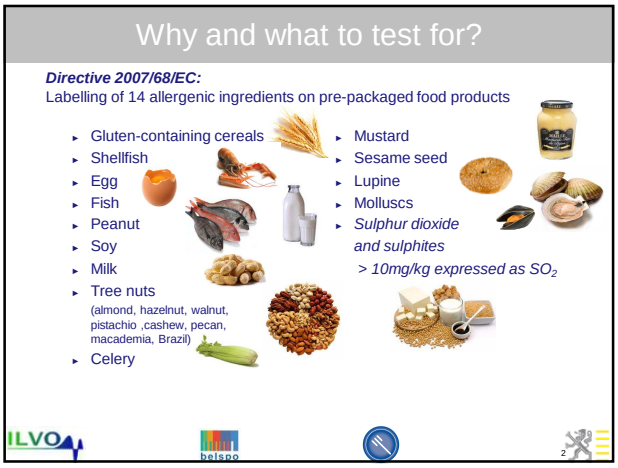
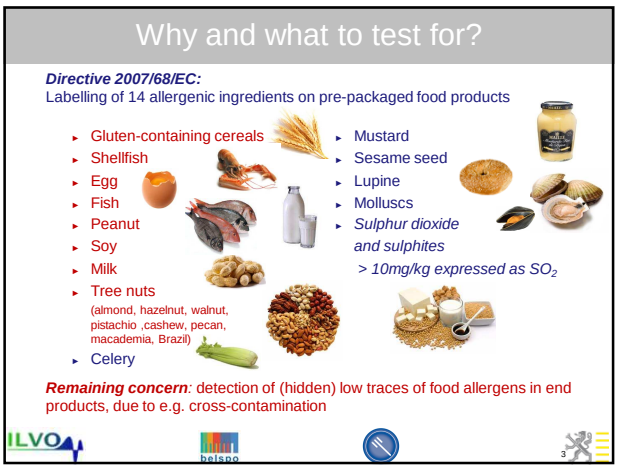

Instituut voor Landbouw- en Visserijonderzoek
PCR and ELISA detection of allergens in food
Bart Van Droogenbroeck, Isabel Taverniers
Marc De Loose
Belgian National Reference Laboratory for Allergens
ILVO, Technology & Food Science
http://www.ilvo.vlaanderen.be
ILVO
belspo


Why and what to test for?
Directive 2007/68/EC:
Labelling of 14 allergenic ingredients on pre-packaged food products
Gluten-containing cereals
Shellfish
Egg
Fish
Peanut
Soy
Milk
Tree nuts
(almond, hazelnut, walnut, pistachio ,cashew, pecan, macademia, Brazil)
Celery
Mustard
Sesame seed
Lupine
Molluscs
Sulphur dioxide and sulphites
> 10mg/kg expressed as SO2
ILVO
belspo
2


Why and what to test for?
Directive 2007/68/EC:
Labelling of 14 allergenic ingredients on pre-packaged food products
Gluten-containing cereals
Shellfish
Egg
Fish
Peanut
Soy
Milk
Tree nuts
(almond, hazelnut, walnut, pistachio ,cashew, pecan, macademia, Brazil)
Celery
Mustard
Sesame seed
Lupine
Molluscs
Sulphur dioxide and sulphites
> 10mg/kg expressed as SO2
Remaining concern: detection of (hidden) low traces of food allergens in end products, due to e.g. cross-contamination
ILVO
belspo
3

Why and what to test for?

Involvement of Hidden Allergens in Food Allergic Reactions

B Añibarro, FJ Seoane, MV Mugica

A retrospective study was carried out in an adult population. Over a five year period, a total of 530 food reactions were reviewed.

One hundred nineteen reactions (22.4%) were considered to be due to hidden allergens. Thirty-two percent of these were anaphylactic reactions.

J Investig Allergol Clin Immunol 2007; Vol. 17(3): 168-172

4

Why and what to test for?

Niet geëtiketteerde allergenen kosten jaarlijks miljarden euro's aan bedrijfsleven in Europa

Door *Hannelore De Maere*
Aangemaakt *16/12/2010 - 09:00*
do, 16/12/2010 - 09:00 — Hannelore De Maere [1]
FoodGate partner:
VLAV [2]

FoodGate netwerk voor innovatie
Gepubliceerd op *Flanders' FOOD*

Uit de jaarlijkse statistieken van het Rapid Alerts system van de EU blijkt dat het overgrote deel (ca. 60%) van de meldingen handelen over allergenen. In de meeste gevallen was de oorzaak van deze Alerts terug te brengen tot niet geëtiketteerde allergenen.

Kost = onderzoeken, advertenties om de consument op de hoogte te stellen van de terughaalactie, terughalen en vernietigen producten, imagoschade, opbrengstverliezen…

5

Monitoring food allergens

Control on primary products, ingredients, processed end products

Allergens
Proteins/peptides

Control and monitoring throughout production chains
Presence of allergens in primary/end products?

Detection in function of labelling & traceability

GMOs
DNA

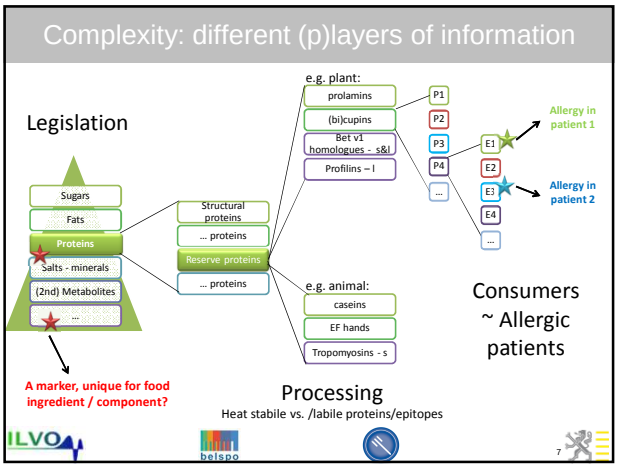
Detection in function of co-existence

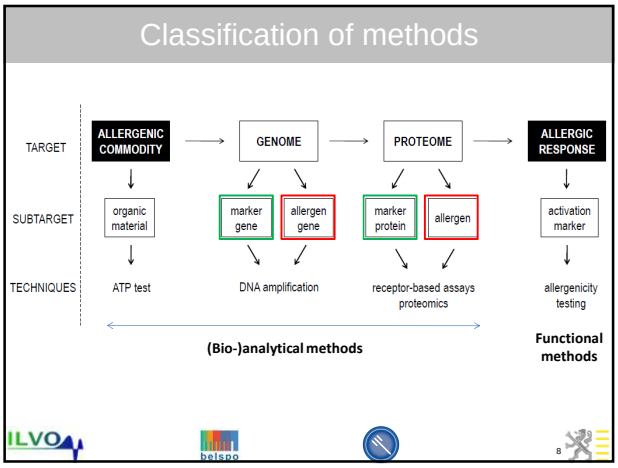
Practical guidelines for sampling, storage and analysis
Field | Container | Silo

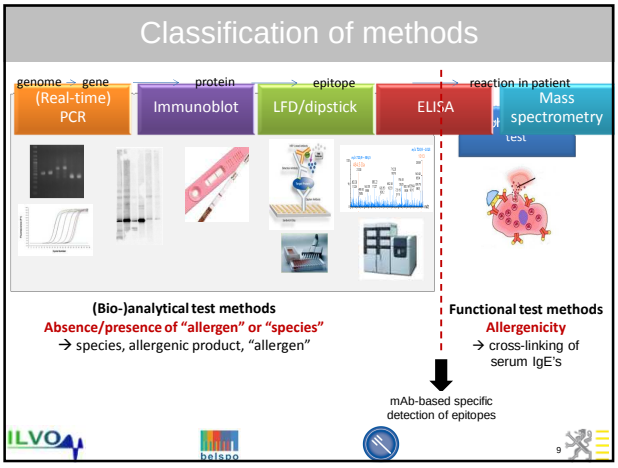
Tools for detection, identification, quantification

Detection in function of labelling & traceability

6


Complexity: different (p)layers of information
Legislation
Sugars
Fats
Proteins
Salts - minerals
(2nd) Metabolites
...
A marker, unique for food ingredient / component?
Structural proteins
... proteins
Reserve proteins
... proteins
e.g. plant:
prolamins
(bi)cupins
Bet v1 homologues - s&l
Profilins – l
e.g. animal:
caseins
EF hands
Tropomyosins - s
P1
P2
P3
P4
...
E1
E2
E3
E4
...
Allergy in patient 1
Allergy in patient 2
Consumers ~ Allergic patients
Processing
Heat stabile vs. /labile proteins/epitopes


Classification of methods
TARGET
SUBTARGET
TECHNIQUES
ALLERGENIC COMMODITY
GENOME
PROTEOME
ALLERGIC RESPONSE
organic material
marker gene
allergen gene
marker protein
allergen
activation marker
ATP test
DNA amplification
receptor-based assays proteomics
allergenicity testing
(Bio-)analytical methods
Functional methods

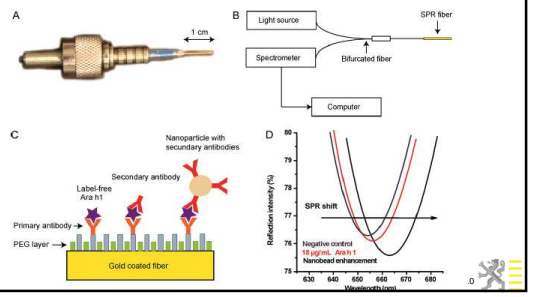

Classification of methods
genome → gene
protein
epitope
reaction in patient
(Real-time) PCR
Immunoblot
LFD/dipstick
ELISA
Mass spectrometry
(Bio-)analytical test methods
Absence/presence of "allergen" or "species"
→ species, allergenic product, "allergen"
Functional test methods
Allergenicity
→ cross-linking of serum IgE's
mAb-based specific detection of epitopes

Technology development: one example

Fast and accurate peanut allergen detection with nanobead enhanced optical fiber SPR biosensor

J. Pollet[a], F. Delport[a], K.P.F. Janssen[a], D.T. Tran[a], J. Wouters[b], T. Verbiest[b], J. Lammertyn[a,*]

[a] BIOSYST-MeBioS, Katholieke Universiteit Leuven, Willem de Croylaan 42, B-3001 Leuven, Belgium
[b] Molecular and Nanomaterials, Katholieke Universiteit Leuven, Celestijnenlaan 200f, B-3001 Leuven, Belgium



Technology development: one example

Fast and accurate peanut allergen detection with nanobead enhanced optical fiber SPR biosensor

J. Pollet[a], F. Delport[a], K.P.F. Janssen[a], D.T. Tran[a], J. Wouters[b], T. Verbiest[b], J. Lammertyn[a,*]

[a] BIOSYST-MeBioS, Katholieke Universiteit Leuven, Willem de Croylaan 42, B-3001 Leuven, Belgium
[b] Molecular and Nanomaterials, Katholieke Universiteit Leuven, Celestijnenlaan 200f, B-3001 Leuven, Belgium

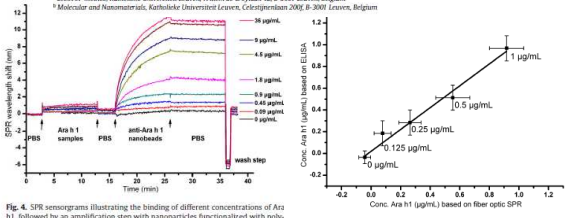


Fig. 4. SPR sensorgrams illustrating the binding of different concentrations of Ara h1, followed by an amplification step with nanoparticles functionalized with polyclonal antibodies. First the fiber was put for 3 min in the main buffer to stabilize and to acquire a baseline signal. Next, the fiber was dipped for 10 min in one of the samples, rinsed and placed again for 3 min in PBS buffer. Finally, the fiber was transferred to the vial with nanobeads to amplify the signal for 10 min. Desorption of the beads was monitored during 10 min of incubation in the PBS buffer. After each measurement the sensor was regenerated with a 2 min acid treatment.

Fig. 6. A correlation between the results of the ELISA kit and the results of the nanobead enhanced SPR fiber for different Ara h1 concentrations. One set of spiked chocolate samples was prepared for both experiments. The error bars represent the standard error of the mean based on three constitutive measurements of the same set of samples.

Monitoring food allergens

Key issue in “allergen” testing: **specificity**

→ ***What does the test used actually measure?***

- “Allergen”?
  - Allergenic activity?
  - Allergenic epitope?
  - Whole protein(s)?
- Another marker (DNA, ATP, …)?

→ **False positive** test results (cross-reactivity?)

Monitoring food allergens

Key issue in allergen testing: **sensitivity**
→ ***How litlle can be detected (ppm)?***

- Qualitative *versus* quantitative tests
- Correlation to actual "allergen" levels (for e.g. DNA)?
- Limit of detection? Ppm (mg/kg) or lower?
- Limit of quantification? Standard curve options
- Impact of food processing/food matrix on target specificity/detection?

→ **False negative** test results

13

ALLERRISK: Integrated strategies for food allergen detection

ISSUES
- increasing number diagnoses
- widely distributed in food chain
- low amounts
- seriousness symptoms

CHALLENGES
- need for detection tools (allergens vs. allergenicity)
- sensitivity (false-negatives)
- specificity (false-positives)
- raw ingredients vs. processed food
- consistent results (reliable tools)

OBJECTIVES
- investigate the analytical performance of detection methods for **hazelnut** and **soy**

Céline Platteau, PhD thesis 2011:
Assessment of ELISA and PCR assays for allergen detection in food:
A comparative study on hazelnut and soy

Finalized research PhD Céline Platteau, 2011

COMPARATIVE EVALUATION OF ASSAYS

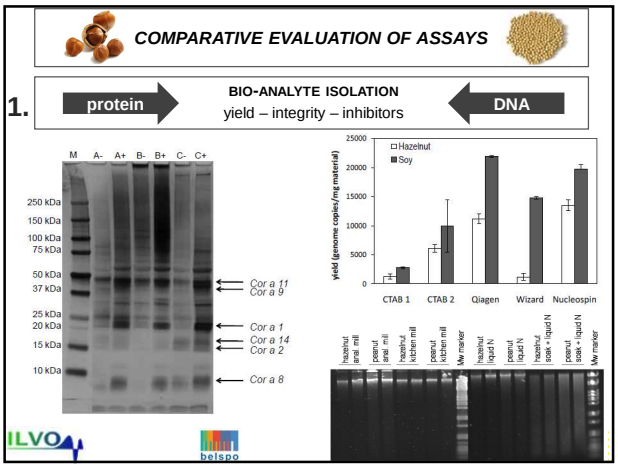
1. protein — BIO-ANALYTE ISOLATION: yield – integrity – inhibitors — DNA

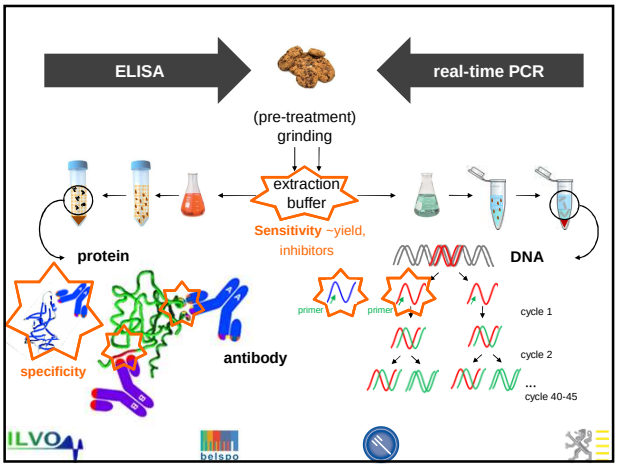
detect target in **composed food** products

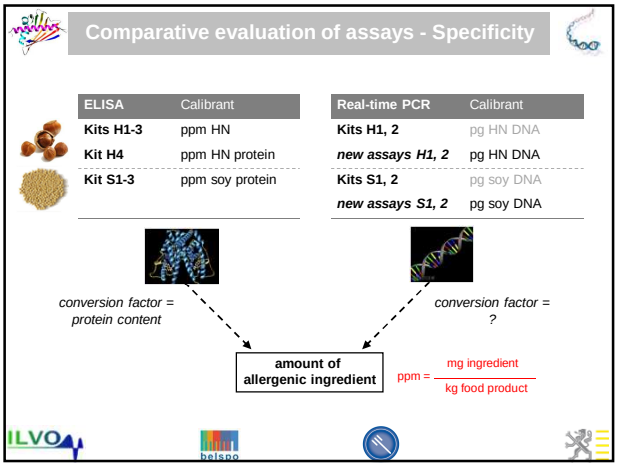
2. ELISA — SPECIFICITY SENSITIVITY: cross-reactivity – LOD/ LOQ — PCR

3. ROBUSTNESS a.f.o. FOOD PROCESSING
   - ELISA — **a.** BUFFERED MODEL SYSTEMS: Maillard reaction – hydrolysis - oxidation
   - ELISA — **b.** FOOD MODEL SYSTEMS (hazelnut) — PCR

specificity to detect target in **processed food** products


COMPARATIVE EVALUATION OF ASSAYS
1.
protein
BIO-ANALYTE ISOLATION
yield – integrity – inhibitors
DNA
M A- A+ B- B+ C- C+
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
Cor a 11
Cor a 9
Cor a 1
Cor a 14
Cor a 2
Cor a 8
Hazelnut
Soy
yield (genome copies/mg material)
CTAB 1
CTAB 2
Qiagen
Wizard
Nucleospin
ILVO
belspo


ELISA
real-time PCR
(pre-treatment)
grinding
extraction buffer
Sensitivity –yield, inhibitors
protein
DNA
specificity
antibody
primer
primer
cycle 1
cycle 2
...
cycle 40-45
ILVO
belspo


Comparative evaluation of assays - Specificity
ELISA
Calibrant
Kits H1-3
ppm HN
Kit H4
ppm HN protein
Kit S1-3
ppm soy protein
Real-time PCR
Calibrant
Kits H1, 2
pg HN DNA
new assays H1, 2
pg HN DNA
Kits S1, 2
pg soy DNA
new assays S1, 2
pg soy DNA
conversion factor = protein content
conversion factor = ?
amount of allergenic ingredient
ppm = mg ingredient / kg food product
ILVO
belspo

Specificity of ELISA assays

Black: >LOD kit
Red: >LOQ kit

	HAZELNUT				*SOY*		
	Kit H1	Kit H2	Kit H3	Kit H4	Kit S1	Kit S2	Kit S3
	ppm hazelnut			ppm HN protein	ppm soy protein		
Almond	0.52	0.26	0.37	0.001	0.59	/	/
Brazil nut	0.06	0.14	/	0.01	/	/	/
Cashew	1.60	0.58	0.79	/	0.55		/
Kamut	/	0.06		/	1.65	/	/
Lettuce	1.61	0.53	0.02	0.02	/	1.82	/
Macademia	0.45	0.14	/	0.001	/	/	/
Oat	/	0.09	0.16	/	10.76	69.10	3.30
Pea	0.37	/	/	/	4.69	/	/
Peanut	0.26	0.15	/	/	0.72	/	/
Pecan nut	2.22	0.34	/	/	/	/	/
Pistachio	0.45	0.12	0,21	/	0.34	/	/
Rapeseed	0.62	0.17	0.63	0.02	0.54	/	/
Spelt	/	0.09	0.17	/	/	22.91	1.05
Walnut	5.41	0.31	0.01	0.02	/	/	/

Specificity of REAL-TIME PCR assays

	HAZELNUT				*SOY*			
	Assay H1	Assay H2	Kit H1	Kit H2	Assay S1	Assay S2	Kit S1	Kit S2
Apple	/	/	/	/	/	/	/	/
Barley	/	/	/	/	/	/	/	/
Grape	/	/	/	/	/	/	/	/
Lettuce	/	/	+	/	/	/	/	/
Maize	/	/	/	/	/	/	/	/
Oat	/	/	/	/	/	/	/	/
Peanut	/	/	/	/	/	/	/	/
Pistachio	/	/	/	/	/	/	/	/
Raspberry	/	/	+	+	/	/	/	/
Rice	/	/	/	/	/	/	/	/
Spinach	/	/	/	/	/	/	/	/
Strawberry	/	/	+	+	/	/	/	/
Walnut	/	/	/	/	/	/	/	/
Wheat	/	/	/	/	/	/	/	/

Specificity of HAZELNUT and SOY assays

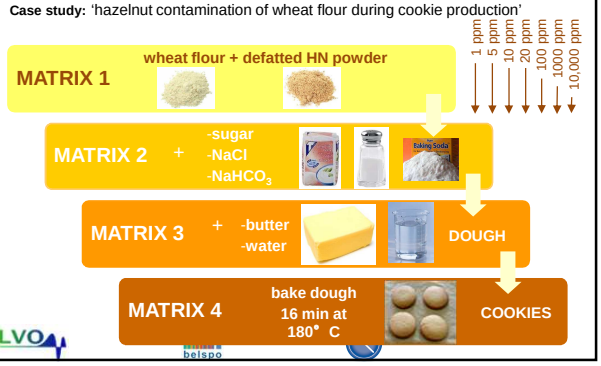
Conclusions

- *in silico* (theoretical) determined **cross-reactivity** in some cases **confirmed experimentally**
- **different kits = different responses !**
- ELISA: cross-reactivity detected, could be problematic in case of **bulk ingredients** (cfr. oat)
- PCR: **contamination** of retail samples with hazelnut/soy revealed
- specificity **ELISA** tests **< real-time PCR** assays

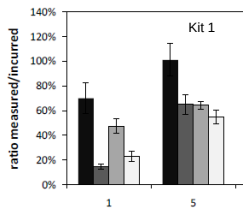
Comparative evaluation of assays - Sensitivity

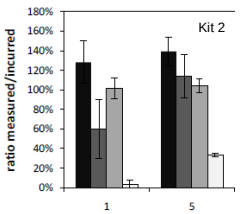
Food model systems: experimental design

Case study: 'hazelnut contamination of wheat flour during cookie production'



Robustness of HAZELNUT ELISA assays





ELISA

- *sensitivity:* 1 ppm
- *mixing/matrix effect:* decrease
- *baking effect:* decrease

Robustness of HAZELNUT and SOY assays

Conclusions

(specific for this case study = these allergens, tests, model systems)

- **results** are **dependent** on the applied **test /kit** (quantification, matrix effect, processing impact)
- hazelnut **ELISA** tests **more sensitive** than hazelnut **real-time PCR** assays
- food **processing** affects **sensitivity** of the assay
  - chemical modification/denaturation
  - **extractability** of the (target) analyte
  - **chemical modification** and/or **denaturation** are nefast for protein detection (buffered model systems – oxidation/hydrolysis/Maillard reaction)

Comparative evaluation of assays

ELISA

- ELISA, lateral flow devices and dipstick tests
- 5-6 hours analytical process
- Sensitive: 10 ppm and lower
- Target: protein(s) / epitope(s)
- Not always specific for allergenic protein
- Antibodies: from animal antisera, batch-to-batch variation, mAbs versus pAbs
- Subject to matrix, extraction, and interference problems
- Not for hydrolyzed, fermented, degraded, processed… products!
- No signal ≠ no allergenic residue present!

PCR

- Classical and real-time PCR
- 5-6 hours analytical process
- Less sensitive than ELISA (100 ppm)
- Target: DNA sequence, either allergenic protein-encoding sequence, or species-specific
- Specificity ~primers
- Subject to matrix, extraction, and inhibition problems
- DNA is more stable than proteins e.g. in processed products
- Does not proove presence or absence of protein
- No signal ≠ no allergenic residue present!

25

GENERAL CONCLUSIONS

- **Extraction procedure** influences quality of isolated bio-analytes (integrity, yield, purity) → **sensitivity**
- Current allergen assays lack **specificity** → **false-positives**
  - (precautionary) labelling of absent allergens
  - unnecessary restriction of allergic consumers' product choice
  - needless economical losses (product recall, restricted sales market)
- Current allergen assays lack **robustness** → **false-negatives**
  - food processing: denaturation, chemical modification, aggregation
- Different tests produce **inconsistent results**

RECOMMENDATIONS

- **More robust** tests with **broad applicability**: stable target molecules
- More **profound assessment** of the applicability of current tests
  → define performance criteria
- Need for assay **validation** + international **harmonisation**
  - pre-set requirements (e.g. sensitivity)
  - international validation protocols
  - reference materials (positive control)
  - international consensus on identity of target molecules
    (cfr. deliver valuable information for both industry and patients)

Thank you for your attention!

"IT'S WHEAT-FREE, DAIRY-FREE, FAT-FREE, NUT-FREE, SUGAR-FREE AND SALT-FREE...ENJOY!"