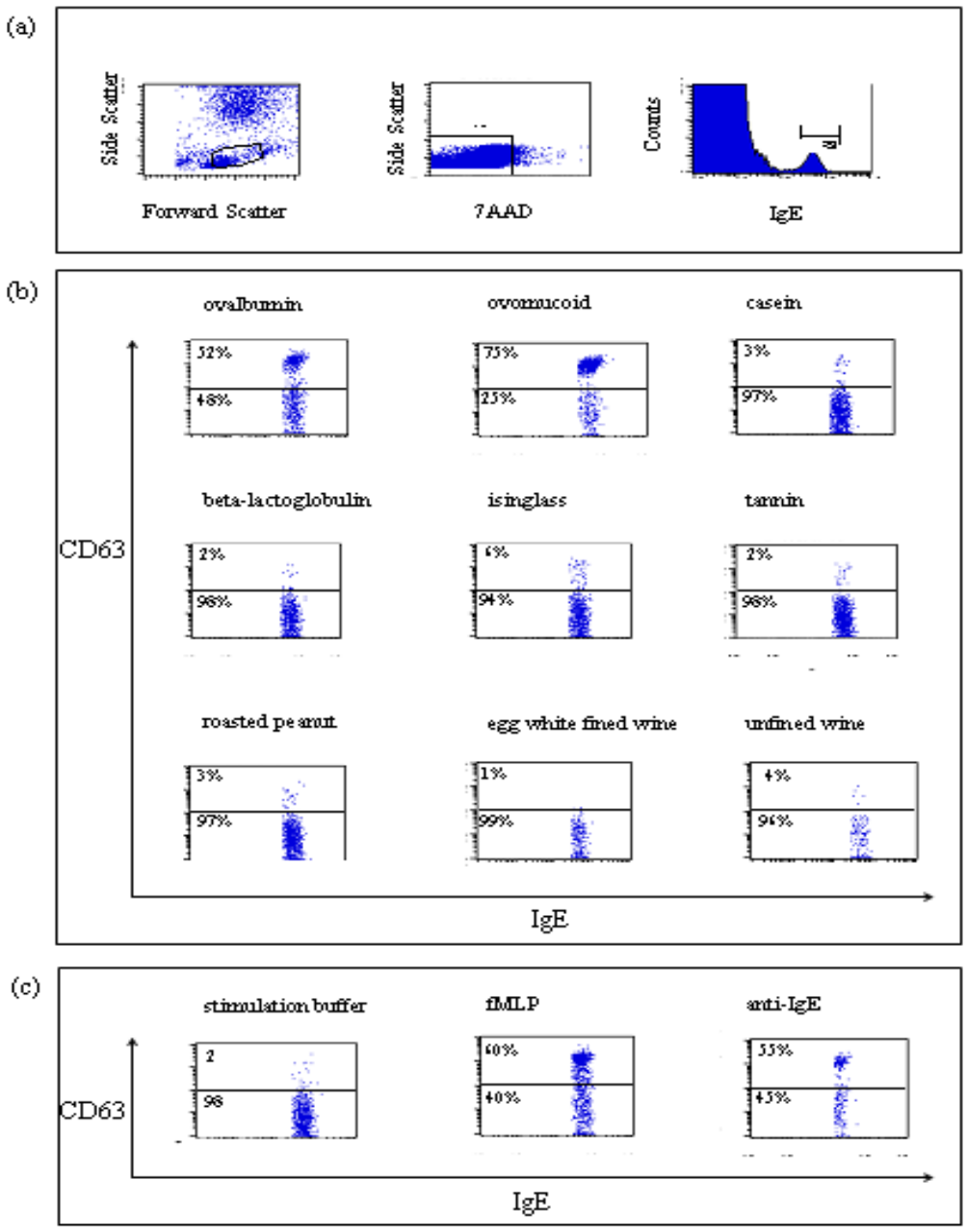

Figure 16 Flow cytometric analysis of activation of blood basophils from an egg-allergic patient

(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-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.

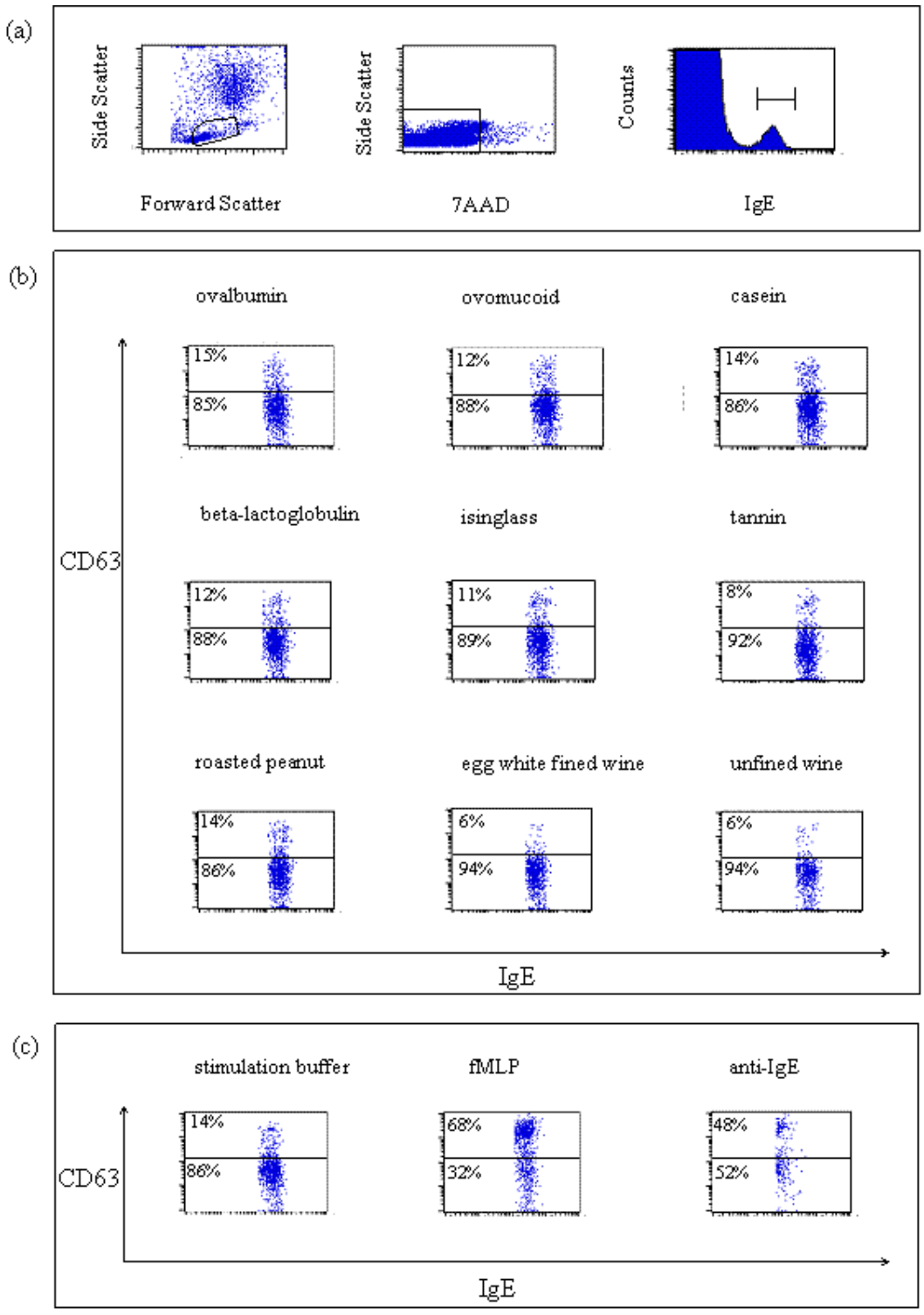

Figure 17 Flow cytometric analysis of activation of blood basophils from a non-allergic patient

(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 study. Table 11 shows the results for one of these studies in which basophil activation was tested with roasted peanut extract at different concentrations for five peanut-allergic subjects.

Table 11 Optimisation of allergen concentration for basophil activation assay using roasted peanut extract

	Basophil activation ratio for a different concentration of roasted peanut extract			
Subject	**0.01 μg/mL**	**0.1 μg/mL**	**1.0 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

Note: Basophil activation ratios (% activated basophils with allergen divided by % activated basophils in stimulation buffer alone) are shown for five peanut-allergic subjects.

7.3.4 Analysis of survey wines by basophil activation assay

As outlined in Section 6.3.3, blood (100 mL) for basophil activation assays was collected and stored from each of the 37 study subjects recruited for the double blind placebo controlled food challenge and described in Chapter 6 prior to the food challenge procedure on the first of their two visits. It was not feasible to test the blood of all of the 37 study subjects with all the 113 panel wines collected and described in Chapter 5. Therefore a chequerboard grid (Figure 18) was established by an ombudsman to ensure that all 113 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 19.

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.

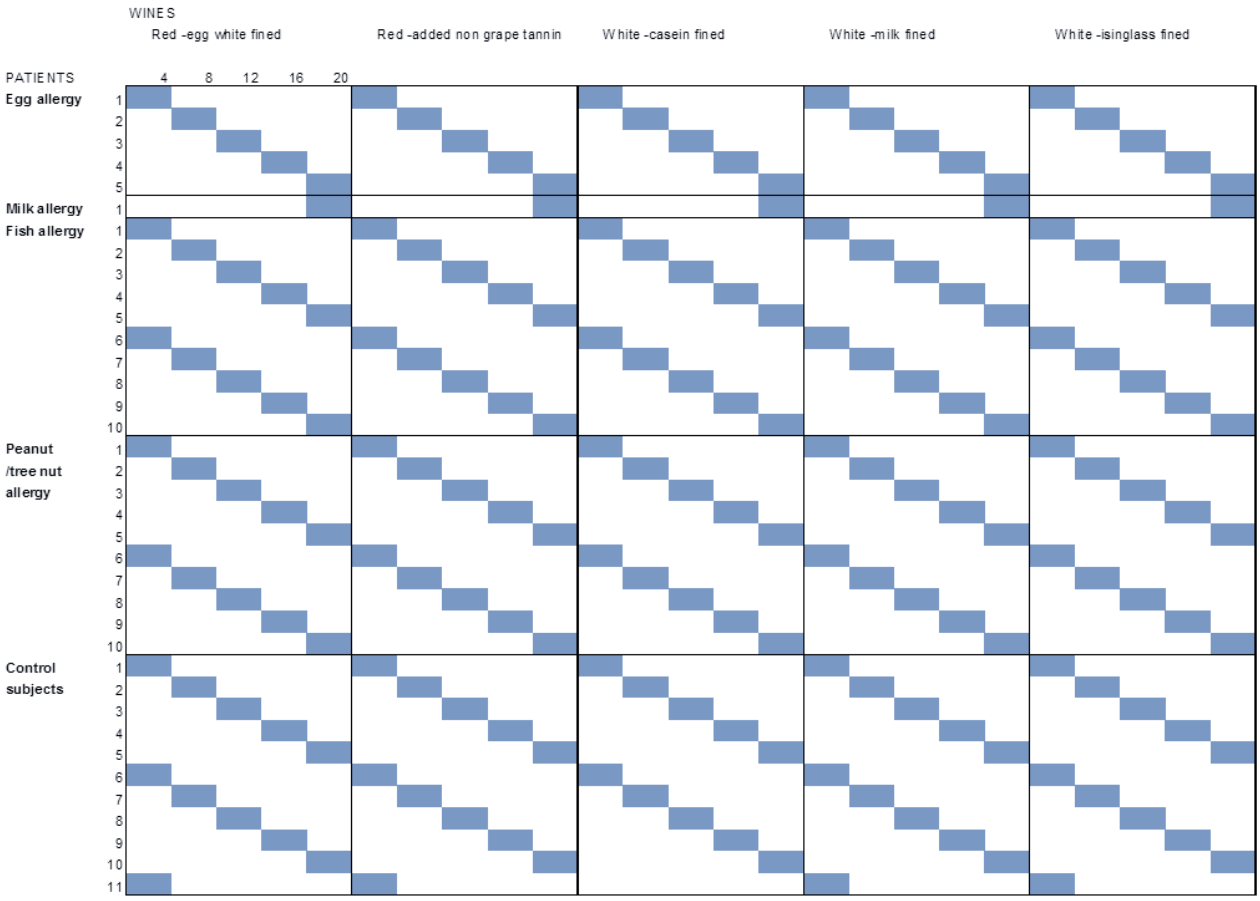

Figure 18 Plan for testing survey wines by basophil activation assay

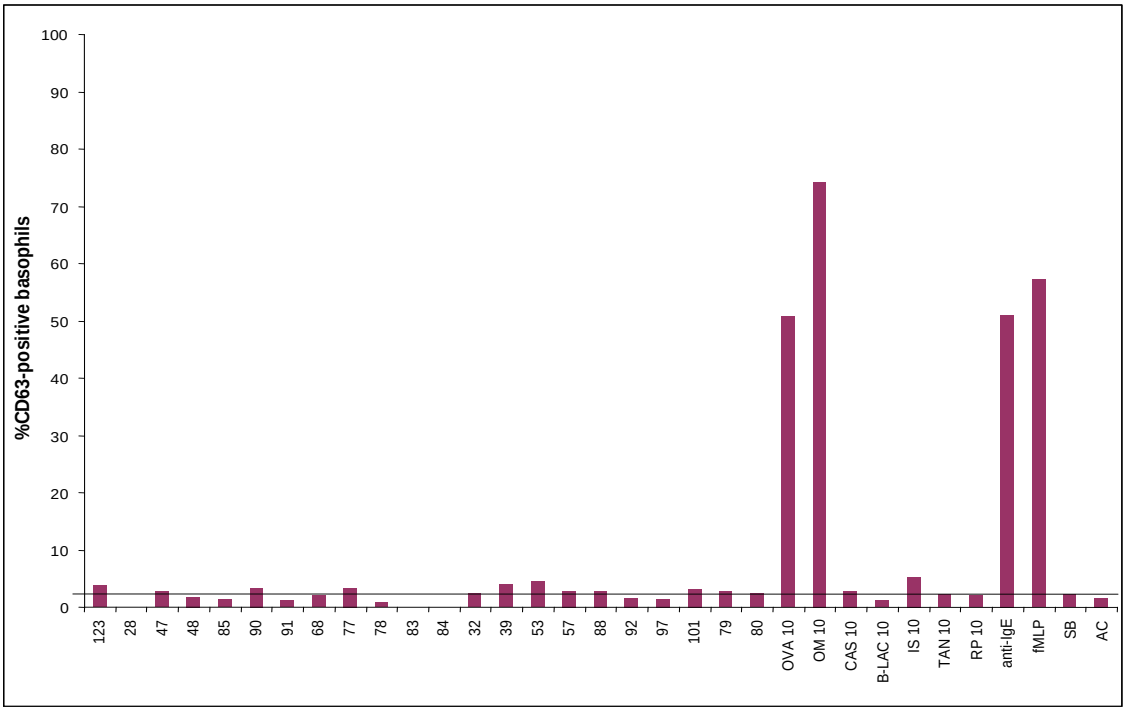

Figure 19 Representative data for basophil activation assay of survey wines

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)

7.3.5 Statistical analysis

Statistical analysis was conducted using InStat 2.0 software. Continuous variables were assessed for normality and log-transformed where appropriate. Comparisons 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.

7.4 Results

The results for testing blood samples from the 37 study subjects against the entire panel of 113 wines are summarised in Figure 20, Figure 21, Figure 22, Figure 23, Figure 24 and Table 12. 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% established benchmark reported in the literature (Erdmann et al., 2004), thus validating each assay. Where the percentage of activated basophils for a test sample (wines and fining agents) 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 test 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 in Figure 20, Figure 21, Figure 22, Figure 23 and Figure 24.

A reference range was established based on the basophil activation ratios for control non-food-allergic subjects tested against test fined 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 were 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, that is wine fined with fish, casein or milk or to which non-grape, nut-derived tannin had been added. 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 similarly for the other wine fining agents. Only nine of the 109 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 12), 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 but non-fish-allergic individual. The two fish-allergic subjects showed no clinical adverse effects to another wine fined with isinglass. One of these two fish-allergic

subjects showed weak basophil activation to a wine made with added non-grape, nut-derived tannin, but this subject was not allergic to peanuts or tree nuts.

Basophils from one egg-allergic subject were activated by a wine fined with casein and milk, and basophils from three fish-allergic subjects responded to five wines fined with casein. None of these subjects was allergic to milk and basophils from the one milk-allergic subject did not respond to any of the casein or milk-fined wines.

One of the control unfined wines 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. None of the wines associated with reported clinical symptoms or physical signs as described in (Table 6, Table 7 and Table 8) elicited a positive basophil activation in any of the same subjects and, except for the one unfined wine, in any other subject.

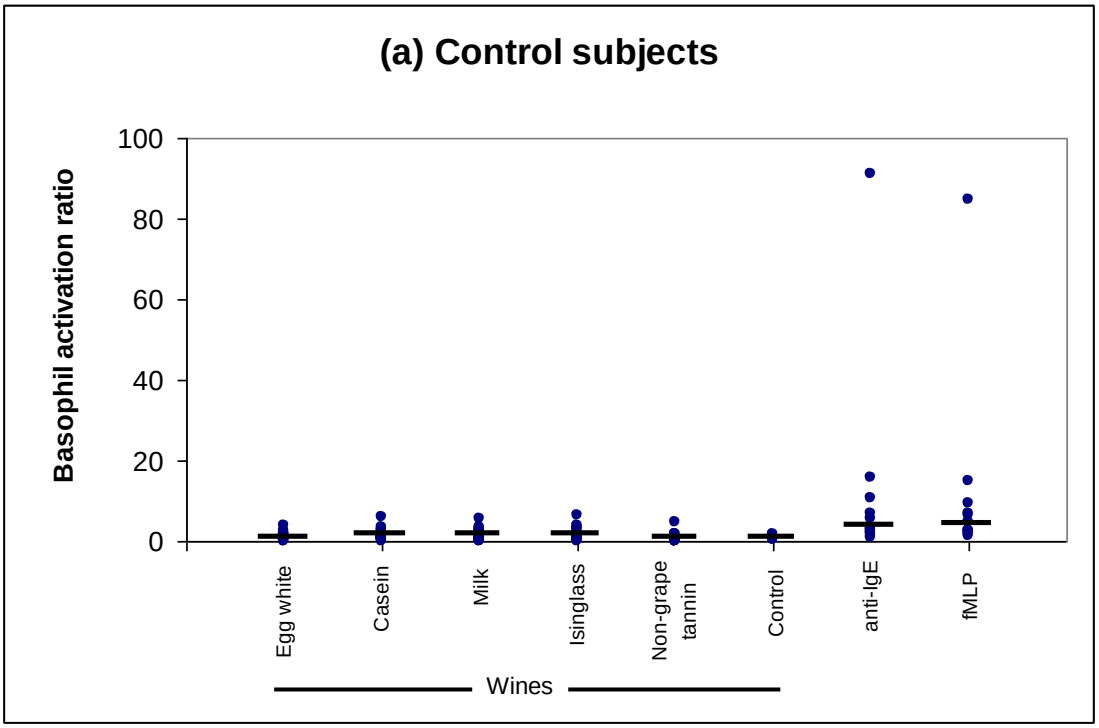

Figure 20 Summary of basophil activation ratios for the control subject group when blood was stimulated with wines from each of the fining agent groups or with control agents

Bars represent median values.

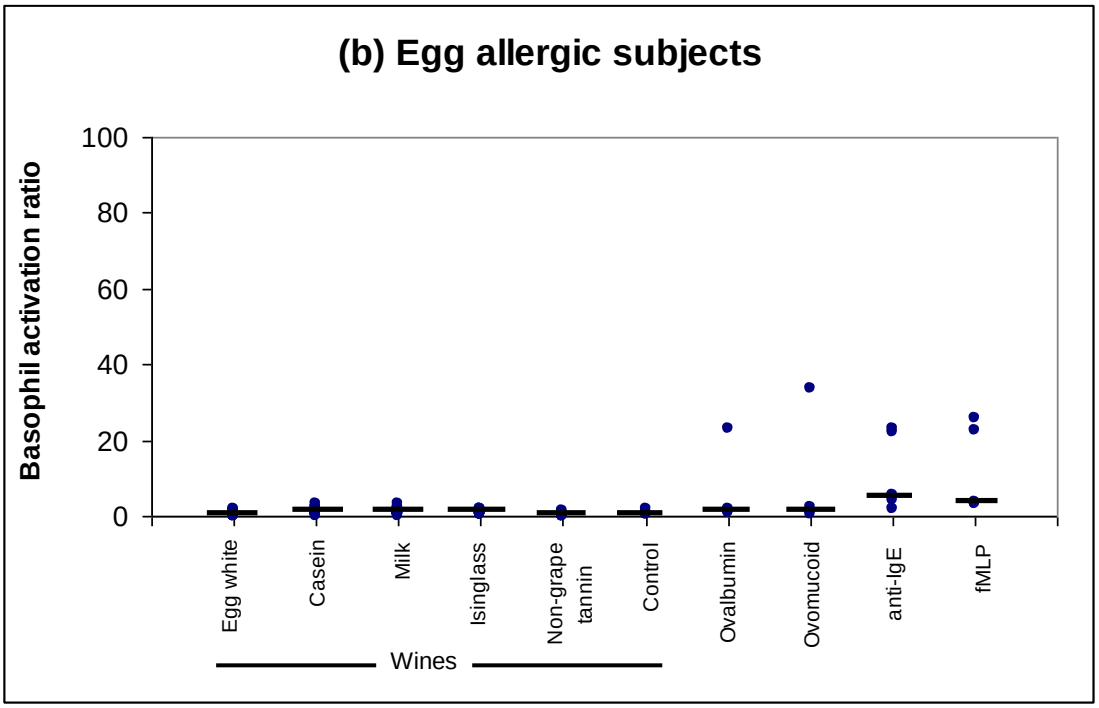

Figure 21 Summary of basophil activation ratios for the egg-allergic subject group when blood was stimulated with wines from each of the fining agent groups or with control agents

Bars represent median values.

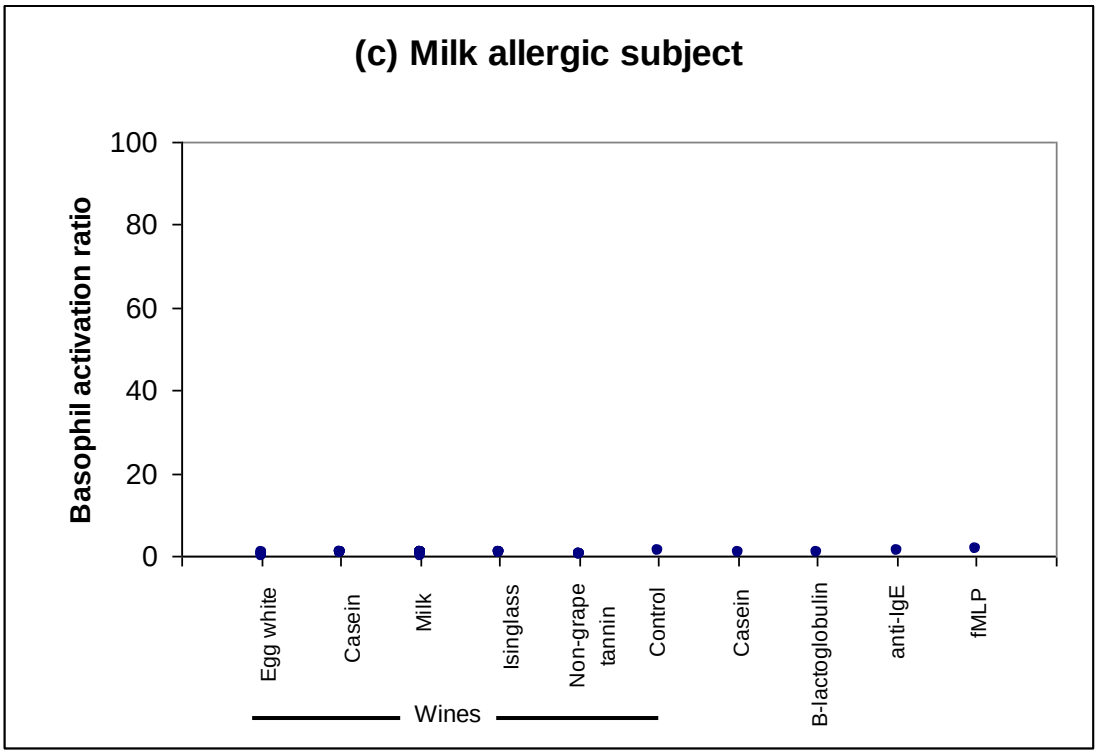

Figure 22 Summary of basophil activation ratios for the milk-allergic subject group when blood was stimulated with wines from each of the fining agent groups or with control agents

Bars represent median values.

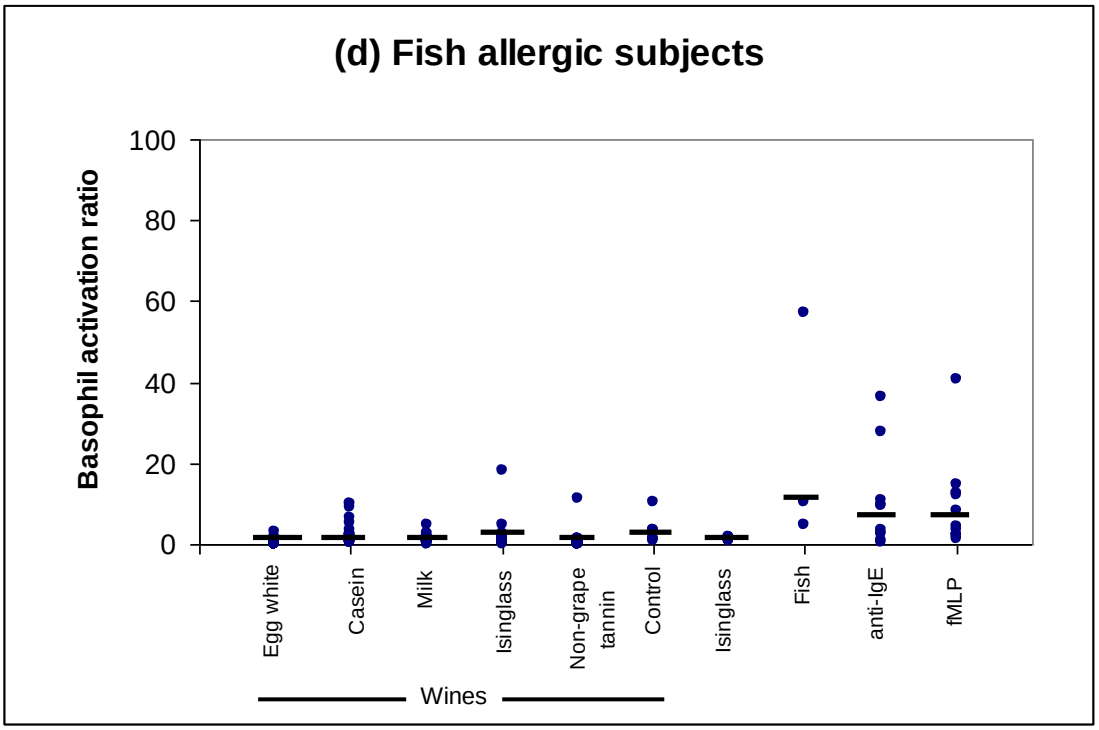

Figure 23 Summary of basophil activation ratios for the fish-allergic subject group when blood was stimulated with wines from each of the fining agent groups or with control agents

Bars represent median values.

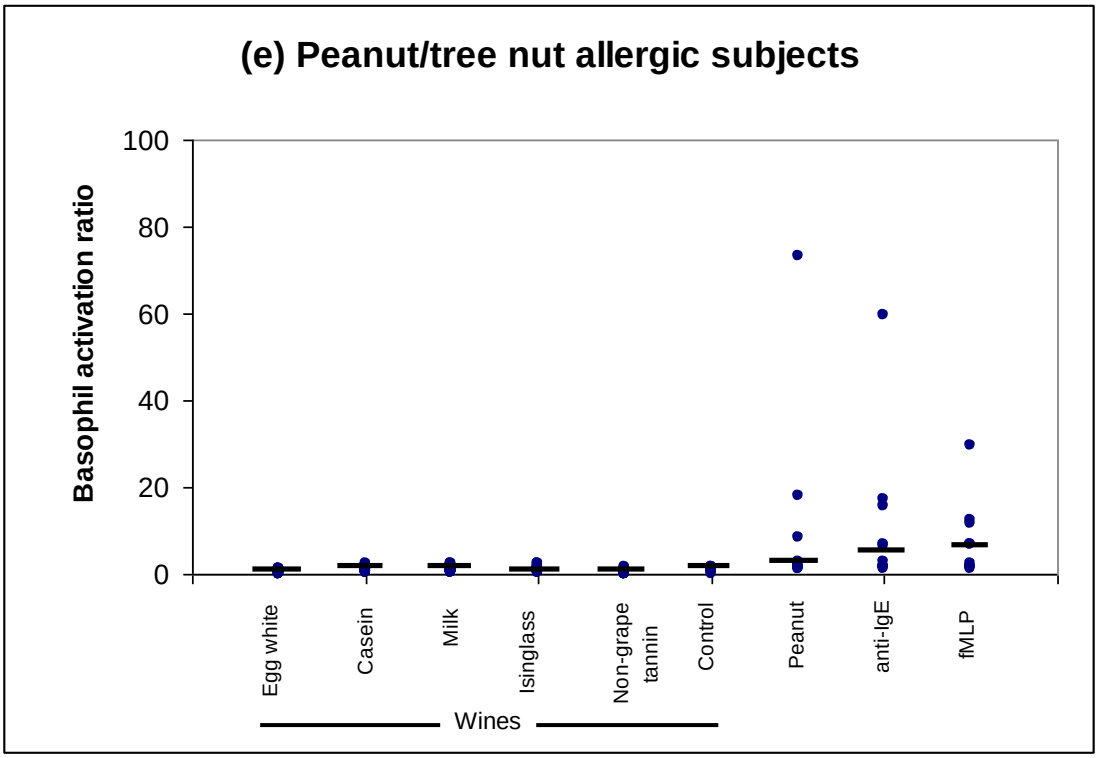

Figure 24 Summary of basophil activation ratios for the peanut/tree nut-allergic subject group when blood was stimulated with wines from each of the fining agent groups or with control agents

Bars represent median values.

Table 12 Summary of basophil activation assays which showed a ratio of >2.85 (upper limit of 95th percentile for control subject assays versus fined and u wine samples).

				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

Note: Data are not shown for assays where <10% basophils were activated i.e. test considered negative. A total of 37 assays were performed on control wines and 730 assays on test wines; some wines were made using more than one fining agent, giving a total of 923 data points.

There was sufficient sample remaining to analyse seven of the nine wines (#8, 20, 22, 26, 60, 68, 71, 88 and 92) that caused weak basophil activation for salicylic acid, histamine and tyramine. There was also sufficient sample remaining to test two of these nine wines for acetaldehyde. These compounds have been associated with adverse reactions in subjects as described in Chapter 3 and/or which might interfere in basophil assays and ELISA, both via non-immunological mechanisms (Table 13). The concentration of salicylic acid, histamine and tyramine in these wines was very low or undetectable; There was, however, no correlation between any of these concentrations and an adverse clinical reaction and/or basophil activation.

Table 13 Chemical analysis of wine samples associated with basophil activation in allergic subjects

Wine sample number	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*				
60*				
68		Trace	Trace	Not detected
71		Trace	2.3	Trace
88		Trace	Trace	Not detected
92		Trace	1.5	Not detected

* Insufficient sample to analyse for acetaldehyde, salicylic acid, histamine and tyramine

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
Acetaldehyde	limit of detection = 1 mg/L trace = 0.2 – 0.8 mg/L

7.5 Discussion

Whole blood basophil activation assays for wine analysis were established 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, nut-derived tannins.

Each of the 113 panel wines described in Chapter 5 was tested by the whole blood basophil activation assay with each of the 37 study subjects described in Chapter 6. Overall there was no statistically significant difference in basophil activation ratios between subject groups or between fining agents. Ninety two of the 113 wines tested gave a basophil activation ratio within the identified reference range. For the 10 wines (nine fined and one unfined) which were associated with basophil activation ratios above the reference range for nine subjects, no pattern could be identified with respect to wine variety, fining agent or geographic indication, or to subject group. Furthermore, none of the wines associated with the reported mild but not clinically significant adverse reactions in Chapter 6 elicited a positive basophil activation assay in any of the same subjects, and except for the unfined control wine (#110), in any other subject.

Basophil activation ratios for all of the egg white fined wine samples were within the reference range for all of the eight subjects tested including one egg-allergic subject. The latter subject's basophils were not activated when incubated with control ovalbumin or ovomucoid (egg) 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 could be considered.

BAT have also relatively recently been observed to be insufficiently sensitive and specific to detect egg and peanut allergy in egg-allergic and peanut-allergic children (Ocmant et al., 2009)

By contrast, in a study subsequently by Vassilopoulou et al. (2011), a small but significant induction of basophil activation was observed in fined wine in comparison with the control wine in subjects with allergies to egg, fish and milk, where the percentage of activated basophils in positive controls was greater than 20%, thus validating each assay (Erdmann et al., 2004). No significant basophil activation was observed in control allergic and non-allergic subjects.

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). Analysis of these and other potentially allergic compounds such as salicylic acid and tyramine in the wines that elicited basophil activation in food-allergic subjects 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). 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 (Bartowsky and Stockley, 2011). There was, however, no correlation between any of these concentrations and an adverse clinical reaction and/or basophil activation.

The findings of the basophil activation assay developed in this study correlates closely with the wine challenge results described in Chapter 6 but its lower specificity than the clinical double blind placebo controlled food challenge suggests that this assay alone would not be a reliable routine testing procedure. Therefore, the BAT is not a suitable alternative to the double blind placebo controlled food challenge to determine whether protein-fined wines would elicit an allergic reaction in sensitive individuals.

7.6 Overall conclusions

This study, in conjunction with the studies undertaken and described in Chapters 4 to 6, suggests that in this food-allergic population of Australian adults wine fined with egg, fish or milk or to which non-grape, nut-derived tannin has been added, poses a low risk of allergic reaction attributable to allergenic residual proteins in wine.

Chapter 8 The ascertainment of risk

Overview

Food safety from allergens in food products such as wine is a relatively recent public health concern, and ascertainment of risk from allergens in food products has only recently been investigated. The first scientific examination and evaluation of risk from egg, fish, milk and nut allergens in wine to food allergic consumers in Australia was undertaken in this body of work.

8.1 Introduction

There has been increasing globalization of food production and distribution, including that of wine. Assessing and managing risks to food safety has become, and will continue to become increasingly important and requires careful scientific examination. The term 'risk' in relation to wine generally encompasses two elements, the nature of the adverse effect and the likelihood that the adverse effect will occur (FSANZ, 2008). When food allergy came to international prominence as an important public health issue in 1995 when flagged by the Codex Alimentarius Commission (Hattersley et al. 2014), no scientific examination of risk had been undertaken for wine although risk management policies and practices, such as mandatory labelling, were put in place. The task of addressing the nature and extent of wine allergens did not occur until after a range of preventive policies and practices had been implemented. The program of research reported here comprised some of the initial work in this area and made a major contribution to the scientific evidence base in regard to wine allergens.

8.2 Ascertaining risk to consumers from wine fined with egg, fish, milk and nut-derived proteins

In order to be able to accurately assess the potential for true IgE-mediated allergic reactions from Australian wine to be elicited by eggs, fish, milk and nuts in an adult population, four studies were designed and executed. From the results of these studies, the risk associated with a defined level of residual egg, milk and nut in wine was assessed, and a safe limit for residual allergens in wine was identified.

The first step in this program of research involved the development of a mechanism to ensure the appropriate analysis of wines fined with egg white, milk or milk proteins casein and potassium caseinate. At the time that this program of work was instigated, there were no commercially-available assays such as ELISAs with which to measure residual protein that were specific to wine and sensitive to below the 1 mg/L level corresponding with calculated LOAELs for egg, fish, milk and nuts and the expected μg-ng/L residual levels in wine (Moneret-Vautrin and Kanny, 2004, Taylor et al., 2004, Hourihane et al., 2005). Therefore, the development of assays that could accurately and reliably measure residual egg, fish, milk and nut-derived proteins in wine was required. Ovalbumin and α-casein, the potent and predominant proteins in egg white and the milk-derived processing aids, respectively, were selected as markers for the presence of any potentially allergenic egg and milk protein in wine.

Having developed sensitive and specific ELISAs to detect and measure levels of the clinically relevant food allergens ovalbumin, casein and peanut-related proteins in wine, the next study undertaken applied the newly developed ELISAs to a diverse panel of 113 wines, 109 of which were protein-fined. This was to determine whether the newly

developed assays could detect residual fining agents in commercially-available bottled Australian wine. The study wines were unfined (n=4) or had been fined (n=109) with one or more of the potentially allergenic processing aids (egg white, isinglass, milk or milk proteins casein and potassium caseinate), or to which non-grape, nut-derived tannins had been added. That study found no residual protein was present in the protein-fined wine. The level of detection of the newly developed ELISAs was 1-8 ug/L. The lowest threshold dose for these potentially allergic food proteins is unknown however, and may be below these levels.

As the most rigorous way to determine whether food-allergic individuals suffer from a true food allergy is via a double blind placebo controlled food challenge (Bock et al., 1988), a food challenge with protein-fined wine was then undertaken. Confirmed egg, fish, milk and nut-allergic individuals (n=26) and non-food-allergic individuals (n=11) were challenged with 100 mL of both fined wine and unfined wine to determine whether wines which did not contain detectable residual protein could elicit an allergic reaction, and in particular, the most clinically significant and potentially life threatening response of anaphylaxis, in susceptible individuals. None of the 26 food-allergic or 11 non-food allergic individuals had any clinically significant adverse effects from the protein-fined wine.

Food challenges unfortunately expose the sensitive individual to the risk of clinically significant adverse reactions. This risk can be avoided by a simple and non-invasive basophil activation assay (BAT) which is a preferable alternative test to a food challenge and where *in vitro* and *in vivo* assays provide contradictory results or are unethical to perform (McGowan and Saini, 2013). Therefore, the final and fourth study

in this program of research was the development of a predictive BAT for egg, fish, milk and non-grape derived tannin. Allergenicity was then tested in a selected subset of the 109 commercially-available Australian wines fined with a potentially allergenic food protein as an alternative to a food challenge. While BAT were established for egg, fish, milk and non-grape derived tannin, the lower specificity than food challenge suggests that this assay alone would not be a reliable routine testing procedure as false positive or negative results could be provided.

8.3 What has this body of work ascertained?

This was the first body of work to establish mechanisms to determine risk of allergic responses to protein-fined wine, and to test for such responses in an Australian food-allergic adult population. It was also the first research to ascertain that for individuals with known allergies to egg, fish, milk or nuts, that protein-fined wine posed a low if not negligible risk of an IgE-mediated allergic reaction.

Three sensitive and specific ELISAs were developed first to detect, with greater precision than previously undertaken, the presence in wine of ovalbumin, α-casein and peanut-derived protein from the traditionally used proteinaceous processing aids. Prior to the development of these three ELISA, no such sensitive or specific assays for ovalbumin, α-casein and peanut-derived protein in wine previously existed commercially or had been previously published in the literature. These three ELISA remain among the most highly sensitive ELISA specific for the potent and predominant egg, milk and peanut-derived proteins developed to date, being at least 25-times more sensitive than those subsequently developed by Restani et al. (2012) and Uberti et al. (2014).

The high sensitivities of the ELISAs developed for egg, milk and peanut-related proteins in this body of work mean that if any residual fining agent is undetectable in wine analysed with these assays it is likely to only be present at low µgL levels. These low levels are unlikely to elicit an allergic reaction in the majority of egg, milk and peanut-allergic individuals according to the calculated 1–2 mg LOAEL for egg, milk and peanut, which represents approximately 100–200 µg of protein (Morriset et al., 2003; Moneret-Vautrin and Kanny, 2004).

When the three sensitive and specific ELISAs were applied to a diverse range of 113 commercially-available Australian wines, 109 of which had been protein-fined, no residual egg, milk or peanut-related proteins were observed except in the two commercially-available wines that had had whole egg added and were labelled as 'egg-marsala'. These 113 wines covered a spectrum of brands, producers, varieties and production protocols and were all made according to good manufacturing practice (GMP) being further fined and/or filtered prior to the final bottling. Therefore, given the diversity of the 113 wines analysed, the finding that minimal, if any, residual potentially allergenic protein remains in finished wine might be extrapolated to most filtered fined and unfined Australian wines made according to GMP with a high degree of confidence. These findings are also supported by the results of a subsequent study by Uberti et al. (2014) that used similar techniques and that found no residual egg proteins in 12 commercially-available filtered and fined Australian wines.

The double blind placebo controlled food challenge that was subsequently undertaken with 37 Australian adults, who were all regular and moderate consumers of wine, found

that none of the 26 individuals with confirmed allergy to these proteins exhibited a clinically significant adverse IgE-mediated allergic reaction (anaphylaxis or laryngeal oedema) on consumption of wine fined with the egg, milk, fish and/or nut-derived proteins made following established manufacturing best practice. Only one milk-allergic individual was recruited which reflects the rarity of IgE-mediated milk allergy in adults, and prevented reliable evaluation of residual allergenic milk proteins in wine by direct challenge. The rarity of IgE-mediated milk allergy in adults is not unique to Australia. Kirschner et al. (2009) in a study undertaken in Germany and Vassilopoulou et al. (2011) in a study undertaken in Greece, Iceland and Spain, were only able to recruit five milk-allergic subjects each for their respective studies involving food challenges and/or skin prick tests with protein-fined wine. However, the lowest dose of milk proteins causing either objective clinical responses or subjective symptoms in any food challenge observed in six studies reported in the literature was approximately 0.10 mg casein (Berstein et al., 1982; Wüthrich et al., 1986; Olalde et al., 1989; Pastrorello et al., 1989; Norgaard et al., 1992; Lam et al., 2008). This LOAEL is greater than the potential amount of residual casein in milk protein-fined wine as described in Chapter 4, and hence milk protein-fined wine is unlikely to elicit an allergic reaction in milk-allergic individuals.

The findings from the food challenge study reported here are consistent with findings from another more recent study of food challenge with protein-fined wine (Kirschner et al., 2009). No clinically significant life threatening adverse reactions were observed by Kirschner et al. (2009) in 14 adult food-allergic individuals in a food challenge with protein-fined wine and no mild subjective adverse reactions were observed.

In conjunction with the high sensitivity of the ELISA, lower than the calculated LOAEL for the majority of egg, fish, milk and peanut-allergic individuals, these findings strongly suggest that a very low risk exists for allergic reactions attributable to residual allergenic food proteins in the wine manufacturing process overseas as well as in Australia.

Although whole blood BAT for wine analysis were established to allow detection of egg white, fish, milk and peanut/tree nut allergenic proteins, which correlates closely with the food challenge results, its lower specificity than the clinical food challenge suggests that this assay alone would not be a reliable routine testing procedure. This lack of sensitivity is consistent with observations of Ocmant et al. (2009). Therefore, this program of work was able to indentify that the established BAT were not a suitable alternative to the clinical food challenge to determine whether protein-fined wines would elicit an allergic reaction in sensitive individuals, These BAT, however could be used as an adjunct to food challenges with wine, initially undertaken to indicate whether confirmatory food challenges with protein-fined wine should be performed (Sato et al. 2011).

Subsequent to this body of work, BAT were successfully commercially established and employed by Vassilopoulou et al. (2011). Although Vassilopoulou et al. (2011) did not detect any residual proteinaceous processing aid in any wine with ELISAs and PCR techniques, they did observe basophil activation in wines fined with excessive amounts of casein. This corresponded with significant basophil activation in the milk-allergic individuals, but only low magnitude basophil activation in egg and fish-allergic individuals with the respective fined wines. When surveyed, however, 89% of food-

allergic individuals had never experienced an adverse reaction to wine, consuming approximately 200 mL wine at least once per week. This implies that although protein-fined wine could still contain residual allergenic protein detected by BAT but not by sensitive in vitro techniques, BAT was not a reliable indicator of an allergic reaction to protein-fined wine.

Thus, this program of research provided the first empirical evidence that traditionally made Australian wine, using common food proteins according to good manufacturing practice, posed a low if not negligible risk of a life-threatening IgE-mediated adverse reaction for egg, milk, fish or nut-allergic Australian consumers. This finding has been subsequently confirmed for European consumers and wines in similar studies. It also provided background data about subjective and IgE-mediated life-threatening allergic reactions to a subset of protein-fined Australian wine in the Australian population. In addition, it provided data pertaining to the likely level of residual potentially allergenic protein in wine. Accordingly, these findings are suitable for use in a quantitative risk assessment (Crevel et al., 2014b), particularly as highly sensitive individuals were among the food challenge subjects.

It has generally been accepted that complete elimination of risk of an adverse reaction from incidental exposure to a food allergen is not feasible (Madsen et al. 2012). The development of appropriate risk management measures requires consensus on what is an acceptable degree of risk (Crevel et al., 2014b). Both frequency of adverse effects and the nature of those effects in regard to severity, duration and reversibility will determine acceptability or tolerability of risk. Severe and irreversible effects will be

tolerated to a lower extent than those with a lesser impact on health and safety (Madsen et al., 2012).

Furthermore, the present program of research has informed subsequent studies undertaken in France (Lilifrani et al., 2009), Germany (Weber et al., 2007a, Weber et al. 2007b; Weber et al., 2009; Kirschner et al. 2009; Deckwart et al. 2014), Greece (Vassilopoulou et al. 2011) and Italy (Restani et al. 2012a; Monaci et al., 2013; Uberti et al., 2014), which have all cited and built on this initial work with improved experimental protocols, with continued investigations into the risk of an allergic reaction from wine.

8.4 Next logical steps

As this body of work comprised studies involving a relatively small panel of wines and studies of a relatively small number of egg, fish, nut and particularly milk allergic individuals, the results cannot be confidently extrapolated to all wines and for all food-allergic consumers. This highlights that further research is undertaken to confirm the results of these studies employing larger panels of protein-fined wines made in Australia and overseas by different production protocols. Further studies of larger numbers of egg, fish, nut and, in particular, milk allergic individuals challenged with larger doses of wine are also suggested. These suggestions are elaborated upon below.

8.4.1. Analysis of wine to measure risk of residue

While the present research was able to establish sensitive and specific ELISAs for ovalbumin, α-casein and peanut-derived protein in wine, it was unable to establish ELISA for the other potential, but less abundant, egg white and milk proteins in wine.

These include ovomucoid, ovotransferrin and lysozyme from egg white, and β- and κ-casein and the whey proteins, α-lactalbumin and β-lactoglobulin, from milk or present as impurities in casein preparations. ELISAs for the detection of other allergenic milk proteins have since been developed and employed by Weber et al. (2009), Restani et al. (2012) and Deckwart et al. (2014). Weber et al. (2007b) and Uberti et al. (2014) also developed and employed ELISAs for other egg white proteins in wine.

This body of work was also unable to establish a sensitive and specific ELISA for isinglass in wine. Although mandatory labelling is no longer required by governments in Australia and the EU for the use of isinglass in winemaking or its presence in wine, Canada does require labelling if isinglass is present in filtered wine above 5 mg/L, and the USA permits voluntary labelling for allergens. Such an ELISA was subsequently established for isinglass in wine by Weber et al. (2010); no isinglass was detected in isinglass-fined wine that had been further bentonite-fined and filtered.

Alcohol, phenolic and other compounds in the wine matrix potentially interfere with the accurate detection of egg and milk proteins by ELISA, with concomitant risk of underestimation of residual protein present in a wine. This was described in Chapter 4 and by both Weber et al. (2007b) and Monaci et al. (2010). Non-immunological analytical methods based on mass spectrometry that could sensitively and simultaneously analyse for both egg and milk proteins in a wine have been investigated by Cereda et al. 2010, Monaci et al. (2010), D'Amato et al. 2010, Monaci et al. (2013) and Matarozzi et al. (2014) as potential alternatives to ELISA. Further investigation of such efficient mass spectrometry-based methods is thus warranted, together with comparisons between immunological and non-immunological methods to ensure

comparable results, so that these methods can be used interchangeably and with confidence by winemakers and surveillance national regulatory bodies.

The validation of commercially-available ELISA kits and other methods for egg white and casein in wine, should be undertaken by collaborative inter-laboratory studies involving multiple laboratories. Such validation studies were recently instigated in the EU by Restani et al. (2012b; 2014). Specific methods for the determination of potentially allergenic residues of fining agent proteins in wine are not prescribed in any national laws and regulations, and a range of ELISAs are already commercially available. Consequently in 2010, the Compendium of International Methods of Analysis[40] of the Organisation International de la Vigne et du Vin (OIV)[41] included reproducibility, repeatability and robustness criteria for methods of quantification of potentially allergenic residues of fining agent proteins in wine that was based on this body of work and the studies that followed.

Allied to this, is the importance of the pursuit of standardised commercially-available assays such as ELISAs for the routine analysis of potential allergens in wine and the interpretation of the results by analysts. Accordingly, the OIV has developed guidelines for the validation of ELISA kits to quantify potentially allergenic residues of fining agent proteins in wine by collaborative trials, and at the time of writing was preparing a conversion table for the specific egg and milk proteins.

[40] http://www.oiv.int/oiv/info/enplubicationoiv#compendium

[41] The International Organisation of Vine and Wine (OIV) is an intergovernmental organisation of a scientific and technical nature with recognised competence for its work concerning vines, wine, wine-based beverages, grapes, raisins and other vine products. It currently comprises 43 member states including 21 Members States of the European Union.

8.4.2 Winemaking practices and procedures to reduce risk of residue

Revisiting a question raised in Chapter 3 of this body of work as to what might happen to the allergenic proteins in the wine production process, the four studies in this body of work strongly suggest that they are substantially removed through the series of fining and filtration steps of the wine production process. These steps, however, were not able to be documented for the wines studied in Chapter 5. Furthermore, while Australian wine is made according to standard good manufacturing practices, but international winemaking practices may differ, particularly in regard to wine clarification fining and filtration practices, internationally-made wine may expose consumers to greater risk. Food challenges with wine undertaken to date have all used bentonite-fined and/or filtered wine post protein fining. For example, residual allergenic protein from was found in a percentage of French and German commercially-available wines which may have not bentonite-fined and/or filtered post protein-fining (Lilfrani et al., 2009; Weber et al., 2009; Weber et al., 2010), at a level which may elicit an allergic reaction in highly sensitive individuals.

By analysing a wine at each stage of the winemaking process, it would become evident as to which winemaking practices remove allergenic protein. Specific practices could correspondingly be codified and considered mandatory by government and/or industry to ensure that risk is negligible to consumers. Initial investigations of the influence of winemaking practices on the content of residual protein in protein-fined wines have been undertaken by Weber et al. (2007a), Weber et al. (2009), Weber et al. (2010), Restani et al. (2012), Uberti et al. (2014) and Deckwart et al. (2014). Practices such as bentonite-fining combined with different filtration techniques appear critical to the removal of residual protein from wine post protein fining and pre bottling. While these practices are considered integral to good manufacturing practice for wine in Australia,

there is no standardised protocol, or procedure for documenting such processes, and hence detailed analysis of the steps that could be included in a refined or standardised protocol is required.

Based on the collective findings of all the investigations undertaken on this issue, including this body of work, in 2014 the Organisation International de le Vigne et du Vin (OIV) established a Code of Good Fining Practice for wine to be applied after the use of proteinaceous [allergenic] wine fining agents [casein and egg white]. This Code stipulates that the filtering of wine post protein fining where the analysis of casein and egg white proteins should always be undertaken on the finished wine product.

8.4.2 Double blind placebo controlled food challenge to assess risk of an allergic reaction

Similar to the other food challenge studies with wine reported in the literature, only low numbers of adult food-allergic individuals could be recruited for the study reported in Chapter 6. These low numbers, particularly of individuals with IgE-mediated egg and milk allergy, allowed only limited investigation of the potential for a clinical challenge with a wine fined with an associated allergenic food protein to induce an adverse clinical response. A multi-national and multicentre study of adults with confirmed IgE-mediated food allergy would strengthen the findings and conclusions cautiously drawn to date. Such a study, with expanded subject numbers could also consider including naive wine consumers in addition to regular wine consumers which have only recruited to date. Further, all studies to date have documented both subjective and objective adverse reactions elicited by wine but have not clarified whether consumers are really prepared to accept the risk of minor, not clinical significant and non life threatening adverse reactions.

Furthermore, as a critical component of risk assessment is exposure (Crevel et al., 2014b), concerning an acceptable or tolerable risk by consumers, four studies in this body of work only assessed the likelihood of a life threatening IgE-mediated allergic reaction occurring from the ingestion of one standard, 10 g alcohol, drink of protein-fined wine; this is a relatively conservative low challenge dose. Consequently, the risk of a range of mild subjective to more severe objective adverse reactions occurring when more than one drink is ingested was not explored. Data from the Australian Institute of Health and Welfare's 2013 National Drugs Strategy Household Survey [42] report that 20% of Australian consumers aged 18-69 years drink two or more standard drinks per day, and approximately 20-25% drink more than four drinks on an occasion at least once a month. Thus, a further studies should be conducted that challenge food allergic consumers with escalating doses from one to four standard drinks to ascertain the risk from this heavier consumption. Furthermore, in real life unlike clinical food challenges, thresholds and subsequent adverse reactions may also be influenced by a number of known and unknown factors such as exercise, infection and medication use and concomitant allergen exposures such as to pollen (Cianferoni and Spergel, 2009; Cochrane et al., 2012; Crevel et al., 2014b). Accordingly, the quantification of these factors will require further research before the current findings from clinical settings can be extrapolated with complete certainty to community settings, and estimates of population-eliciting doses are correspondingly conservatively calculated (Crevel et al., 2014b).

[42] http://www.aihw.gov.au/alcohol-and-other-drugs/ndshs/2013/alcohol/

8.6 Implications of this body of work for public health policy

Avoidance diets are the primary approach for prevention of an allergic reaction (Taylor et al. 1986). Consequently, regulatory risk management strategies for food allergic individuals have predominantly focused on communication of the presence of food allergens via packaged food label declarations. To effectively manage an allergy, food allergic individuals are dependent on the availability, accuracy and quality of information provided in foods that they want to buy and consume. Australia was the first country to label for the 10 major or priority allergens in food. A number of other countries and regulatory bodies have since recognized the importance of providing this information by enacting laws, regulations or standards for food allergen labelling. They have, however, taken different approaches to designing labelling declaration regulatory frameworks (Gendel, 2012, Allen et al. 2014b). Food allergic consumers increasingly appear to ignore these labelling declarations (Helfe et al., 2007), which may reflect concern of the accuracy of declarations especially those that use the precautionary phrase 'may contain' and are ambiguous and meaningless (Barnett et al., 2011a, 2011b).

Only a small number of governments and national regulatory bodies have actually addressed the issue of food allergen risk assessment and risk management for wine apart from labelling declarations (Allen et al., 2014b). despite recognising that complete absence of risk is unrealistic (Madsen et al., 2010; Madsen et al., 2021), no internationally consistent regulations have been established for maximum tolerable levels of allergen in wine above which labelling declarations are mandatory, but below which labelling is not necessary. This has resulted in analytical limits of detection for food allergens often being adopted as defacto thresholds by both national regulatory agencies and food producers. Low µg/L level defacto threshold can be a more

conservative assessment of risk than that ascertained from a food challenge which is generally mg/L (Threshold Working Group, 2008), and hence unrelated to real life. This problem is exacerbated by improvements in analytical techniques and technologies such that lower and lower levels of allergen can be detected (Diaz-Amigo and Popping 2010).

Standard 1.2.3 of the Australia New Zealand Food Standard Code requires food producers to declare the presence of a food when detected. With the advent of commercially-available analytical techniques with lower limits of detection, wine producers often declare 'may contain', or 'made with egg or milk products and trace may remain'. This is precautionary labelling with limited usefulness for food-allergic wine consumers. Accordingly, national regulatory bodies such as FSANZ need to provide guidance on maximum tolerable levels of allergen in wine below which labelling is unnecessary, and above which 'contains' can be accurately and meaningfully declared.

Whilst not provided in Australia as yet for wine, the results generated in this and other subsequent studies have been translated by national bodies in Canada and the EU into their regulations, providing such guidance. For example, after reviewing the published literature, in 2012 Health Canada[43] concluded that "the use of allergen-derived fining agents does not normally result in any appreciable amount of protein from food allergens remaining in the wine, particularly when usual manufacturing practices such as filtration steps are employed. As such, the use of food allergen-derived fining agents in wine production, following good manufacturing practices, is not expected to produce

[43] http://www.hc-sc.gc.ca/fn-an/label-etiquet/allergen/vintage-wine-vin-millesimes-eng.php

wine that would pose a risk to egg, milk, or fish allergic consumers. Therefore, in most cases of allergen-derived fining agent use, the new labelling regulations for priority allergens would not be triggered. However, if the use of a food allergen-derived fining agent resulted in a wine which contained a significant amount of residual protein from the food allergen, such as a wine that was not filtered following the addition of the fining agent, then there could be some risk to an allergic consumer and the new allergen labelling regulations would apply" (Health Canada, 2012). The significant amount of residual protein and thus threshold level has been deemed to be between 1 and 5 mg/L, which corresponds with the LOD of certain commercially-available ELISA.

The EU in Commission Implementing Regulation (EU) no. 579/2012 of 29 June 2012[44], however, has adopted the threshold limits prescribed in the OIV resolutions 427–2010 (OIV 2010) modified by OIV/COMEX 502–2012 (OIV 2012), as its default threshold limit. The regulation states that "It is therefore necessary to establish detailed rules for labelling these beverages, including a mention of the substances referred to in Annex IIIa to Directive 2000/13/EC and used when making the beverages, if their presence can be detected in the final product using the analysis methods referred to in Article 120g of Regulation (EC) No 1234/2007 and if they consequently must be considered ingredients within the meaning of Article 6(4)(a) of Directive 2000/13/EC." Effectively this means that protein-fined wine is considered negative for the presence of residues when analytical methods are used with detection and quantification limits of 0.25 and 0.5 ppm, respectively, according to the analytical requirements that were defined in the OIV resolutions.

[44] Commission Implementing Regulation (EU) no. 579/2012 of 29 June 2012. Official Journal of the European Union L171/4 dated 30.6.2012

In addition, the present work has informed and was cited by the 2014 draft *Scientific opinion on the evaluation of allergenic foods and food ingredients for labelling purposes*[45] by the EFSA Panel on Dietetic Products, Nutrition and Allergies.

Although no similar threshold limit has been prescribed for wine in Australia, the present results supported a successful application (A490) from the New Zealand Brewer's Association to amend the Table to clause 4 of Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations of the ANZFSC to exempt isinglass from labelling on beer and wine product labels when used as a clarifying agent. Specially, the supporting submission suggested that exposure to isinglass through the consumption of clarified or fined wine would be very low and, based on the oral food challenge studies, would not be expected to provoke reactions in fish-allergic wine consumers. Therefore, FSANZ considered that consumption of isinglass-fined wine was not likely to present a risk of allergic reactions in fish-allergic consumers. Subsequently, the isinglass exemption for beer and wine labelling was gazetted on 28 May 2009[46] as follows: "Schedule [1] Standard 1.2.3 is varied by omitting from the Table to clause 4, the entry for Fish and fish products, substituting – Fish and fish products, except for isinglass derived from swim bladders and used as a clarifying agent in beer and wine".

Additional risk management measures that could be considered by regulatory bodies include determining critical control points in food production, such as that being defined in the OIV Code of Good Fining Practice for wine to be applied after the use of

[45] http://www.efsa.europa.eu/fr/consultations/call/140523.pdf

[46] http://www.foodstandards.gov.au/_srcfiles/Gazette%20Notice%20Amendment%20No%20108%20WEB%20VERSION.pdf

proteinaceous [allergenic] wine fining agents [casein and egg white], in order to contain potential contamination or alternatively to remove residual allergens as in wine production have been prepared and published by the UK Food Standards Agency (2006, 2013).

An adjunctive approach, however, to labelling declarations for wine and guidance on critical control points in wine production is to ensure adequate awareness and training of winemakers, to provide safe wine products to their allergic and non-allergic consumers alike.

8.7 Conclusions

Given the increasing incidence of food allergy worldwide, combined with the globalisation of our food supply, it has become important to evaluate foods in the food supply that could elicit and allergic reaction in food allergic individuals. This had not previously been undertaken for wine, which can be produced with proteinaceous processing aids, and thus be a hidden source of allergens.

A series of four studies were the first undertaken to ascertain the risk of an allergic reaction in sensitive individuals from Australian wine fined with egg, fish or milk and products derived thereof, and/or to which nut-derived non-grape tannins were added. The four studies comprised: the development of sensitive and specific ELISAs for the candidate allergens in wine; the analysis of a diverse panel of 113 wines, 109 of which were produced with these proteinaceous processing aids; the development of an alternative *in vitro* assay (BAT) to predict the potential allergenicity of protein fined-

wines; and a food challenge of protein-fined and un-fined wines in 37 individuals, 26 of whom were food-allergic.

Sensitive and specific ELISAs were developed for the most abundant potent egg and milk allergens, and for peanut-derived allergens in wine. The level of detection was between 1-8 µg/L and is among the lowest for such assays. When the ELISA were applied to the panel of wines, no residual egg, milk or peanut-related protein was detectable in the protein-fined wines. Residual egg was only detected in two wines to which whole eggs had been added and these wines were labelled as containing egg. In the food challenge with protein-fined and unfined wines, no clinically significant life threatening adverse reactions were elicited by the wines in the 37 individuals. The subsequently developed BAT was, however, insufficient sensitive to be an alternative to the ELISA or BAT but may be considered as an adjunctive tool to predict potential allergenicity.

In risk assessment terms, the relationship of the analytical values of residual allergen in wine to the human threshold data from food challenge studies described in the literature review (Chapter 3, Section 3.5.1), suggests that the µg/L amounts of residual ovalbumin, α-casein and peanut-derived protein in protein-fined Australian wine made according to good manufacturing practice is below the threshold values of a life threatening allergic reaction for egg, fish, milk and nut-allergic adults who consume light to moderate amounts of wine. Therefore, there is low if not negligible risk of an allergic reaction in an adult food allergic population to Australian wine fined with egg, fish or milk and products derived thereof, and/or to which nut-derived non-grape tannins were added.

In view of the globalisation of the food supply, good practice in allergen risk management also needs to be implemented consistently across all jurisdictions worldwide by all stakeholders. This body of work can, and has been, used to inform a variety of stakeholders. It has informed national and international public health policy on food allergens in wine as to what may be appropriate risk management approaches to ensure the healthy and safety of food-allergic individuals, such as regulations relating to mandatory allergen labelling. This included the revoking of mandatory allergen labelling for isinglass-fined wine in Australia and the EU, as well as the establishment of appropriate levels of detection and quantification of residual food protein as determined by ELISAs and other analytical methods in Canada and the EU.

Remaining to be undertaken is research into wine production, as to which practices and procedures should be performed to remove residual protein from wine, and proscribed in codes of good fining/manufacturing practice for wine. It is also important to validate the assays developed and the food challenge data in larger groups of food allergic individuals and in different populations with different extrinsic, environmental and genetic risk factors for food allergy, and with multiple doses, that is, standard drinks of wine in amounts above moderation. The aim of ongoing research and the provision of increasingly robust data into the risk of an allergic reaction from protein-fined wine, is to provide an accurate assessment for food allergic individuals. An outcome of regular reviews of public health policy regarding this risk should be clear and credible communication to protect allergic wine consumers.

In conclusion, this body of research is the first to ascertain and inform public health policy that Australian wine made with traditional proteinaceous processing aids, according to good manufacturing practice, poses little risk to the health of food-allergic adult consumers.

Appendices 1-7

Appendix 1 Declaration

Role and responsibilities of Creina Stockley

PhD Project Task/Responsibility	Percentage of CSS contribution
The initial concept originated with Creina Stockley and the full project was based on her original concept.	100%
Scientific literature review	100%
Instigated collaboration with The Alfred and Monash University	100%
Grant proposal preparation and submission Creina Stockley was formally co-chief investigator with Professor Robyn O'Hehir of The Alfred, and co-supervisor with Professor Robyn O'Hehir of The Alfred and A/Prof Jenny Rolland of Monash University.	75%
Chapter 1 Introduction to general concepts and conclusions	100%
Chapter 2 Risk analysis concepts for food and wine	100%
Chapter 3 Analysis of risk related to potential allergens in food and wine	100%
Chapter 4 (Study 1) Development of ELISA for detection of proteinaceous allergens in wine including collection and assessment of wine panel Project design Project supervision Wine sample collection and analysis Laboratory ELISA experiments Data collection Data analysis Data interpretation Conclusions drawn Discussion	 50% 33.3% 100% 5% 20% 33.3% 33.3% 70% 90%
Chapter 5 (Study 2) Analysis of wine by ELISA and other relevant wines analyses Project design Project supervision Wine sample collection and analysis Laboratory ELISA experiments Data collection Data analysis Data interpretation Conclusions drawn Discussion	 50% 33.3% 100% 5% 20% 33.3% 33.3% 70% 90%

Chapter 6 (Study 3) DBPCFC study for protein-fined wine Project design Project supervision Wine sample analysis Clinical challenges Data collection Data analysis Data interpretation Conclusions drawn Discussion	 50% 33.3% 100% 20% 5% 33.3% 33.3% 70% 90%
Chapter 7 (Study 4) Development of BAT and other relevant wine analyses Project design Project supervision Wine sample analysis Laboratory BAT experiments Data collection Data analysis Data interpretation Conclusions drawn Discussion	 50% 33.3% 100% 5% 5% 33.3% 33.3% 70% 90%
Chapter 8 Risk assessment for wine based on the 4 studies and translation of research to practice The program of research was used to design further clinical and experimental studies, and facilitate and support changes to national and international regulations and standards on potential allergens in wine.	100%
Preparation of peer-reviewed papers in international journals CSS co-authored two manuscripts reporting the results of the above studies and provided critical appraisal of data presentation and interpretation.	50%
Preparation of project reports	100%
Presentation of results at international conferences and meetings	100%

Project group

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18 June, 2014

Ref: Creina Stockley PhD Thesis Submission, Flinders University SA

We write in support of the PhD thesis submission by Creina Stockley. We confirm that Creina was instrumental in the initiation of our joint research project investigating the potential presence of residual food allergens in wine, and that she played a major role in all steps of the project from literature review and experimental design to data interpretation and presentation as well as report and manuscript preparation. She was essential for the collection and blinding of wine samples for the study, for obtaining chemical analyses on the wines, and for the industry perspective on relevant risk assessment issues.

We consider it appropriate that this joint study forms the basis of Creina's PhD thesis and that the percentage contributions by Creina in the table showing her roles and responsibilities are reasonable. With the substantial introductory review material in chapters 1-3 and the final risk assessment chapter written entirely by Creina, as well as additional discussion sections for each experimental chapter prepared by Creina, we believe that overall, it can be considered that at least 50% of each chapter is Creina's sole contribution.

At all times, Creina's contribution to the project was extremely competent, efficient and professional and we give our unreserved support to her PhD application.

Yours sincerely,

Professor Jennifer Rolland
Professor Robyn O'Hehir
Department of Immunology, Monash University, and
Department of Allergy, Immunology and Respiratory Medicine, Monash University and Alfred Hospital

Appendix 2 Description of the panel of 113 Australian wines

Wine sample number	Processing aid*	Addition rate (mg/L)	Timing of addition	Grape 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	Red
29	1		Pre cold	Red

			stabilisation	
30	5		During fermentation and clarification	Red
31	3		During fermentation	White
32	2 4		Pre clarification and fermentation During cold stabilisation	White
33	2		Pre clarification and fermentation	White
34	4		During cold stabilisation	White
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 1			Shiraz
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	200		Merlot

	3	50		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	Shiraz

			stabilisation, pre filter and 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
110	Control	—	Not applicable	Riesling
111	Control	—	Not applicable	Riesling
112	Control	—	Not applicable	Grenache
113	Control	—	Not applicable	Grenache

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

Appendix 3 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
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	129	2	1	nd	nd

					urticaria, facial angioedema					
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 4 Eligibility criteria for subject selection

Wine Challenge Candidates Questionnaire Form

No.	Question	Response
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 5 Exclusion criteria for subject selection

Wine challenge study: Data collection sheet – baseline
Medical Condition Exclusions Form

No.	Medical Condition	Exclusions (x [cross], if absent)
1.	asthma	☐
2.	urticaria	☐
3.	angioedema	☐
4.	GIT upset	☐
5.	URTI	☐
6.	anti-histamines in last 2 days	☐
7.	ventolin use in last 4 hours	☐
8.	seretide/oxis in AM	☐
9.	alcohol in last 3 days	☐
10.	ischaemic heart disease	☐
11.	stroke	☐
12.	diabetes mellitis	☐
13.	epilepsy	☐
14.	renal failure	☐
15.	liver disease	☐

Appendix 6 Visual analogue scale (VAS)

Wine challenge study: Data collection sheet – baseline
Visual analogue scale (VAS) History Results Form

None	Visual analogue scale (VAS)	Worst
Not wheezy at all		Worst possible wheeze
No chest tightness at all		Worst possible chest tightness
Not short of breath at all		Worst possible shortness of breath
No cough at all		Worst possible cough
No difficulty swallowing		Worst possible difficulty swallowing
No lump in throat at all		Worst possible lump in throat
No nausea at all		Worst possible nausea
No itch at all		Worst possible itch
No body swelling at all		Worst possible body swelling
No light headedness		Worst possible light headedness

Appendix 7 Physical Examination

Wine challenge study: Data collection sheet – baseline
Physical Examination Results Form

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		

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