Residual Protein and Potential Allergenicity in Processed Products from Allergenic Source Materials

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RESIDUAL PROTEIN AND POTENTIAL ALLERGENICITY IN PROCESSED PRODUCTS FROM ALLERGENIC SOURCE MATERIALS 2010-2011

Prepared for Ministry of Agriculture and Forestry
under project CFS 10/06 – Evidence for Allergenicity of Processed Foods,
as part of overall contract for scientific services

Client Report No. FW11045

by

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August 2011

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SUMMARY

The Australia New Zealand Food Standards Code requires labelling of all foods containing ingredients, ingredients of compound ingredients, food additives or components of food additives, or processing aids or components of processing aids from specified source materials. The regulatory process allows parties to seek an exemption from the mandatory labelling requirements of the standard if it can be demonstrated that the inclusion of material from an allergenic source is not likely to present a risk of allergic reactions in allergic consumers.

The current project analysed three food products from allergenic sources for residual levels of protein. These food products represent three distinct food processes; recrystallisation, distillation and oil extraction and refining. Results were placed in context by summarising previous literature information on allergenicity and protein content of equivalent products and the conclusions of EFSA regulatory assessments.

Recrystallisation

Doubly recrystallised lactose from whey

Evidence from current analytical investigations

Testing of ten doubly recrystallised refined edible lactose samples, produced in New Zealand, found whey-specific protein at concentrations in the range 1.9-5.3 mg/kg in nine out of ten samples. Total soluble protein (Bradford method) was detected in all samples, with concentrations in the range 5.9-11.3 mg/kg. While there is limited literature information on the protein content of lactose from whey, these concentrations appear to be consistent with or lower than previous reports.

Evidence from the literature and other sources

Clinical trials with lactose or lactitol (a polyol derived from lactose) administered to cows' milk allergy cases produced no allergic response following oral administration, although the amount of protein ingested was not reported in some studies.

No regulatory assessments of the allergenic potential of lactose from whey have been carried out. The EFSA Panel on Dietetic Products, Nutrition and Allergies has assessed an application for exemption from allergen labelling requirements for lactitol and concluded that it is not very likely that lactitol will trigger adverse reaction in cow's milk allergic individuals under the conditions of use specified by the applicant. The reported protein content of lactitol samples was similar to those seen in doubly recrystallised lactose in the current study.

Distillation

Distilled ethanol from wheat starch

Evidence from current analytical investigations

Testing of ten food-grade grain ethanol samples, produced in Australia, did not detect gluten protein (limit of detection = 1 mg/L) or general soluble protein (limit of detection = 0.12 mg/L). These results are consistent with the results of protein testing reported in the scientific literature and in an EFSA assessment.

Analytical evidence supports the proposition that gluten proteins and peptides are not carried over in the distillation process and are not present in grain ethanol.

Evidence from the literature and other sources

No reports of clinical trials on the allergenicity of grain ethanol or spirits derived from grain ethanol were found in the scientific literature. The EFSA Panel on Dietetic Products, Nutrition and Allergies has assessed an application for exemption from allergen labelling requirements for cereals used in distillates for spirits and concluded that proteins and peptides are not carried over into the distillate during a properly controlled distillation process, at least not in amounts higher than 1 mg/L for total proteins and 0.4 mg/kg for gluten. The panel considered that distillates made from cereals are unlikely to trigger severe allergic reactions in susceptible individuals.

Oil extraction and refining

Refined oil from soybeans

Evidence from current analytical investigations

Testing of six retail samples of soy oil, produced in Australasia, did not detect soy-specific protein (limit of detection = 1 mg/kg). The Bradford general soluble protein method did not detect protein in any oil sample above the method limit of detection (0.5 mg/kg). The scientific literature includes reports of quite widely varying protein concentrations for soybean oil. However, the results of the current study are largely consistent with recent reports for refined soybean oil, which generally contain lower protein concentrations than crude or cold-pressed soybean oils.

Evidence from the literature and other sources

Several clinical studies have been carried out involving administration of soybean oil to soy allergic individuals, with no adverse effects reported. One study included a ‘worst case’ dose scheme involving ingestion of 84 ml of soybean oil. While some mild symptoms were reported by participants, symptoms were no more prevalent following soybean oil administration than following control administration.

EFSA Panel on Dietetic Products, Nutrition and Allergies has assessed applications for labelling exemption on fully refined soybean oil and fat on two occasions. The notifications covered edible neutralised (alkali refined) bleached and deodorised (N/RBD) soybean oils and hydrogenated and interesterified soybean oils and fats. In the most recent opinion, EFSA considered information on the protein content of N/RBD soybean oil and, more particularly, two clinical challenge studies. The Panel considered that it is not very likely that N/RBD soybean oils will trigger a severe allergic reaction in susceptible individuals under the conditions of production and use stated by the applicant.

1 INTRODUCTION

The Australia New Zealand Food Standards Code requires labelling of all foods containing ingredients, ingredients of compound ingredients, food additives or components of food additives, or processing aids or components of processing aids from specified source materials (FSANZ, 2009a). The source materials specified in Standard 1.2.3 are:

- Cereals containing gluten and their products, namely, wheat, rye, barley, oats and spelt and their hybridised strains other than where these substances are present in beer and spirits standardised in Standards 2.7.2 and 2.7.5 respectively;
- Crustacea and their products;
- Eggs and egg products;
- Fish and fish products, except for isinglass derived from swim bladders and used as a clarifying agent in beer and wine;
- Milk and milk products;
- Peanuts and soybeans and their products;
- Added sulphite in concentrations of 10 mg/kg or more; and
- Tree nuts and sesame seeds and their products other than coconut from the fruit of the palm *Cocos nucifera*.

The regulatory process allows parties to seek an exemption from the mandatory labelling requirements of the standard if it can be demonstrated that the inclusion of material from an allergenic source is not likely to present a risk of allergic reactions in allergic consumers. FSANZ has already assessed an application and granted an exemption for the use of isinglass as a fining agent in the production of beer and wine (FSANZ, 2009b).

Allergic reactions occur through an immunological response to specific proteins from the allergenic source material. Exemption from allergen labelling requirements for processed foods from allergenic sources is generally sought on the basis that food processing has reduced the concentration of allergenic proteins (or proteins in general) in the product to a point where they no longer constitute a risk of eliciting an allergic reaction under likely conditions of use.

1.1 Evidence of Potential Allergenicity

Two sources of exemplar assessments of potential allergenicity of products were identified:

- The FSANZ assessment of isinglass (FSANZ, 2009b); and
- Various assessments carried out by the European Food Safety Authority's (EFSA) Scientific Panel on Dietetic Products, Nutrition and Allergies (http://www.efsa.europa.eu/EFSA/ScientificPanels/efsa_locale-1178620753812_NDA.htm)

Aspects covered by the assessments listed above include:

- Characteristics of the allergy (prevalence, natural history) and the allergen (concentration and distribution in the source material, physical and chemical characteristics);
- Characteristics of the source material and processes used to derive the product to be assessed from the source material.

- Methods and analytical data on protein, and particularly allergenic protein, from the source material in the finished product.
- Clinical information on the allergenicity (or non-allergenicity) of the product under assessment from skin prick tests or oral challenges. Literature searches to demonstrate lack of reported cases of allergic reaction to the product have also been presented as evidence, although this is generally viewed as weak evidence, as products assessed are rarely consumed as foods on their own.
- Estimates of potential dietary exposure to allergens through consumption of the product, through normal use patterns

The current study will, wherever possible, present or produce information on these aspects for the products under consideration that is relevant in the context of allergenicity.

1.2 Products to be Considered

Three products were chosen for the current project year, specifically:

- Lactose from milk
- Alcohol from grain
- Refined oil from soy

These food products represent three distinct food processes; recrystallisation, distillation, and oil extraction and refining.

2 RECRYSTALLISATION

The product considered in this section is lactose derived from whey, a milk fraction.

2.1 Background Information

2.1.1 Cows' milk proteins

Bovine milk contains 3-3.5% protein with the proteins divided into two main classes, caseins that constitute approximately 80% of the total milk proteins and whey proteins, that make up the remaining 20% (Monaci *et al.*, 2006). Whey proteins remain soluble after acidic precipitation of casein at pH 4.6.

Casein proteins are made up of a number of classes designated αS1 (approximately 40% of total casein), αS2 (12.5%), β (35%), κ (12.5%) and γ-caseins (Monaci *et al.*, 2006). The γ-caseins are secondary products, formed by proteolytic cleavage of β-caseins.

The protein in whey is more homogeneous, with 50% of the protein contributed by β-lactoglobulin (β-LG), a 18.3 kDa lipid-binding protein (Monaci *et al.*, 2006). Other whey proteins include α-lactalbumin (25%), bovine serum albumin (5%), immunoglobulins (6%) and lactoferrin.

2.1.2 Cows' milk allergens

The most abundant proteins in cows' milk have also been demonstrated to be the major allergenic proteins, including caseins, lactoglobulins and α-lactalbumin (Monaci *et al.*, 2006).

2.1.3 Prevalence of cows' milk allergy (CMA)

CMA has been reported to be the most common food allergy in infants and young children (Skripak *et al.*, 2007). Estimates of self-reported hypersensitivity to cows' milk in the very young of greater than 10% have been reported in some studies (Rona *et al.*, 2008). However, objective assessment based on food challenges has generally given point estimates in the range 0.3-5% (Cressey, 2007; Rona *et al.*, 2008), with a prevalence of 2-3% being widely accepted (Skripak *et al.*, 2007). Most of the very young who develop CMA will outgrow the allergy by about three years of age (Skripak *et al.*, 2007). Estimates of the prevalence of CMA in adult populations are generally less than 0.5% (Cressey, 2007; Rona *et al.*, 2008).

2.1.4 Source material (whey)

The term whey is used to refer to the liquid remaining after milk has been curdled and strained. As such, it is a common by-product of cheese or casein production. Utilisation of this whey is a significant issue, as approximately 9 kg of whey are produced for every 1 kg of cheese produced (González Siso, 1996). Whey has a biological oxygen demand (BOD) of 35-45 g/L, largely due to its lactose content, which makes its disposal as an effluent stream problematic (Mawson, 1994)

Whey typically contains 0.5-0.6% protein and 4-5% lactose (Archer, 1998). Key compositional aspects of whey from various production sources are given in Table 1.

Table 1: Typical composition of New Zealand wheys (Mawson, 1994)

Component	Concentration (%w/w)			
	Cheddar cheese whey	**Lactic acid casein whey**	**Sulphuric acid casein whey**	**Rennet casein whey**
Total solids	5.6	5.6-6.4	6.3	5.8-6.5
Protein	0.55	0.56	0.56	0.62-0.73
Lactose	4.0	3.8-4.4	4.7	4.5-5.2
Ash	0.5	0.66-0.76	0.8	0.42-0.49
Lactate	0.08	0.63-0.73	-	0.02

2.2 Lactose Doubly Recrystallised from Whey

Whey is produced as a by-product from cheese or casein production. In the late 1990s, New Zealand produced approximately four billion litres of whey (Archer, 1998). Whey can be deproteinated to produce whey protein concentrate (WPC) or lactalbumin (Archer, 1998). The remaining liquor contains approximately 4-5% lactose, (Hamilton, 1998; Mawson, 1994). Lactose production from whey in New Zealand has increased from approximately 29,000 tonnes in the late 1990s[1] to 110,000 tonnes in 2006 (Affertsholt-Allen, 2007).

Lactose is recovered from whey by crystallisation, following concentration of the solids in the whey (Kellam, 1998). The lactose is then redissolved with heating and purified before being recrystallised.

2.2.1 Evidence from current analytical investigations

Ten samples (approximately one kilogram each) of refined edible lactose were obtained from a New Zealand manufacturer. Each sample was from a different production day during the period 17-30 January 2011. Refined edible lactose is used mainly for nutritional products. Pharmaceutical grade lactose differs little in composition, but is whiter and is milled to a more specific particle size.

All samples were analysed for residual protein by Enzyme-Linked Immunosorbent Assay (ELISA), and Bradford colourimetric method. Details of analytical methods used are included in Appendix 1.

2.2.1.1 ELISA

All samples were analysed by Biokit β-lactoglobulin (β-LG) ELISA. The analytical procedure was validated by spike recovery from a lactose matrix. Spike recoveries were within acceptable limits, as defined by the kit manufacturer. The limit of detection for the method was 1.3 mg/kg.

[1] http://www2.stats.govt.nz/domino/external/web/nzstories.nsf/0/b88ff0f2aa375339cc256b1f0000ebc1?OpenDocument

Low concentrations of whey protein (β-LG) were detectable in nine of the 10 samples. Concentrations were in the range 1.9-5.3 mg/kg, with a mean for the nine samples of 3.2 mg/kg.

2.2.1.2 Micro protein (Bradford method)

All samples were prepared as 2% lactose and were spiked with bovine serum albumin (BSA) at a concentration of 4 mg/kg. Spike recoveries were in the range 87.8-103.1% (mean = 93.6%). All samples were analysed in triplicate. The limit of detection of the method was in the range 3.7-4.3 mg/kg, based on the standard deviation of blank determinations.

Protein was detected at concentration above the limit of detection in all samples. Concentrations were in the range 5.9-11.3 mg/kg, with a mean for the 10 samples of 8.7 mg/kg.

2.2.2 Evidence from the literature and other sources

2.2.2.1 Previous regulatory assessments

No regulatory assessments of the potential allergenicity of lactose derived from whey were found.

EFSA has twice considered applications for exemption from labelling for lactitol, a polyol sweetener produced by reduction of lactose (EFSA, 2004c;2007b). In the more recent assessment, the applicant based evidence that lactitol preparations do not trigger cow's milk allergic reactions on the low residual milk protein levels in lactitol preparations and a double blind placebo controlled food challenge (DBPCFC) with five cow's milk allergic children, which found no adverse reactions to lactitol. The scientific panel considered that "it is not very likely that lactitol will trigger adverse reaction in cow's milk allergic individuals under the conditions of use specified by the applicant" (EFSA, 2007b).

Concentrations of milk proteins in lactitol (up to 3.2 mg/kg casein and up to 9.7 mg/kg β-LG) were lower than concentrations reported for the corresponding lactose (up to 9.7 mg/kg casein and up to 118 mg/kg β-LG). The lactose used in the production of lactitol was reported to be of pharmaceutical grade or food grade (recrystallised, refined or purified) (EFSA, 2004c). The concentration of β-LG measured in lactitol is slightly higher than that measured in lactose samples in the current study.

2.2.2.2 Clinical studies

Twenty-four children with confirmed immediate CMA were assessed for clinical tolerance to cow's milk, soy infant formula and soy infant formula with added lactose (Fiocchi *et al.*, 2003). Lactose, extracted from cow's milk whey, was added to soy infant formula either for skin prick testing (lactose concentrations of 0.01, 0.1, 1 and 10%) or double-blind placebo-controlled food challenge (DBPCFC; maximum lactose dose of 11.6 mg). Challenges with soy infant formula containing lactose were negative in all cases. The lactose was reported to

contain no residual protein, as determined by SDS-PAGE and immunoblotting. The limits of detection of these methods were not reported.

In a clinical study, lactitol derived from lactose, containing 3.2 mg/kg casein protein and 9.7 mg/kg β-LG protein, was used in challenge studies with five patients with CMA (EFSA, 2007b). None of the patients developed signs or symptoms of an allergic reaction during or after the lactitol challenge. However, the dose of lactitol used in challenges was not reported.

Reactions to injected medications, containing lactose as an excipient, have been reported in patients with CMA (Eda *et al.*, 2009). The β-LG content of the lactose was reported as 1.35 mg/kg. However, the authors commented that direct injection of lactose containing any amount of milk protein would be likely to produce an adverse reaction, while the equivalent amount of protein would usually be safe if exposure was by ingestion.

Lactose is also used as an excipient in dry powder inhalers (DPIs) used by asthmatics (Nowak-Wegrzyn *et al.*, 2004). These products represent potential for both inhalation and ingestion exposure, as more than 98% of inhaled lactose settles in the oropharynx and is swallowed (Nowak-Wegrzyn *et al.*, 2004). While one study identified no reactions attributable to milk proteins in 21 patients with CMA using lactose-containing inhalers (Spiegel and Anolik, 2010), cases of allergic reaction to lactose-containing DPIs have been reported (Morisset *et al.*, 2006; Nowak-Wegrzyn *et al.*, 2004). Milk proteins have been detected in extracts from DPIs, with whey proteins present at higher levels than casein (Nowak-Wegrzyn *et al.*, 2002). However, actual protein concentrations were not reported.

2.2.2.3 Protein content of lactose from whey

Lactose used in the production of the polyol lactitol was reported to be of pharmaceutical grade or food grade (recrystallised, refined or purified) (EFSA, 2004c). The protein content of lactose used in this process was usually in the range 50-200 mg/kg, although some batches contained up to 500 mg/kg.

ELISA analysis of lactose for casein and β-LG proteins found 1.4-9.7 mg/kg casein and 11.8-118 mg/kg β-LG (EFSA, 2004c).

Analysis of peptide fragments in one lactose sample by Matrix-Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) mass spectrometry demonstrated that all peptides present had molecular weights less than 2600 Da, with most less than 1500 Da (EFSA, 2007b). While molecular weights depend on the particular amino acids present, peptides in this size range are likely to be no more than 10-20 amino acids in length.

2.2.3 Conclusions

Testing of ten doubly recrystallised lactose samples, produced in New Zealand, found whey-specific protein at concentrations in the range 1.9-5.3 mg/kg in nine out of ten samples. Total soluble protein (Bradford method) was detected in all samples, with concentrations in the range 5.9-11.3 mg/kg. While there is little literature information on the protein content of lactose from whey, these concentrations appear to be consistent with or lower than previous reports for protein in lactose.

Clinical trials with lactose or lactitol (a polyol derived from lactose) administered to cows' milk allergy cases produced no allergic response following oral administration, although the amount of protein ingested was not reported in some studies.

No regulatory assessments of the allergenic potential of lactose have been carried out. The EFSA Panel on Dietetic Products, Nutrition and Allergies has assessed an application for exemption from allergen labelling requirements for lactitol and concluded that it is not very likely that lactitol will trigger adverse reaction in cow's milk allergic individuals under the conditions of use specified by the applicant. The reported protein content of lactitol samples was similar to those seen in doubly recrystallised lactose in the current study.

3 DISTILLATION

The product considered in this category is ethanol produced from wheat starch (grain ethanol).

3.1 Background Information

Two distinct immunologically-mediated diseases are associated with ingestion of proteins from wheat and some related cereals. Wheat allergy is an IgE-mediated 'classical' food allergy, while Coeliac disease is an autoimmune inflammatory response in the small intestine leading to nutrient malabsorption (EFSA, 2004d).

3.1.1 Wheat proteins

Wheat proteins are conventionally classified according to their solubility, molecular weight, function and location within the wheat grain. Albumins (water soluble) and globulins (salt soluble) are generally functional (enzymes, etc.) low-molecular proteins, concentrated in the bran and germ of the wheat grain and constituting approximately 20% of total grain protein (EFSA, 2004d). The remainder of wheat protein is referred to as gluten protein and is involved in the formation of the rubbery gluten complex that enables wheat's use for breadmaking. Gluten proteins are the major storage proteins of the wheat grain (Battais *et al.*, 2008). Gluten contains approximately equal amounts of alcohol soluble gliadin proteins and alcohol insoluble glutenin proteins (EFSA, 2004d). Gliadin is monomeric, while glutenin is a highly viscous, heterogeneous polymer. These are high molecular weight storage proteins and are located predominantly in the starchy endosperm of the wheat grain. Consequently, gluten proteins are the main proteins present in white wheaten flour.

Gliadins are further sub-divided into α, β, γ and ω-gliadins. These classes have decreasing electrophoretic mobility or increased molecular weight, respectively. The subunits of the glutenin polymers are classified as either high molecular weight (HMW) or low molecular weight (LMW).

3.1.2 Wheat allergens

3.1.2.1 Wheat allergy

A number of proteins have been identified as allergens. Identification is generally by binding to IgE from individuals with wheat allergy. Identified allergens include water/salt-soluble proteins of the α-amylase/trypsin inhibitor family, with molecular weights of 12-17 kDa, and lipid transfer proteins, with molecular weights of 7-9 kDa (Battais *et al.*, 2008).

While a wide range of gliadins and glutenins have been associated with wheat allergy, there is evidence to suggest two different profiles of wheat allergy. Water/ salt-soluble proteins and α, β and γ gliadins appear to be more important allergens for children, while ω-gliadins are the major wheat allergens for adults (Battais *et al.*, 2008).

3.1.2.2 Coeliac disease

The role of wheat in Coeliac disease has been shown to be due to the proline and glutamine-rich gliadins, particularly the α, γ and ω-gliadins (EFSA, 2004d). A 33 amino acid peptide with high resistance to protease enzymes has been identified and is believed to be a primary initiator of the inflammatory response in Coeliac disease (Shan *et al.*, 2002). Food grain homologues to this peptide were only identified in wheat gliadin, barley hordeins and rye secalins. Wheat, barley and rye may all elicit adverse reactions in Coeliac disease sufferers.

3.1.3 Prevalence of disease

3.1.3.1 Wheat allergy

Despite the huge quantity of cereals consumed worldwide, cereal allergies in adults are reported to be rare (EFSA, 2004d). Estimates of the prevalence of wheat allergy in children (0-14 years) have ranged from 0.0 to 0.5% (Zuidmeer *et al.*, 2008).

3.1.3.2 Coeliac disease

General estimates for prevalence of Coeliac disease in Europe of approximately one in 200 (0.5%) have been made (EFSA, 2004d). Prevalence of Coeliac disease in the Asia-Pacific region has been reported to be in the range 0.2-2.0% (Cummins and Roberts-Thomson, 2009).

Several estimates of the prevalence of Coeliac disease in New Zealand have been made (Carrington *et al.*, 1987; Cook *et al.*, 2000; Cook *et al.*, 2004; Ussher *et al.*, 1994). Estimates have generally increased over time, but it is uncertain whether this reflects a true increase in prevalence or improvements in detection and diagnosis. A large long-term study in Christchurch estimated the prevalence of Coeliac disease to be in the range 0.6-1.2% (Cook, 2000).

3.1.4 Source Material (Wheat Starch)

In general terms, wheat starch is produced by mixing milled flour to produce a dough, followed by washing of the dough to separate starch and solubles from gluten. Starch is then separated from the solubles by sieving and refining. While the majority of the wheat proteins will be in the insoluble gluten or the solubles phase, some protein will remain in the starch. Protein in starch has been described as composed of two main categories (Kasarda *et al.*, 2008):

- Internal (intrinsic) proteins. Mainly proteins involved in starch synthesis (e.g. starch synthases); and
- Surface-associated proteins. A diverse array of storage proteins (gluten proteins) and proteins involved in the management of biotic and abiotic stresses.

The protein content of commercial wheat starch, determined by oxidation/combustion (Leco), has been reported to be in the range 0.11-0.20% (1100-2000 mg/kg) (Kasarda *et al.*, 2008). Skerritt and Hill found a wider range of protein contents, determined by Kjeldahl analysis, in

starches (0.20-0.54%), but noted that the 'first 0.25%' did not appear to be gluten (storage) proteins, as determined by ELISA (Skerritt and Hill, 1992).

3.2 Ethanol from Wheat Starch

Carbohydrate-rich residues from the gluten-starch separation process undergo enzymatic hydrolysis, followed by fermentation and distillation. The distillate is further purified to increase the alcohol content.

3.2.1 Evidence from current analytical investigations

Ten samples of finished grain ethanol were provided by an Australian manufacturer, following a FSANZ visit. Samples were taken during the period 21-27 April 2011 and included five samples of 95% alcohol and five samples of 100% alcohol.

All samples were analysed for residual protein by ELISA and Bradford colourimetric method. Details of analytical methods used are included in Appendix 1.

3.2.1.1 Gluten ELISA

The gluten ELISA method has been previously validated (Cressey and Jones, 2005). Applicability of the method to grain alcohol was confirmed by adding the gluten control material provided with the kit to alcohol samples. Results were in the acceptable range for the gluten control. The gluten ELISA method has a limit of detection of 1 mg/L.

No samples contained detectable gluten above the detection limit of 1 mg/L.

3.2.1.2 Micro protein (Bradford method)

Ethanol interferes with the Bradford micro protein method. All samples were evaporated to dryness and the residues extracted with phosphate-buffered saline, containing 0.2% Triton X. All samples were spiked with bovine serum albumin (BSA; approximately 4 mg/kg) to determine the efficiency of protein recovery. Spike recoveries were in the range 68-103%.

Protein was not detected in any ethanol sample above the method limit of detection of 0.12 mg/kg.

3.2.2 Evidence from the literature and other sources

3.2.2.1 Previous regulatory assessments

EFSA has considered information provided by the European Spirits Organisation (CEPS) on two occasions on cereals used in distillates for spirits (EFSA, 2004b;2007a). The opinion of the EFSA panel was largely the same on both occasions and at the latter assessment it was concluded that "proteins and peptides are not carried over into the distillate during a properly controlled distillation process, at least not in amounts higher than 1 mg/L for total proteins and 0.4 mg/kg for gluten. The panel considers that distillates made from cereals are unlikely to trigger severe allergic reactions in susceptible individuals" (EFSA, 2007a).

3.2.2.2 Clinical studies

The most recent EFSA assessment of grain ethanol reported that no systematic skin prick testing studies, DBPCFC studies or epidemiological studies had been reported on adverse immunological reactions to grain ethanol (EFSA, 2007a). A review of the subsequent scientific literature using the SCOPUS[2] and PUBMED[3] databases did not identify any more recent clinical investigations.

3.2.2.3 Protein content of distilled grain ethanol

Residual solids were determined gravimetrically in two grain distillates (one from corn mash and one from rye mash) (Campbell, 1988). Residues were less than 0.2 mg/L. The authors concluded that the protein content of the spirits would be less than 0.05 mg/L.

ELISA analysis of dried residues of ethanol derived from wheat did not detect gluten above a detection limit of 0.006 mg/L (Oldani *et al.*, 2001).

ELISA tests on material from several points in the distillation process for neutral grain spirits and from three crude alcohol samples concluded that concentrations of cereal proteins were less than 2.4-3.1 mg/kg (EFSA, 2007a).

As part of an industry application to EFSA, information was provided on analysis of 39 bottled products and 76 samples of distillates produced using cereal as a raw material (EFSA, 2007a). Of these, 86 samples were analysed for total protein and 45 samples were analysed for gluten. Total protein was determined by the Bradford microassay (Bradford, 1976). Samples with a positive response in the Bradford assay were also tested by the AAA Direct analysis[4]. The methods both have limits of detection for protein of approximately 0.5 mg/L. Fifteen samples gave positive responses under the Bradford assay. These were confirmed by the AAA Direct assay, with protein concentrations in the range 0.5-1 mg/L. One sample had a measured protein concentration greater than 1 mg/L (1.3 mg/L), but this was not confirmed by repeat analysis. No samples tested positive for gluten by ELISA, with a limit of detection of 10 mg/kg.

3.2.3 Conclusions

Testing of ten food-grade grain ethanol samples, produced in Australia, did not detect gluten protein (limit of detection = 1 mg/L) or general soluble protein (limit of detection = 0.12 mg/L). These results are consistent with the results of protein testing reported in the scientific literature and in an EFSA assessment.

Analytical evidence supports the proposition that gluten proteins and peptides are not carried over in the distillation process and are not present in grain ethanol.

[2] http://www.scopus.com/home.url
[3] http://www.ncbi.nlm.nih.gov/pubmed/
[4] http://www.dionex.com/en-us/webdocs/7442-AN163_V22.pdf

No reports of clinical trials on the allergenicity of grain ethanol or spirits derived from grain ethanol were found in the scientific literature. The EFSA Panel on Dietetic Products, Nutrition and Allergies has assessed an application for exemption from allergen labelling requirements for cereals used in distillates for spirits and concluded that proteins and peptides are not carried over into the distillate during a properly controlled distillation process, at least not in amounts higher than 1 mg/L for total proteins and 0.4 mg/kg for gluten. The panel considered that distillates made from cereals are unlikely to trigger severe allergic reactions in susceptible individuals.

4 OIL EXTRACTION AND REFINING

The product considered in this section is refined edible oil from soybean (*Glycine max*).

4.1 Background Information

4.1.1 Soybean proteins

Soybeans contain approximately 40% protein, on a dry-weight basis (Koppelman *et al.*, 2004). Proteins in soybean include:

- Metabolic proteins;
- Structural proteins; and
- Storage proteins.

In soybeans the storage proteins are globulin (salt-soluble) proteins and make up 80-90% of the total proteins in the soybean (Koppelman *et al.*, 2004). Soybean storage proteins are conventional classified on the basis of their sedimentation coefficients (a measure of the rate at which particles sediment under defined conditions e.g. centrifugation in a defined buffer, at a defined temperature) into four groups; 2S, 7S, 11S and 15S (EFSA, 2004d). These fractions make up approximately 8-22%, 35%, 31-52% and 5% of the soluble soybean protein, respectively. Alternatively, soybean globulins can be classified on the basis of immunological differences into glycinin (in 11S), β-conglycinin (in 7S), trypsin inhibitors (in 2S) and other less abundant proteins (EFSA, 2004d; Koppelman *et al.*, 2004). Glycinin and β-conglycinin are the dominant seed storage proteins, making up approximately 40% and 25% of total protein, respectively (Gagnon *et al.*, 2010).

4.1.2 Soybean allergens

Allergenicity of a number of soybean proteins has been established through *in vitro* IgE-binding studies (EFSA, 2004d). The International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee recognizes six allergenic proteins from soybeans[5]. Details of these proteins are given in Table 2.

Table 2: Recognised allergens from soybean (*Glycine max*)

Allergen designation	Protein	Molecular weight (kDa)	Reference
Gly m 1	Hydrophobic lipid transfer protein	8	(Gonzalez *et al.*, 1991)
Gly m 2	Defensin (storage protein)	8	(Codina *et al.*, 1997)
Gly m 3	Profilin	14	(Rihs *et al.*, 1999)
Gly m 4	PR-10 protein (Kunitz trypsin inhibitor)	20	(Moroz and Yang, 1980)
Gly m 5	β-conglycinin	140-180	(Ogawa *et al.*, 1995)
Gly m 6	Glycinin	320-360	(Djurtoft *et al.*, 1991)

[5] http://www.allergen.org/

A further soybean protein not included in the IUIS database has been designated Gly m Bd 30K and is a 30 kDa vacuolar serine protease (Ogawa *et al.*, 1995).

4.1.3 Prevalence of soybean allergy

Little information is available on the prevalence of soybean allergy in the general population. However, where available, estimates of self-reported soybean allergy and soybean allergy determined by DBPCFC are similar and usually less than 1% (Zuidmeer *et al.*, 2008). Two challenge studies established soybean allergy in 0/486 (0.0%, 95th percentile confidence interval 0.0-0.8%) Danish children (3 years of age) (Osterballe *et al.*, 2005) and in 4/598 (0.7%, 95th percentile confidence interval 0.2-1.7%) German children (14 years of age or less) (Roehr *et al.*, 2004). Two studies in children in the US found soybean allergy in 3/632 (0.5%, 95th percentile confidence interval 0.1-1.4%) (Halpern *et al.*, 1973) and in 4/480 (0.8%, 95th percentile confidence interval 0.2-2.1%) of cases (Bock, 1987).

Studies of self-reported soybean allergy in adults generally produced estimates of prevalence of less than 0.1% (Björnsson *et al.*, 1996; Niestijl Jansen *et al.*, 1994; Osterballe *et al.*, 2005; Yoneyama and Ono, 2002).

4.1.4 Source material

Soy oil is extracted from flaked soybeans.

4.2 Refined Oil from Soybeans

Soy oil is usually extracted from flaked or sliced soybeans by solvent extraction, usually with hexane. After removal of the solvent, the crude oil is further purified by various processes, which may include:

- Filtration
- Degumming
- Neutralisation
- Bleaching
- Deodorisation

4.2.1 Evidence from current analytical investigations

Samples of soy oil (2 L) from an Australasian manufacturer were obtained from retail sources. Over a sampling period of five months, six unique batches were found, as identified by ‘best before’ dates. The best before dates covered a period of greater than one year.

All samples were analysed for residual protein by ELISA and Bradford colourimetric method. Details of analytical methods used are included in Appendix 1.

4.2.1.1 Soy ELISA

The soy ELISA method has been previously validated (Cressey and Jones, 2005). The method was further validated for use with soy oil as the test matrix, by spiking soy oil with

non-fat soy flour to a soy protein content of 3.5 mg/kg. Soy protein was successfully recovered from the soy oil. The soy ELISA method has a limit of detection of 1 mg/kg.

Soy protein was not detected in any soy oil sample analysed above the limit of detection of 1 mg/kg.

4.2.1.2 Micro protein (Bradford method)

The Bradford micro protein method cannot be performed directly on an oil substrate. Protein was recovered from oil samples using the low temperature acetone precipitation method (Paschke *et al.*, 2001; Rigby *et al.*, 2011).

This method demonstrated 95% recovery of a 3.5 mg/kg soy protein spike (non-fat soy flour).

Soy protein was not detected in any soy oil sample above the method limit of detection (0.5 mg/kg)

4.2.2 Evidence from the literature and other sources

4.2.2.1 Previous regulatory assessments

EFSA have considered information provided by FEDIOL (EC Seed Crushers' and Oil Processors Federation) and IMACE (International Margarine Association for the Countries of Europe) on fully refined soybean oil and fat on two occasions (EFSA, 2004a;2007d). The notifications covered edible neutralised (alkali refined), bleached and deodorised (N/RBD) soybean oils and hydrogenated and interesterified soybean oils and fats.

In the earlier opinion, the EFSA Panel expressed concerns about:

- Insufficient clinical characterisation of patients studied in clinical challenge trials and their clinical reactivity to soy at the time of the challenge;
- Absence of N/RBD soybean oil dose escalation studies in highly allergic individuals;
- Absence of clinical studies with N/RBD soybean oils in highly peanut allergic patients;
- Reliability and validity of methods used for protein determination in oils; and
- Absence of statutory and voluntary protein limits in N/RBD soybean oils.

In the most recent opinion, EFSA considered information on the protein content of N/RBD soybean oil and, more particularly, two clinical challenge studies, which largely addressed the concerns raised at the earlier assessment. The Panel considered that "it is not very likely that N/RBD soybean oils will trigger a severe allergic reaction in susceptible individuals under the conditions of production and use stated by the applicant". Despite the concerns raised, this same conclusion was expressed in the earlier EFSA assessment.

4.2.2.2 Clinical studies

Seven patients with a history of immediate hypersensitivity reaction to soybean were recruited from allergy clinics (Bush *et al.*, 1985). Three challenge soybean oils (a partially hydrogenated oil, a non-hydrogenated oil and a cold-pressed oil) and a placebo (olive oil)

were placed in separate gelatin capsules (1 ml of oil per capsule). Patients were challenged with each of the four oils in a randomised, double-blinded scheme with six days between challenge days. Oils were administered in a dose escalation manner; receiving 2, 5 and then 8 ml doses, with each dose followed by a 30 minute observation period. No adverse responses to any of the soy oil varieties or olive oil were seen at any of the dose levels used. The protein content of the oils was not reported.

Twenty nine patients (1.1-59 years old) were recruited in a multi-centre study, based on; a convincing history of soybean allergy (29), a positive DBPCFC (5), a positive IgE CAP-RAST (7) or a positive skin test (20) (EFSA, 2007d). No adverse reactions were reported to challenge with soybean oil up to a 16 ml cumulative dose. The protein content of the challenge oil was not reported.

Two further clinical studies were conducted in response to concerns raised by EFSA's Scientific Panel on Dietetic Products, Nutrition and Allergies (EFSA, 2007d):

- A dose escalation study in peanut-allergic individuals to determine whether N/RBD soybean oil represents a risk in the event of cross-allergenicity or cosensitisation to soy; and
- A dose escalation study in soy-allergic individuals, using worst-case intake dose levels.

N/RBD soybean oils with the highest protein concentrations were blended and used as challenge material. The protein content of the blended material was 150 µg/kg. N/RBD rapeseed oil was used as a control. Challenge doses of 12, 24 and 48 ml of oil were administered at 30 minute intervals, with at least three hours between active challenge series and control series. The full active challenge of 84 ml of N/RBD soybean oil delivered approximately 12 µg of soy protein. Consumption of 84 ml of N/RBD soybean oil was considered to be a worst case scenario. Administration of active and control challenge oil series was randomised across participants.

Amongst the peanut-allergic cohort (30 individuals, 8-57 years), 28 completed full challenge with soybean oil and 27 with control oil. Mild symptoms (itch in the mouth, hoarseness, nausea) were experienced by two individuals following active challenge and by four individuals following control administration. The EFSA Panel considered that these results were difficult to interpret.

Amongst the soy-allergic cohort (32 individuals, 12-62 years), 27 challenged with soybean oil and 24 challenged with control reported no symptoms at all. Two reported mild symptoms with both soybean and control challenge (oral allergy syndrome; OAS). Three reported OAS with soybean challenge only, while three reported OAS with control only. A further three reported gastrointestinal symptoms with control only. The EFSA Panel did not consider that the reported symptoms indicated severe allergic reactions.

Minimum eliciting doses of soy protein were determined for the soy-allergic cohort and were in the range 1.5-11.47 mg. This is 2-3 orders of magnitude higher than the worst case dose (12 µg) used in the challenge studies.

4.2.2.3 Protein content of soybean oil

Table 3 summarises literature information on the protein content of soybean oils.

Table 3: Protein content of soybean oil

Description of oil	Analytical method	Results	Reference
Soy oil, refined and deodorised	Solvent fractionation. Amino acid analysis.	0.96 mg/kg	(Tattrie and Yaguchi, 1973)
Soy oil, nfd	ELISA	Soy protein detected in 3/8 oils (110- 3,300 mg/kg)	(Porras *et al.*, 1985)
Soy oil, crude (A) and refined (B)	Extraction with PBS, Bradford protein method	A = 1.9 mg/kg B = 0.72 mg/kg	(Klurfeld and Kritchevsky, 1987)
Soy oil, nfd	Extraction with saturated ammonium sulphate, solvent precipitation. Lowry protein method.	Mean = 0.023 mg/kg (n=5, range 0.014-0.040 mg/kg)	(Awazuhara *et al.*, 1998)
Soy oil, refined and crude	Acetone precipitation, PBS extraction. Bradford protein method.	Crude: 0.090-0.138 mg/kg (n=3) Refined: 0.033-0.035 mg/kg (n=2)	(Paschke *et al.*, 2001)
Soy oil, deodorised (A) and cold pressed (B)	PBS extraction. Protein determination method not stated.	A = 0.32 mg/L B = 1.8 mg/L	(Errahali *et al.*, 2002)
Soy oil, cold pressed	Solvent extract-precipitation, dissolution in 6M HCl, amino acid analysis	1.44 mg/kg (n=1)	(Martin-Hernandez *et al.*, 2008)
Soy oil refined	Extraction with PBS, dialysis. Bradford or bicinchoninic acid protein method or total amino acid analysis.	0.16-0.19 mg/kg (n=2, colourimetric) 0.96-1.66 mg/kg (n=2, total amino acid)	(Ramazzotti *et al.*, 2008)
Soy oil -Crude degummed -Neutralised -Neutralised, bleached -Neutralised, bleached, deodorised	Extraction with borate buffer. CBQCA protein / total amino acid analysis	 0.3-16.2 /ND-18.6 mg/kg 0.06-1.7/0.1-5.4 mg/kg 0.03-0.32/0.03-2.9 mg/kg 0.05-0.70/0.03-0.43 mg/kg	(Rigby *et al.*, 2011)

nfd = not further described PBS = Phosphate buffered saline

4.2.2.4 Dietary intake of soy oil

An estimate of potential dietary exposure to neutralised (alkali refined), bleached and deodorised (N/RBD) soy oil was included in the 2007 EFSA assessment (EFSA, 2007d). This estimate was based on consumption of a meal containing a serving of the four main foods in which soy oil is used (margarine, salad dressing, French fries and mayonnaise). This approach produced an estimate for soy oil consumption from a hypothetical meal of 80.5 g. Using a protein level of 0.15 mg/kg, this equates to intake of 12.1 µg of soy protein.

An analysis of data from the 1997 National Nutrition Survey (Russell *et al.*, 1999) by the Australia New Zealand Food Authority (now FSANZ), using a standard set of recipes,

concluded that an average adult New Zealander consumer of soy oil would consume 9.7 g/day, while a high consumer (97.5th percentile) would consume 55.8 g/day (ANZFA, 2001).

4.2.3 Conclusions

Testing of six retail samples of soy oil, produced in Australasia, did not detect soy-specific protein (limit of detection = 1 mg/kg). The Bradford general soluble protein method did not detect protein in any oil sample above the method limit of detection (0.5 mg/kg). The scientific literature includes reports of quite widely varying protein concentrations for soybean oil. However, the results of the current study are largely consistent with recent reports for refined soybean oil, which generally contain lower protein concentrations than crude or cold-pressed soybean oils.

Several clinical studies have been carried out involving administration of soybean oil to soy allergic individuals, with no adverse effects reported. One study included a 'worst case' dose scheme involving ingestion of 84 ml of soybean oil. While some mild symptoms were reported, they were no more prevalent following soybean oil administration than following control administration.

EFSA Panel on Dietetic Products, Nutrition and Allergies has assessed applications for labelling exemption on fully refined soybean oil and fat on two occasions. The notifications covered edible neutralised (alkali refined) bleached and deodorised (N/RBD) soybean oils and hydrogenated and interesterified soybean oils and fats. In the most recent opinion, EFSA considered information on the protein content of N/RBD soybean oil and, more particularly, two clinical challenge studies. The Panel considered that it is not very likely that N/RBD soybean oils will trigger a severe allergic reaction in susceptible individuals under the conditions of production and use stated by the applicant.

5 REFERENCES

Affertsholt-Allen T. (2007) Market developments and industry challenges for lactose and lactose derivatives. Accessed at: http://lactose.ru/present/1Tage_Affertsholt-Allen.pdf. Accessed: 8 July 2011.

ANZFA. (2001) Raw commodity consumption figures. Canberra: ANZFA.

Archer RH. (1998) Whey products. Accessed at: http://nzic.org.nz/ChemProcesses/dairy/3G.pdf. Accessed: 4 December 2009.

Awazuhara H, Kawai H, Baba M, Matsui T, Komiyama A. (1998) Antigenicity of the proteins in soy lecithin and soy oil in soybean allergy. Clinical and Experimental Allergy; 28(12): 1559-1564.

Battais F, Richard C, Jacquenet S, Denery-Papini S, Moneret-Vautrin DA. (2008) Wheat grain allergies: an update on wheat allergens. European Annals of Allergy and Clinical Immunology; 40(3): 67-76.

Björnsson E, Janson C, Plaschke P, Norrman E, Sjoberg O. (1996) Prevalence of sensitization to food allergens in adult Swedes. Annals of Allergy, Asthma and Immunology; 77(4): 327-332.

Bock SA. (1987) Prospective appraisal of complaints of adverse reactions to foods in children during the first 3 years of life. Pediatrics; 79(5): 683-688.

Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry; 72(1-2): 248-254.

Bush RK, Taylor SL, Nordlee JA, Busse WW. (1985) Soybean oil is not allergenic to soybean-sensitive individuals. Journal of Allergy and Clinical Immunology; 76(2, Part 1): 242-245.

Campbell JA. (1988) The question of gluten in distilled alcohol. Journal of the Canadian Dietetics Association; 49(3): 204.

Carrington JM, Hewitt CJ, Dowsett LR, Barbezat GO. (1987) The prevalence of coeliac disease in Otago. New Zealand Medical Journal; 100(828): 460-462.

Codina R, Lockey RF, Fernandez-Caldas E, Rama R. (1997) Purification and characterization of a soybean hull allergen responsible for the Barcelona asthma outbreaks. II. Purification and sequencing of the Gly m 2 allergen. Clinical and Experimental Allergy; 27(4): 424-430.

Cook HB. (2000) Coeliac disease: A New Zealand perspective. Australian Journal of Medical Science; 21(2): 60.

Cook HB, Burt MJ, Collett JA, Whitehead MR, Frampton CM, Chapman BA. (2000) Adult coeliac disease: Prevalence and clinical significance. Journal of Gastroenterology and Hepatology; 15(9): 1032-1036.

Cook HB, Oxner RB, Chapman BA, Whitehead MR, Burt M. (2004) A thirty-year (1970-1999) study of coeliac disease in the Canterbury region of New Zealand. New Zealand Medical Journal; 117(1189): U772.

Cressey P, Jones S. (2005) Food allergen capability development. Summary of investigations 2004-2005. ESR Client Report FW0569. Christchurch: ESR.

Cressey P. (2007) Stochastic risk model: Allergens in processed foods. ESR Client Report FW0732. Christchurch: ESR.

Cummins AG, Roberts-Thomson IC. (2009) Prevalence of celiac disease in the Asia-Pacific region. Journal of Gastroenterology and Hepatology; 24(8): 1347-1351.

Djurtoft R, Pedersen HS, Aabin B, Barkholt V. (1991) Studies of food allergens: Soybean and egg proteins. Advances in Experimental Medicine and Biology; 289: 281-293.

Eda A, Sugai K, Shioya H, Fujitsuka A, Ito S, Iwata T, Funabiki T. (2009) Acute allergic reaction due to milk proteins contaminating lactose added to corticosteroid for injection. Allergology International; 58(1): 137-139.

EFSA. (2004a) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from FEDIOL and IMACE on fully refined soybean oil and fat pursuant to Article 6, paragraph 11 of Directive 2000/13/EC. Accessed at: http://www.efsa.europa.eu/en/scdocs/doc/147.pdf. Accessed: 14 December 2010.

EFSA. (2004b) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from CEPS on distillates made from cereals pursuant to Article 6 paragraph 11 of Directive 2000/13/EC. EFSA Journal; 130: 1-6.

EFSA. (2004c) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from EPA on lactitol pursuant to Article 6 paragraph 11 of Directive 2000/13/EC. EFSA Journal; 158: 1-6.

EFSA. (2004d) 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. EFSA Journal; 32: 1-197.

EFSA. (2007a) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from CEPS on cereals used in distillates for spirits, pursuant to Article 6 paragraph 11 of Directive 2000/13/EC. EFSA Journal; 484: 1-7.

EFSA. (2007b) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from EPA on lactitol pursuant to Article 6, paragraph 11 of Directive 2000/13/EC - for permanent exemption from labelling. EFSA Journal; 565: 1-6.

EFSA. (2007c) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to the notification from CEPS on whey used for spirits pursuant to Article 6 paragraph 11 of Directive 2000/13/EC. EFSA Journal; 483: 1-6.

EFSA. (2007d) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from FEDIOL and IMACE on fully refined soybean oil and fat pursuant to Article 6, paragraph 11 of Directive 2000/13/EC - for permanent exemption from labelling. EFSA Journal; 570: 1-9.

Errahali Y, Morisset M, Moneret-Vautrin DA, Kanny G, Metche M, Nicolas JP, Frémont S. (2002) Allergen in soy oils. Allergy; 57(7): 648-649.

Fiocchi A, Restani P, Leo G, Martelli A, Bouygue GR, Terracciano L, Ballabio C, Valsasina R. (2003) Clinical tolerance to lactose in children with cow's milk allergy. Pediatrics; 112(2): 359-362.

FSANZ. (2009a) Australia New Zealand Food Standards Code. Accessed at: http://www.foodstandards.gov.au/_srcfiles/Standard_1_3_1_Additives_Part_1_v111.pdf. Accessed: 30 March 2008.

FSANZ. (2009b) Final assessment report. Application A490. Exemption of allergen declaration for isinglass. Accessed at: http://www.foodstandards.gov.au/_srcfiles/A490%20Isinglass%20FAR%20FINAL.pdf#search=%22isinglass%22. Accessed: 3 December 2009.

Gagnon C, Poysa V, Cober E, Gleddie S. (2010) Soybean allergens affecting North American patients identified by 2D gels and mass spectrometry. Food Analytical Methods; 3(4): 363-374.

Gonzalez R, Zapatero L, Caravaca F, Carreira J. (1991) Identification of soybean proteins responsible for respiratory allergies. International Archives of Allergy and Applied Immunology; 95(1): 53-57.

González Siso MI. (1996) The biotechnological utilization of cheese whey: A review. Bioresource Technology; 57(1): 1-11.

Halpern SR, Sellers WA, Johnson RB, Anderson DW, Saperstein S, Reisch JS. (1973) Development of childhood allergy in infants fed breast, soy, or cow milk. Journal of Allergy and Clinical Immunology; 51(3): 139-151.

Hamilton R. (1998) The manufacture of ethanol from whey. Accessed at: http://nzic.org.nz/ChemProcesses/dairy/3H.pdf. Accessed: 4 December 2009.

Kasarda DD, Dupont FM, Vensel WH, Altenbach SB, Lopez R, Tanaka CK, Hurkman WJ. (2008) Surface-associated proteins of wheat starch granules: Suitability of Wheat starch for celiac patients. Journal of Agricultural and Food Chemistry; 56(21): 10292-10302.

Kellam S. (1998) The manufacture of lactose. Accessed at: http://nzic.org.nz/ChemProcesses/dairy/3F.pdf. Accessed: 3 November 2010.

Klurfeld D, Kritchevsky D. (1987) Isolation and quantitation of lectins from vegetable oils. Lipids; 22(9): 667-668.

Koppelman SJ, Lakemond CM, Vlooswijk R, Hefle SL. (2004) Detection of soy proteins in processed foods: literature overview and new experimental work. Journal of AOAC International; 87(6): 1398-1407.

Martin-Hernandez C, Benet S, Obert L. (2008) Determination of proteins in refined and nonrefined oils. Journal of Agricultural and Food Chemistry; 56(12): 4348-4351.

Mawson AJ. (1994) Bioconversions for whey utilization and waste abatement. Bioresource Technology; 47(3): 195-203.

Monaci L, Tregoat V, van Hengel A, Anklam E. (2006) Milk allergens, their characteristics and their detection in food: A review. European Food Research and Technology; 223(2): 149-179.

Morisset M, Moneret-Vautrin D, Commun N, Schuller A, Kanny G. (2006) Allergy to cow milk proteins contaminating lactose, common excipient of dry powder inhalers for asthma. Journal of Allergy and Clinical Immunology; 117(2, Supplement 1): S95-S95.

Moroz LA, Yang WH. (1980) Kunitz soybean trypsin inhibitor. A specific allergen in food anaphylaxis. New England Journal of Medicine; 302(20): 1126-1128.

Niestijl Jansen JJ, Kardinaal AFM, Huijbers G, Vlieg-Boerstra BJ, Martens BPM, Ockhuizen T. (1994) Prevalence of food allergy and intolerance in the adult Dutch population. Journal of Allergy and Clinical Immunology; 93(2): 446-456.

Nowak-Wegrzyn A, Shapiro GG, Beyer K, Bardina L, Sampson HA. (2004) Contamination of dry powder inhalers for asthma with milk proteins containing lactose. Journal of Allergy and Clinical Immunology; 113(3): 558-560.

Nowak-Wegrzyn AH, Bardina L, Beyer K, Shreffler WG, Sampson HA. (2002) Detection of milk proteins in dry powder inhalers containing lactose. Journal of Allergy and Clinical Immunology; 109(1, Supplement 1): S258-S259.

Ogawa T, Bando N, Tsuji H, Nishikawa K, Kitamura K. (1995) Alpha-subunit of beta-conglycinin, an allergenic protein recognized by IgE antibodies of soybean-sensitive patients with atopic dermatitis. Bioscience, Biotechnology and Biochemistry; 59(5): 831-833.

Oldani A, Iametti S, Bonomi F. (2001) In alcoholic cereal derived beverages are there still gluten traces? Tecnica Molitoria: 641-646.

Osterballe M, Hansen TK, Mortz CG, Host A, Bindslev-Jensen C. (2005) The prevalence of food hypersensitivity in an unselected population of children and adults. Pediatric Allergy and Immunology; 16(7): 567-573.

Paschke A, Zunker K, Wigotzki M, Steinhart H. (2001) Determination of the IgE-binding activity of soy lecithin and refined and non-refined soybean oils. Journal of Chromatography B: Biomedical Sciences and Applications; 756(1-2): 249-254.

Porras O, Carlsson B, Fallstrom SP, Hanson LA. (1985) Detection of soy protein in soy lecithin, margarine and, occasionally, soy oil. International Archives of Allergy and Applied Immunology; 78(1): 30-32.

Ramazzotti M, Mulinacci N, Pazzagli L, Moriondo M, Manao G, Vincieri FF, Degl'Innocenti D. (2008) Analytic investigations on protein content in refined seed oils: implications in food allergy. Food and Chemical Toxicology; 46(11): 3383-3388.

Rigby NM, Sancho AI, Salt LJ, Foxall R, Taylor S, Raczynski A, Cochrane SA, Crevel RW, Mills EN. (2011) Quantification and partial characterization of the residual protein in fully and partially refined commercial soybean oils. Journal of Agricultural and Food Chemistry; 59(5): 1752-1759.

Rihs H-P, Chen Z, Ruëff F, Petersen A, Rozynek P, Heimann H, Baur X. (1999) IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. Journal of Allergy and Clinical Immunology; 104(6): 1293-1301.

Roehr CC, Edenharter G, Reimann S, Ehlers I, Worm M, Zuberbier T, Niggemann B. (2004) Food allergy and non-allergic food hypersensitivity in children and adolescents. Clinical and Experimental Allergy; 34(10): 1534-1541.

Rona RJ, Keil T, Summers C, Gislason D, Zuidmeer L, Sodergren E, Sigurdardottir ST, Lindner T, Goldhahn K, Dahlstrom J, McBride D, Madsen C. (2008) The prevalence of food allergy: A meta-analysis. Journal of Allergy and Clinical Immunology; 120(3): 638-646.

Russell DG, Parnell WR, Wilson NC, Faed J, Ferguson E, Herbison P, Horwath C, Nye T, Reid P, Walker R, Wilson B, Tukuitonga C. (1999) NZ Food: NZ People. Wellington: Ministry of Health.

Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C. (2002) Structural basis for gluten intolerance in Celiac sprue. Science; 297(5590): 2275-2279.

Skerritt JH, Hill AS. (1990) Monoclonal antibody sandwich enzyme immunoassays for determination of gluten in foods. Journal of Agricultural and Food Chemistry; 38(8): 1771-1778.

Skerritt JH, Hill AS. (1992) How "free" is "gluten free"? Relationship between Kjeldahl nitrogen values and gluten protein content for wheat starches. Cereal Chemistry; 69(1): 110-112.

Skripak JM, Matsui EC, Mudd K, Wood RA. (2007) The natural history of IgE-mediated cow's milk allergy. Journal of Allergy and Clinical Immunology; 120(5): 1172-1177.

Spiegel WA, Anolik R. (2010) Lack of milk protein allergic reactions in patients using lactose containing dry powder inhalers (DPIs). Journal of Allergy and Clinical Immunology; 125(2, Supplement 1): AB69-AB69.

Tattrie NH, Yaguchi M. (1973) Protein content of various processed edible oils. Canadian Institute of Food Science and Technology Journal; 6(4): 289-290.

Ussher R, Yeong ML, Stace N. (1994) Coeliac disease: incidence and prevalence in Wellington 1985-92. New Zealand Medical Journal; 107(978): 195-197.

Yoneyama K, Ono A. (2002) Study of food allergy among university students in Japan. Allergology International; 51(3): 205-208.

Zuidmeer L, Goldhahn K, Rona RJ, Gislason D, Madsen C, Summers C, Sodergren E, Dahlstrom J, Lindner T, Sigurdardottir ST, McBride D, Keil T. (2008) The prevalence of plant food allergies: A systematic review. Journal of Allergy and Clinical Immunology; 121(5): 1210-1218.e1214.

APPENDIX 1 ANALYTICAL TECHNIQUES

1.1 Micro-Protein Determination

Soluble protein at parts per million levels was determined by the colourimetric dye-binding Bradford method (Bradford, 1976). The method was calibrated against bovine serum albumin (BSA).

Lactose samples were analysed as 2% aqueous solutions. Standards were also prepared in a 2% lactose solution (matrix matched).

Ethanol interferes with the Bradford method (EFSA, 2007c). Ethanol samples were evaporated to dryness and redissolved in deionised water for analysis.

The Bradford micro protein method cannot be performed directly on an oil substrate. Protein was recovered from oil samples using the low temperature acetone precipitation method (Paschke *et al.*, 2001; Rigby *et al.*, 2011). This involves combining equal volumes (10 ml) of oil and acetone and freezing for at least 24 hours at -80ºC. Samples are defrosted and filtered (0.2 µm Millipore GV) and extracted with phosphate-buffered saline (PBS; 4 ml). Analyses were performed on the PBS extract.

1.2 Source-specific Protein

Protein originating from specific allergenic source materials was determined by Enzyme-Linked ImmunoSorbent Assay (ELISA). Gluten and whey proteins were determined using Neogen Biokits (Neogen Corporation, Auchincruive, Scotland)[6] and soy protein was determined using Elisa Systems food allergen test kits (Elisa Systems, Windsor, Australia)[7].

1.2.1 Whey (β-lactoglobulin)

The method is an indirect competitive ELISA to β-lactoglobulin, which accounts for approximately 50% of total whey protein. Samples (2 g) were extracted with 20 ml extraction buffer (0.05 M carbonate/bicarbonate, pH 9.6) and analysed according to manufacturers instructions. Matrix spikes were prepared by spiking 100 µL of provided β-lactoglobulin control into the test matrix and then analysing as for other samples.

Standards equivalent to 2.5, 5, 10, 20 and 40 ppm β-lactoglobulin protein were also analysed. Levels of β-lactoglobulin in unknowns were determined by linear interpolation from the standards.

1.2.2 Gluten

The method is a direct sandwich ELISA, based on reaction of extracted proteins with monoclonal antibodies to ω-gliadins (Skerritt and Hill, 1990). Samples (2 ml) were mixed with 20 ml extraction solution (40% v/v ethanol in water) and analysed according to

[6] http://www.neogen.com/foodsafety/BK_Index.html
[7] http://www.elisasystems.net/contact/index.htm

manufacturers instructions. Method performance was checked by analysis of provided gluten control.

Standards equivalent to 3, 5, 10, 20 and 50 ppm gluten protein were also analysed. Levels of gluten in unknowns were determined by linear interpolation from the standards.

1.2.3 Soy

The method is a direct sandwich ELISA to the heat-stable soy trypsin inhibitor. Oil samples (2 ml) were combined with extraction solution (18 ml) and placed in a water bath at 60ºC for 15 minutes, with shaking for one minute every five minutes. Samples were brought to room temperature and the aqueous phase collected for analysis. Satisfactory extraction of protein from oil was checked by spiking an oil sample with non-fat soy flour.

Standards equivalent to 1, 2 and 5 ppm soybean protein were also analysed. Levels of soy protein in unknowns were determined by linear interpolation from the standards.