safefood

Food allergens in prepacked foods

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Foreword and acknowledgements

The unintended presence of food allergens in prepacked food products continues to present a challenge for food hypersensitive consumers – people who are allergic to or intolerant of certain food ingredients – on the island of Ireland and elsewhere.

Recent legislation has ensured that accurate information about deliberately added allergenic ingredients must be displayed on the product label. However, the possibility of unintentional cross-contamination of food with allergens on the production line, and the associated widespread but often unjustified use of precautionary allergen labelling such as "may contain", is still problematic.

A number of recent surveys have called into question the approach taken by the food industry in applying precautionary allergen labelling and its reliability for the user. Consumers with food hypersensitivity find precautionary labelling confusing and there is evidence to suggest that many are now ignoring precautionary allergen labelling statements, or even ranking the different statements in order of importance. This could have negative consequences for the risk management of their condition.

Continued vigilance on the part of the regulatory regime is essential for controlling and highlighting allergen cross-contamination and ensuring that prepacked food products contain accurate information on both intended and unintended allergenic ingredients.

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Executive summary

Accurate labelling of prepacked food products is essential for food hypersensitive consumers to make safe food choices. In this study, food products were selected for gluten (a group of proteins found in grains such as wheat, rye, oats and barley) or peanut allergen analysis (sometimes both), based on an analysis of product recalls in the United Kingdom and the Republic of Ireland between 2011 and 2017.

The samples were analysed using commercially available enzyme-linked immunosorbent assays – two different kits per allergen. This analytical technique uses antibody specifically to detect and quantify the presence of certain proteins in a sample (in this case peanut protein or gluten). Real-time quantitative polymerase chain reaction was used for verification purposes. This analytical technique detects and quantifies specific DNA sequences in a sample. Definitive liquid chromatography–mass spectrometry allergen analysis was not part of the project plan. This technique combines physical separation using liquid chromatography with the sample identification capacity of mass spectrometry (MS).

The outcome of the peanut analyses was inconclusive as a cross-reaction with other allergenic proteins in the food matrices (the chemical make-up of the sampled food) may have occurred. The food products that tested positive for peanut listed some form of tree nuts or legumes as ingredients on the label. Nonetheless, the findings from the first enzyme-linked immunosorbent assay screening using 2 different kits were a cause for concern. This is because several products had peanut levels in excess of 20 parts per million and any precautionary allergen labelling referred only to nuts.

The gluten analyses showed that the use of the advisory statement “gluten-free” was robust and reliable. Just 1 product had detectable gluten in both screenings and this was well below the threshold for “gluten-free” labelling. Real-time quantitative polymerase chain reaction proved to be an ineffective method for quantifying peanut allergen or gluten in this study.

Glossary of abbreviations

AOAC	Association of Analytical Communities
DNA	Deoxyribonucleic acid
ED	eliciting dose
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FHS	food hypersensitivity
FSAI	Food Safety Authority of Ireland
gLC–MS	liquid chromatography coupled to mass spectroscopy
M&S	Marks and Spencer
mg	milligrams
nm	nanometre
PAL	precautionary allergen labelling
ppm	parts per million
q-PCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROI	Republic of Ireland
RTE	ready-to-eat
UK	United Kingdom
µl	Microlitres
VITAL	Voluntary Incidental Trace Allergen Labeling Program

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1 Introduction

Food hypersensitivity (FHS) is an increasingly important health issue globally. It covers a broad range of adverse reactions to food but for the purposes of this study "food hypersensitivity" is defined as food allergy, coeliac disease and food intolerance (Surojanametakul et al., 2012).

Food allergy is a result of a "hyperimmune" response (an excessive autoimmune system reaction) involving Ig-E antibodies[1]. The prevalence, or extent, of food allergy alone has risen by 50% in the past decade with over 17 million people suffering from food allergy in Europe (European Academy of Allergy and Clinical Immunology, 2014). Closer to home, it is estimated that in the UK between 1 and 2% of adults and 5 to 8% of children have a food allergy (Food Standards Agency, 2016).

Coeliac disease is an autoimmune enteropathy (a disease of the intestines) triggered by ingestion of gluten and related cereal proteins in genetically predisposed individuals (Ludvigsson et al., 2013). The disease now affects about 1% of most populations (Rubio-Tapia and Murray, 2010). Getting prevalence estimates for different types of food intolerance, such as "non-coeliac gluten sensitivity", is currently hampered by a lack of measurable biomarkers[2] and associated objective clinical diagnostic criteria (Lundin and Alaedini, 2012). Whatever the type of FHS, the affected consumer needs to avoid the food allergen that triggers a reaction and this is achieved largely by adhering to an avoidance diet.

The vast majority of food allergens are proteins. Many different approaches have been taken to their analysis. The most commonly used is the enzyme-linked immunosorbent assay (ELISA), which enables the detection and semi-quantification of specific allergens. Additionally, an alternative gene-based approach such as polymerase chain reaction (PCR) has also been used for allergen analyses.

There are 14 major food allergens (Barnett et al., 2011) controlled under European Union (EU) legislation. According to a 2011 web survey by the Food Safety Authority of Ireland (FSAI), peanut allergy and coeliac disease are the most prevalent in the Republic of Ireland (ROI). This is supported by the findings of reports from ***safe*food** on the challenge for those with a food allergy or food intolerance when dining outside the home. In a study focussed on food allergy sufferers only, the

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[1] Anitbodies are blood proteins that are produced to defend the body from allergens, viruses and so on

[2] A biomarker is a naturally occuring process or substance that can be identified and therefore measured

most prevalent food allergens in both the ROI and Northern Ireland were peanut, nuts and egg (*safe*food, 2013).

There is no cure for FHS. Strict avoidance of foods containing the offending allergen is the cornerstone of risk management strategies in the prevention of FHS reactions. Food allergen labelling and regulatory policies are recognised as tools to manage and lower the risk of allergen exposure. Since 2003, EU allergen labelling legislation has been applied to prepacked foods. This legislation has been updated by Regulation (EU) No. 1169/2011, commonly referred to as the *Food Information for Consumers* legislation that came into force in December 2014. Among other aspects of this legislation was an extension of the allergen labelling obligations to non–prepacked foods, or foods sold "loose". However, the Regulation does not completely address the issue of allergen cross-contamination in the process of food production that can potentially make foods unsafe for consumption (Scaravelli et al., 2009).

Cross-contamination with food allergens can occur at any point during production or along the food supply chain. It can be caused by different products being made in the same facilities, on the same equipment or during transportation and handling. Cross-contamination remains the biggest risk for FHS consumers, particularly for those with a severe food allergy such as an allergy to peanut. Unfortunately, this also seems to be one of the most prevalent food allergies around the world (Barnett et al., 2011).

Consumers with FHS rely heavily on accurate food labels to make safe food choices. However, even with sufficient labelling there can be uncertainty posed by the possibility of trace amounts of food allergens accidentally contaminating a food product. Allergic reactions are triggered depending on the quantity of allergen present in the food product and the proportion of product consumed, and the sensitivity of the individual to the allergen. An "eliciting dose" (ED) is the minimal amount of allergen consumed that triggers a reaction (Zurzolo et al., 2013). The establishment of EDs is currently a work in progress. However, the absence of agreed eliciting dose data has resulted in a lack of "reference doses" for allergens in food products to be measured against. This has prompted food manufacturers to voluntarily provide allergy advice for unintentional contamination in manufacturing by using precautionary allergen labelling (PAL) statements such as "may contain". The use of PAL is voluntary and is not covered under existing legislation. As a result, its use across different food businesses and on various food products is inconsistent (DunnGalvin et al., 2015).

According to the FSAI (2011), 28 of the 67 food alerts issued in 2016 concerned food allergens. (Four alerts concerned peanut and 3 concerned gluten). In 2015 there were also 67 food alerts of which 36 concerned food allergens. (Five alerts concerned peanut and 12 concerned gluten). Key reasons for the occurrence of such incidents include

1. Mislabelling of the food product
2. Poor traceability of ingredients along the food supply chain resulting in incomplete labelling
3. Lack of allergen management planning or allergen control
4. Cross-contamination.

This study focusses on the analysis of prepacked food on the island of Ireland for the unintended presence of food allergens, specifically peanut and gluten.

2 Aims and objectives

The aim of this research was to screen prepacked food products available at retail level throughout the island of Ireland for the presence of undeclared or unlabelled peanut or gluten. These products were purchased in Cork, Dublin, Galway and Newry.

Objective 1: Prepacked food products were selected to be investigated for the presence of peanut protein and/or gluten. These included biscuits, breakfast mixes, confectionary products, crisps, protein powders, sauces, snack bars and soups. All products were selected based on an analysis of the product recall literature in the ROI and the United Kingdom (UK) from 2011 to 2017. A database of all the products and brands to be analysed was created.

Objective 2: Initial screening for the presence of peanut and gluten in the food samples using ELISA-based test kits was carried out. Two different kits from Neogen (Veratox® Peanut and Veratox® Gliadin R5) and R-Biopharm (RIDASCREEN®FAST Peanut and RIDASCREEN®FAST Gliadin) were used[3]. All were accredited by the Association of Analytical Communities (AOAC). Following the initial screening, product samples that tested positive were repurchased from different locations and retested to verify the level of allergen.

Objective 3: Real-time quantitative PCR (q–PCR) was carried out on the samples that tested positive during the initial screening and the retest, to confirm and quantify the amount of peanut or gluten present.

Objective 4: The data was analysed and the final report submitted to ***safe*****food**.

[3] Gliadin is a constituent protein of gluten and is found in wheat and other cereals

3 Results

Sample collection

- Thirty-nine different pre-packaged food products were purchased in Cork, Dublin, Galway and Newry at several retailers.
- Twenty products were screened for the presence of peanut and 22 products were screened for gluten. (Five products were screened for both allergens).
- A wide range products was purchased including biscuits, breakfast mixes, confectionary products, crisps, protein powders, sauces, snack bars and soups. These were selected following an analysis of the foods involved in product recalls in the ROI and the UK from 2011 to 2017.
- The products were stored at 4 degrees Celsius (°C) and categorised as “solid”, “semi-solid” or “liquid”.
- Food products were initially screened using ELISA-based assays and subsequently any positive food products were resampled from different locations (sometimes from the same retailer but a different store) and retested for either peanut or gluten using both ELISA and q–PCR assays.

Allergy labelling

- Of the products analysed in the initial ELISA screening, two – Fajita Seasonings and Mexican Style Rice – did not contain any form of PAL.
- In contrast, most of the ready-to-eat (RTE) foods and bakery products had some form of PAL.
- On those products that carried PAL, this predominantly referred to gluten or nuts (either for “nuts” in general or for specific types of nuts.)

Sample analysis

- Sample extraction for both gluten and peanut analyses was done in duplicate (that is, 2 samples of each food product were taken) and each sample was tested in duplicate. This was performed according to the protocols provided by the kit manufacturers.

- Two ELISA-based test kits were used for peanut analysis and 2 were used for gluten analysis: the Veratox® Peanut and Veratox® Gliadin R5 test kits from Neogen, and the RIDASCREEN®FAST Peanut and RIDASCREEN®FAST Gliadin test kits from R-Biopharm.
- The peanut analysis kits contain specific "polyclonal" antibodies. These are a collection of antibodies that react to different features of the same allergen – in this case a range of peanut proteins.
- The gluten (gliadin) analysis kits contain "monoclonal" antibodies. These are antibodies made by identical clones of other immune-response cells that recognise the same single feature of an allergen – in this case, prolamins (proteins) from wheat, rye and barley.
- To ensure analytical performance, an allergen-free sample (negative control) and an allergen-containing sample (positive control or "spiked" sample) were also tested with the product samples. Food products containing more than 0 parts per million (ppm) of allergen were considered positive for the presence of the allergen.

Peanut allergen analysis

- In the initial screening of food products for peanut allergen, 8 products tested positive.
- One product showed a concentration above 30 ppm, 2 samples above 10 ppm and 5 samples showed a concentration of 10 ppm or less (Table 1).
- The sample that showed a concentration of above 30 ppm had a disclaimer of being produced in a "nut-free environment".

Table 1: Results of initial screening of food products for presence of peanut

Sample	Veratox® Peanut (ppm)	RIDASCREEN®FAST Peanut (ppm)
Almond Fingers	1.82	3.98
Cacao Crunch	11.31	27.55
Flame Raisin Granola	3.93	Negative
Garlic and Coriander Mini Naans	2.76	11.32
Hazelnut Cream Wafers	1.27	6.03
Mexican Style Rice	Negative	1.01
Moroccan Falafel	35.03	36.12
Moroccan Medley Couscous	Negative	3.93

- The samples listed in Table 1 were purchased in different locations. Different samples of these same food products all tested negative for peanut (Table 2).
- The Garlic and Coriander Mini Naans were not resampled due to a change in the supplier of the product; the product was substituted with a different brand from the same retailer.
- The lack of reproducibility in the results could be a consequence of cross-reactivity in the initial ELISA tests or of different batches being tested in the second screening.

Table 2: Results of second screening of food products for presence of peanut

Sample	Veratox® Peanut (ppm)	RIDASCREEN®FAST Peanut (ppm)
Almond Fingers	Negative	Negative
Cacao Crunch	Negative	Negative
Flame Raisin Granola	Negative	Negative
Garlic and Coriander Mini Naans	Negative	Negative
Hazelnut Cream Wafers	Negative	Negative
Mexican Style Rice	Negative	Negative
Moroccan Falafel	Negative	Negative
Moroccan Medley Couscous	Negative	Negative

Gluten analysis

Of the food products analysed, most were labelled “gluten-free”.

Twenty samples did not contain detectable gluten.

Two products that did not have a gluten-free label, or any listing of gluten in the ingredients, tested positive. These were tortilla chips and Fajita Seasonings. However, the gluten concentration in both products was below 10 ppm indicating the manufacturer could justifiably have included the label “gluten-free” on these particular samples at least (Table 3).

Table 3: Results of initial screening of food products for presence of gluten

Sample	Veratox® Gliadin R5 (ppm)	RIDASCREEN®FAST Gliadin (ppm)
Fajita Seasonings	2.12	1.81
Tortilla chips	Negative	6.12

- The gluten-positive food products were resampled and analysed with 2 different ELISA kits.
- The Fajita Seasoning tested positive but with a concentration below 10 ppm, while the tortilla chips tested negative (Table 4).

Table 4: Results of second screening of food products for presence of gluten

Sample	Veratox® Gliadin R5 (ppm)	RIDASCREEN® FAST Gliadin (ppm)
Fajita Seasoning	2.39	4.02
Tortilla chips	Negative	Negative

Real-time quantitative polymerase chain reaction

- Food products found to contain undeclared gluten or peanut were further examined by q-PCR[4]. Deoxyribonucleic acid (DNA) from the food products was extracted using phenol: chloroform: isogamy alcohol method (Kang & Yang, 2004). The extraction procedure yielded a high concentration of DNA with minimal presence of contaminants such as ribonucleic acid (RNA), proteins, salts and other organic compounds.
- The food products that tested positive during the first ELISA screening (and during the second, in the case of 1 gluten test) were shown to be negative with q-PCR.

The amplification in Figures 1 and 2 depicts the inhibition control containing the samples of both test positive gluten and peanut. The expression of amplification of the inhibition for samples showing no contamination in the reaction or in sample.

[4] Real-time Polymerase Chain Reaction, also known as quantitative Polymerase Chain Reaction (hence 'q-PCR'), permits monitoring of the amplification of a targeted DNA molecule while this is actually happening without having to wait until the process has concluded as with conventional PCR. q-PCR can be used quantitatively or semi-quantitatively. Quantification is achieved through measurement of fluorescence. q-PCR can be used to detect DNA from allergenic foods such as peanut or egg. However, a positive result is not necessarily indicative of the presence of an allergen (and vice versa). Consequently, q-PCR is used in conjunction with other tests such as those based on ELISA.

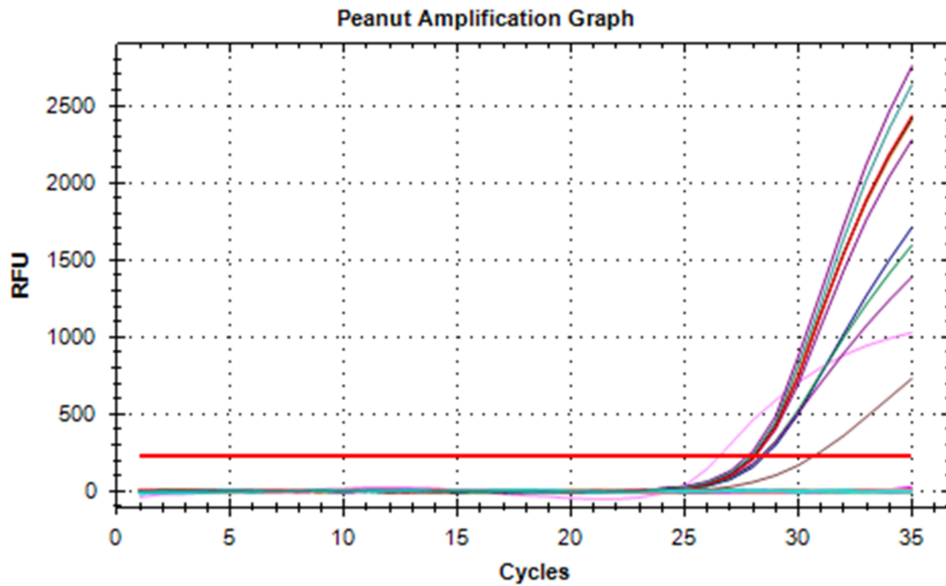

Figure 1: Peanut amplification graph. This shows the amplification plot of inhibition control of samples and no amplification for test samples: Pink (positive control), Purple (inhibition control), Grey (negative control), Blue (Moroccan Couscous), Dark Blue (Couscous inhibition), Green (Hazelnut wafers), Dark Green (Hazelnuts Wafers inhibition), Yellow (Mini Naans), Orange (Mini Naans inhibition), Purple (Granola), Dark Purple (Granola inhibition), Brown (Cacao Crunch), Dark Brown (Caco Crunch inhibition), Grey (Mexican Rice), Dark Grey (Mexican Rice inhibition), Pink (Almond Fingers), Red (Almond Fingers inhibition), Mint (Moroccan Falafel) and Dark Mint (Falafel inhibition.)

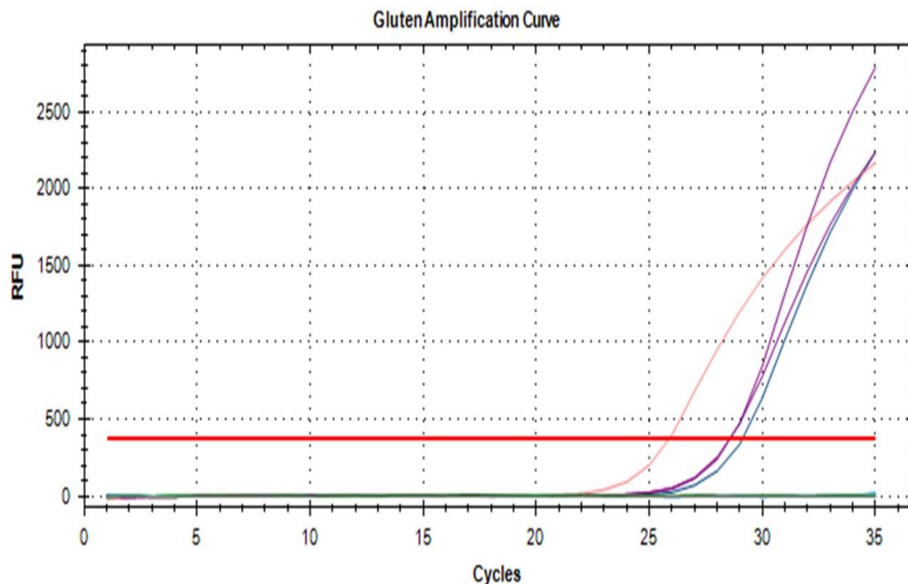

Figure 2: Gluten amplification graph. This shows the amplification plot of inhibition control of samples and no amplification for test samples: Pink (positive control), Purple (inhibition control), Grey (negative control), Blue (Tortilla), Dark Blue (Tortilla inhibition), Green (Fajita Seasoning), Dark Green (Fajita Seasoning inhibition.)

4 Discussion

In this study, 42 food products were purchased, 20 for peanut analysis and 22 for gluten analysis. (Five underwent both peanut and gluten analyses.) These were screened using both Veratox® and RIDASCREEN® ELISA assay kits for peanut or gluten. Ten of the 42 food products (24%) investigated tested positive for the allergen in the first ELISA screening.

The constraint in the investigation was that the occurrence of false positives was an indication of cross-reactivity of the test kit. To confirm that samples were positive, repeat ELISA tests were performed in alongside q-PCR tests on the positive samples from the first screening and the second-purchase confirmatory samples.

Eight samples were positive for peanut – 5 by both Veratox® and RIDASCREEN® kits, 1 by Veratox® only and 2 by RIDASCREEN® only. Subsequently, new samples of these 8 food products were purchased and tested using both ELISA kits. These were negative for peanut allergen. All 31 samples from both the first and second ELISA screenings were negative with q-PCR. On this basis, the peanut-positive samples from the initial ELISA screening were considered to be false positives.

Two samples were positive for gluten – 1 by both Veratox® and RIDASCREEN® kits and 1 by RIDASCREEN® only. On the subsequent testing of new samples of these products, 1 was positive for the presence of gluten using both ELISA kits. Both food products were negative with q-PCR. Because 1 sample was positive in the initial and second screenings with ELISA, it was considered to be positive for the presence of gluten.

Peanut analysis

Eight of the 20 samples (40%) tested for peanut were positive - 5 by both Veratox® and RIDASCREEN® kits, 1 by Veratox® only and 2 by RIDASCREEN® only. Subsequently, new samples of these same food products tested negative for peanut allergen when screened using both ELISA kits. All positive samples from the first screening and the new samples for the second screening were negative with q-PCR. Therefore, the peanut-positive samples from the initial ELISA screening were considered to be false positives.

Compared with the initial ELISA screening, the complete absence of any detected peanut in the second samples of the same food products was striking. The kit manufacturers advertise the specificity of their kits. However, both kits are specific for several allergenic peanut proteins including

Ara h 1, *Ara h 2*, *Ara h 3* and so on, and many of these share sequence homology with proteins from other plant species[5]. *Ara h 1* alone shares sequence homology with other allergenic vicilins (proteins that are found in legumes) including *Gly m 5* in soybean, *Pis s 1* in pea, *Ana o 1* in cashew nut, *Jug r 2* in walnut, *Len c 1* in lentil, *Ses i 3* in sesame and *Cor a 11* in hazelnut (Schein et al., 2005; Bublin and Breiteneder, 2014). According to the ingredients listed on the labels, the food products that tested positive for peanut contained some form of tree nuts or legumes.

A similar suggestion was made by the Food Standards Agency in the UK (2014) when they conducted an investigation of undeclared allergens in prepacked food. False positive results identified by AOAC-approved ELISA kits were attributed to cross-reactivity of peanut allergens to that of soya. Such cross-reactivity occurs due to changes in the structure of the target molecule (the allergen of interest) caused by food processing or sample extraction methods (Walker et al., 2015). “Matrix effects” of the food products themselves (that is, the physical and chemical nature of the food) can have a significant influence on the sample extraction and preparation steps and subsequently the detection of allergens, particularly in the RTE foods (Scaravelli et al., 2009).

That said, it seems strange that on subsequent screening of new samples of the same food products with the same label, using the same extraction and preparation procedure, none returned a positive result by either ELISA kit. If the outcome of the second screening was an artefact (i.e. due to the experimental process itself) of kit storage and therefore a false negative, then the levels of peanut detected in the initial screening justify further investigation, particularly where levels exceeded 2 ppm (0.2 milligrams [mg] per 100 grams [g] of food).

The expert panel for the establishment of reference doses for allergenic food residues, as a part of the Voluntary Incidental Trace Allergen Labeling (VITAL®) Program of the Allergen Bureau of Australia and New Zealand, established a reference dose of 0.2 mg protein for peanut (Taylor et al., 2014). This was based on “ED01”, which is the dose of peanut allergen that elicits a reaction in 1% of the peanut-allergic population or those that are highly sensitive to peanut.

The VITAL® system advocates PAL for peanut levels of between 2 and 20 ppm, and full ingredient labelling for levels greater than 20 ppm (Zurzolo et a., 2013). Taking average values from the 2 ELISA kits, 7 of the positive samples would justify the use of PAL and 1 sample would require “peanut” to be included in the ingredients list under this system. However, for this to apply, definitive LC–MS analyses of each sample would be required.

[5] “Shared sequence homology” means these proteins have evolved at some point from the same genetic source and so have similar DNA, and therefore amino acid, sequences.

Gluten analysis

Two (10%) samples tested positive for gluten – 1 (Fajita Seasoning) by both Veratox® and RIDASCREEN® kits and 1 (tortilla chips) by RIDASCREEN® only. On the subsequent screening of new samples of these same food products, the Fajita Seasoning was found to be positive for the presence of gluten, again by both ELISA kits. Both food products were negative with q-PCR. However, because Fajita Seasonings was positive in the initial and second screenings with ELISA, it was considered to be positive for the presence of gluten.

Eighteen food products did not contain detectable gluten. Most of these carried the "gluten-free" label advisory, highlighting its reliability for those consumers who need to (or wish to) avoid gluten.

The results from the ELISA assays were more reproducible for the gluten (gliadin) analyses and the samples of Fajita Seasonings were positive in both the initial and second screenings. No sample tested positive with q-PCR, which may not be an effective method for the detection of allergens in processed food products (Khuda et al., 2012; Fu and Maks, 2013). This may be because the method involves a thermal processing step and heat treatment that can cause denaturation and degradation of DNA (Scaravelli et al., 2009; Costa et al., 2014).

The highest level of gluten measured was 4.02 ppm, which is well below the threshold for using a "gluten-free" label advisory. It is worth noting that this product did not carry such an advisory. This highlights the possibility that many other commercially available food products could carry a "gluten-free" indication on the label. This would extend the range of products that are accessible to FHS consumers by providing extra assurance that the product is safe for them to eat.

However, the presence of gluten also indicates the potential for cross-contamination with food allergens. The unintentional presence of allergens in foods may be a result of contamination at some stage during the manufacturing process or maybe even during product distribution (Surojanametakul et al., 2012; Walker et al., 2015). This can result from contact with inadequately cleaned equipment or with allergen-containing products. The hygiene principles used to control bacterial contamination can also be applied to the management of allergen cross-contamination.

This research showed that the use of commercial allergen test kits and different allergen testing methodologies still requires considerable interpretation and carries an amount of uncertainty. Other methods being developed for the identification and quantification of allergens in food include

microarray[6] and surface Plasmon resonance[7] (Scherf et al., 2016). Methods based on LC–MS have also been developed and, while these methods are considered definitive for allergen analyses, they are slow, high cost and require considerable expertise.

There is now an obvious need for the development of robust, sensitive and cost-effective methods for the detection of allergens in processed foods.

[6] A microarray is a device used to detect the expression of thousands of genes at the same time. For instance, a DNA microarray consists of a microscope slide containing known DNA genes or sequences. These act as probes to detect specific gene expression in a sample.

[7] Surface plasmon resonance (SPR) is a detection method based on optical changes to thin metal films that are triggered by molecular interactions such as protein-protein or protine-DNA binding. These interactions can be measured in real-time with high sensitivity and without the need of labels.

5 Recommendations

1. Food manufacturers should include allergen advice statements such as PAL only where absolutely necessary.

2. Where feasible, adding an advisory “free from” statement would give food-hypersensitive consumers more confidence in making safe choices of food products, as well as making a wider range of food products more accessible to them.

3. Food manufacturers should develop and implement specific Allergen Management Plans, separate to their existing Hazard Analysis and Critical Control Plans, to manage allergenic ingredients and the potential for cross-contamination. This should extend from pre- to post-production of packaged food products – from the raw food supplier to the retailer or food outlet.

4. Allergic consumers must always investigate the ingredients list for the presence of allergens to which they react. They also need to consider any PAL present.

5. Currently, ELISA is the method of choice employed for the detection of allergens in foods. However, this technique has its drawbacks, such as cross-reactivity of peanut allergens with those from soy, chickpeas, almonds and hazelnut. There is now a clear need to develop a robust, reliable and inexpensive method for detection of peanut, gluten and other allergens in pre-packaged and processed foods.

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7 Appendices

Appendix 1: Food products analysed for the presence of undeclared peanut and gluten

Table 5: Products tested for undeclared gluten

Products	Purchase location	Free from undeclared gluten?
White rolls	Newry	Yes
Pork pies	Newry	Yes
Ginger Crunch Cookies	Cork	Yes
Salami sticks	Cork	Yes
Gluten-free bread	Cork	Yes
Moroccan Falafel	Cork	Yes
Gluten-free Mini Rice Cakes	Cork	Yes
Cheddar and Shallot Handcooked Crisps	Cork	Yes
Hazelnut Cream Wafers	Galway	Yes
Mature Cheddar and Red Onion Crisps	Galway	Yes
Mexican Style Rice	Galway	Yes
Tortilla chips	Galway	Possibly
Hand Cooked Vegetable Crisps	Galway	Yes
Sour Cream and Onion Lentil Curls	Galway	Yes
Fruity Couscous Salad	Dublin	Yes
Salt Marsh Lamb and Mint Hand Cooked Crisps	Dublin	Yes
Gluten-free soy sauce	Dublin	Yes
Scotch eggs	Dublin	Yes
Cacao Crunch	Dublin	Yes
Lightly Salted Rice Cakes	Dublin	Yes
Wasabi peas	Dublin	Yes
Fajita Seasoning	Dublin	No

Table 6: Products tested for undeclared peanut

Products	Purchase location	Free from undeclared peanut?
Chia Pod	Newry	Yes
Harissa Style Couscous	Newry	Yes
Mango and Passion Fruit Yogurt	Newry	Yes
Oaty Flapjacks	Cork	Yes
Energy Bar	Cork	Yes
Cacao powder	Cork	Yes
Organic Yacon Granola	Cork	Yes
Garlic and Coriander Mini Naans	Cork	Possibly
Moroccan Falafel	Cork	Possibly
Flame Raisin Granola	Cork	Possibly
Cacao Crunch	Cork	Possibly
Roasted Nut and Seed Granola	Galway	Yes
Red pesto	Galway	Yes
Weetabix On the Go Breakfast Drink	Galway	Yes
Hazelnut Cream Wafers	Galway	Possibly
Almond Fingers	Galway	Possibly
Mexican Style Rice	Galway	Possibly
Caramel and Pecan Cookies	Dublin	Yes
Pilau Basmati Rice	Dublin	Yes
Moroccan Medley Couscous	Dublin	Possibly

Appendix 2: Analytical methods

The method used for initial analysis of the products was based on 2 commercial ELISA kits obtained from Neogen and R-Biopharm.

A2.1 Veratox® Peanut ELISA kit

A2.1.1 Sample preparation

1. Samples were homogenised into fine particles using liquid nitrogen. ("Homogenisation" is a process that breaks a substance down and suspends it evenly throughout a solution.) Five grams of sample or 5 ml of liquid sample was transferred into a sterile extraction bottle to which 1 scoop of extraction additive was added.
2. One hundred and twenty-five millilitres of preheated (60 °C) extraction solution (phosphate "buffer" saline, which helps to maintain a constant pH, in 1 litre of distilled water) was transferred to the extraction bottle.
3. Samples were extracted by placing the extraction bottle containing the sample in a shaker water bath at 60 °C at 150 revolutions per minute (rpm) for 15 minutes.
4. The sample, once settled, was centrifuged (spun) at 4000 rpm for 20 minutes and the clear supernatant (the liquid left lying above the settled sediment) was used for analysis.
5. The supernatant was used immediately for analysis.

A2.1.2 Sample analysis

Before the start of analysis all the reagents (the compounds to be added to create a reaction) were brought to room temperature. They were stored at 2 to 4 °C after use. Microwells were not left to dry or left in direct exposure to sunlight. ("Microwells" are flat plates covered in many "wells" that are used as very small test tubes.)

1. The wash buffer provided as 10 times (10x) concentrate was reconstituted into distilled water.
2. The supernatant obtained from the sample preparation was mixed well and 100 microlitres (µl) of this solution was directly added onto the antibody-coated microwells. Each extraction was analysed in duplicate to ensure high analytical performance.
3. As standard control a concentration of peanut allergen was used – 0 ppm, 2.5 ppm, 5.0 ppm, 10.0 ppm and 25.0 ppm. One hundred microlitres of each standard solution was pipetted into the antibody-coated microwell strip.
4. The microwell strip was covered and incubated for 10 minutes.

5. Unbound compounds were washed off using wash buffer.
6. One hundred microlitres of enzyme conjugate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
7. The microwell strip was then covered and incubated for 10 minutes.
8. Unbound compounds were washed off using wash buffer.
9. One hundred microlitres of substrate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
10. The microwell strip was then covered and incubated for 10 minutes.
11. One hundred microlitres of Neogen's Red Stop Solution (a reagent) was added onto each well. The microwell strips were read using a Stat Fax® microwell plate reader with 650 nanometre (nm) filter.

A2.2 RIDASCREEN®FAST Peanut kit

A2.2.1 Sample preparation

1. Five grams of food sample was homogenised using liquid nitrogen. One gram of the ground sample was transferred to a 50 ml centrifuge tube. Furthermore, 1 g of skimmed milk was added to food products containing spices and chocolate.
2. Twenty millilitres of preheated extraction buffer (60 °C) was then added.
3. The extract was extensively mixed in a shaker water bath for 10 minutes.
4. The sample, once cooled, was centrifuged at 4000 rpm for 10 minutes.
5. The clear supernatant obtained was used immediately for analysis.

A2.2.2 Sample analysis

Before the start of analysis all the reagents were brought to room temperature. They were stored at 2 to 4°C immediately after use. Microwells were not left to dry or left in direct exposure to sunlight.

1. The wash buffer provided as 10x concentrate was reconstituted into distilled water.
2. The supernatant obtained from the sample preparation was mixed well. One hundred microlitres of this solution was directly added on to the antibody-coated microwells. Each extraction was analysed in duplicate to ensure high analytical performance.
3. As standard control a concentration of peanut allergen was used – 0 ppm, 2.5 ppm, 5.0 ppm, 10.0 ppm and 20.0 ppm. One hundred microlitres of each standard solution was pipetted into the antibody-coated microwell strip.
4. The microwell strip was covered and incubated for 10 minutes.

5. Unbound compounds were washed off using 250 µl of wash buffer.
6. One hundred microlitres of enzyme conjugate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
7. The microwell strip was then covered and incubated for 10 minutes.
8. Unbound compounds were washed off using 250 µl of wash buffer.
9. One hundred microlitres of substrate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
10. The microwell strip was then covered and incubated for 10 minutes.
11. One hundred microlitres of Neogen's Red Stop Solution was added onto each well. The microwell placed on microwell titre plate were read at an absorbance of 450nm.

A2.3 Veratox® Gliadin R5 kit

A2.3.1 Sample preparation

1. Samples were homogenised into fine particles using liquid nitrogen.
2. One-quarter of a gram of processed sample was weighed into a 50 ml screwcap centrifuge tube.
3. Two-and-a-half millilitres of Cocktail Solution from Neogen was added to heat-processed samples and incubated in a water bath at 50 °C for 40 minutes.
4. Once the samples were cooled, 7.5 ml of 80% ethanol was added and vortexed (mixed) for 20 seconds.
5. Samples were shaken at room temperature on a shaker incubator for 1 hour.
6. The samples were centrifuged at 4000 rpm for 10 mins.
7. The supernatant was diluted in 1:12.5 (that is, 200 µl in 2 ml) of phosphate buffer solution and vortexed for 5 seconds.
8. The diluted samples were analysed using antibody-coated microwells.

A2.3.2 Sample analysis

Before the start of analysis all the reagents were brought to room temperature. They were stored at 2 to 4°C immediately after use. Microwell strips were not let to dry or left in direct exposure to sunlight.

1. The wash buffer provided as 10x concentrate was reconstituted into distilled water.
2. The supernatant obtained from the above sample preparation was mixed well and 100 µl of this solution was directly added onto the antibody-coated microwells. Each extraction was analysed in duplicate to ensure high analytical performance.

3. As standard control a concentration of peanut allergen was used – 0 ppm, 2.5 ppm, 5.0 ppm, 10.0 ppm, 20.0 ppm and 40.0 ppm. One hundred microlitres of each standard solution was pipetted into the antibody-coated microwell strip.
4. The microwell strip was covered and incubated for 10 minutes.
5. Unbound compounds were washed off using wash buffer.
6. One hundred microlitres of enzyme conjugate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
7. The microwell strip was then covered and incubated for 10 minutes.
8. Unbound compounds were washed off using wash buffer.
9. One hundred microlitres of substrate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
10. The microwell strip was then covered and incubated for 10 minutes.
11. One hundred microlitres of Neogen's Red Stop Solution was added onto each well. The microwell strips were read using a StatFax® microwell plate reader with 650 nm filter.

A2.4 RIDASCREEN®FAST Gliadin kit

A2.4.1 Sample preparation

1. Samples were homogenised into fine particles using liquid nitrogen.
2. One-quarter of a gram of processed sample was weighed into a 50 ml screwcap centrifuge tube. A further 0.25 g of skimmed milk was added for samples containing spices and chocolate.
3. Two-and-a-half millilitres of Cocktail Solution from Neogen was added to heat-processed samples and incubated in a water bath at 50 °C for 40 minutes.
4. Once the samples were cooled, 7.5ml of 80% ethanol was added and vortexed for 20 seconds.
5. Samples were shaken at room temperature on a shaker incubator, upside down, for 1 hour.
6. The samples were centrifuged at 4000 rpm for 10 minutes.
7. The supernatant was diluted in 1:12.5 (that is, 80 µl in 920 µl) of sample diluent and vortexed for 5 seconds.
8. The diluted samples were analysed immediately using antibody-coated wells.

A2.4.2 Sample analysis

Before the start of analysis all the reagents were brought to room temperature. They were stored at 2 to 4°C immediately after use. Microwells were not left to dry or left in direct exposure to sunlight.

1. The wash buffer provided as 10x concentrate was reconstituted into distilled water.
2. The supernatant obtained from the above sample preparation was mixed well. One hundred microlitres of this solution was directly added onto the antibody-coated microwells. Each extraction was analysed in duplicate to ensure high analytical performance.
3. As standard control a concentration of peanut allergen was used – 0 ppm, 10 ppm, 20 ppm, 40 ppm and 80 ppm. One hundred microlitres of each standard solution was pipetted into the antibody-coated microwell strip.
4. The microwell strip was then covered and incubated for 10 minutes.
5. Unbound compounds were washed off using 250 µl of wash buffer.
6. The enzyme conjugate was reconstituted in 1:11 ratio with distilled water (that is, 1 ml of concentrate to 11 ml of distilled water).
7. One hundred microlitres of enzyme conjugate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
8. The microwell strip was then covered and incubated for 10 minutes.
9. Unbound compounds were washed off using 250 µl of wash buffer.
10. One hundred microlitres of substrate was added to each well. The well contents were mixed thoroughly by sliding the strips back and forth on a flat surface for 20 seconds.
11. The microwell strip was then covered and incubated for 10 minutes.
12. One hundred microlitres of Neogen's Red Stop Solution was added onto each well. The microwell placed on microwell titre plate were read at an absorbance of 450 nm.

NOTE:

1. Each sample was read within 30 minutes of adding Red Stop Solution.
2. A standard curve of absorbance against the concentration of the standard was analysed. The concentration of unknown sample was read from this graph.

Appendix 3: Analysis of products for undeclared peanut and gluten using q-PCR

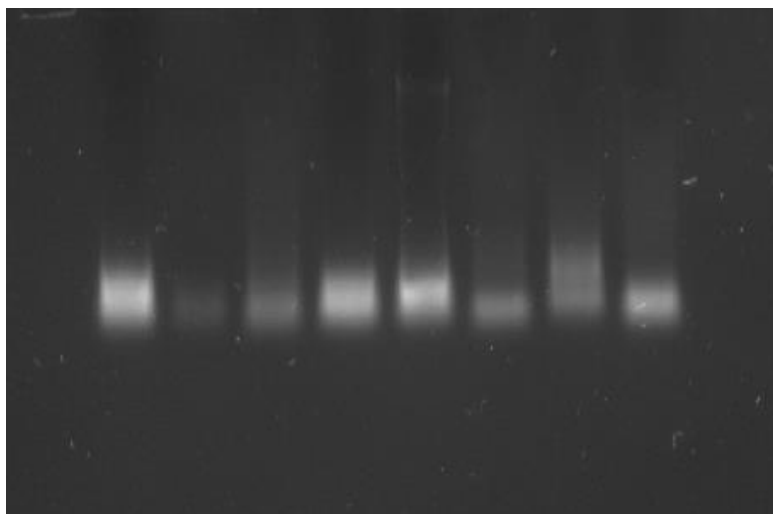
Genomic DNA extraction of the food products was done by Phenol: Chloroform: Isoamylalcohol (25:24:1) (Iniesto et al., 2013; López-Calleja et al., 2013). The amount of starting material used for the extraction was 1 g, and the same was maintained for all food products that were positive in the initial screening for undeclared peanut and gluten.

The concentration of the samples was checked using a NanoDrop™ spectrophotometer (which tests the light density of samples) and a high yield of genomic DNA was obtained from the food products tested for gluten and peanuts (Table 7 and Table 8). There was minimal contamination of RNA, proteins, salts and other organic compounds obtained from the 260/280 ratio and 260/230 ratio that was obtained from the NanoDrop™. Agarose gel electrophoresis (a technique that is used to identify DNA fragments) was later used to verify the results obtained from the NanoDrop™ (Figure 3 and Figure 4).

Table 7: Genomic DNA concentration of product analyses for undeclared peanut

Food Products	Concentration (ng/µl)
Hazelnut Cream Wafers	76.2
Garlic and Coriander Mini Naans	176.2
Cacao Crunch	284.5
Almond Fingers	231.5
Moroccan Falafel	286.6
Mexican Style Rice	307.3
Moroccan Medley Couscous	285.7
Flame Raisin Granola	339.7

Figure 3: Agarose gel electrophoresis of genomic DNA of products analysed for the presence of undeclared peanut



Lane 1: Moroccan Medley Couscous

Lane 2: Hazelnut Cream Wafers

Lane 3: Garlic and Coriander Mini Naans

Lane 4: Flame Raisin Granola

Lane 5: Cacao Crunch

Lane 6: Mexican Style Rice

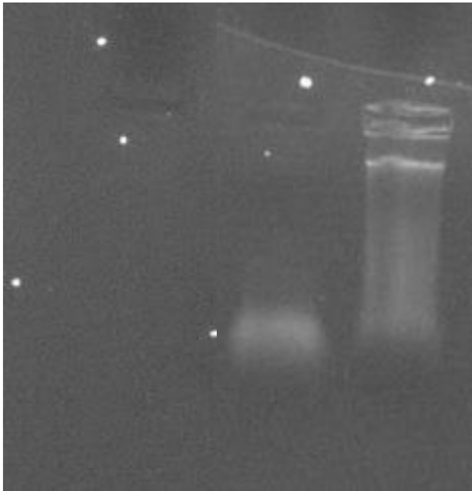
Lane 7: Almond Fingers

Lane 8: Moroccan Falafel

Table 8: Genomic DNA concentration of product analyses for undeclared gluten

Food Products	Concentration (ng/µl)
Fajita Seasoning	320.1
Tortilla chips	355.7

Figure 4: Agarose gel electrophoresis of genomic DNA of products analysed for the presence of undeclared gluten



Lane 1: Tortilla chips

Lane 2: Fajita Seasonings

The extracted genomic DNA of all the products was analysed using q-PCR kits from R-Biopharm (SureFood® ALLERGEN ID Peanut and SureFood® ALLERGEN ID Gluten).

Components for q-PCR for both peanut and gluten were

1. Reaction mix
2. Inhibition control
3. Taq polymerase
4. Dilution buffer
5. Standard DNA.

Control assay for every PCR included

1. Positive ontrol
2. Negative control
3. Inhibition control for every sample
4. Positive control of inhibition control.

Preparation of real-time PCR mix for peanut:

1) Twenty microlitres of the master mix was pipetted into appropriate wells on a 96-well plate.
2) Five microlitres of sample DNA test was pipetted into the designated wells.
3) A positive control of 5 µl was added into the designated wells.
4) The 96-well plate was run on the real-time PCR instrument for a total of 35 cycles. The “reporter dye” used – a fluorescent dye that is easily seen – was 6-carboxy fluorescein (“FAM”).

Preparation of real-time PCR mix for gluten:

1) Twenty microlitres of the master mix was pipetted into appropriate wells on a 96-well plate.
2) Five microlitres of sample DNA was pipetted into the designated wells.
3) A positive control of 5 µl was added into the designated wells.
4) The 96-well plate was run on the real-time PCR instrument for a total of 35 cycles. The reporter dye was FAM.

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