Australian Government
National Measurement Institute

National Measurement Institute

Allergen Analysis

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17th August 2015 – EPHA Allergen training Day - Melbourne

NMI – Allergen Background

- **National Measurement Institute – 20 years of food allergen analysis**

- Immunological ELISA expertise:
  Gluten, Peanut, Sesame, Soy, Egg, Milk (Casein, Beta lactoglobulin), Crustaceans, Almonds, Hazelnuts, Walnut, Pistachio, Macadamia, Cashew, Fish, Brazil nut, Lupin, Mustard including Lateral Flow Devices

- PCR experience:
  GMO, food pathogens, qPCR, digital PCR, next gen sequencing
  PCR reference materials, Food- borne virus and food allergens

- Chromatography and Mass Spec expertise and equipment:
  Triple Quad and TOFs

- Proficiency Testing & Quality Control Materials

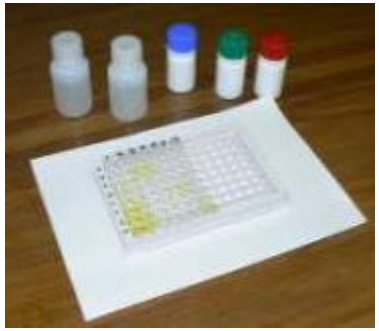
Technologies

Technologies - ELISA

- Enzyme Linked ImmunoSorbant Assay - Screen
- Immunological - Specific antibody as the detector for the analyte or allergen

- **Majority of analysis is Immunologically based - ELISAs and LFDs (Lateral Flow Devices)**

- Availability for a range of allergen proteins
- Generally specific for allergenic proteins
- Cost effective
- Ease of use
- Field portable
- Low detection levels - ppm to sub ppm
- Simple sample preparation – time effective
- High affinity for target



ELISA – Analysis Challenges

- Limited validation data – or International Accreditation
- Conflicting results from different kits for the same allergen
- Inconsistent allergen targets in different kits
- Cross-reactivity and Antibody specificity – caramel current issue
- Extraction efficiency – matrix specific or tailoring required
- Food matrix and processing impacts
- Lack of confirmation options
- Lack of reference materials
- Uncertainty of approx. 20-30% at best
- Usually require multiple epitopes and intact molecule

ELISA – Food matrix and processing impacts

- Fermented and hydrolysed food
  - beer, starch syrup, starch, malt extract, sourdough, soy sauce
- Often claimed or assumed to be gluten free
- Gluten molecules are partly or wholly broken down in the production process
- Small protein fragments remain dangerous to Coeliac patients after digestion in the stomach
- Potentially toxic sequence QQPFP occurs repeatedly in the prolamin molecules
- **Not recognised by Standard - Sandwich ELISAs**

- We use a competitive ELISA that targets the penta-peptide QQPFP

ELISA – Cross Reactivity

Members of the Rosaceae (Rose) family include:
- Almond, Peach, Plum, Cherry, Apricot, Mahlab

- Mahlab is an aromatic spice made from the seeds of a species of cherry, Prunus mahaleb.
- It has been used for centuries in the Middle East and the surrounding areas

- Almond in spices issue late 2014

- False positive??

- I would be cautious to ignore the cross reactivity here

- Likely an almond allergic individual could react to the this product as a kit does.

Options to deal with cross-reactivity or false positives

- Retests
- Product information
- Component analysis
- Different kits, different antibodies
- Different techniques
- Product family tree and allergen relatives
- Nonlinear responses
- Known interfering compounds
- Discuss with Lab

Technologies - PCR

- Commercial kits available for:
  Soy, peanut, milk, almond, hazelnut, lupine, gluten, celery, walnut, sesame, mustard, fish, crustaceans and molluscs.

- Include optimised and simplified DNA extraction protocols and reagents

- Promoted as option for verifying immunoassay results
  Skills and equipment becoming more common in Food testing labs

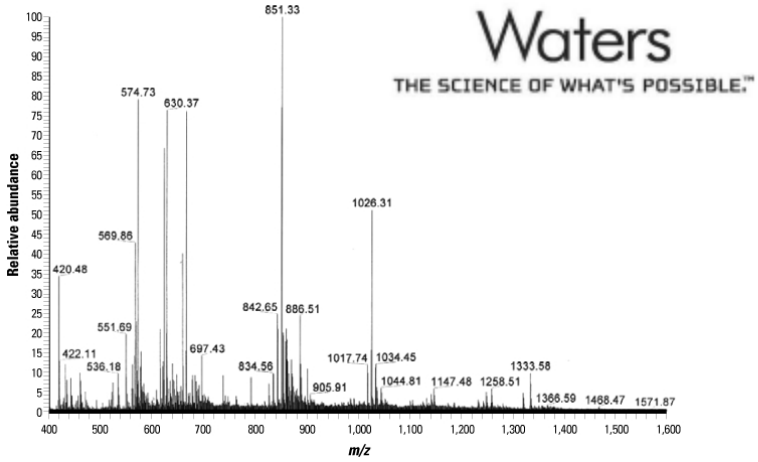
PCR – Analysis Challenges

- **No longer targeting allergenic protein**
  - Levels of detection – not consistent with ELISA
  - Qualitative or at best semi-quantitative

- **Species distinction vs food matrix - limitation**
  - Conversion from copies numbers to mg/kg of protein – are interesting discussions
  - High precision required with small and single microlitre volumes
  - Absence of DNA does not guarantee absence of allergenic protein
    Presence of DNA does not guarantee presence of allergenic protein

Technologies
- Mass Spectrometry

- Development of soft-ionising techniques (MALDI & ESI) allow the measurement of large biomolecules (i.e. proteins)
- MS measures mass-to-charge (*m/z*) ratios for ions allowing accurate molecular mass determinations
- **Predominant technology for peptide and protein identification**
- **Can provide protein composition, structure and sequence information**
- **Traceable to known amino acid sequences**
- High sensitivity and resolving power
- With appropriate ion selection confirmation is assured – unmatched level of confidence
- **Single extraction multi-analyte potential**



Mass Spectrometry – Analysis Challenges

- **High cost of equipment**
- Output data complex
- **Higher level of expertise and skill required**
- No routinely applicable methods
- **Extraction challenges**

Waters
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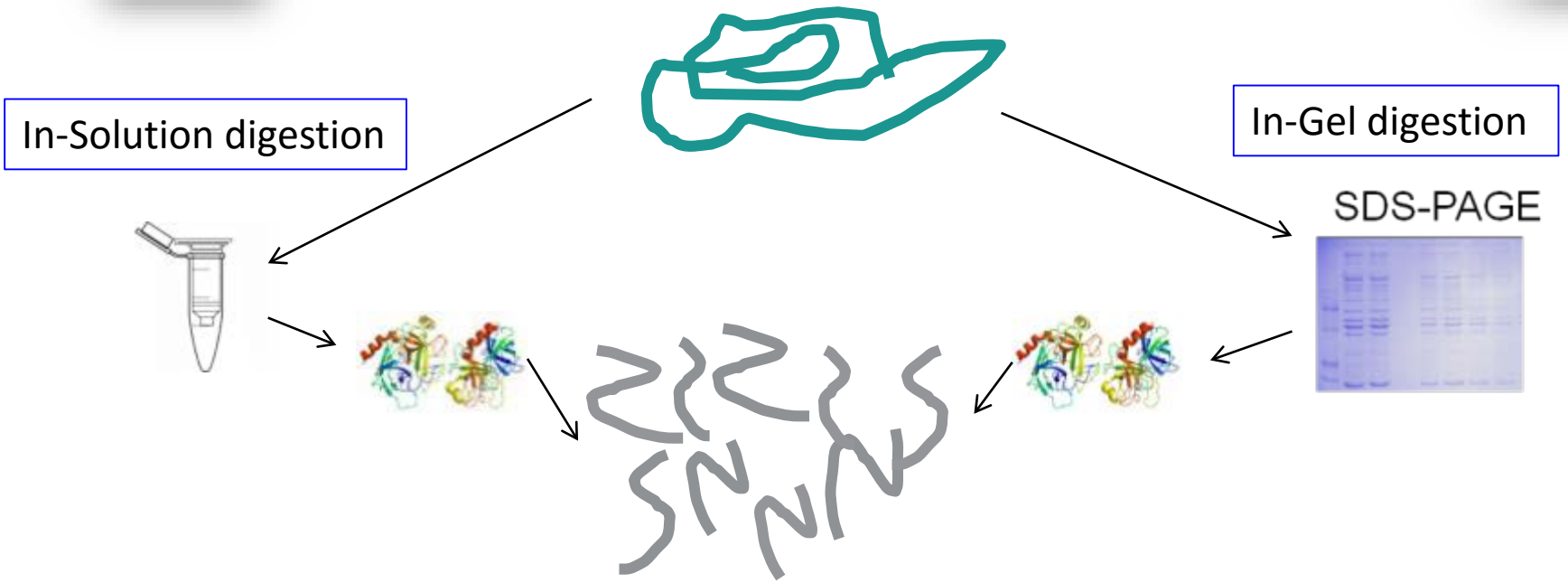
How to identify good signature peptides



Protein Extraction | Protein Digestion | Peptide Analysis | Protein/Peptide Identification | Signature Peptide Identification

How to identify good signature peptides

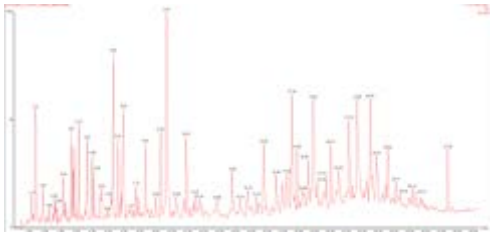
MS

MALDI

LC/(q)TOF

LC/IT

LC/MS Chromatogram



Database search

Protein Extraction | Protein Digestion | Peptide Analysis | Protein/Peptide Identification | Signature Peptide Identification

Mass Spectrometry - Data

- Trypsin digested Peanut (Arachis Hypogaea) extract
- Q-TOF Mass Spec – MSe and DDA
- MASCOT – Targeting Ara H1 – Peanut Allergen
- Sequence coverage 38% from 35 peptides
- Peptide data to generate optimised Triple Quad MS MRM transitions

Precursor MH+ (Da)	Sequence
1817.9656	(R)IFLAGDKDNVIDQIEK(Q)
1572.7292	(R)VLLEENAGGEQEER(G)
1738.818	(R)NTLEAAFNAEFNEIR(R)
1714.8606	(K)KGSEEEDITNPINLR(D)
1586.7478	(K)GSEEEDITNPINLR(D)
1073.5518	(K)DNVIDQIEK(Q)
1320.579	(R)DGEPDLSNNFGR(L)
1128.641	(K)GTGNLELVAVR(K)
1560.6787	(R)EGEQEWGTPGSEVR(E)
983.5251	(K)EHVQELTK(H)
2227.0344	(K)ISMPVNTPGQFEDFFPASSF
972.5996	(K)AMVIVVVNK(G)
1343.6967	(K)EGALMLPHFNSK(A)
1376.6597	(K)DLAFPGSGEQVEK(L)
1047.5349	(R)SSDNEGVIVK(V)
1080.4254	(R)QPGDYDDDR(R)
896.4418	(R)HDNQNLR(V)
2437.9087	(R)REQEWEEEEEDEEEEGSNR(
1019.4603	(R)EREEDWR(Q)
975.5834	(R)LFEVKPDK(K)
812.4173	(R)WGPAEPR(E)
2281.7842	(R)EQEWEEEEEDEEEEGSNR(E)
1293.5366	(R)GRQPGDYDDDR(R)
1141.5482	(R)NNPFYFPSR(R)
1321.5646	(R)DGEPDLSNNFGR(L)
2620.3062	(K)HADADNILVIQQGQATVTV
3331.6194	(K)NPQLQDLDMMLTCVEIKEG
1720.8254	(R)NTLEAAFNAEFNEIR(R)
2541.2568	(R)VAKISMPVNTPGQFEDFFP/
1728.842	(R)RVLLEENAGGEQEER(G)
2619.308	(K)HADADNILVIQQGQATVTV
1972.9604	(K)NPQLQDLDMMLTCVEIK(E)
2822.063	(R)REQEWEEEEEDEEEEGSNRE
703.357	(R)REEGGR(W)
526.2307	(R)EREE(D)

ALL11_ARAHY Coverage Map

1	MRGRVSPLML	LLGILVLASV	SATQAKSPYR	KTENPCAQRC	LQSCQQEPDD
51	LKQKACESRC	TKLEYDPRCV	YDTGATNQRH	PPGERTRGRQ	PGDYDDDRRQ
101	PRREEGGRWG	PAEPRERERE	EDWRQPREDW	RRPSHQQPRK	IRPEGREGEQ
151	EWGIPGSEVR	EETSRNNPFY	FPSRRFSIRY	GNQNGRIRVL	QRFDQRSKQF
201	QNLQNHRIVQ	IEARPNTLVL	PKHADADNIL	VIQQGQAIVT	VANGNNRKSF
251	NLDEGHALRI	PSGFISYILN	RHDNQNLRVA	KISMPVNTPG	QFEDFFPASS
301	RDQSSYLQGF	SRNTLEAAFN	AEFNEIRRVL	LEENAGGEQE	ERGQRRRSTR
351	SSDNEGVIVK	VSKEHVQELI	KHAKSVSKKG	SEEEDITNPI	NLRDGEPDLS
401	NNFGRLFEVK	PDKKNPQLQD	LDMMLTCVEI	KEGALMLPHF	NSKAMVIVVV
451	NKGIGNLELV	AVRKEQQQRG	RREQEWEEEE	EDEEEEGSNR	EVRRYTARLK
501	EGDVFIMPAA	HFVAINASSE	LHLLGFGINA	ENNHRIFLAG	DKDNVIDQIE
551	KQAKDLAFPG	SGEQVEKLIK	NQRESHFVSA	RPQSQSPSSP	EKEDQEEENQ
601	GGKGPLLSIL	KAFN			

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Crustacean/ Shellfish	Egg	Fish	Milk	Peanut	Soy	Tree nuts	Wheat
Myosin light chain	Lyozyme C	β-pavalbumin (and isoforms)	S100 calcium binding protein A7	Lipid transfer protein	Defensin	2S albumin	Lipid transfer protein
Troponin C	Ovomucoid (α, β)	Aldolase A	α-lactalbumin	Defensin	Profilin	Lipid transfer protein	α-purothionin
Sarcoplasmic calcium binding protein	Ovalbumin	β-enolase	β-lactoglobulin	Oleosin	PR-10, Bet v 1 family member	Ribosomal protein P2	Thioredoxin
Triosphosphate isomerase	Serum albumin		Caseins (α, β, γ, κ)	Profilin	Hydrophobic protein	S albumin	Profilin
Tropomyosin	Ovotransferrine		Lipocalin	α-amylase	Glycinin (legumin, 11S globulin)	Profilin	Trypsin inhibitor
Glyceraldehyde -3-phosphate dehydrogenase	Vitellogenin II		Serum albumin	Conglutin (2S albumin)	β-conglycinin (vicilin, 7S globulin)	2S albumin	Amylase Inhibitor (α, β)
α-actin	Ovomucin			PR-10, Bet v 1 family member	Biotinylated protein	Oleosin	Agglutinin Isolectin
Arginine kinase				Agglutinin		PR-10, Bet v 1 family member	Gliadin (α, γ, ω)
SERCA/ Ca2+ATPase				Cupin (Vicilin-type, 7S globulin)		Manganese superoxide dismutase	Peroxidase 1
Myosin heavy chain				Cupin (11S globulin, Glycinin)		11S globulin	LMW Glutenin
				Phospholipase D		Vicilin-like protein	Serpin
				Conarachin I		Legumin-like protein	β-D gulcan exohydrolase
				α-arachin		Vicilin	HMW Glutenin
						Prunin	
						Amandin	

Molecular weight (lowest 8 kDa)

Molecular weight (highest 170kDa)

Yellow boxes highlight allergens investigated by MS systems
Red circles highlight allergens with signature peptides investigated

Comparison of Technologies

	Set up cost AUD	Training time	Interpretation complexity/simplicity
LFD	$100-200	minutes	line or no-line
ELISA	$10-20K	hours	Absorbance or colour intensity over a threshold
PCR	$40-60K	Days	response over a threshold
Mass Spec	$500K -1M	Months+	complex spectra, data conversion, data base searches, sequence uniqueness

Research

NMI Food Allergen Activities

- **Methodology Activities – Research**
  - NMI-James Cook University Collaboration
  - NMI-Victoria University Collaboration
  - Development of Instrument based methods
    - Allergen Protein identification via sequence proteomics
    - QQQ-TOF – Mass Spectrometry
    - Sample extraction
  - 3 PhD projects
    - Orthogonal confirmation techniques
    - Signature peptides in shellfish
    - FODMAPS

Proficiency Testing & Quality Control Materials

Proficiency Testing & Quality Control Materials

Benefits:

- Satisfy accreditation requirements
- Verify methods and procedures
- Satisfy regulators
- Identify problems
- Enhance accuracy
- Monitor analysis
- Compare with peers
- Demonstrate and build confidence

NMI Allergen Proficiency Round on Gluten in Custard Powder AQA 10-21

Gluten in Custard Powder AQA 10-21

- 11 labs participated
- Commercially prepared - Gluten containing & gluten free product
- *Different results from different kit manufacturers*
- *All correctly identified positive and negative samples*

Sesame in Chocolate Cake Mix AQA 12-10

- recalled product with history of reactions in allergic individuals
- 7 labs participating
- *All correctly identified positive and negative samples*
- *MU quoted ranged from 7.7 to 290%*

Quality Control Materials

- Used with every kit run

- Provide:
  - Within run control checks – assay drift
  - Between run variations
  - Batch to batch kit variations
  - Comparison between kits
  - Comparison between labs
  - Measurement uncertainty data

Allergen Testing – Special Interest group

AT-SIG

Allergen Testing - Special Interest Group (AT-SIG)

Target audience

- Testing Laboratories
- Accreditation bodies
- Regulators
- Industry Bodies
- Consumer Advocates
- Kit manufacturers
- Food Manufacturers

Allergen Bureau

AT-SIG: Group Aim

- **Improve food allergen analysis**
- **Share challenges and issues - Address common or reoccurring issues**
- Keep labs talking – particularly on overlapping issues
- Provide a forum for formal and informal networking
- Encourage input from all parties

- **Forum to present emerging technology applicable to food allergen analysis**
- Promote and assist Laboratory accreditation
- Guide – Proficiency, Reference Materials and Confirmation Methods
- Keep up to date with International (and local) Allergen Activities

Allergen Bureau

AT-SIG: Activities

- First meeting held:
  October 2010 @ NMI, Port Melbourne
- Second meeting held:
  June 2011 @ NMI, Port Melbourne
- Third meeting held:
  April 2012 @ NMI, Port Melbourne
- Fourth meeting held:
  Nov 2013 @ 24th CRC Victoria University, Melbourne
- Fifth meeting held
  Feb 2014 @ FSS QHSS, Brisbane
- Sixth meeting held
  May 2015 @ NMI, North Ryde

AT-SIG - Meeting Topics

- Case studies
- Proficiency Testing
- Laboratory Accreditation for Allergens
- Reporting Issues
- Up coming Food Allergen events (Local and International)
- Reference Materials
- New technology
- **Regional summaries**

Benefits and Outcomes

- Precompetitive environment to discuss critical issues
- Guidance documents
- Links participants into National and International activities
- Directly linked into industry issues – valuable information and shared experiences
- Forum for immerging issues that brings experts into a single space - a resource to address immediate and longer term food allergen concerns
- Conscious of confidentiality issues
- Informal opportunities to raise concerns
- Opportunity to be involved in shared research and development of allergen relevant protocols and documents
- Drive and guide future Allergen activities
  – PT, Reference Materials and Confirmation Methods

Allergen Bureau

Guidance Documents

Guidance Documents

Requested by Allergen Bureau

- Basic definitions related to allergen analysis
- Key methods (i.e. ELISA; PCR; Mass spectrometry)
- Guidelines around interpreting results
- Documentation of recognised issues associated with analytical results (i.e. false results; matrix effects)
- Information matrix around methods and commercially available kits and protocols (note this information is not endorsed but for information only)
- References and resources

Comments from Stakeholders

- 'greater than' results - all but useless for risk assessment purposes
- lack of clarity around 'less than' results i.e. less than the LOD, or detected but less than the LOQ
- lack of clarity around the whether the kit is calibrated/reporting against the allergenic protein, total protein or whole food
- variability of results, including detections with different kits - significant in survey work where a number of labs may be involved
- Guidance through and to…. appropriate analysis

Guidance Documents

What we can provide in manageable chunks:

- **Basic definitions** related to allergen analysis – building on existing documents
- **Methods summaries** (i.e. ELISA; PCR; Mass spectrometry) – description of techniques and applications – research papers
- **Sampling guidance** – sampling plans and sub-sample size etc
- **Data interpretation and Reporting Document**
- **Analysis issues/method limitations**: (i.e. false results; matrix effects) – how to collate and present this?

Consider target audience for documents

3 Current Documents

1. Sampling Document
2. Data Interpretation & Reporting Document
3. Method Summaries & Definitions Document

Sampling Document

Sampling guidance

- Sampling plans - How many samples should I submit?.....
- Sub-sample size
- Product considerations
- Problem matrices

- Swabs –
- Swab choice
- Area choice

- Interpretation considerations
- Larger uncertainties

Data Interpretation & Reporting Document

- Reporting results below LOQ but above LOD - NQ
- Numbers vs ranges
- 2 significant figure or 1 decimal place accuracy
- Analyte or measurand – target
- Units (ppm,mg/kg)
- Assumptions or exclusions
- Known or suspected cross-reactivities
- Measurement Uncertainty
- What to do with unexpected / disputed results
- Minimum information on report
- Conversion factors – assumptions
- Swabs – limitations

Method Summaries & Definitions Document

- **Basic definitions** related to allergen analysis
- **Methods summaries - explanations**
  - ELISA
  - PCR
  - Mass spectrometry … from recent published papers
- **Analysis issues/method limitations**:
  - False positive/negative results
  - Matrix effects
  - Cross reactivity's

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