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

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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 allergenic 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 two distinct food processes; distillation and enzymatic hydrolysis/separation. Results were placed in context by summarising previous literature information on allergenicity and protein content of equivalent products and the decisions of EFSA regulatory assessments.

Distillation

Distilled ethanol from whey (whey ethanol)

Testing of 35 food-grade whey ethanol samples, produced in New Zealand, did not detect whey-specific protein (limit of detection = 2.5 mg/L) or general soluble protein (limit of detection = 0.6-2.3 mg/L). Analysis of whey-specific proteins was carried out by Enzyme-Linked Immunosorbent Assay (ELISA), while general soluble protein was measured by the Bradford colourimetric method. These results are consistent with the results of protein testing reported in an EFSA assessment. Further to this, analysis of samples by liquid-chromatography mass spectrometry did not detect any residues of major whey proteins.

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

No reports of clinical trials on the allergenicity of whey ethanol or spirits derived from whey 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 whey used in distillates for spirits and concluded that proteins, peptides and lactose are not carried over in the distillation process during properly controlled operation, at least not to levels above the limits of detection quoted, and distillates made from whey are unlikely to trigger a severe allergic reaction in susceptible individuals.

Vinegar from distilled whey ethanol (whey vinegar)

Whey vinegar is produced from secondary fermentation of distilled whey ethanol and all findings with respect to distilled whey ethanol should be applicable to whey vinegar.

Testing of seven retail samples of whey vinegar did not detect whey-specific protein (limit of detection = 2.5 mg/L). The Bradford general soluble protein method detected a very low level of protein (0.6 mg/L) in one white vinegar sample and higher levels (3.3 and 5.4 mg/L) in two spiced vinegar samples. The higher levels are likely to be due to protein associated with the spice material. The origins of the protein in the low level (0.6 mg/L) sample are

uncertain. However, given that the vinegar is produced from a starting material that has been demonstrated to be free of whey proteins or peptides, the extremely low level of protein, and the fact that four other production lots of the same product from the same manufacturer contained no detectable protein, contamination of the sample appears to be a more likely explanation than carry over of whey protein. This finding is unlikely to be significant with respect to the allergenicity of the vinegar. Analysis of samples by liquid-chromatography mass spectrometry did not detect any residues of major whey proteins.

No literature or regulatory information was found on assessment of the potential allergenicity of whey vinegar.

Enzymatic hydrolysis/separation

Glucose syrup from wheat starch

Gluten-specific (ELISA) and general soluble (Bradford) protein measurements carried out on finished glucose syrups as part of the current project confirmed that the protein content of glucose syrups from wheat starch is generally about or less than 20 mg/kg.

Clinical trials with wheat allergy cases and Coeliac disease cases reported in the scientific literature and in EFSA regulatory assessment reports, involving challenge with wheat starch glucose syrup, found no statistical difference between responses to glucose syrup and responses to placebo.

Regulatory assessment by EFSA's Panel on Dietetic Products, Nutrition and Allergies for exemption from allergen labelling requirements for glucose syrups derived from wheat starch or barley starch concluded that while glucose syrups from these sources contained low residual levels of proteins, peptides or fragments thereof, it is not known what level of glucose syrup intake could cause allergic reactions in susceptible individuals. The Panel considered that it was not very likely that the glucose syrups under assessment would cause severe allergic reactions in most allergic individuals. For Coeliac disease, the Panel considered that the glucose syrups under assessment were unlikely to cause adverse reactions in individuals with Coeliac disease provided that the (provisional) value of gluten considered by Codex Alimentarius for food rendered gluten-free (20 mg/kg) is not exceeded. It should be noted that the Australia New Zealand Food Standards Code requires foods claimed to be gluten free to contain no detectable gluten.

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 allergenic 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 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

In order to test the utility of the proposed evidence gathering process, three products were chosen for the current project year, specifically:

- Alcohol from whey (milk)
- Vinegar from whey (milk)
- Glucose syrup from wheat

These food products represent two distinct food processes; distillation and enzymatic hydrolysis/separation.

2 DISTILLATION

The products considered in this section are both 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 Distilled Ethanol from Whey (Whey Ethanol)

Whey is produced as a by-product from cheese or casein production. In the late 1990s, New Zealand produced approximately 4 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, which can be fermented by yeasts, such as *Kluveromyces marxianis,* to produce ethyl alcohol (ethanol) (Hamilton, 1998; Mawson, 1994).

The yeast produces β-galactosidase, an enzyme that splits lactose into its component sugars; galactose and glucose, which are then fermented to produce ethanol (Hamilton, 1998). Fermentation typically produces a 'beer' containing 1.8-2.0% ethanol (Mawson, 1994). Ethanol is then recovered by distillation. The ethanol produced from this process can be used in food, medical, cosmetic and industrial applications (Hamilton, 1998).

2.2.1 Evidence from current analytical investigations

Thirty-five samples of finished food-grade whey ethanol were provided by a New Zealand manufacturer, following a FSANZ visit. These represented:

- Samples taken from each of three distillation columns on each of nine production days, taken at 12:00 (7-15 January 2010); and
- In line samples taken at 09:00 on eight production days (8-15 January 2010).

All samples were analysed for residual protein by Enzyme-Linked Immunosorbent Assay (ELISA), Bradford colourimetric method and by liquid chromatography with mass spectrometric detection (LC-MS). 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 an alcohol matrix. Spike recoveries were within acceptable limits, as defined by the kit manufacturer.

No samples contained detectable β-LG at a detection limit of 2.5 ppm (mg/L).

2.2.1.2 Micro protein (Bradford method)

All samples were spiked with bovine serum albumin (BSA) at a spike concentration of 6 mg/L. Spike recoveries were in the range 40.0-85.2% (mean = 68.0%). All samples were analysed in quadruplicate, with within batch coefficients of variation (CV) all less than 1%. The limit of detection of the method was in the range 0.6-2.3 mg/L, based on the standard deviation of blank determinations.

No samples contained detectable soluble protein.

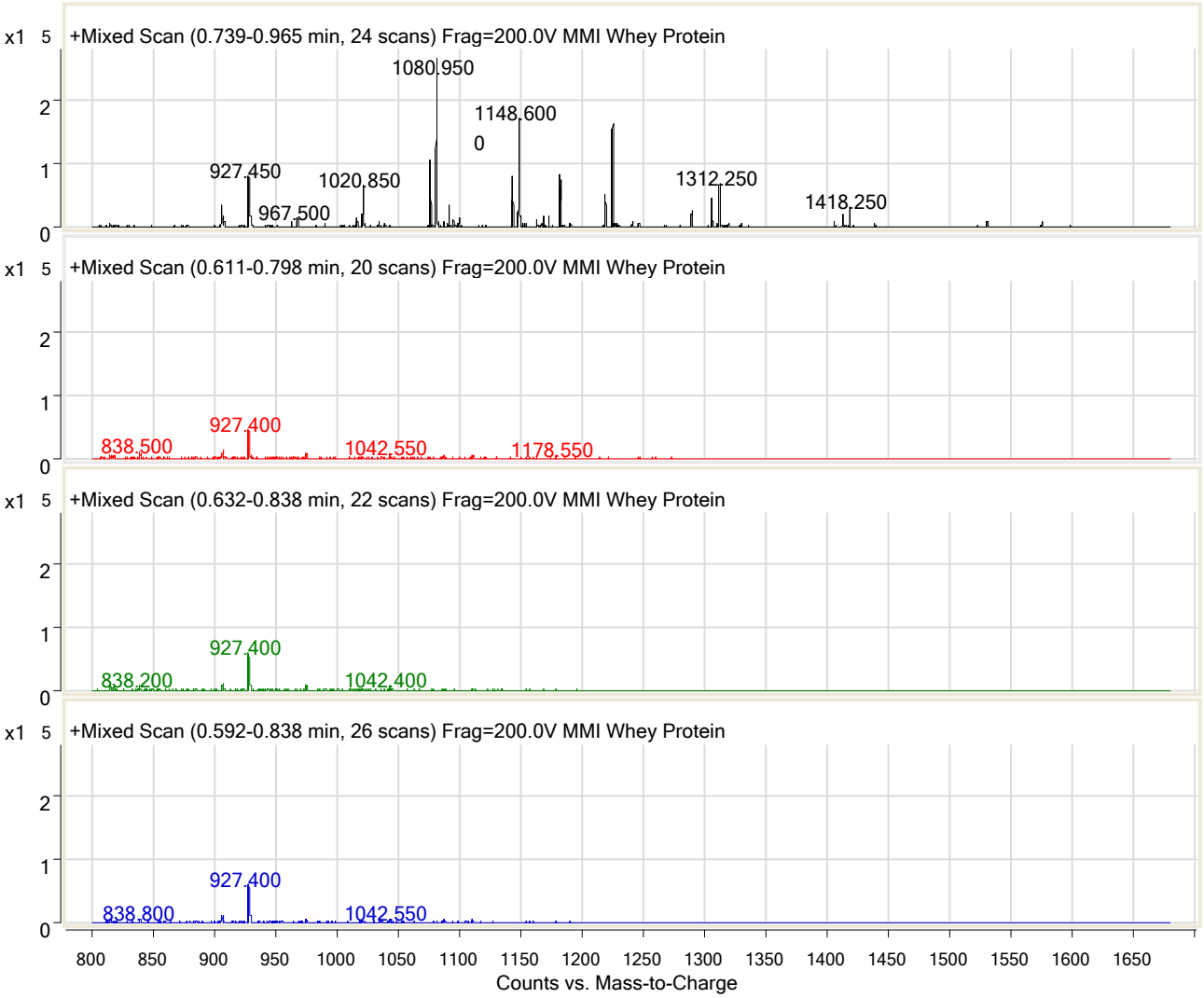
2.2.1.3 Liquid chromatography-mass spectrometry (LC-MS)

Unlike the ELISA and Bradford methods, mass spectrometric detection is not dependent on the presence of particular structural features of the proteins. This allows LC-MS to potentially detect any proteins or protein fragments present.

Figure 1 shows mass spectra of a mixed α-lactalbumin, α-lactoglobulin and β-lactoglobulin standard and three food grade ethanol samples. It should be noted that the peak at *m/z* 927.4 is an artefact peak present in all spectra. Details of peak assignments for the three individual whey proteins are given in Appendix 1, section 1.3.

No residues of whey proteins were detected in any of the 35 ethanol samples at a limit of detection of 3 mg/kg.

Figure 1: Mass spectra of mixed whey protein standard (3.3 mg/kg α-lactalbumin, α-lactoglobulin, β-lactoglobulin) and three food-grade ethanols



2.2.2 Evidence from the literature and other sources

2.2.2.1 Previous regulatory assessments

EFSA's Panel on Dietetic Products, Nutrition and Allergies has assessed an application for exemption from allergen labelling requirements for whey used in distillates for spirits (EFSA, 2007c).

The assessment noted that:

- A literature survey had failed to reveal reports of allergic reactions after consumption of distillates made from whey, although under-reporting could not be excluded;
- No protein was detected in 24 spirit samples by the Bradford method to a limit of detection of 0.5 mg/L;
- No β-LG was detected in 24 spirit samples by ELISA to a limit of detection of 0.5 mg/L; and
- No epidemiological studies, or double-blind placebo-controlled food challenge studies in clinical settings have been carried out.

The panel concluded that proteins, peptides and lactose are not carried over in the distillation process during properly controlled operation, at least not to levels above the limits of detection quoted, and distillates made from whey are unlikely to trigger a severe allergic reaction in susceptible individuals.

2.2.2.2 Literature survey

Allergenicity of whey alcohol

The literature survey carried out and reported to EFSA in the application discussed in section 2.2.2.1 was repeated to determine if any relevant literature had been published in the intervening period (EFSA, 2007c). The original searches were carried out in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). In the current study searches were also carried out in Scopus (http://www.scopus.com/search/form.url). Results are summarised in Table 2.

Table 2: Summary of literature searches in PubMed and Scopus for information on allergenicity of ethanol from whey

Search Terms	Number of References Found (number with obvious relevance)	
	PubMed	Scopus
MILK or WHEY and ALLERGY	4477	5177
(MILK or WHEY and ALLERGY) and (GIN or VODKA or DISTILLED SPIRITS)	0	0
ALCOHOL and (MILK and ALLERGY)	74 (0)	37 (0)
ALCOHOL and (WHEY and ALLERGY)	4 (0)	0

The literature survey reported by EFSA failed to recover any literature related to allergic reactions after consumption of distillates made from whey (EFSA, 2007c) and only one reference that discussed potential allergenicity to alcoholic drinks (Vally and Thompson, 2003). The current search also recovered this reference, but did not recover any new references of relevance to the issue of allergenicity of whey alcohol.

Using wildcard search terms (e.g. allerg*) or using the term ETHANOL, rather than ALCOHOL, did not result in recovery of any relevant references.

Protein content of whey alcohol

Further literature searches were carried out to retrieve any available information on protein carryover into distilled whey alcohols. No information was recovered using a wide range of search strategies in PubMed and Scopus.

A single paper was found on characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation (Dragone *et al.*, 2009). Compounds detected included higher alcohols and esters, aldehydes, organic acids and terpenes.

Data provided to EFSA included protein measurements on whey spirit by the Bradford method (Bradford, 1976), which reported no detectable protein in 24 whey spirit samples at a limit of detection of 0.5 mg/L (EFSA, 2007c).

2.3 Vinegar from Distilled Whey Ethanol (Whey Vinegar)

Although direct production of vinegar from whey has been reported (Tuckett *et al.*, 1996), in New Zealand whey vinegar is produced by a two-step fermentation process. Whey is converted to whey ethanol, which is distilled to produce food-grade ethanol. Food Grade ethanol is used as the substrate for fermentative oxidation of ethanol to ethyl (acetic) acid (Parrondo *et al.*, 2003). In terms of the characteristics of the allergy and the allergenic source material, all of the information on ethanol from whey is relevant to vinegar from whey.

2.3.1 Evidence from current analytical investigations

Seven samples of vinegar labelled "brewed from fermented spirit derived from milk" were purchased from local supermarkets. While all samples were of the same brand (the only brand to declare milk as the ultimate source of their product), two different varieties (white and spiced) were purchased, with all samples of the same variety having different production date codes.

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

2.3.1.1 ELISA

All samples were analysed by Biokit β-lactoglobulin (β-LG) ELISA. The analytical procedure was validated by spike recovery from a vinegar matrix. Spike recoveries were within acceptable limits, as defined by the kit manufacturer. No samples contained detectable β-LG at a detection limit of 2.5 ppm (mg/L).

2.3.1.2 Micro protein (Bradford method)

All samples were spiked with bovine serum albumin (BSA) at a spike concentration of 6 mg/L. Spike recoveries were in the range 86-111.7% (mean = 103.5). All samples were analysed in quadruplicate, with within batch coefficients of variation (CV) all less than 2%. The limit of detection of the method was 0.5 mg/L.

Protein was not detected in four of the seven vinegar samples analysed. Two samples of spiced vinegar contained protein at concentrations of 3.3 and 5.4 mg/L. The negative ELISA results for these samples suggest that the protein is probably associated with the spice flavouring, rather than associated with the vinegar.

One sample contained protein at a concentration of 0.6 mg/L. This result is very close to the limit of detection and its significance is uncertain. However, given that the vinegar is produced from a starting material that has been demonstrated to be free of whey proteins or peptides, the extremely low level of protein, and the fact that four other production lots of the same product from the same manufacturer contained no detectable protein, contamination of

the sample at the factory or in the laboratory appears to be a more likely explanation than carry over of whey protein. Procedures in place for dealing with allergen analyses suggest that contamination in the laboratory is unlikely. This finding is unlikely to be significant with respect to the allergenicity of the vinegar. This result was also below the limit of detection of β-LG ELISA method and this sample gave a negative result in the ELISA assay.

2.3.1.3 Liquid chromatography-mass spectrometry

No residues of whey proteins were detected in any of the seven vinegar samples at a limit of detection of 3 mg/kg. While spectra from spiced vinegars showed qualitative difference to white vinegars, there was no evidence of whey protein material.

2.3.2 Evidence from the literature and other sources

2.3.2.1 Previous regulatory assessments

No previous regulatory assessment of whey vinegar, with respect to allergen labeling was found.

2.3.2.2 Literature survey

No relevant literature was found on the allergenicity, protein content or protein composition of whey vinegar.

2.4 Conclusions

2.4.1 Distilled ethanol from whey

Testing of 35 food-grade whey ethanol samples, produced in New Zealand, did not detect whey-specific protein (limit of detection = 2.5 mg/L) or general protein (limit of detection = 0.6-2.3 mg/L). These results are consistent with the results of protein testing reported in the EFSA assessment. Further to this, analysis of samples by liquid-chromatography mass spectrometry did not detect any residues of major whey proteins.

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

No reports of clinical trials on the allergenicity of whey ethanol or spirits derived from whey 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 whey used in distillates for spirits (EFSA, 2007c) and concluded that proteins, peptides and lactose are not carried over in the distillation process during properly controlled operation, at least not to levels above the limits of detection quoted, and distillates made from whey are unlikely to trigger a severe allergic reaction in susceptible individuals.

2.4.2 Vinegar from distilled whey ethanol

Whey vinegar is produced from secondary fermentation of distilled whey ethanol and all findings with respect to whey ethanol should be applicable to whey vinegar.

Testing of seven retail samples of whey vinegar did not detect whey-specific protein (limit of detection = 2.5 mg/L). A general soluble protein method (Bradford, 1976) detected a very low level of protein (0.6 mg/L) in one white vinegar sample and higher levels (3.3 and 5.4 mg/L) in two spiced vinegar samples. The higher levels are likely to be due to protein associated with the spice material. The origins of the protein in the low level (0.6 mg/L) sample are uncertain. However, given that the vinegar is produced from a starting material that has been demonstrated to be free of whey proteins or peptides and given the extremely low level of protein, contamination of the sample appears to be a more likely explanation than carry over of whey protein. This finding is unlikely to be significant with respect to the allergenicity of the vinegar. Analysis of samples by liquid-chromatography mass spectrometry did not detect any residues of major whey proteins.

No literature or regulatory information was found on assessment of the potential allergenicity of whey vinegar.

3 ENZYMATIC HYDROLYSIS/SEPARATION

The product considered in this category is glucose syrup produced from wheat starch.

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, 2004c).

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, 2004c). 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. These 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, 2004c). 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 toxicity 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, 2004c). 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. These cereals are all toxic to 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, 2004c). 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, 2004c). 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 Glucose Syrup from Wheat Starch

In very general terms, glucose syrup is produced from wheat starch by saccharifying the starch with amylase enzymes and then cleaning the resultant product through a series of filtration and ion exchange steps. The saccharification process is likely to result in release of both internal and surface-associated proteins.

3.2.1 Evidence from current analytical investigations

A total of 12 finished product samples from a wheat starch glucose syrup plant were provided for analysis. These were made up of six paired samples, one of finished product before evaporative concentration (eluent from ion exchange column) and one after evaporative concentration (finished glucose syrup). Further samples were provided, representing intermediate stages in the production process for glucose syrup.

Duplicates of all samples were provided, to allow the possibility of exogenous gluten contamination to be checked in positive samples.

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 and Micro protein (Bradford method)

The gluten ELISA method has been previously validated (Cressey and Jones, 2005). Applicability of the method to glucose syrup substrates was confirmed by adding the gluten control material provided with the kit to glucose syrup samples. Results were in the acceptable range for the gluten control. The gluten ELISA method has a limit of detection of 3 mg/kg.

For validation of the Bradford micro protein method, all samples were spiked with bovine serum albumin (BSA) at a spike concentration of 6 mg/L. Spike recoveries were in the range 75.4-118.1% (mean = 92.8%). All samples were analysed in quadruplicate, with within batch coefficients of variation (CV) all less than 3%. The limit of detection of the method was in the range 0.6-0.7 mg/L, based on the standard deviation of blank determinations. Due to the viscosity of some samples, samples were analysed at a ten-fold dilution, meaning that the detection limit equates to 6-7 mg/L in the product as received.

A subset of seven samples from the wheat starch glucose syrup factory were identified as representing the sequential stages of the normal process. These stages and the analytical results from gluten ELISA and Bradford micro protein method are included in Table 3.

Table 3: Soluble protein content at various stages in the production of glucose syrup, determined by gluten ELISA and Bradford micro protein method

Processing stage	Protein content (mg/kg)	
	Gluten ELISA[1]	Bradford micro protein[2]
Raw wheat starch slurry	29	97
Amylase starch hydrolysis (heated)	82	32
Additional enzymatic hydrolysis	24	30
Vacuum drum filter	27	25
Physical screening	29	12
Ion exchange	<3	9
Finished glucose syrup	8	9

[1] The gluten ELISA method has a coefficient of variation of 5%, equating to a standard deviation of 1 mg/kg at a concentration of 20 mg/kg

[2] The Bradford micro protein method has an overall coefficient of variation of 16% equating to a standard deviation of 3.2 mg/kg at 20 mg/kg

Results from the gluten ELISA testing suggest that not all of the starch associated protein was available under the extraction conditions used for the ELISA test. This is plausible, as some proteins are internalised within the starch granules or attached to the exterior of the starch granules (Kasarda *et al.*, 2008) and an increase in measured gluten was seen at the saccharification step by gluten ELISA. However, this increase was not apparent in the total soluble protein (Bradford) measurements. There was a substantial reduction in gluten at the beginning of the purification process for gluten ELISA measurements, but little further removal until the ion exchange step. Total soluble protein (Bradford) measurements suggest a more gradual reduction in the protein present in the process.

While the two protein determination methods (ELISA and Bradford) show quite different results for some samples, it is not possible to draw conclusions from these differences at this time. Given the completely different chemistry involved in the two assays, differences in results are not surprising.

Table 4 gives detailed results of protein determinations for six sets of finished product i.e. the eluent from the ion exchange column and the same material after concentration to give the finished glucose syrup.

Table 4: Protein content (mg/kg) in glucose syrups, before (eluent from ion exchange column) and after (finished glucose syrup) final concentration, determined by gluten ELISA and Bradford micro protein method

Run number	Protein content (mg/kg)			
	Eluent from ion exchange column		Finished glucose syrup	
	Gluten ELISA[1]	Bradford[2]	Gluten ELISA[1]	Bradford[2]
1	<3	9	8	9
2	<3	8	22	12
3	<3	12	<3	15
4	<3	11	15	16
5	<3	7	<3	8
6	<3	9	<3	16

[1] The gluten ELISA method has a coefficient of variation of 5%, equating to a standard deviation of 1 mg/kg at a concentration of 20 mg/kg

[2] The Bradford micro protein method has an overall coefficient of variation of 16% equating to a standard deviation of 3.2 mg/kg at 20 mg/kg

In terms of gluten ELISA results, there was some variability in the gluten content of the finished (concentrated) glucose syrup, with no detectable gluten present for 50% of samples and 8-22 mg/kg present for the remaining three samples. This is consistent with results reporting in the literature and with data provided to EFSA. The most recent application to EFSA reported "no detected gluten level higher than 25.3 mg/kg (limit of detection 3.1 mg/kg, LOD) in glucose syrups and dextrose sample" (EFSA, 2007a). Total soluble protein (Bradford) results were more consistent across runs, both for the concentrated (finished glucose syrup) and unconcentrated (eluent from ion exchange column) product.

While absolute agreement between results from gluten ELISA and Bradford micro protein method is generally not apparent, both methods provide a consistent message. The Bradford method would be expected to give higher concentration results, as it is not dependent on the presence of a particular amino acid sequence. This is the case for all samples except finished glucose syrup run 2. However, the results from the two methods (22 mg/kg by ELISA and 12 mg/kg by Bradford) are of a similar order of magnitude.

3.2.2 Evidence from the literature and other sources

3.2.2.1 Previous regulatory assessments

The EFSA's Panel on Dietetic Products, Nutrition and Allergies has assessed four applications for exemption from allergen labelling requirements for glucose syrups derived from cereal starches; two derived from wheat starch (EFSA, 2007a;2004a) and two derived from barley starch (EFSA, 2007b;2004b).

In all cases the Panel concluded that while glucose syrups from these sources contained low residual levels of proteins, peptides or fragments thereof, it is not known what level of glucose syrup intake could cause allergic reactions in susceptible individuals. EFSA considered that it was not very likely that the glucose syrups under assessment would cause severe allergic reactions in most allergic individuals. For Coeliac disease, EFSA considered that the glucose syrups under assessment were unlikely to cause adverse reactions in individuals with Coeliac disease provided that the (provisional) value of gluten considered by Codex Alimentarius for food rendered gluten-free (20 mg/kg) is not exceeded.

It should be noted that the Australia New Zealand Food Standards Code requires foods claimed to be gluten free to contain no detectable gluten.

3.2.2.2 Clinical studies

Wheat/cereal allergy

Fifteen subjects with allergy to one or more cereal by double-blind placebo-controlled food challenge (DBPCFC) were administered 30 g of barley starch syrup (glucose syrup) per day for 5 days (Nermes *et al.*, 2009). In DBPCFC with barley starch syrup, none of the fifteen subject exhibited allergic symptoms. Atopic dermatitis scores for the subjects were not statistically different between barley starch syrup and placebo.

Two separate challenge studies were carried out to assess the potential allergenicity of wheat starch glucose syrup in a cohort of 36 patients with confirmed (DBPCFC) wheat allergy (EFSA, 2007a). Fifteen subjects received either 13.85 g (children) or 27.7 g (adults) of dried glucose syrup in three doses separated by 30-minute intervals. One subject had symptoms after ingesting glucose syrup, while two had symptoms after ingesting placebo. A second challenge involved 15 subjects receiving glucose syrup or placebo for 10 days each in a blinded manner. Five subjects exhibited symptoms after glucose syrup administration and three after placebo. The difference was not statistically significant.

Coeliac disease

Adult subjects with biopsy-diagnosed Coeliac disease were challenged with either glucose syrup, maltodextrins or placebo for 24 weeks in a DBPCFC study (EFSA, 2007a; Kaukinen *et al.*, 2008). Subjects receiving glucose syrup consumed an average of 27.7 g of dried glucose syrup per day, with a gluten content of 4.3 mg/kg, as determined by HPLC (Kaukinen *et al.*, 2008). No significant differences were found between glucose syrup and placebo groups with respect to small bowel clinical measures, gastrointestinal symptoms, quality of life and laboratory parameters.

3.2.2.3 Protein content of wheat starch glucose syrup

Protein content of wheat starch glucose syrups was determined as Kjeldahl nitrogen x 6.25 (EFSA, 2004a). A range of commercial products contained protein in the range 0.15-0.38% (1500-3800 mg/kg) on a dry weight basis. However, it was noted that the majority of nitrogen in wheat starch was present in the form of phospholipids and only approximately 30% of nitrogen is likely to be protein-based.

The R5 enzyme-linked immunosorbent assay (ELISA), specific for a particular gluten epitope (QQPFP) was used to examine wheat starch and glucose syrups from wheat starch (EFSA, 2007a). While wheat starch was found to contain up to 280 mg/kg of gluten by this method, the highest level measured in glucose syrups was 25.3 mg/kg. In another study glucose syrups from wheat and maize were analysed by R5 and normal gliadin ELISA, with gluten proteins not detected by either method above the detection limit of 3 mg/kg in any of 20 products (Dostalek *et al.*, 2010).

Nine glucose syrups, prepared from wheat starch by a number of different processes, were analysed for protein content by the Bradford method (Iametti *et al.*, 2004). Protein contents were in the range <10 – 344 mg/kg. ELISA methods failed to detect gluten protein in six samples and in the remaining three found gluten concentrations of 0.4, 20 and 325 mg/kg.

Analysis of glucose syrups by mass spectrometry found some intact gliadin proteins and some fragments from degradation of gluten, with total concentrations in the range 1-40 mg/kg (EFSA, 2007a). Matrix-Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) mass spectrometry of glucose syrups did not detect any intact gluten proteins or fragments of gluten proteins (Dostalek *et al.*, 2010). However, the limit of detection in this study was quite high (200 mg/kg).

3.2.2.4 Dietary exposure to gluten from wheat starch glucose syrup

A European dietary exposure assessment was reported as part of an EFSA opinion (EFSA, 2007a). The main sources of exposure (the main usage areas for glucose syrup) were soft drinks, dairy desserts, yoghurt drinks, candy, canned food, soups and savoury sauces. Based on a gluten content in glucose syrup of 10-20 mg/kg (from mass spectrometry), the 95th percentile exposure to gluten from this source for an adult Dutch male was 3.5 mg/day. Exposure was lower for all other age-gender groups examined.

3.2.3 Conclusions

Gluten-specific and general soluble protein measurements (Bradford) carried out on finished glucose syrups as part of the current project confirm that the protein content of syrups is generally about or less than 20 mg/kg.

Clinical trials with wheat allergy cases and Coeliac disease cases reported in the scientific literature and in EFSA regulatory assessment reports, involving challenge with wheat starch glucose syrup, found no statistical difference between responses to glucose syrup and responses to placebo (EFSA, 2007a; Kaukinen *et al.*, 2008; Nermes *et al.*, 2009).

Regulatory assessment by EFSA's Panel on Dietetic Products, Nutrition and Allergies for exemption from allergen labelling requirements for glucose syrups derived from wheat starch (EFSA, 2007a;2004a) or barley starch (EFSA, 2007b;2004b) concluded that while glucose syrups from these sources contained low residual levels of proteins, peptides or fragments thereof, it is not known what level of glucose syrup intake could cause allergic reactions in susceptible individuals. The Panel considered that it was not very likely that the glucose syrups under assessment would cause severe allergic reactions in most allergic individuals. For Coeliac disease, the Panel considered that the glucose syrups under assessment were unlikely to cause adverse reactions in individuals with Coeliac disease provided that the (provisional) value of gluten considered by Codex Alimentarius for food rendered gluten-free (20 mg/kg) is not exceeded. It should be noted that the Australia New Zealand Food Standards Code requires foods claimed to be gluten free to contain no detectable gluten.

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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).

Vinegar samples were analysed directly. All glucose syrup samples, except finished (viscous) syrups, were analysed directly. Finished glucose syrups were dilute 1 to 10 with dionised water before analysis (Iametti *et al.*, 2004).

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

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)[1].

1.2.1 Gluten

The method is a direct sandwich ELISA, based on reaction of extracted proteins with monoclonal antibodies to ω-gliadins (Skerritt and Hill, 1990). Clarified samples (2 g) were mixed with 20 ml extraction solution (40% v/v ethanol in water) and analysed according to manufacturers instructions. Method performance was checked by analysis of provided gluten control.

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

1.2.2 Whey (β-lactoglobulin)

The method is an indirect competitive ELISA to β-lactoglobulin, which accounts for approximately 50% of total whey protein. Sample (2 ml) was mixed 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] http://www.neogen.com/foodsafety/BK_Index.html

1.3 Liquid Chromatography-Mass Spectrometry

Ethanol and vinegar samples were analysed for trace whey proteins by liquid chromatography-mass spectrometry (LC-MS) (Monaci and van Hengel, 2008). Ethanol samples were evaporated to dryness and resuspended in deionised water. The integrity of this process was checked by spiking samples with α-lactalbumin, α-lactoglobulin and β-lactoglobulin at 5 mg/kg each. Good recovery of the spikes after evaporation, resuspension and chromatrography was observed. Vinegar samples were analysed unaltered.

Proteins were separated on an Agilent 1200 LC liquid chromatograph, fitted with an Agilent "zorbax" SB-C18 column, 2.1 x 50 mm, 1.8 micron particle size. The mobile phase was 5% acetonitrile/water (solvent A) and 95% acetonitrile/water (solvent B). A constant ratio of 55% solvent A and 45% solvent B was used. Both solvents contained 0.02% formic acid to promote analyte ionization in the mass spectrometer source. A flow rate of 0.2 ml/min was used.

A 5 µL sample was injected into the liquid chromatograph.

The liquid chromatograph was coupled to an Agilent 6410 triple quadrupole mass spectrometer fitted with an Agilent multimode source. The mass spectrometer was operated in single quadrupole mode, scan range 800 *m/z* to 1680 *m/z*. The source was operated in a predominately electrospray ionisation (ESI) mode. The three whey proteins monitored each produced a distinctive fragmentation pattern. α-Lactalbumin produced ions at *m/z* 1091.1, 1182.1, 1289.6, and 1418.5. α-Lactoglobulin produced ions at *m/z* 1020.8, 1080.9, 1148.4, 1224.9, and 1312.4. β-Lactoglobulin produced ions at 1016.1, 1075.9, 1143.1, 1219.2, and 1305.9.