

Allergen Bureau 2010 Conference,
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Mt Wellington

Allergen testing – what's new?

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Technical Director - FACTA

Food Allergen Control Training Analysis

FACTA is an Australian company formed to provide expert advice and consultative services on food borne allergens for the food manufacturing, food service and associated supply industries. We provide specialist training in food allergen awareness, specialist analysis services for food borne allergens and consultation in strategies for allergen control within factory and food service environments. We have had experience in this area over the past six years under the umbrella of EML Consulting Services Qld and holding National accreditation NATA – National Association of Testing Authorities - for the performance of peanut and now gluten analysis .

We work closely with both industry and regulatory laboratories and have been involved in Industry working groups and the Australian Standards committee and provided expert advice and witness where required .

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FACTA
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In Brief

- Analysis Basics
- The Good , the Bad and the Ugly
- Proficiencies
- Sampling plans (there's a concept !)
- Future

Techniques Applicable for Routine Analysis

- Non specific
 - ATP – bioluminescence
- Specific
 - Target either the allergen itself or a marker that indicates the presence of the allergenic food .
- Markers include
 - Specific proteins
 - DNA fragments

Detection Methodology Requirements

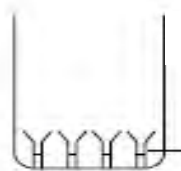
- **Sensitive** (ppm to ppb)
- **Quantitative**
- **Specific** (does not give false positives)
- **Widely Applicable** (does not give false negatives in complex food matrices)
- **Rapid**
- **Technically Straight-forward**
- **Economical**
- **Validated** (independently to ensure all of the above true!)



ELISA (Enzyme-Linked Immunosorbent Assays) are the current **analytical method of choice for food allergen detection**

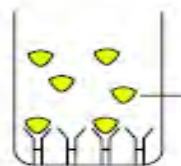
Sandwich ELISA

Microwells coated with specific antibody to allergen



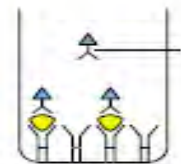
Antibody

Sample and controls added to respective wells



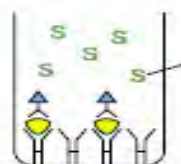
Allergen

Conjugate binds to second epitope of allergen



HRP-Antibody Conjugate

Colourless substrate enzymatically converted to blue product (amplification step)



Substrate

Spectrophotometer used to measure OD

Linear range an order of magnitude

Negative



Positive

Key Analytical Considerations

(1) Type of test kit

- what does it detect
- is kit VALIDATED
- how long does it take?

(2) Objectives of the testing

- will processing and type of sample matrix affect detection of allergenic protein?
- is sampling plan appropriate?

(3) Result Interpretation

- what does my result mean?

Enzyme Linked Immuno Sorbent Assays Available

- Almond
 - Buckwheat
 - Crustacea (tropomyosin)
 - Gluten
 - Lupin
 - Peanut
 - Soy
 - Walnut
- Beta Lactoglobulin
 - Casein
 - Egg
 - Hazelnut
 - Mustard
 - Sesame
 - Total Milk
- Increasing number of reputable kit producers responsive to industry needs

Results expressed as

Allergen Kit Name	LOD/ LOQ/ LOR (ppm)	Results expressed as	Antibody Target	Sample
Neogen Veratox for Egg Allergen	No LOD / 2.5 / 2.5	ppm of Whole Dried Egg	Unprocessed and heat processed egg protein residue	Product & Environmental Samples (pasta, salad dressing, cake mix, ice cream)
ELISA Systems Enhanced Egg Residue	No LOD / 1 / 1	ppm Egg Powder	Ovomucoid (heat stable)	Product samples
Neogen Veratox for Peanut Allergen	No LOD / 2.5 / 2.5	ppm of Total Peanut (detects peanut protein)	Peanut proteins	Product & environmental samples (cookies, crackers, chocolate bars , ice cream and cereals)
ELISA Systems Enhanced Assay Soy Protein Residue	No LOD / 2.5 / 2.5	ppm of Soy Flour Protein	Soy Trypsin Inhibitor and other soy proteins	Product & Environmental Samples
Neogen Veratox Total Milk Allergen	1 / 2.5 / 2.5	ppm of Non Fat Dried Milk (NFDM)	Casein and whey proteins from cow, goat sheep	Product & Environmental Samples (juices, cake mixes, cookies , sauces and sorbets)
ELISA Systems Beta-Lactoglobulin Residue	No LOD / 0.1 / 01	ppm of BLG (x by 31.2 to express as ppm of NFDM)	Whole Milk OR Whey (Ruminants and Pigs)	Product & Environmental Samples
ELISA Systems Casein Residue	No LOD / 1 / 1	ppm of Non Fat Dried Milk (NFDM)	Alpha S Casein from mammalian species (including cow)	Product Samples
Neogen Veratox for Almond Allergen	No LOD / 2.5 / 2.5	ppm of total almond	Almond protein (cross reacts with apricot seed)	Product & Environmental Samples(food products)
ELISA Systems Almond Residue	No LOD / 0.5 / 0.5	ppm of Almond Protein (x by ~4.8 to express as ppm of Total Almond)	Specific Almond Protein (heat stable)	Product Samples
ELISA Systems Sesame Seed Protein residue	No LOD / 0.5 / 0.5	Sesame seed protein	2S – albumin protein (heat stable)	Product & Environmental Samples
ELISA Systems Crustacean -	NO LOD / 0.05 / 0.05	ppm of Crustacean	Tropomyosin	Product & Environmental Samples



The Good , The Bad and the Ugly .

- Not all kits are created equal
 - Gluten
 - Antibodies are important
 - Skerrit, Mendez and ... The list goes on
 - Competitive assay
 - For the peptide not the protein
 - QQFPF
 - » difficult to convert this to an equivalent gluten level due to variability in the degree of hydrolysis between samples
 - » Starches and washing
 - Gliadin/ Glutelins relationship altered by the degree of washing



Analytical Considerations: Type of Test Kit

Critical determinant of gluten assay sensitivity, specificity and applicability is the source of antibody

Two main commercial antibodies used:

Antibody Name	Ref	Description	Epitope	Prolamins Detected		Kit Suppliers using Antibody
Mendez	Valdés <i>et al</i> 2003	Monoclonal R5	QQFPF	Gliadin (wheat)	100%	R-Biopharm
				Secalin (rye)	100%	Neogen
				Hordein (barley)	100%	
				Avenin (oats)	0%	
Skerritt	Skerritt <i>et al</i> 1991	Monoclonal	-	Gliadin (wheat)	100%	Tepnel
				Secalin (rye)	120%	ELISA Systems
				Hordein (barley)	5%	
				Avenin (oats)	0%	

Note that neither antibody detects prolamins from oats

The Codex Committee on Methods of Analysis and Sampling (CCMAS) approved Mendez ELISA assay as a **type I** method that is **the recommended method for the detection of gluten in food** (CAC, 2006).



Proficiencies

- Efficacy
- Comparability
 - Reference Materials
- Programs
 - FAPAS
- And possibilities
 - NMI

In the absence of peanut reference standards, proficiency programs essential for comparing kit performance

2009 FAPAS round for peanut in chocolate

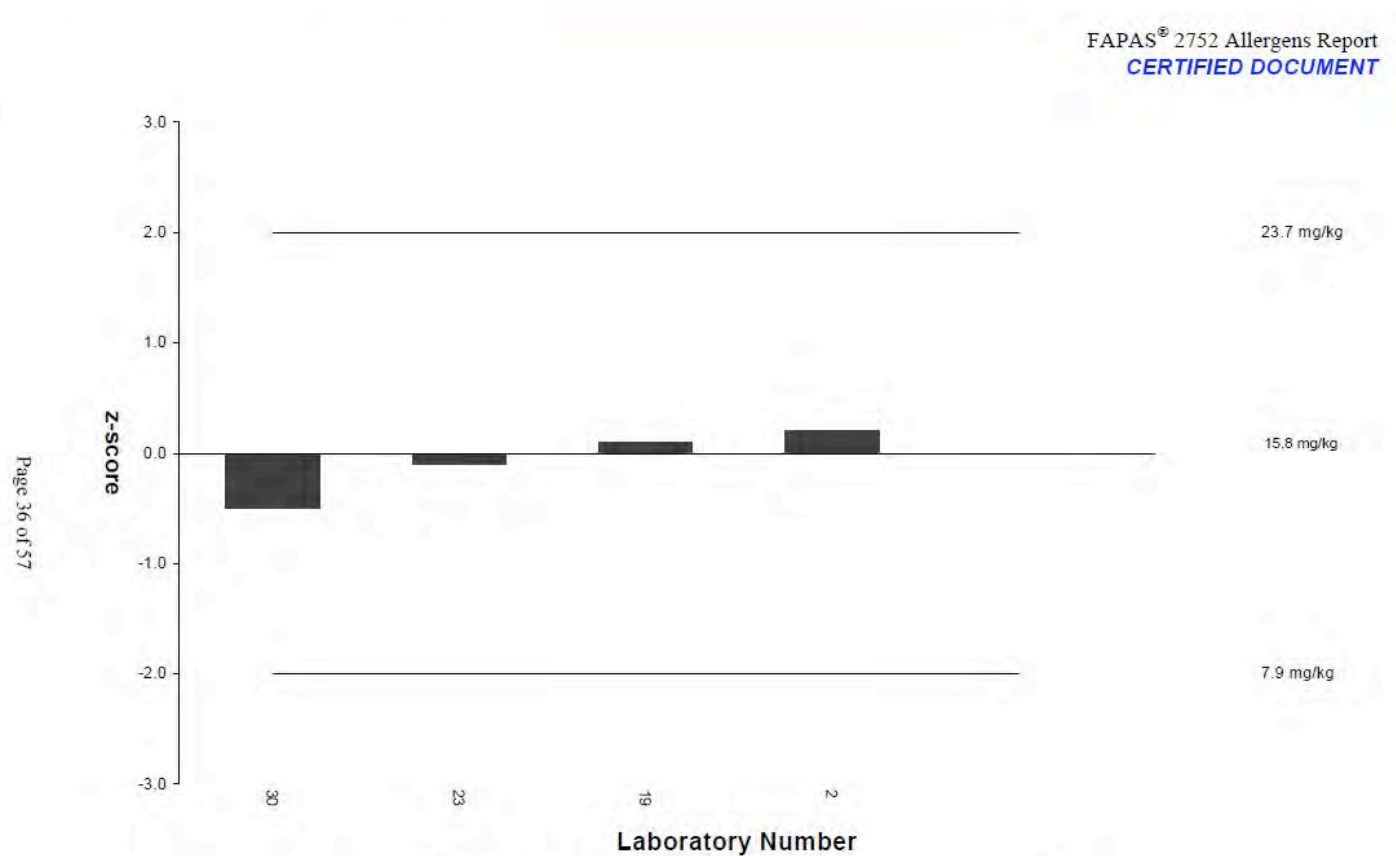


Figure 4: z-Scores for 'Neogen' Peanut Data (15.8 mg/kg) for Chocolate Test Material 2752-B

Reported Results Dependent on Kit Source

FAPAS[®] 2752 Allergens Report
CERTIFIED DOCUMENT

9. Quantitative results for this round are summarised as follows:

test material	analyte	assigned value \bar{X} , mg/kg	number of scores within $ z \leq 2$	total number of scores	%
2752-B	peanut protein 'ELISA Systems'	2.6	4	4	100
	peanut 'Neogen'	15.8	4	4	100
	peanut 'R-Biopharm'	20.2	8	10	80
	peanut 'Tepnel'	16.0	20	20	100

Valid Comparison Dependent on Knowing Standards Used and how Results Reported

:

Kit Manufacturer	Detects	Results Format	LOQ (ppm)	Adjusted LOQ* Peanut Protein (ppm)
Neogen	Peanut protein	Total Peanut	2.5	0.625
ELISA SYSTEMS	Ara h1 Ara h2 + other peanut proteins	Peanut Protein	1.0	1

* assumes 25% of peanut weight is protein

http://www.allergenbureau.net/downloads/vital/VITAL_Allergenic_Protein_Levels_10_11_08.pdf

Results Dependent on Kit Source

FAPAS[®] 2752 Allergens Report
CERTIFIED DOCUMENT

9. Quantitative results for this round are summarised as follows:

test material	analyte	assigned value \bar{X} , mg/kg	number of scores within $ z \leq 2$	total number of scores	%	Corrected assigned value mg/kg peanut PROTEIN
2752-B	peanut protein 'ELISA Systems'	2.6	4	4	100	2.6
	peanut 'Neogen'	15.8	4	4	100	4.0
	peanut 'R-Biopharm'	20.2	8	10	80	5.1
	peanut 'Tepnel'	16.0	20	20	100	4.0

Residual difference likely reflects different source of calibration standards

Critical to know reporting units for application to VITAL

Gluten assay not so technically straightforward

FAPAS[®] 2747 Allergens Report
CERTIFIED DOCUMENT

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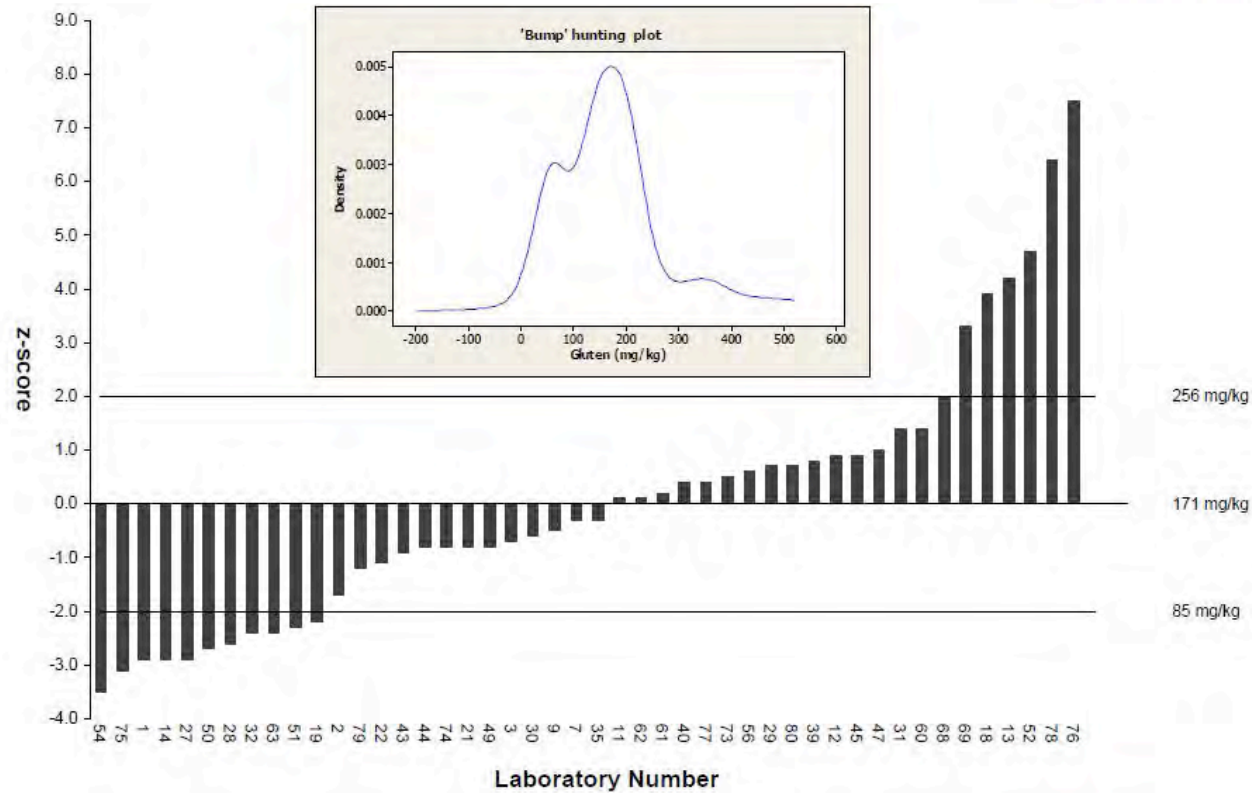


Figure 5: z-Scores for 'R-Biopharm' Gluten Data (171 mg/kg) for Chocolate Cake Mix Test Material 2747-A
 This histogram is shown for information only

Considerations for Analysis

Nature of sample matrix:

When using testing to assess potential cross-contact of an allergenic product within a facility, verify that allergen itself can in fact be detected. Particularly relevant for highly processed samples e.g. peanut oil.

Effect of processing/matrix on allergen:

Need to be aware that processing and certain matrices may render proteins undetectable by antibodies used in ELISA assay, yet may still be allergenic.

Eg, a matrix that is highly acidic or basic is likely to cause protein hydrolysis (may also affect performance of assay itself)

CIP/COP Solution monitoring

Considerations for Analysis

Effect of sampling:

Representative sampling crucial for e.g gluten assay as only analyse 0.25g (5g for peanut assay).

large homogenous sample to ensure representative of whole product.

Effect of sample processing:

Various chemical and enzymatic procedures can lead to the hydrolysis of gluten proteins into small peptide fragments

E.g. beer, soy sauces and glucose syrups

These peptides are still toxic for coeliac patients but such small fragments do not have two epitopes necessary for Sandwich ELISA.

Solution:

Competitive ELISA (R5 antibody only binds to one site)

Batch Analysis Example

it all depends on how you look at it

- Previous product run contained peanut
- Equipment cleaned, swabbed then swabs and finished product analysed:

	Equipment Swabs				Composite Sample	Individual Sample			
	Swab 1	Swab 2	Swab 3	Swab 4	Products 1-4	Product 1	Product 2	Product 3	Product 4
Peanut Residue (ppm)	N.D.	N.D.	N.D.	N.D.	<2.5 (2.2)	10	<2.5	<2.5	<2.5

CIP/COP Solutions and Peanut Analysis

Can be analysed BUT often highly caustic/acidic

Potentially problematic matrixes are examined by 'spiking' experiments, eg:

- Incubated diluted peanut butter spike with caustic wash solution (30min @ RT) and compared results with spike incubated with water only:

-

Diluted peanut spike

Peanut Residue

5 ppm

Diluted peanut spike + COP solution (pH>10)

<2.5 ppm

- Experiments with several caustic wash solutions showed ~ 10-fold decrease in signal.
- May be preferable to use post-wash rinses or swabs in such circumstances

Dipstick tests

- Almond
- Cashew
- Crustacean
- Hazelnut
- Milk
- Peanut
- Rye
- Walnut
- LOD in buffer 1-5 ppm in matrix 2-10 ppm in Bakery, Icecream, Rinse waters , Surfaces
- Bioavid from r biopharm
- Some known cross reactivities
- Strip format also available form other Kit manufacturers

Barley

Celery

Egg

Lupin

Molluscs

Pistachio

Soy

Brazil Nut

Coconut

Wheat/ Gluten

Macadamia

Mustard

Pine nut

Sesame

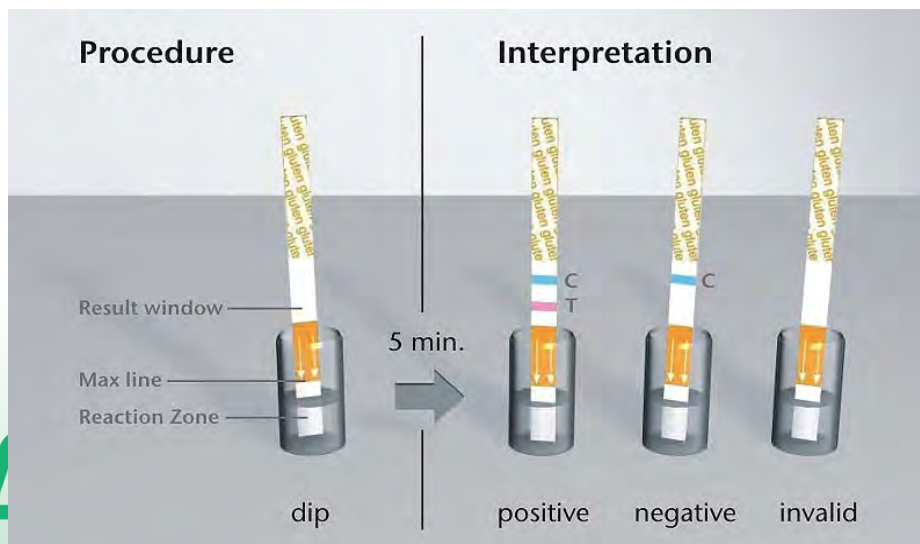
RIDAQUICK Gliadin Assay (Immunochromatographic 'dipstick' Test)

Qualitative

- Non-heated and non-processed food (Ethanol extraction): $\geq 5\text{ppm}$ Gluten
- All food samples (Cocktail extraction): $\geq 20\text{ppm}$ Gluten

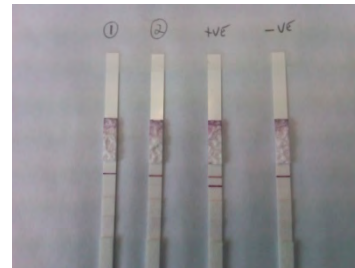
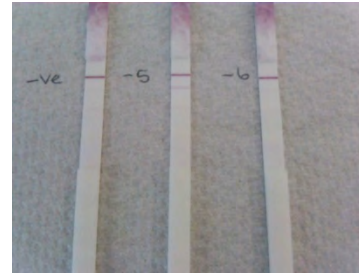
False Negatives

- Highly gluten positive samples overload strip: 'Hook Effect'



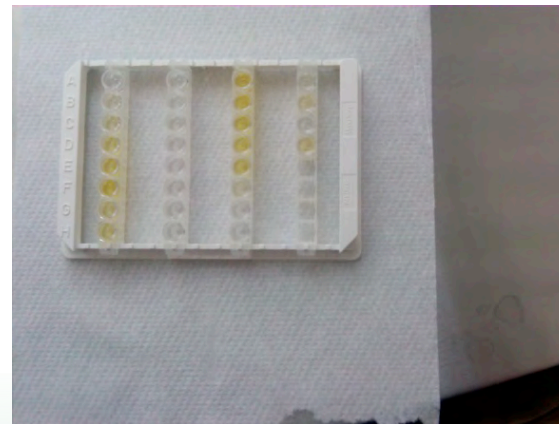
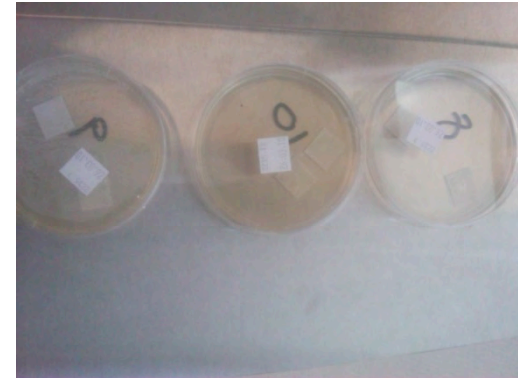
Macadamia Strips – a possibility

- Whole fresh Macadamia
- Ground to a fine paste
- Serial Dilutions
- Convincing positive at 10^{-5}
- Detectable positive at 10^{-6}



Settling down to settle plates

- To allow a more representative sample related to time
- Swab the plate and give a semi quantitative value



Sampling Plan

Distribution usually not homogenous

- May concentrate in first part of the run due to presence of previous product
- Hang ups in the system may result in “random” dumping of allergen
- May be particulate and therefore distributed irregularly in the sample itself

Test multiple samples at different points of production run

Avoid batching of samples for analysis as dilution may prevent identification of push through allergen from previous product run.

Sanitation swabs for aerial contamination– apply the target principle.

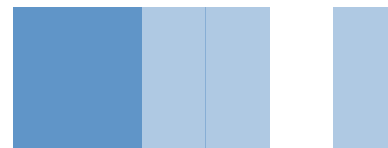
Distribution !!!



Random distribution.



Regular, non-random distribution. i.e. contaminated filler head.



Irregular non-random distribution i.e. contaminated RM added to continuous production system



Irregular non-random distribution i.e. unsanitary equipment.



Sampling the 10th column.



Random distribution.



Regular, non-random distribution. i.e. contaminated filler head.



Irregular non-random distribution i.e. contaminated RM added to continuous production system



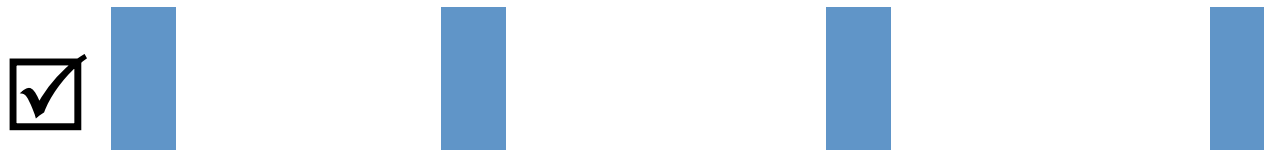
Irregular non-random distribution i.e. unsanitary equipment.



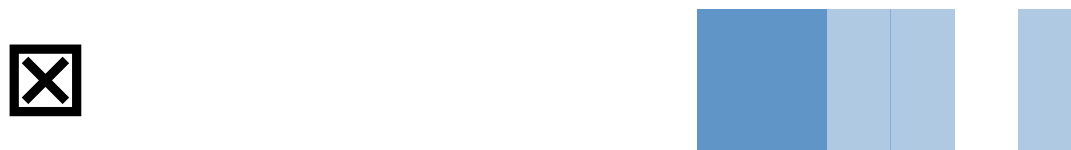
Sampling the 5th, 10th & 15th column.



Random distribution.



Regular, non-random distribution. i.e. contaminated filler head.



Irregular non-random distribution i.e. contaminated RM added to continuous production system



Irregular non-random distribution i.e. unsanitary equipment.



Sampling Plan

Type of health hazard	Hazard is unchanged	Examples
No hazard	n=0	Products produced in a mixed environment where the allergen is declared as an ingredient
Low hazard	Case A1 n=5, c=0	<ul style="list-style-type: none"> ▪ where an allergen is declared as a justified “may contain claim “ (testing is to ensure claims are correct and control measures in place) ▪ in an “Excluded “ facility where allergen is not handled on facility but no specific labeling claim is made
Moderate hazard	Case A2 2 class n=10, c=0	Products manufactured in allergen excluded premises, with no specific allergen free claims. (not specifically targeting sensitive population .
Severe hazard	Case A3 2 class n=30, c=0	<ul style="list-style-type: none"> ■ A product produced and labeled as ALLERGEN FREE would be expected to comply with the label claim and is actively targeting a sensitized population.

Draft Plan Stringency (Case) in relation to degree of health hazard and conditions of use.

Modified for Food borne allergen testing.

Reference

Stuttard, E.J. Jenson, I. and Best, J. Sampling for Microbiological Analysis, in Foodborne Microorganisms of Public Health Significance, 5th Ed AIFST, 1997.

VITAL Table in mg/kg (ppm) **TOTAL PROTEIN**

A voluntary risk based methodology for food producers to use in assessing the impact of allergen cross contact and provide APPROPRIATE precautionary allergen labelling (May Be Present:)

Uses data from clinical studies to calculate a 'safe' level of allergen

VITAL GRID
as at 02 July 2007

	Allergen Milk*	Egg*	Soy*^	Fish*	Peanuts*	Tree Nuts*	Sesame Seed*	Crustacea*	Gluten#
Action Level 1 (ppm)	<5	<2	<10	<20	<2	<2	<2	<2	<20
Action Level 2 (ppm)	5 - 50	2 - 20	10 - 100	20 - 200	2 - 20	2 - 20	2 - 20	2 - 20	20 - 100
Action level 3 (ppm)	>50	>20	>100	>200	>20	>20	>20	>20	>100

* mg/kg (ppm) of total protein

Gluten includes all gluten type proteins as defined in the Food Standards Code

Interpreting Results

- LOQ for Gliadin assay is 2.5ppm which equates to 5ppm Gluten
- Measurement of Uncertainty:
 - FACTA Positive control run with every assay: 18.8 ± 1.8 ppm
 - Kit lot # variation a potentially greater source of uncertainty:
 - 20-30% variation is regarded as acceptable
 - Result given as a range to reflect this.
- Competitive assay result is expressed as ug/g peptide in sample
 - Currently difficult to convert this to an equivalent gluten level due to variability in the degree of hydrolysis between samples
 - In the next couple of months, the competitive assay will incorporate additional standards to aid conversion of units to ppm Gluten

Table 1 Commercially available kits for the isolation and detection of food allergens using real-time or endpoint PCR authentication of species-specific DNA

Company (Australian Distributor)	Kit Name	Allergen	Assay type	Company website
R-biopharm (Lab Diagnostics)	SureFood Prep Allergen/Plant/PlantX (processed foods)		DNA extraction kits – 50 preps	www.r-biopharm.com
	SureFood Allergen	Soya, Hazelnut, Peanut, Almond, Celery, Gliadin, Walnut, Sesame, Mustard, Fish, Lupine, Crustaceans, Molluscs	Real-time PCR detection – 100 rxns	
Tepnel Biosystems (BioSys Australia)	BIOKITS DNA extraction kit (GMO & Allergen)		DNA extraction kit	www.tepnel.com
	BIOKITS Allergen selection module	Peanut, Soya, Cow's milk	Endpoint PCR	
The Food Safety Laboratory Limited	Food-Ex, Ex plus, Ex magnetic		DNA extraction kits	www.foodsafetylabs.com
	Watcher Series – Food allergen detection collection	Almond, Brazil nut, Cashew nut, Celery, Crustacea, Gluten, Hazelnut, Macadamias, Mustard, Peanut, Pistachio nut, Sesame, Shrimp, Soya, Walnut, Wheat	Endpoint PCR	
InCura srl	GREES DNA Kit FOOD		DNA extraction kit	www.incura.it
	EC Directive allergen plus maize endpoint and real-time PCR kits	Almond, Apricot, Celery, Corn, Crustacea, Gluten, Hazelnut, Lupin, Molluscs, Peach, Peanut, Pistachios, Sesame, Soya, Cashew	endpoint and real-time PCR detection kits available	
Generon srl	DNA Extractor IonForce		DNA extraction kit	www2.generon.it
	REX Allergen kits	Almond, Barley, Brazil nut, Cashew, Celery, Hazelnut, Lupin, Mustard, Oats, Peanut, Pecan nut, Pistachios, Rye, Sesame, Spelt, Soya, Walnut, Wheat	Real-time PCR detection kit with reference DNA for relative quantification	

Allergen Detection Methods - The Compendium of Food Allergen Methodologies- Health Canada

Analyte of Interest	Method Evaluated	Date Evaluated
Sesame	Elisa Systems Sesame	March 2010
Casein	Elisa Systems Casein	December 2006
Beta Lactoglobulin	Elisa Systems Beta Lacto.	March 2006
Egg	Neogen Veratox for Egg	July 2004
Almond	Neogen Veratox for Almond	December 2004

- <http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/allergen/index-eng.php#list>

ELISA Not the Complete Solution

Confirmatory/complimentary methods:

Immunological – Western blot

PCR – Real-Time technology

- Still issues with converting DNA to protein levels
- Used for confirmation and where no antibody

Mass Spectrometry - The future

analysis and confirmation LC-ESI MS

Biosensors

Chromatographical techniques.

Immunocapture MS

Multiplex

The Initial Questions

- When the code was introduced the industry requirement for analysis was relatively simple
 - Can you test my product ?
 - Does it contain any allergen ?
 - How much is enough to declare ? What's a trace anyway ?
 - If you cant find it do I still have to declare it ?

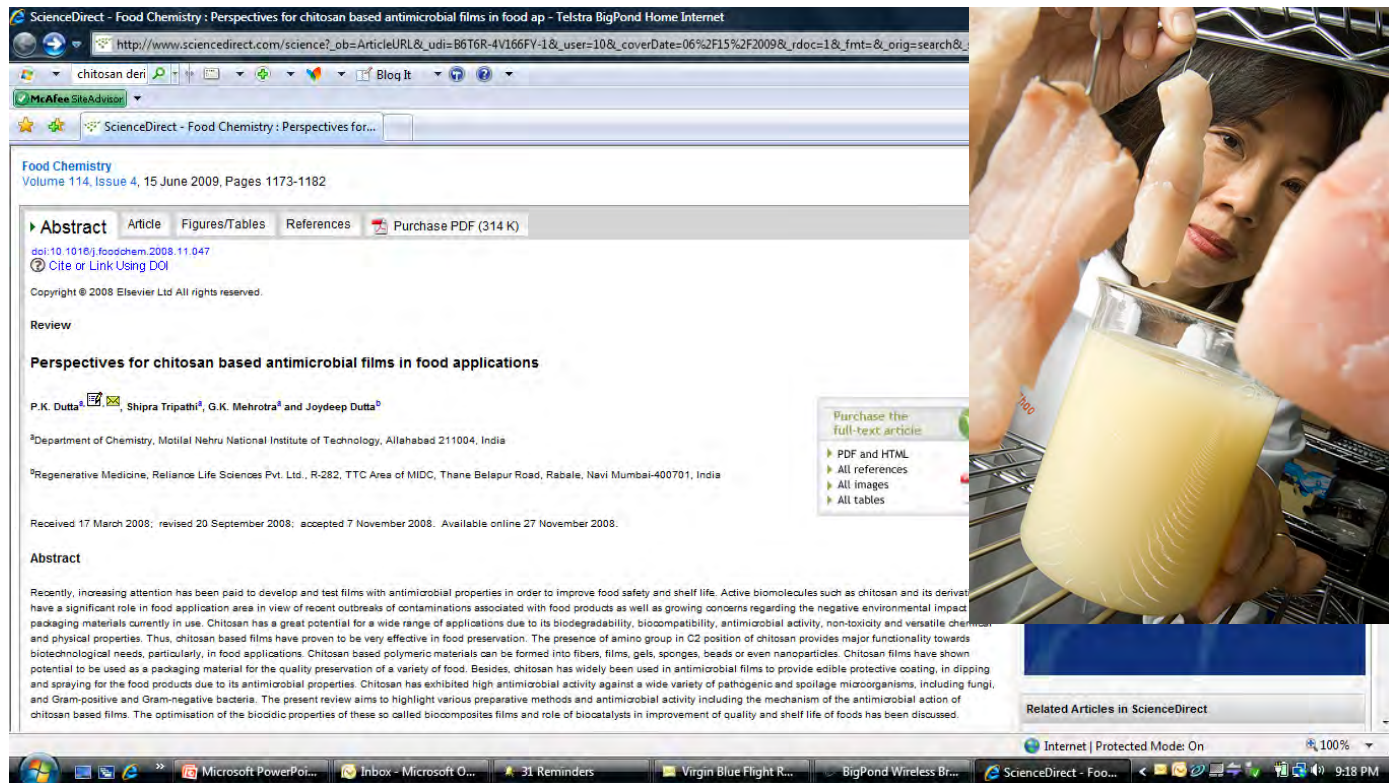
Today's Questions

- Which protein are you detecting
- How are you reporting it – is it representative of the commodity or the allergenic protein
- Can it be more sensitive ? : analytical limbo
- What's the best method ? What about MS , Biosensors, Nanosensors , Intelligent Packaging
- And crucially
 - How does that relate to the VITAL action grid
 - What will this mean when I export .
 - Harmonisation of techniques – agreement on methodologies , reference materials and standards

New Working Groups

- **VTAG** **VITAL Technical Advisory Group**
 - to oversee VITAL review and provide technical and industry advice
- **ATAG** **Analytical Technical Advisory Group**
 - To examine the relationship between the action levels as detailed in the VITAL grid and analytical results

Food but not as we know it !!!!



The image shows a screenshot of a ScienceDirect article page on the left and a photograph on the right. The photograph shows a woman dipping a piece of fish (lingcod fillet) into a glass beaker containing a yellow liquid coating. The article page on the left is titled "Perspectives for chitosan based antimicrobial films in food applications" and lists authors P.K. Dutta, Shipra Tripathi, G.K. Mehrotra, and Joydeep Dutta. The abstract discusses the use of chitosan-based films for food preservation.

Yanyun Zhao, a professor at Oregon State University, dips a lingcod fillet into a liquid coating that makes the fish longer-lasting and possibly healthier.

CONCLUSIONS

- ELISA is robust, cost-effective technique for the measurement of gluten and peanut levels with a sensitivity that is clinically relevant
- Need to consider the effects of matrix and food processing on allergens to ensure test objectives can be achieved
- Data interpretation must take into account the particular target specificities of antibodies, standards used (reporting units), and the quantitative limitations of ELISA.

