



Allergenicity as a future challenge

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Background

Food allergy is an increasing health problem.

About 6-8 % of children and 2 % of adults are affected in industrialised countries.

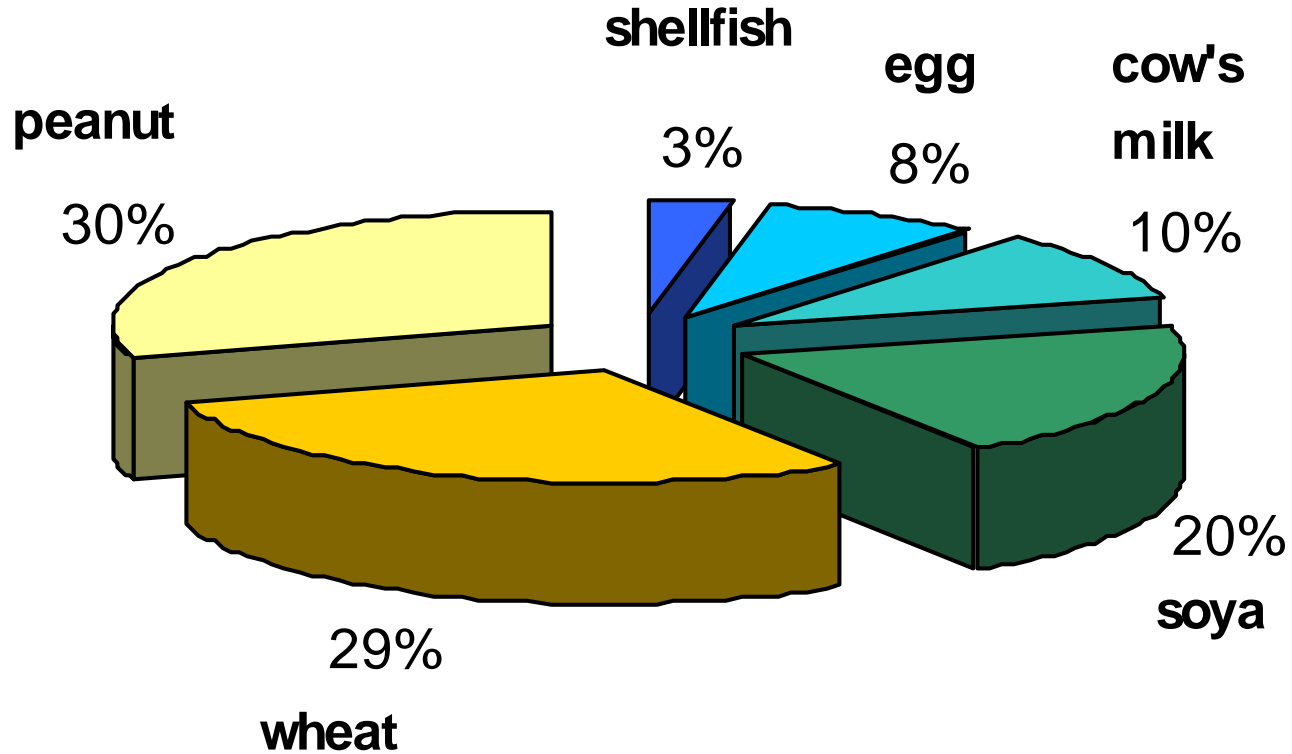
About 160 food products are known to cause allergic reactions.





Background

Joint Research Centre



Peanut allergy is the major problem due to ubiquity and severity of reactions





Background

In the EU: mandatory labelling of ingredients that are considered to be the most common allergens

There is an urgent need for specific, sensitive methods to control allergen-free products





Background

➤ Directive 2003/89/EC

Cereals	Nuts
Fish & crustaceans	Celery
Egg	Mustard
Peanut	Sesame seed
Soy	Sulphites
Milk	

➤ Methods should detect allergens at low ppm levels
(discussed in CEN TC 275/WG 12)





Challenges

Speculaaskoeken
met amandelen

Ingrediënten: tarwebloem
(gecontroleerde teelt), suiker,
plantaardige oliën en vetten,
geroosterde amandelstukjes
(10%), tarwezetmeel,
rijsmiddelen (E500, E336),
speculaasknuiden, zout,
emulgator (lecithine), gebrande
suiker, specerij.

Een E-nummer is een door
de Europese Gemeenschap
goedgekeurde hulpstof.

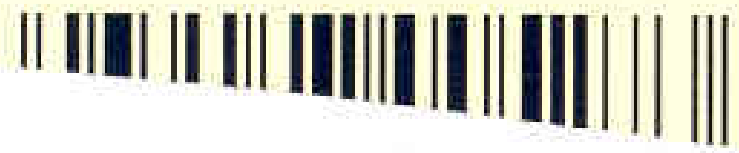
Gemaakt in een productie-
ruimte waar ook andere noten
en sesamzaad worden verwerkt.





Challenges

PRODUCT OF *Scotland*
IMPORTE D'ECOSSE
HERGESTELLT IN SCHOTTLAND



STORE IN A COOL DRY PLACE
TENIR AU FRAIS ET AU SEC
KÜHL UND TROCKEN LAGERN

NOT SUITABLE FOR NUT ALLERGY SUFFERERS.
NE CONVIENT PAS AUX PERSONNES ALLERGIQUES
AUX FRUITS À COQUE. NICHT GEEIGNET FÜR
MENSCHEN MIT NUSSALLERGIE.





Challenges

Caramels with Milk Chocolate Centres

Ingredients: Glucose syrup, Sugar, Milk chocolate (18%) (Milk, Sugar, Cocoa mass, Cocoa butter, Vegetable fat, Emulsifier (E442), Flavourings), Dried whole milk, Hydrogenated vegetable oil, Emulsifier (E471), Salt, Flavouring.

CONTAINS SULPHITES. MAY CONTAIN TRACES OF PEANUT, NUT AND WHEAT.

Nutrition Information		Per Sweet	Per 100g
Energy	kJ	135	1910
	kcal	35	455
Protein	g	0.3	4.5
Carbohydrate	g	5.0	68.9
Fat	g	1.3	17.9





Challenges

- Non appropriate labelling (“may contain” is not a solution).
- Cross-contamination during production and transport
- Re-work in food processing.
- Hidden allergens.
- One food species (e.g. milk, peanuts) can contain various allergenic substances.
- Not enough knowledge on thresholds.
- Different sensitivity of allergic patients.
- Critical values for labelling not known yet.
- Food processing may lead to non detectability, but allergenicity is still present





Requirements on methods

- **Detection and quantification
at low mg/kg range**
- **Comparability of quantitative
results**
- **Matrix effects**
- **Appropriate calibration**





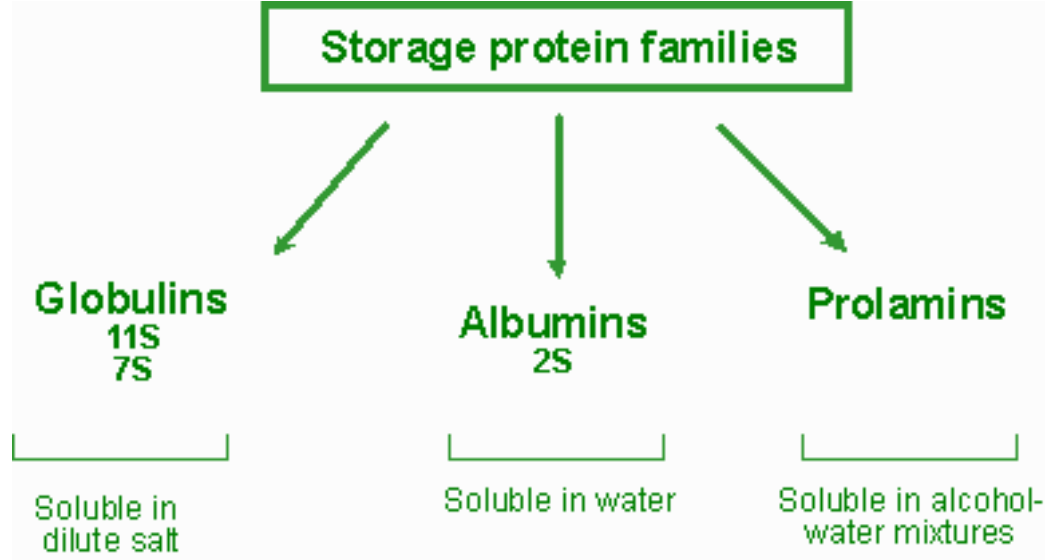
Example: Peanut allergy





Challenges

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Cupin superfamily

Prolamin superfamily

- Ara h1**
- Ara h3**
- Ara h4**
- Ara h5**

- Ara h2**
- Ara h6**
- Ara h7**





Challenges

Parameters influencing detection

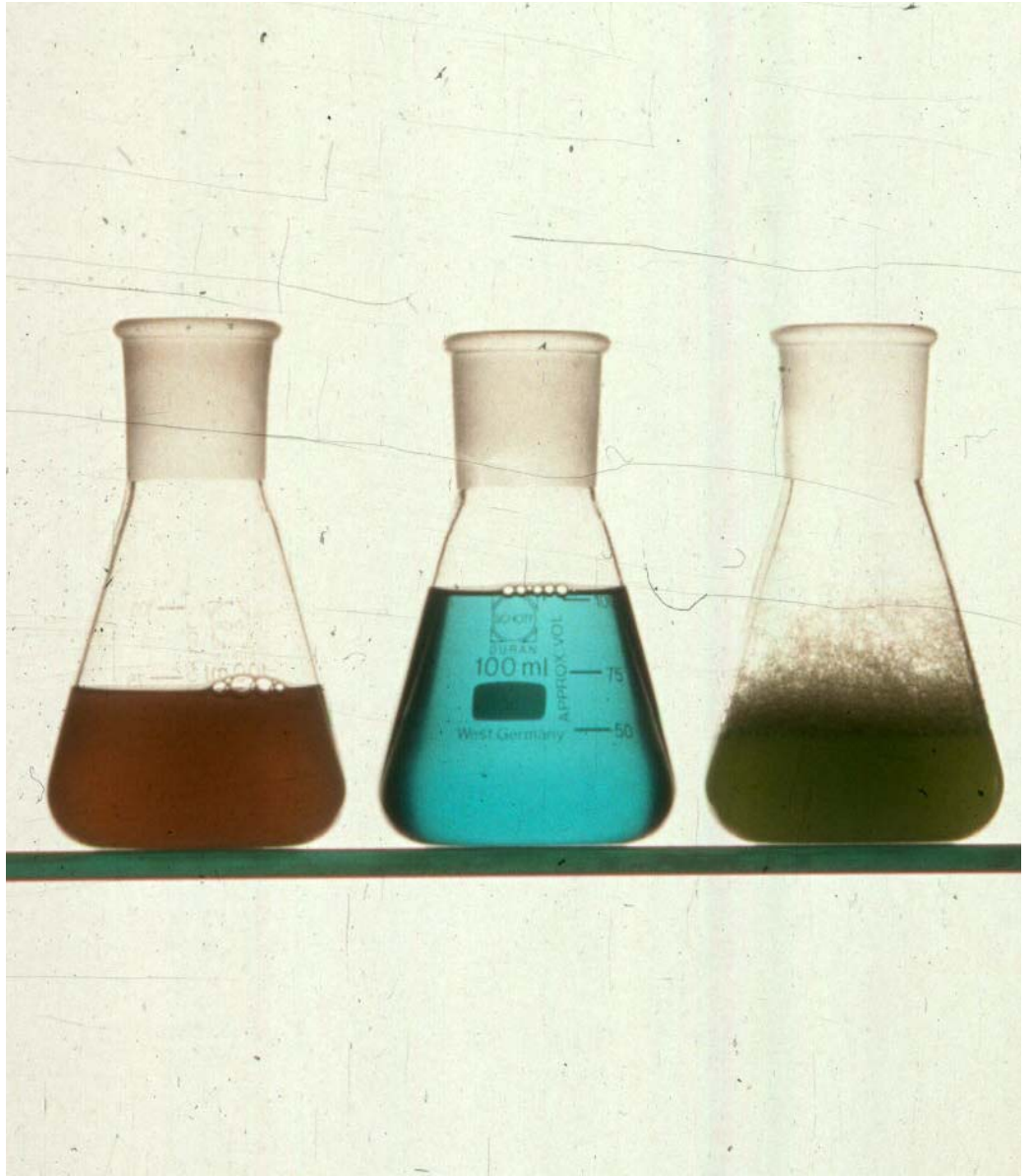
- **Extraction efficiency**

- **The effect of processing**
 - **protein / DNA degradation**
 - **protein deformation**
 - **Maillard reaction**



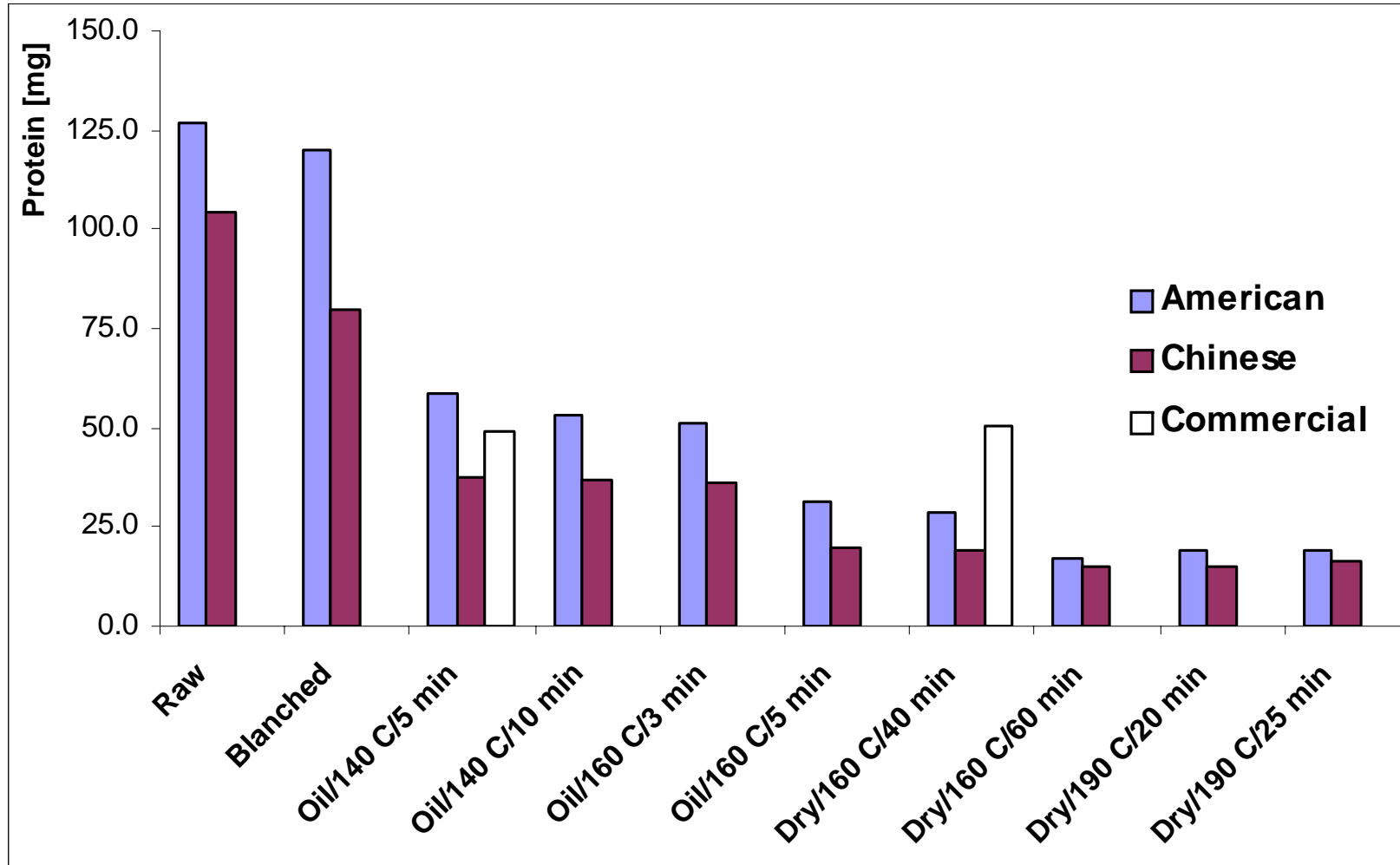


Extraction



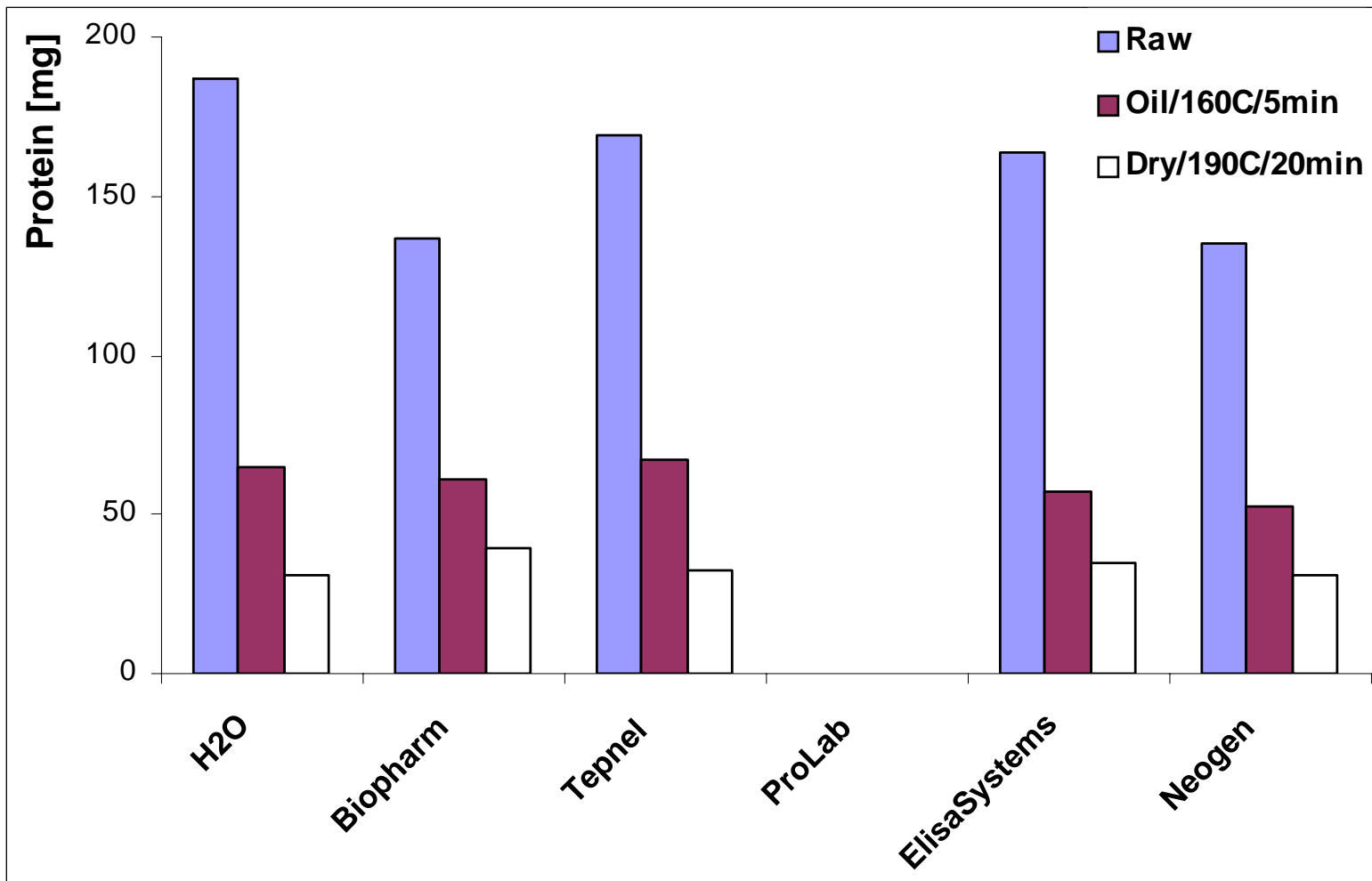


Extraction





Extraction



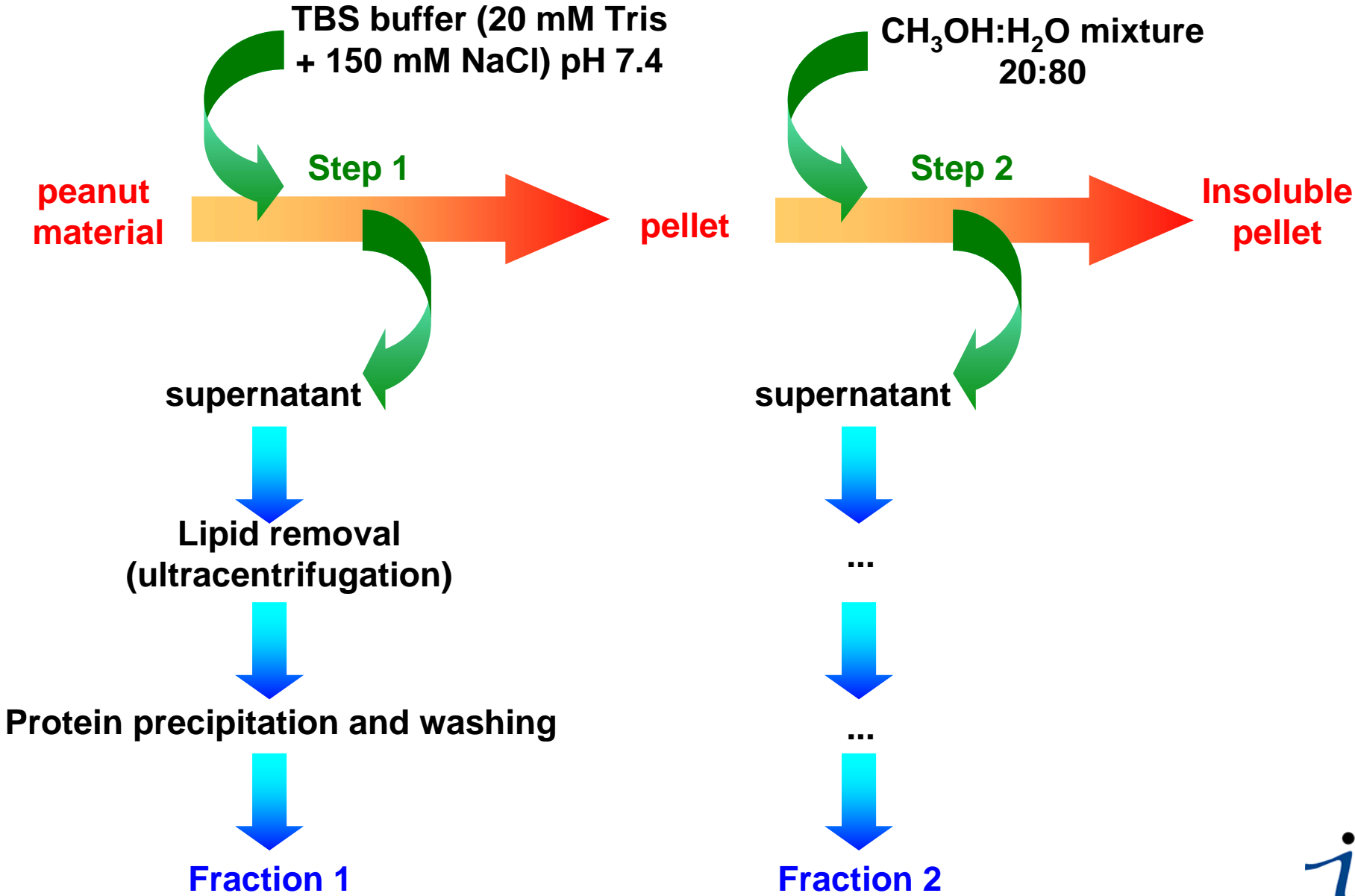
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Sequential extraction

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Determination of protein concentration

Raw peanut (Chinese variety): 25% proteins, 46% lipids

	Protein amount in extract (mg/ml)	Extraction yield (%)
(1) Extract in TBS buffer pH 7.4	2.7 ± 0.6	21.0 ± 3.0
(2) Extract in EtOH:H₂O, 20:80	3.5 ± 0.3	26.6 ± 2.7
(1) + (2)		47.6 ± 2.1

results for 5 independent extractions, 5 replicates





Challenges

Methods available

Immunological methods

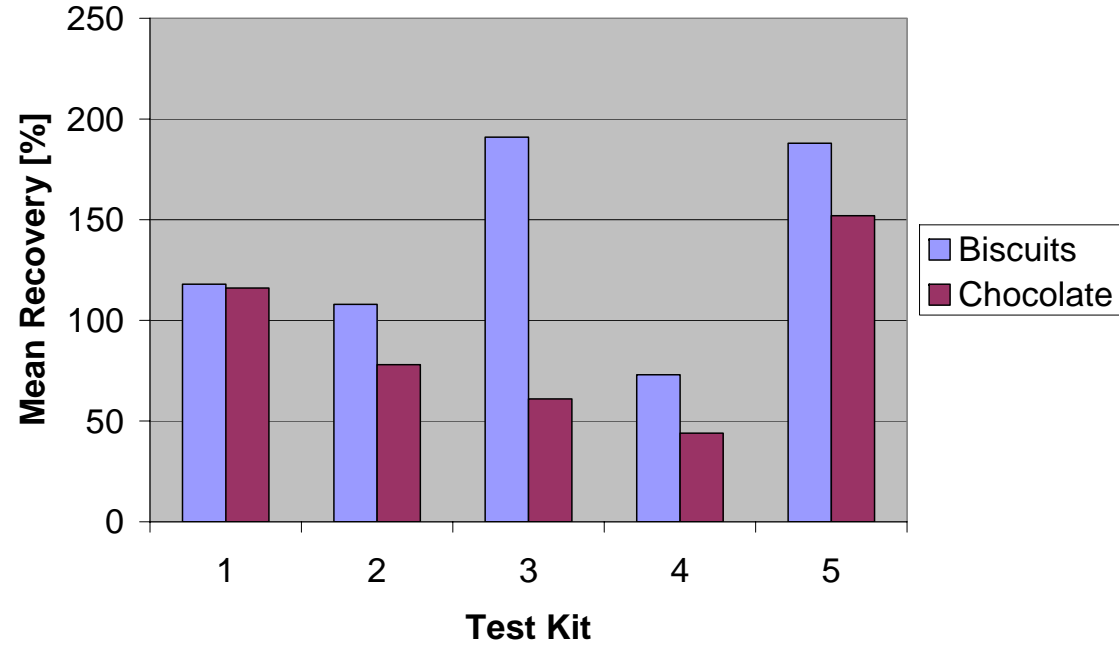
ELISA test kits

Dip stick methods

Molecular biological methods

DNA (PCR) methods

PCR-ELISA methods





Commercial peanut kits

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Sandwich ELISA

- BioSystems from Tepnel, UK (Ara h 1)
- Elisa Systems, Australia (Ara h 2)
- Prolisa from Pro-Lab, Canada (soluble peanut protein)
- Ridascreen from R-Biopharm, D (soluble peanut protein)
- Veratox from Neogen, USA (soluble peanut protein)

Dipstick tests

- Tepnel, UK
- Neogen, USA
- r-Biopharm, Germany (Hazelnut & peanut)

PCR

- SureFood from Congen, Germany (RT-PCR, DNA-ELISA)
- Biosystems from Tepnel, UK (PCR and Agarose-Gel)





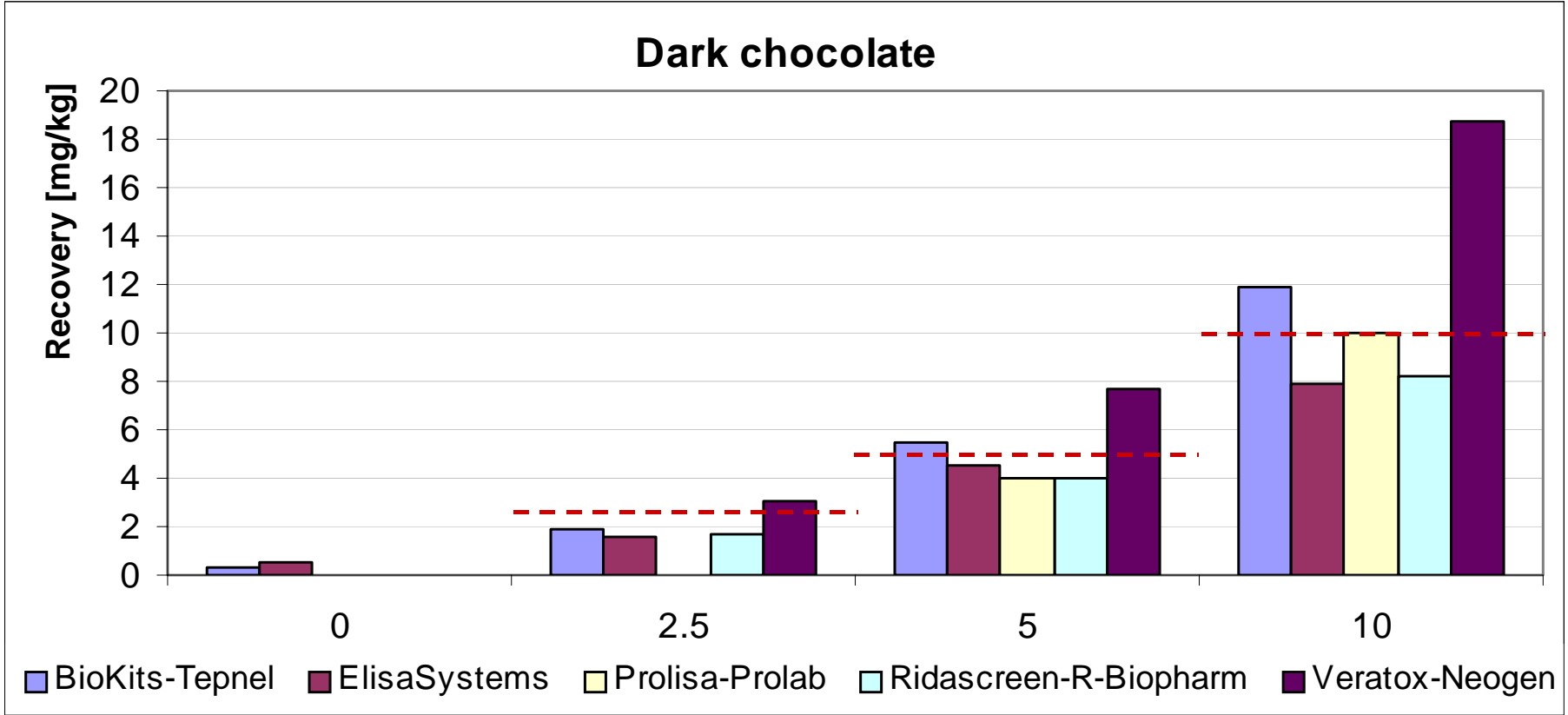
Results from validation study

Method	Matrix	Average recovery (%)	False negatives %	RSD _r %	RSD _R %
BioKits-Tepnel	Cookie	118.0	1.9	32.1	37.7
	Dark chocolate	116.2	0	16.5	26.5
ElisaSystems	Cookie	108.2	6.9	72.2	72.2
	Dark chocolate	78.4	5.9	17.9	52.9
Prolisa-Prolab	Cookie	190.9	3.3	48.2	50.5
	Dark chocolate	60.5	25.5	40.2	77.3
Ridascreen-R-Biopharm	Cookie	72.9	18.6	71.4	86.3
	Dark chocolate	43.7	17.3	25.6	67.2
Veratox-Neogen	Cookie	188.3	2.1	30.3	37.4
	Dark chocolate	151.8	0	11.7	22.3



Recoveries from ELISA test kits

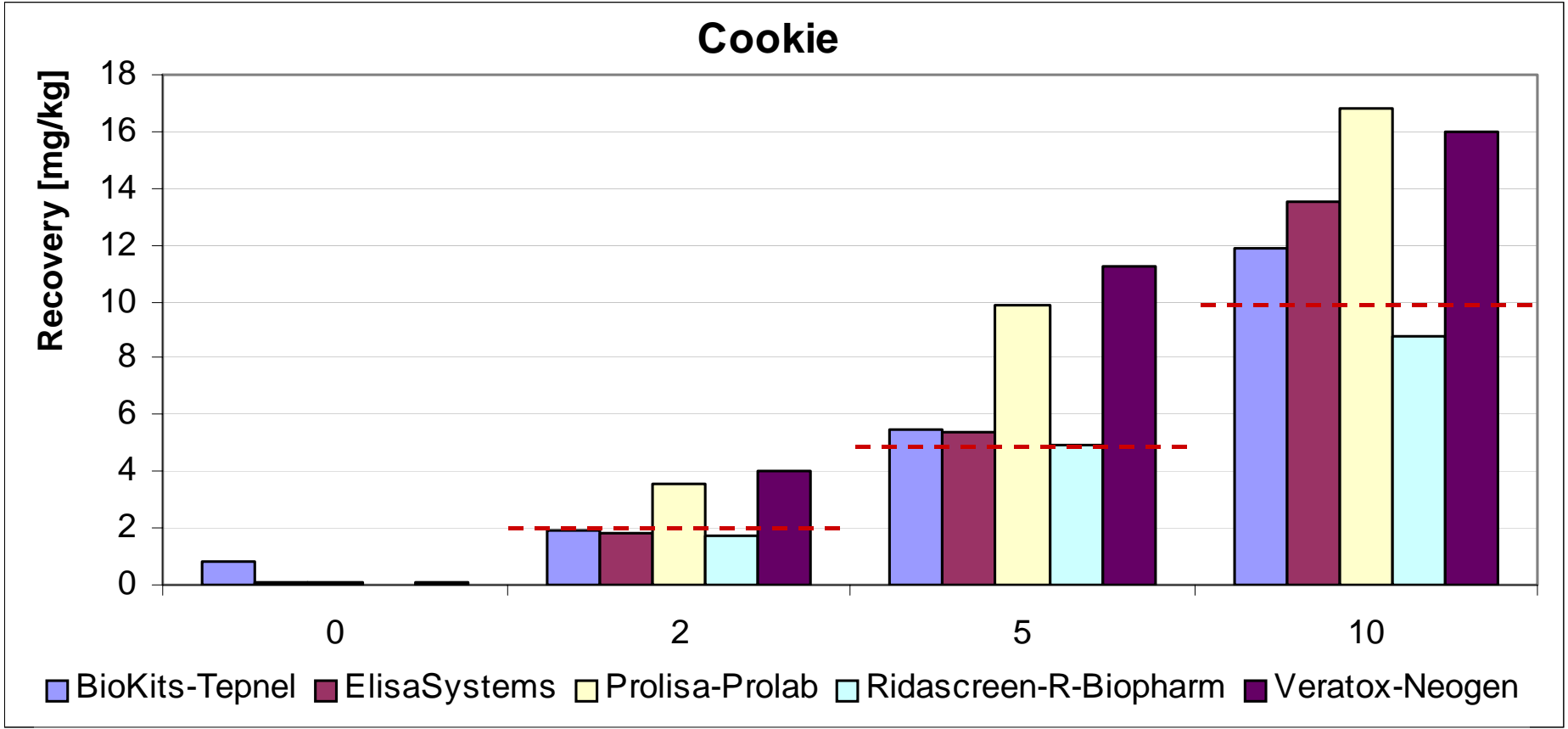
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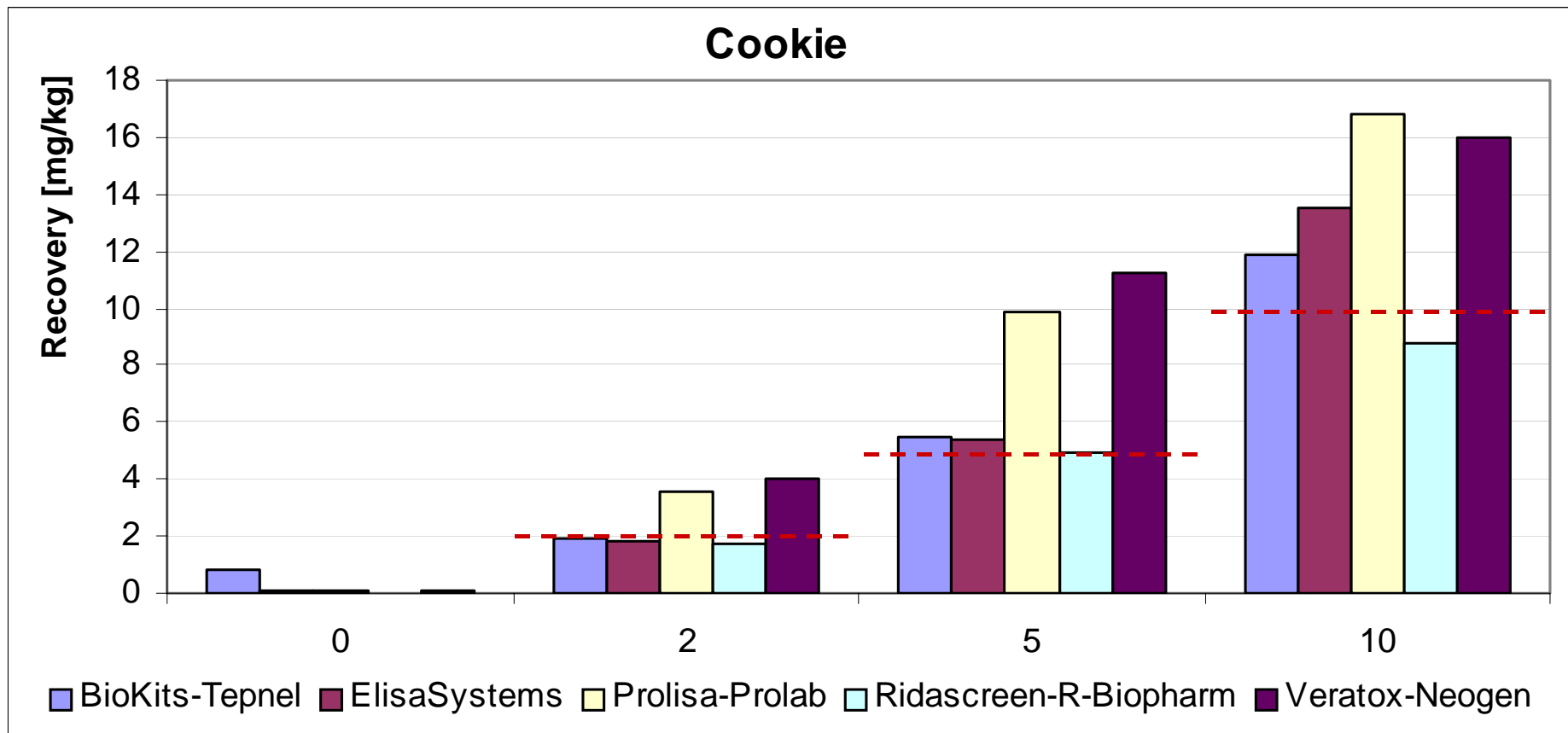
Recoveries from ELISA test kits

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Recoveries from ELISA test kits



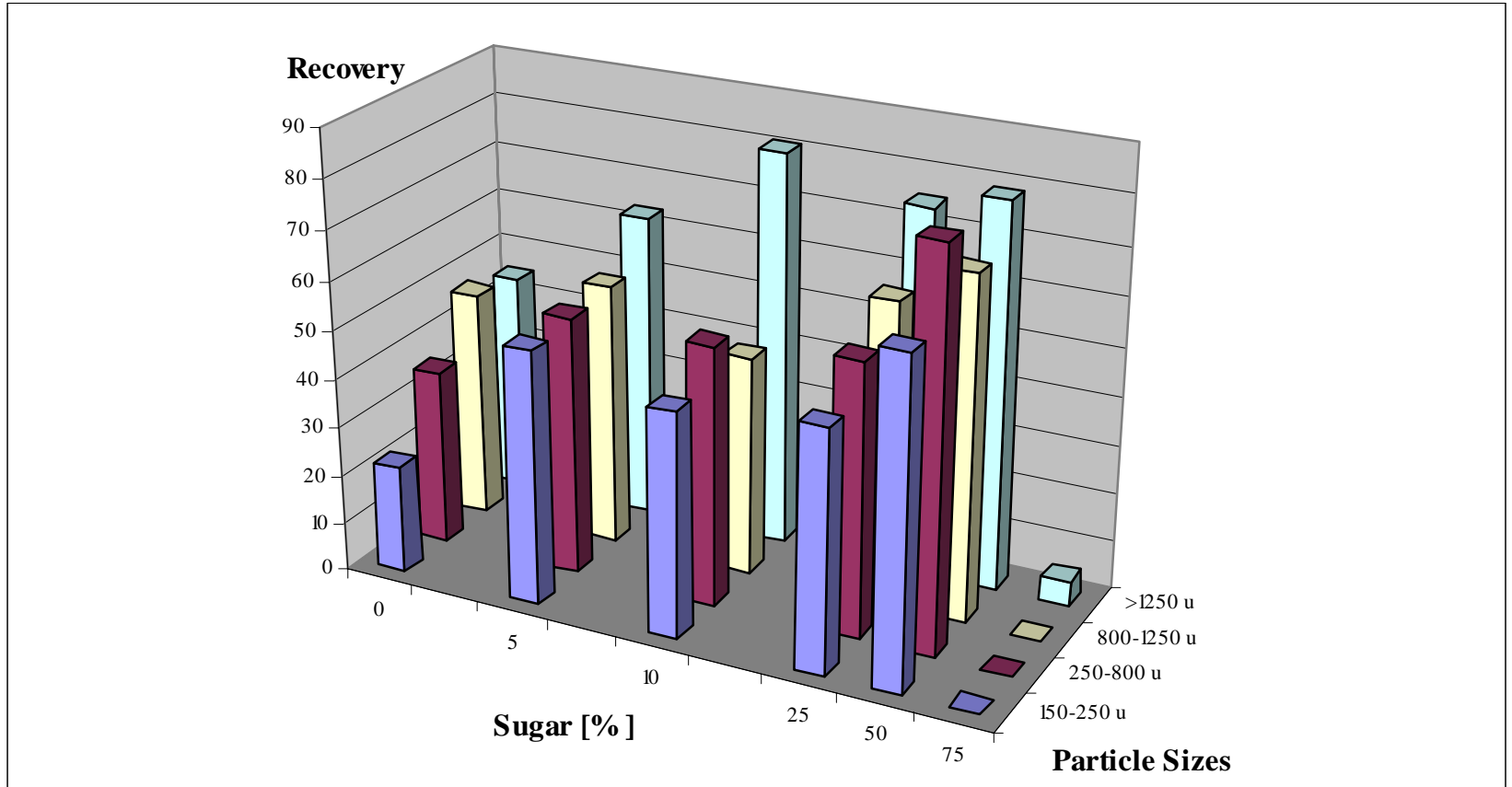


Processing



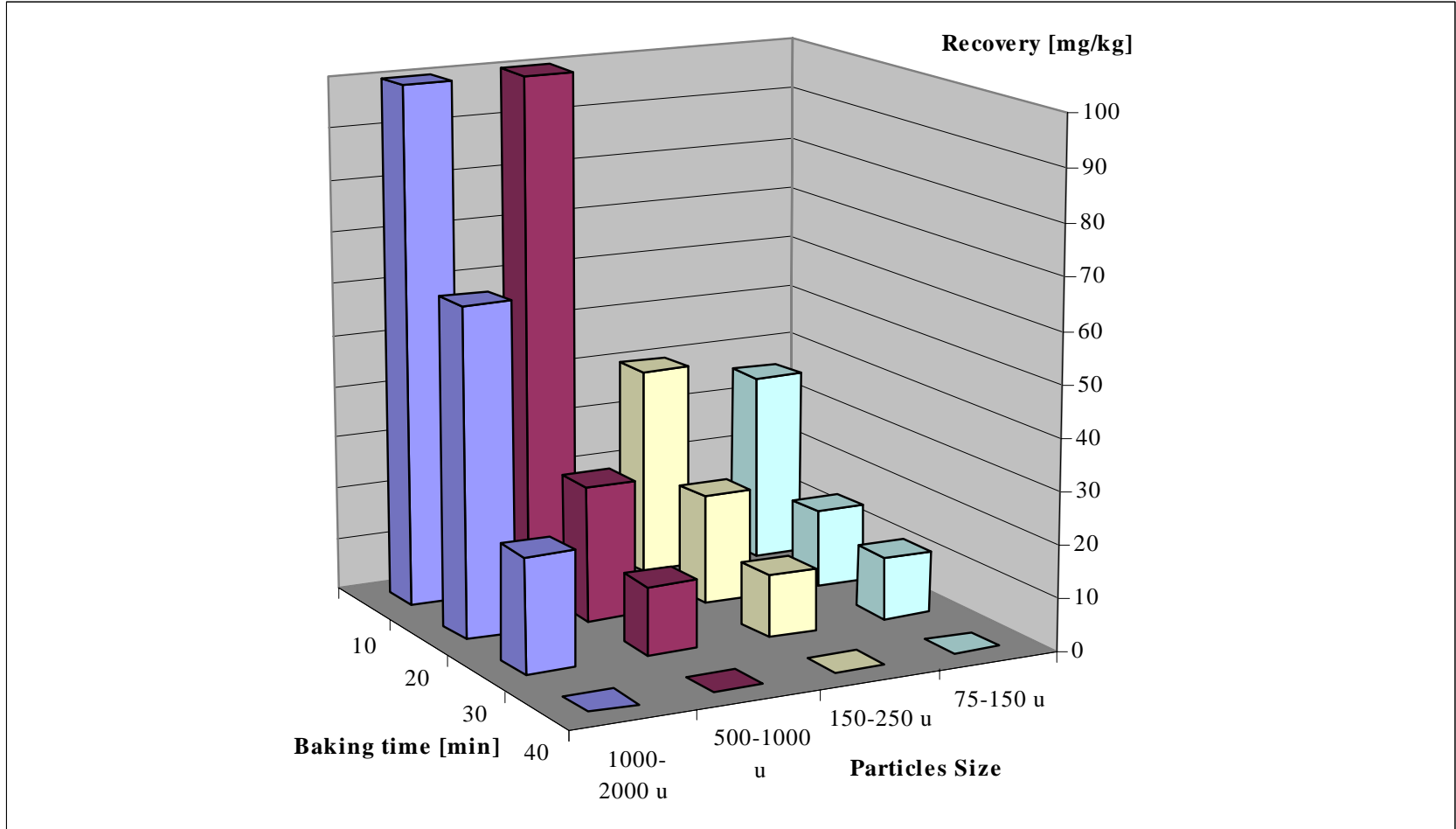


Effect of roasting



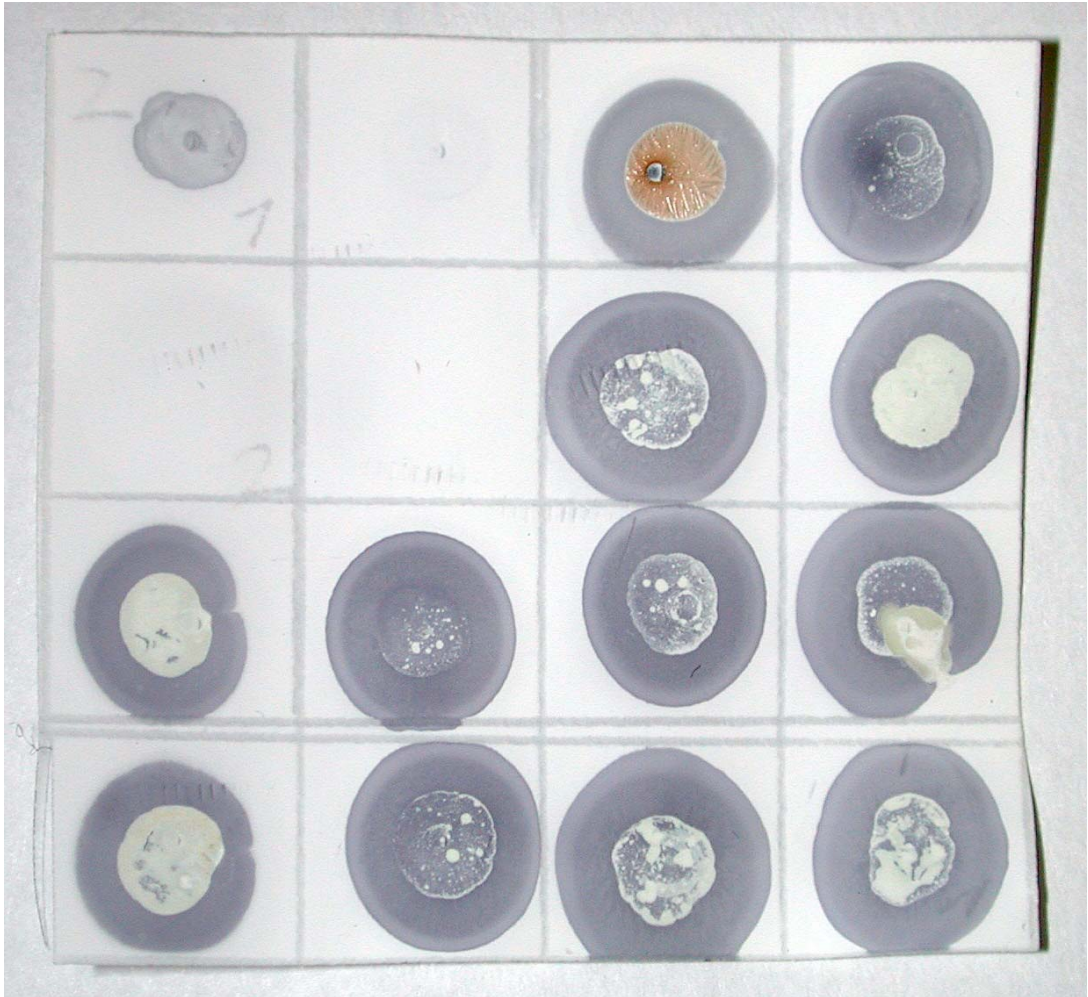


Effect of roasting





Effect of roasting

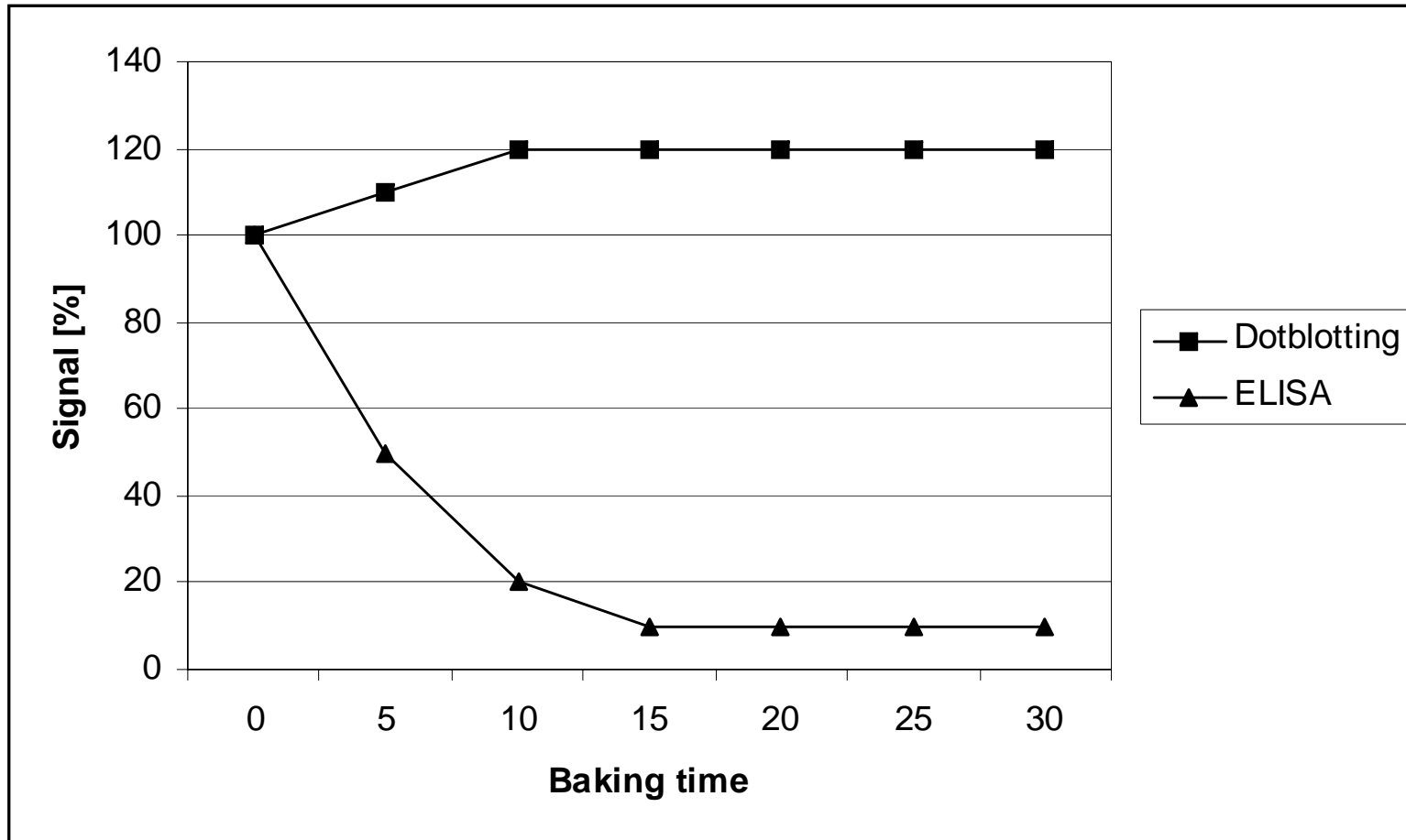


IgE binding capacity of various peanut containing cookie samples determined by Dotblot analysis.





Effect of roasting





Challenges for confirmatory methods based on a “proteomics” approach





Integrated “proteomics” approach for allergens

2D Gel Electrophoresis

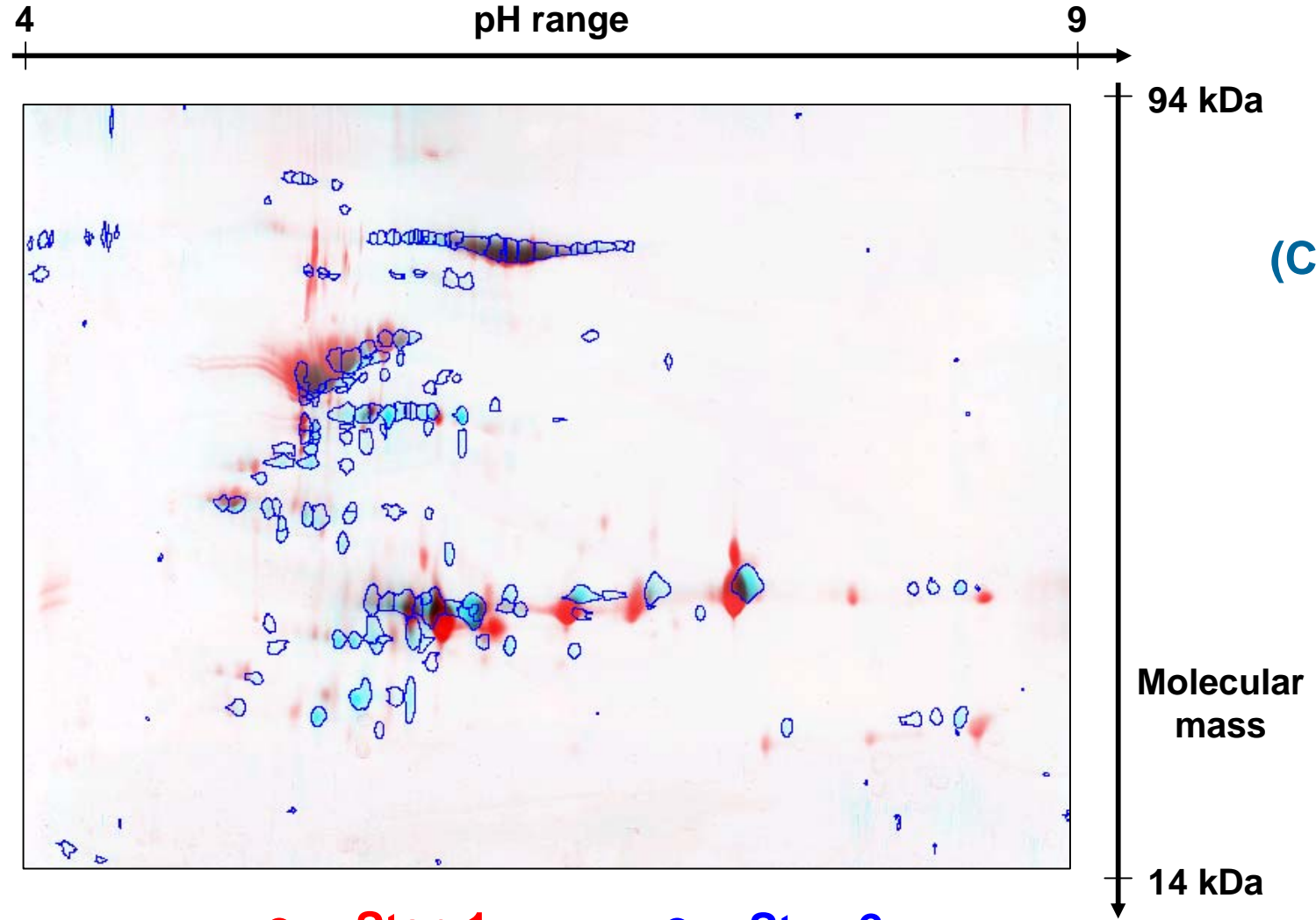
Immunochemical analysis (e.g. Western blotting)

Capillary LC-MS/MS (Q TOF)



2D gel-based approach

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Raw peanut
(Chinese variety)

● Step 1
(TBS extract)

● Step 2
(EtOH extract)

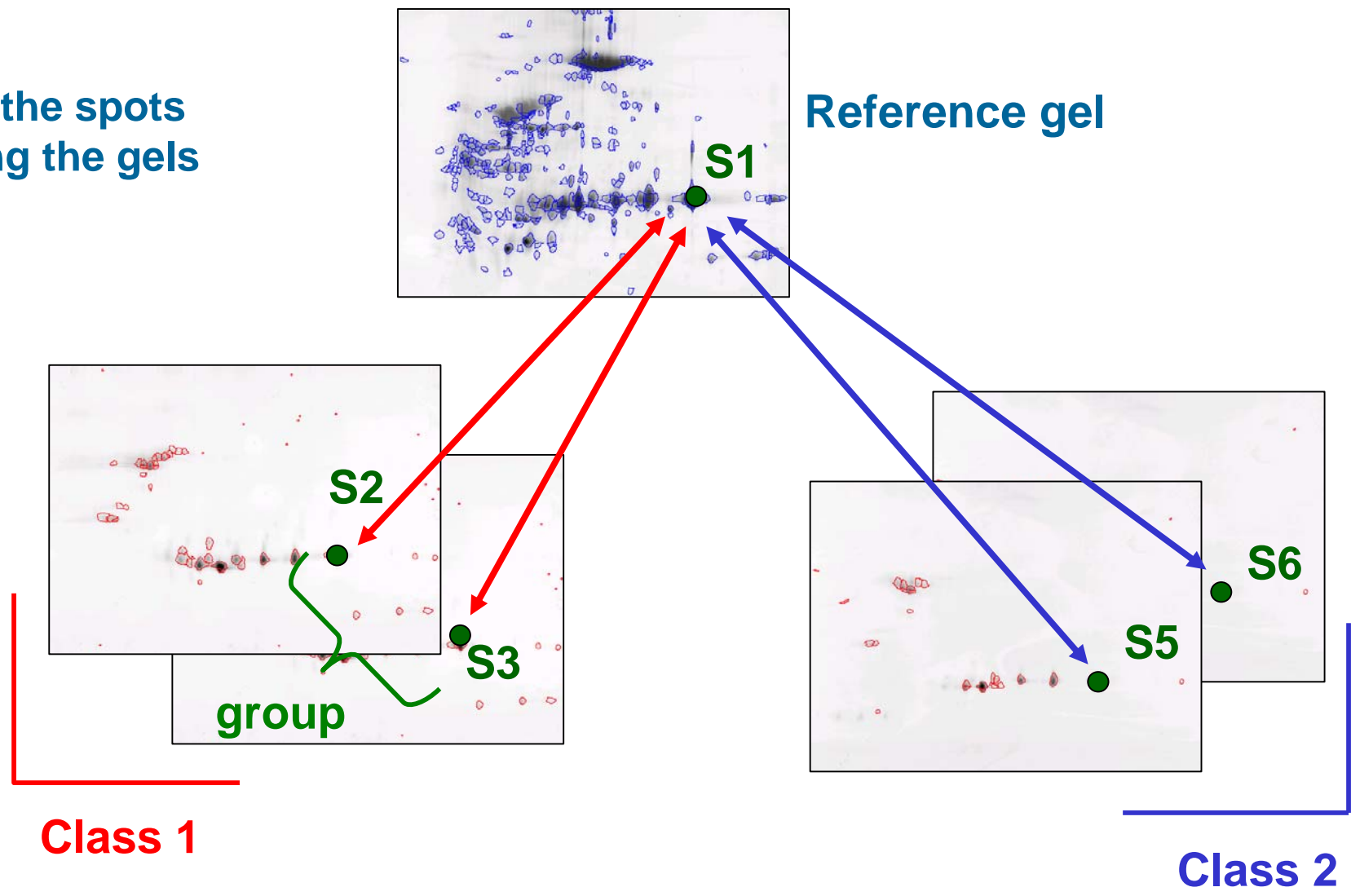




Protein detection and gel matching

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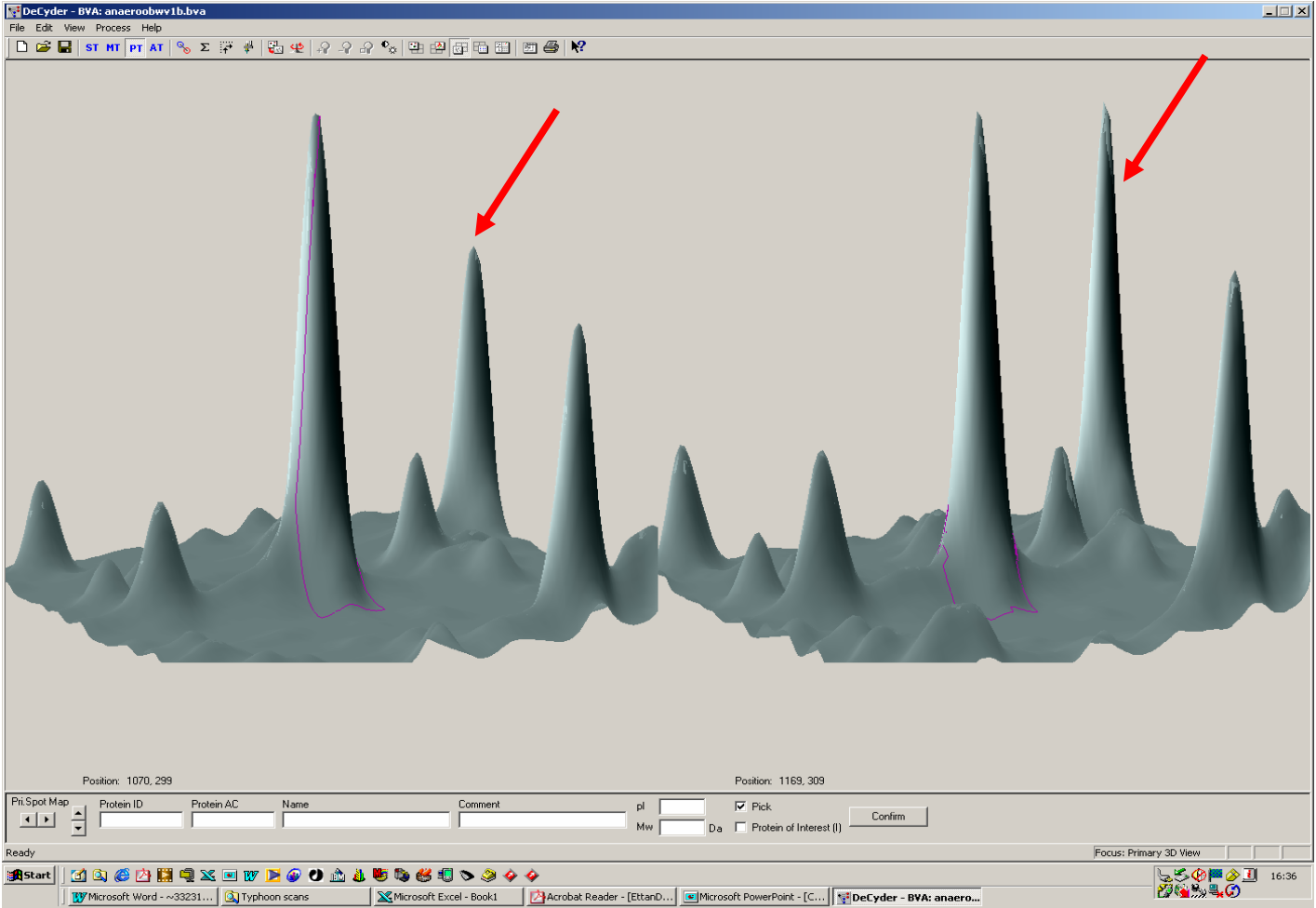
- Pairing the spots
- Matching the gels





Classical 2D Electrophoresis

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Same sample
different gels,
Protein specific
differences
occur

- Problem: gel-to-gel variation, due to variability in fixation, staining etc.
- Not possible to differentiate real variations between samples

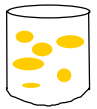




2D Difference Gel Electrophoresis (DIGE)

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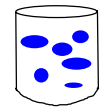
Internal standard
Label with Cy2



Protein extract 1
Label with Cy3



Protein extract 2
Label with Cy5

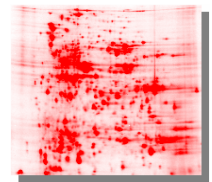


Mix labelled samples

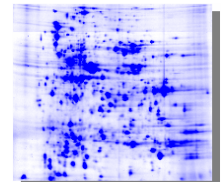
Separate by 2-D PAGE



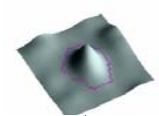
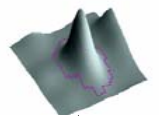
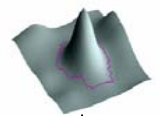
Cy2 excitation wavelength



Cy3 excitation wavelength



Cy5 excitation wavelength



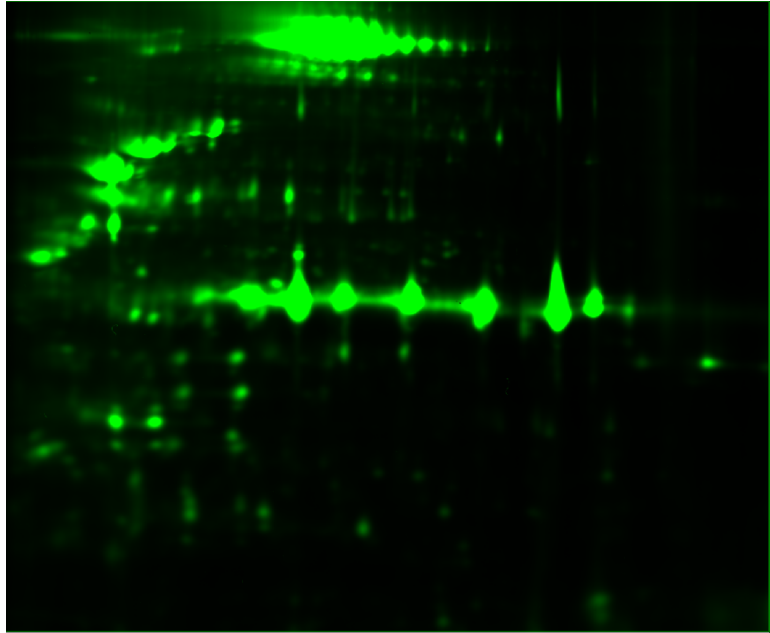
Volumes expressed as ratios relative to pooled internal standard

Protein extract 1:
raw peanut
Protein extract 2:
oil roasted peanut
Internal standard:
pooled extracts 1 & 2

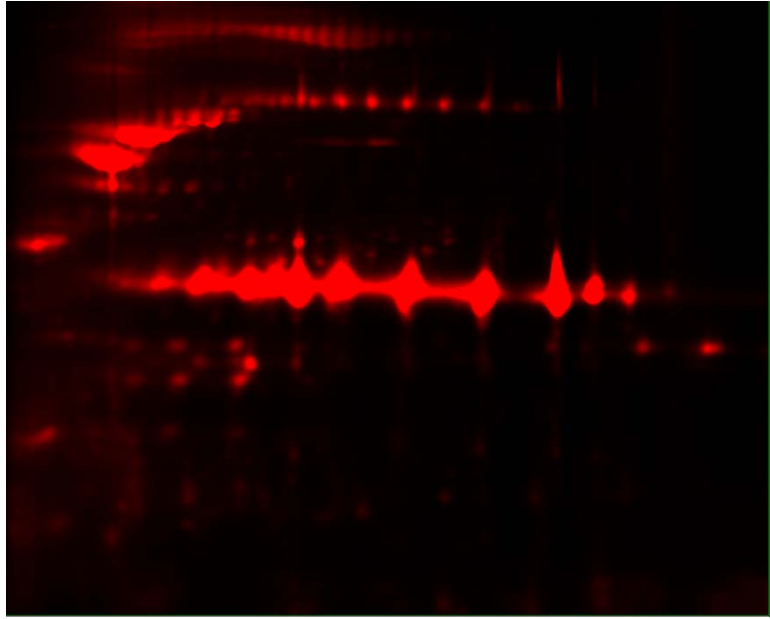


2D DIGE Detection

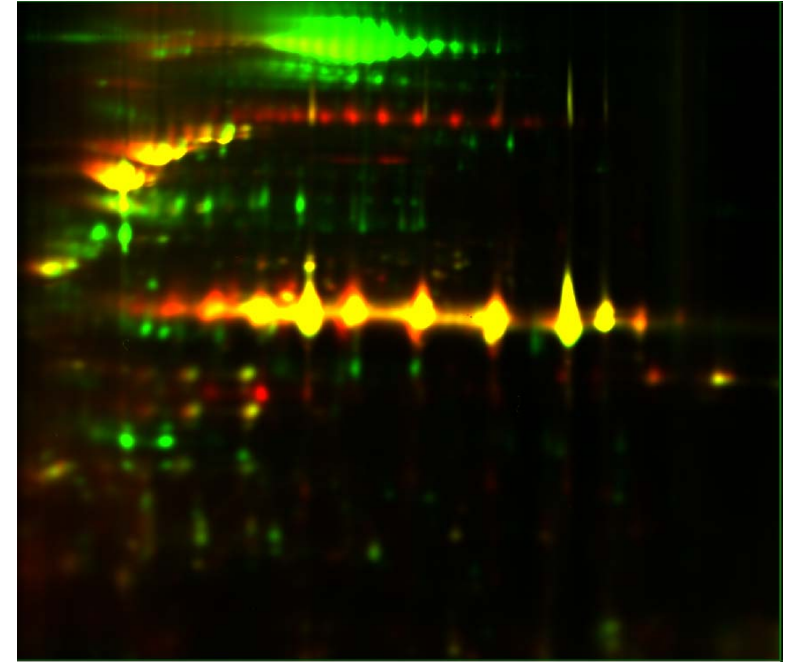
Cy3



Cy5



Cy3 = raw peanut
Cy5 = oil roasted peanut



- Ara h 1 (63.5 kDa), Ara h 3/4 (60 kDa)
- Different isoforms
- Different glycoforms

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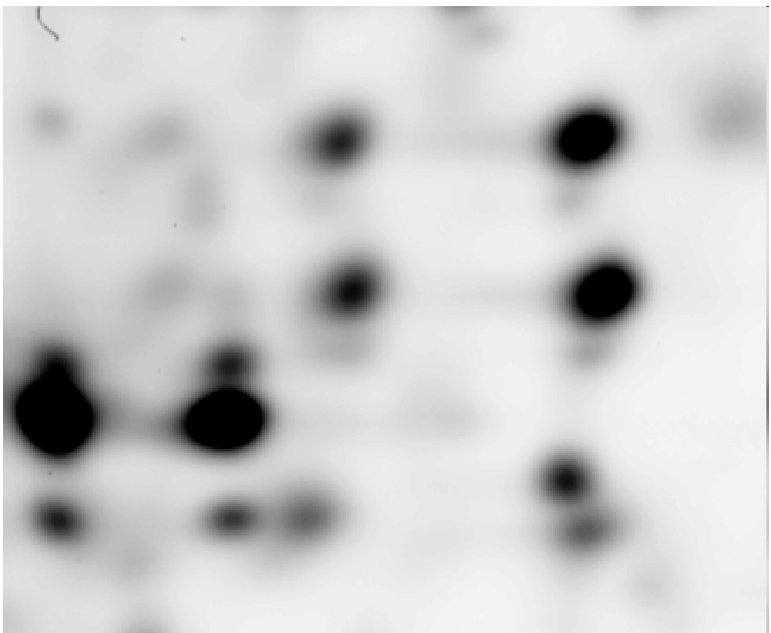
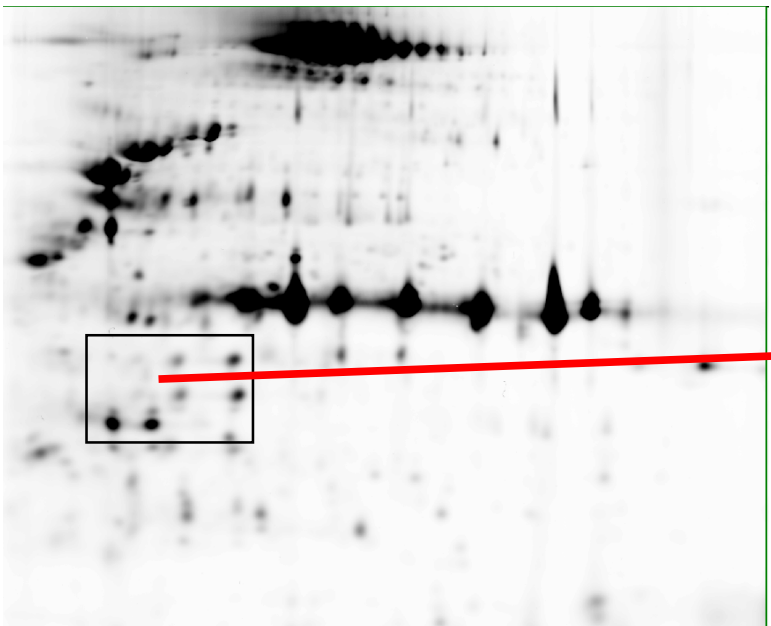




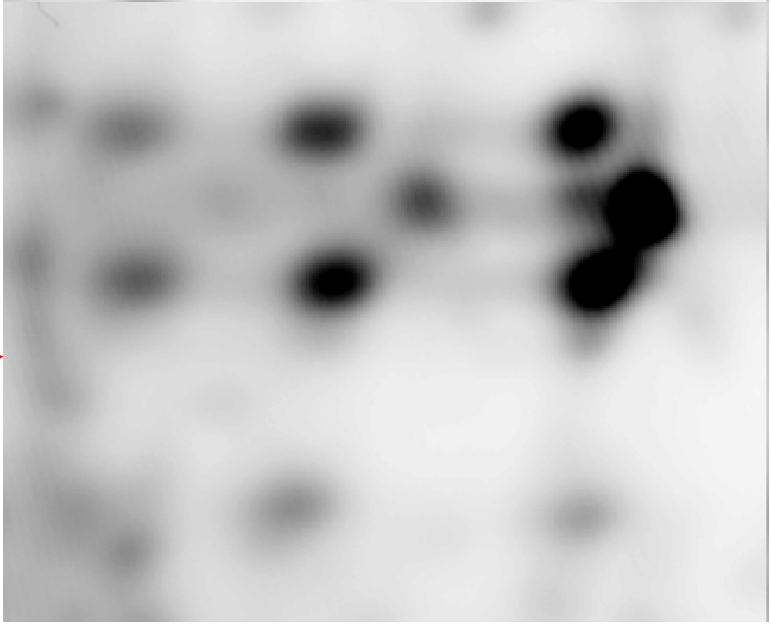
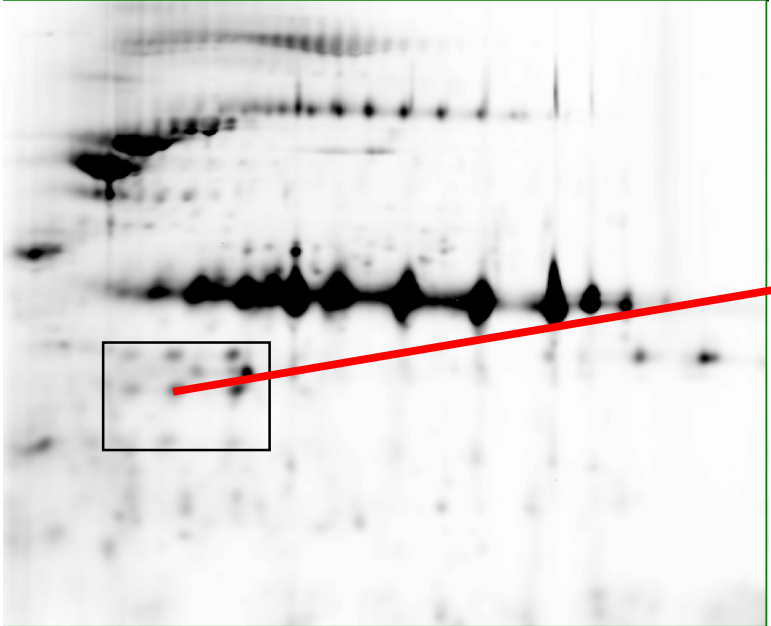
2D DIGE Detection

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Cy3
(raw)

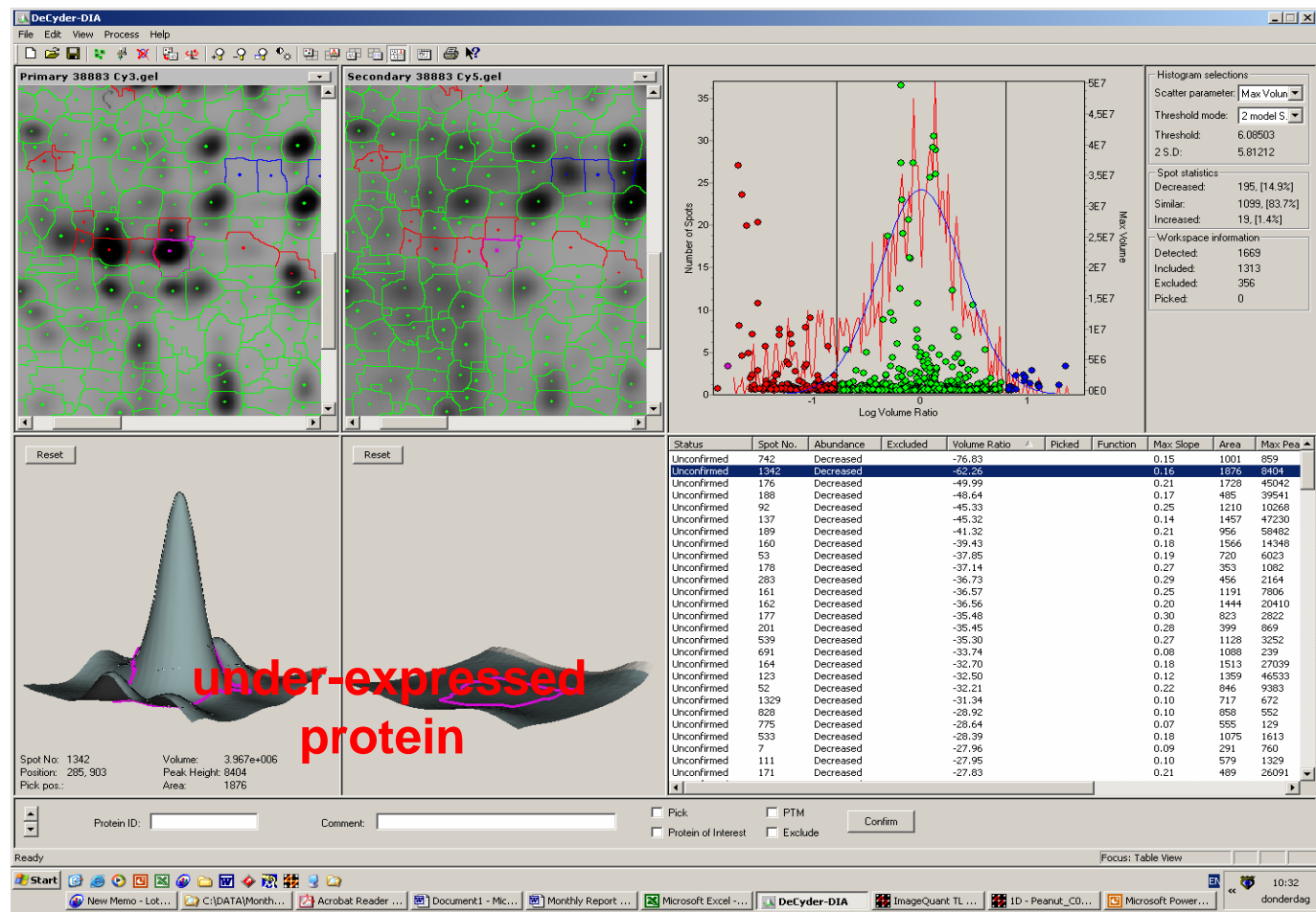


Cy5
(roasted)



Differential In Gel Analysis (DIA)

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highlights differences between samples (here raw and oil roasted peanut)
 in a single gel

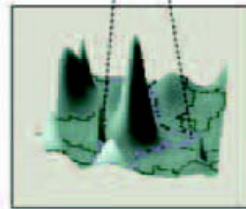
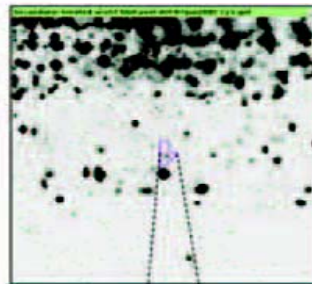




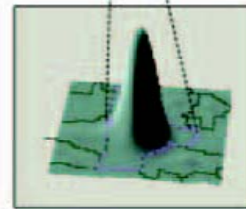
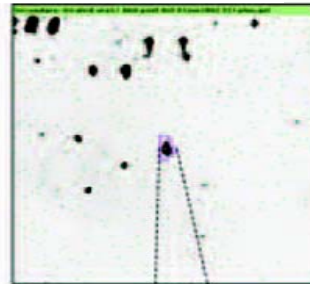
Immunochemical detection

Data obtained by differential fluorescence protein analysis will be linked to the detection of peanut allergens on 2D Western blots

Protein stain



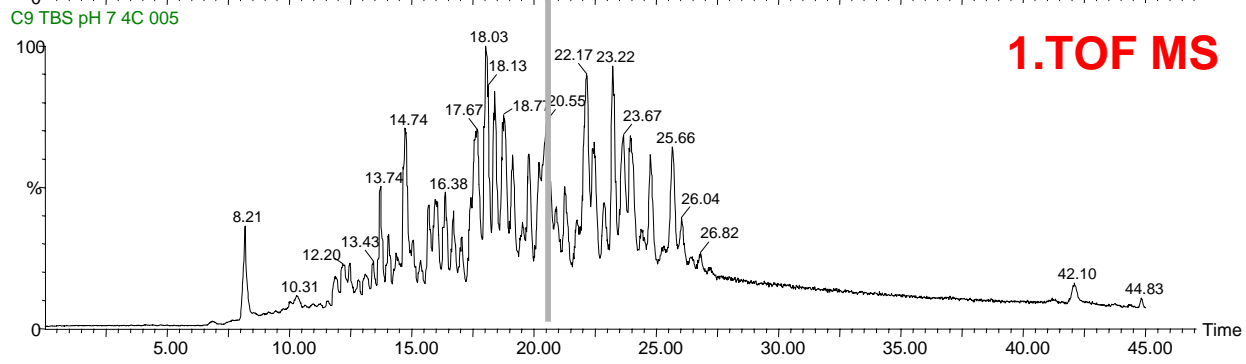
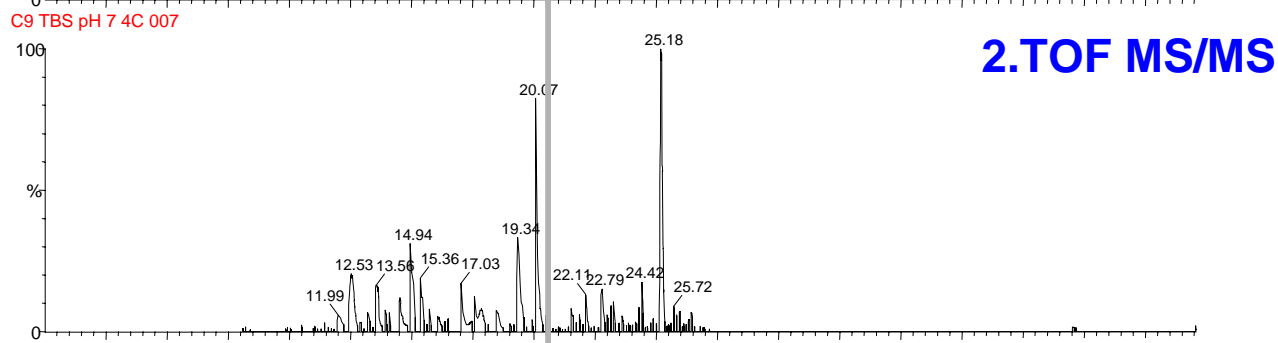
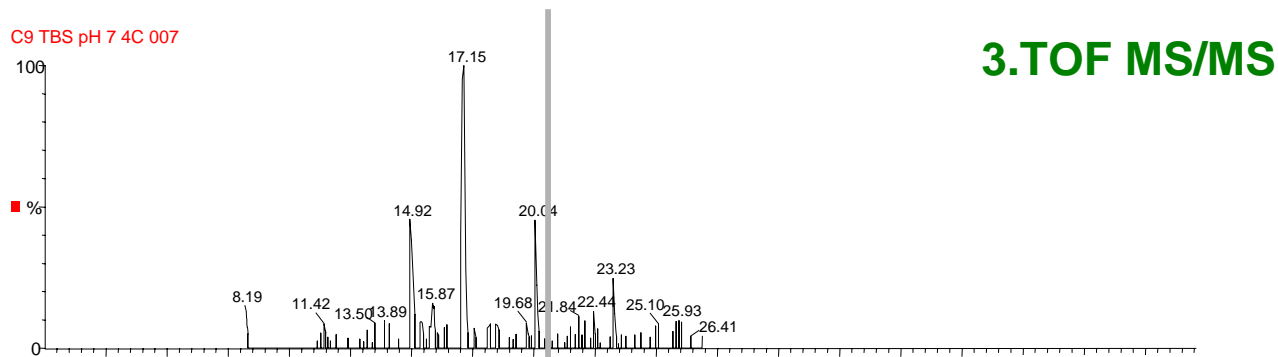
Protein Blot



- antibodies raised against peanut protein
- antibodies raised against peanut allergens
- IgE from allergic individuals

Capillary LC with Q-TOF detection

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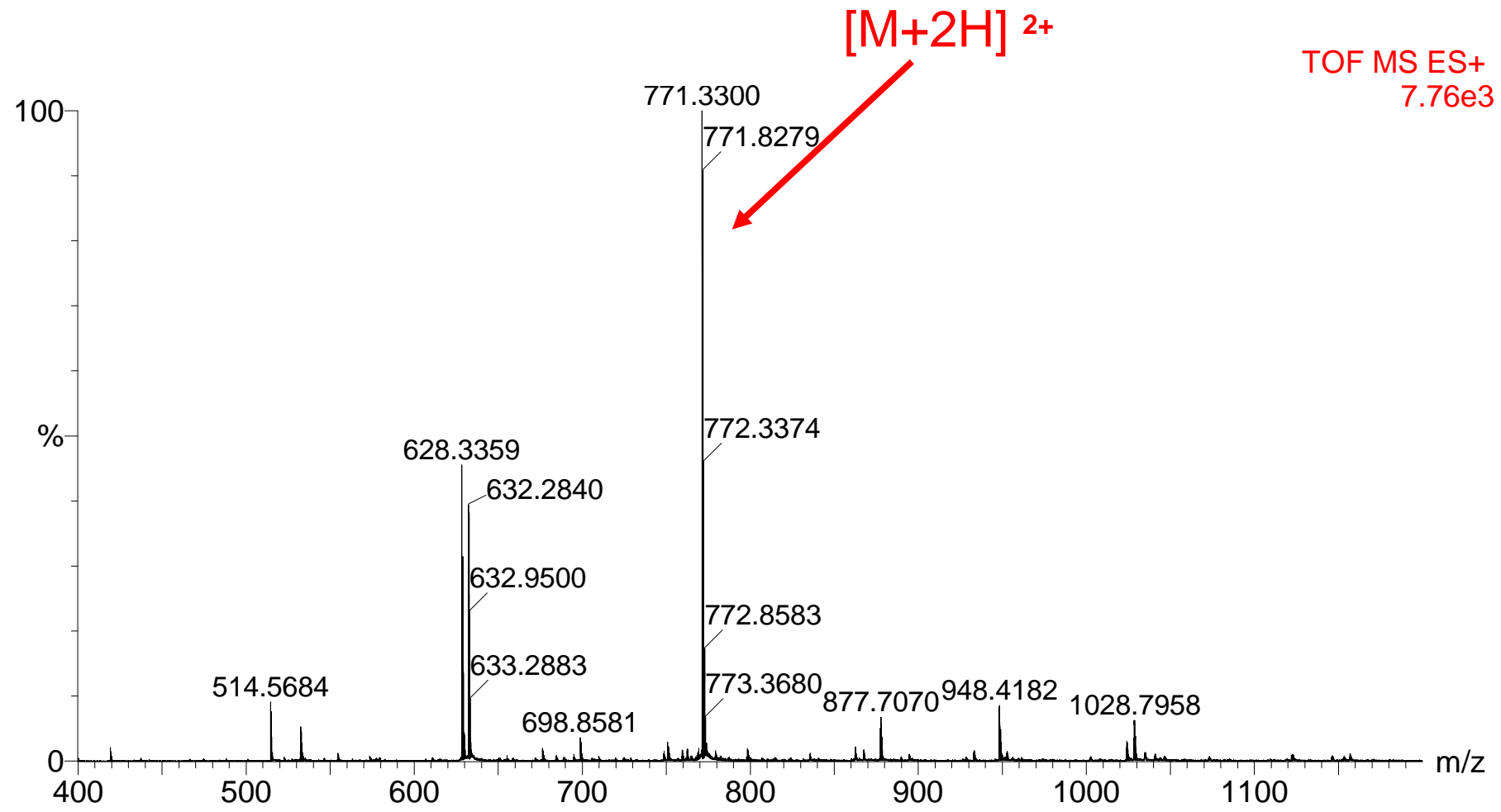
**Oil roasted peanut
 (Chinese variety)
 Extraction with TBS**

**Capillary column:
 C₁₈ 300 μm
 Flow rate 5 μl/min
 Z spray source**



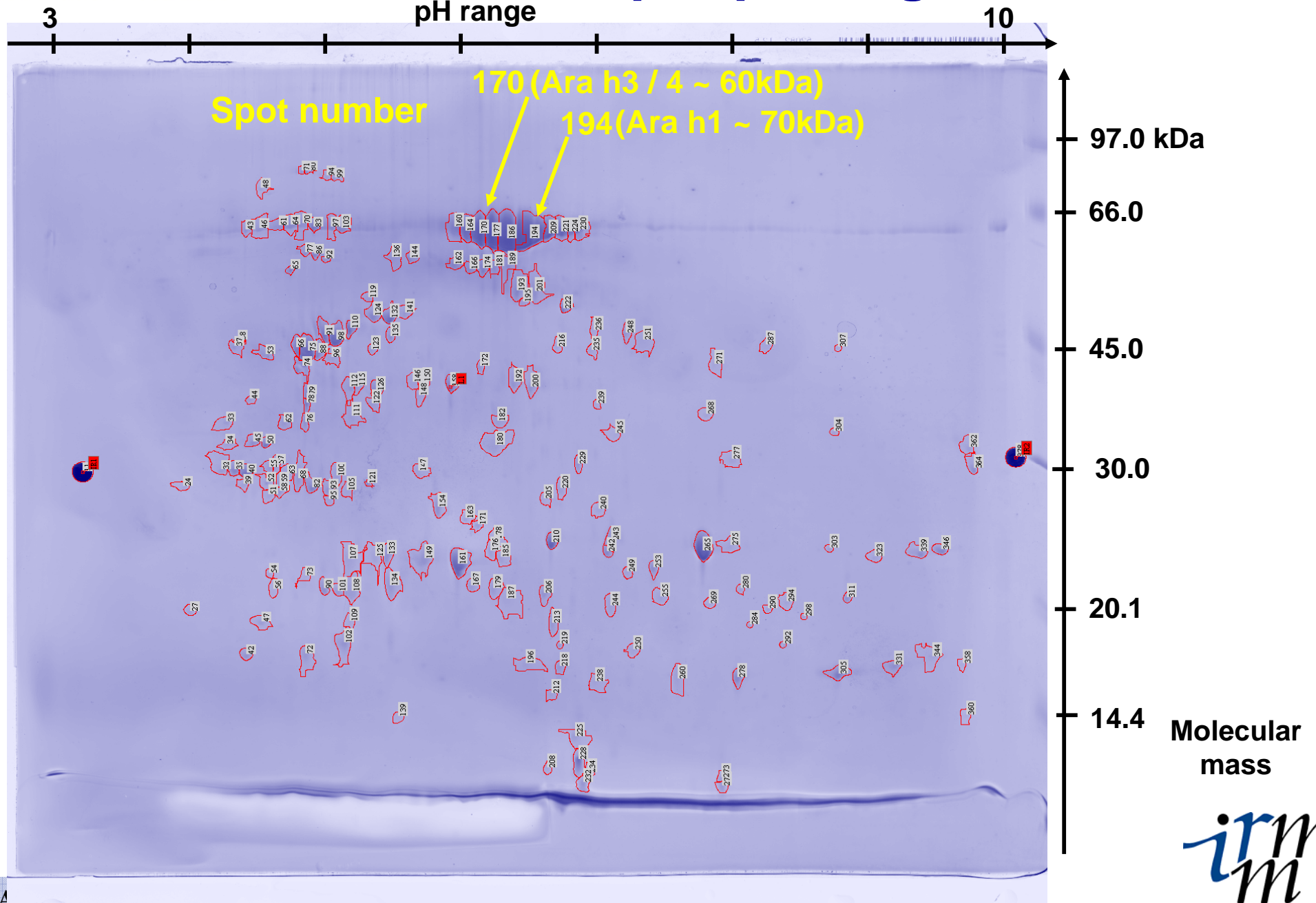
MS scan at t = 20 min

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Protein spot picking and MS

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Challenges

**Availability of Reference Materials,
especially real food matrices containing allergens**





Reference materials

Requirements to gain reliable results

- Availability of appropriate standards for calibration
- Availability of matrix matched materials to understand matrix problems, e.g. interference
- If possible, certified reference materials





Reference materials

Matrix CRMs for method quality assurance



Example
Agricultural food commodity, cookies,
chocolate, breakfast cereals
Traceability ensured through
gravimetric approach

CRMs as Calibrants for instrument calibration



Example
Pure analyte (DNA or proteins),
certified calibrants
Traceability requires a reliable
quantification possibility





Reference materials in production in JRC

- **Gliadin certified reference material (finalisation)**
- **Peanut mixture reference material (in preparation)**
- **Matrix matched reference material (future work)**





Future JRC method validation studies

➤ Establishment of an international and harmonised validation protocol

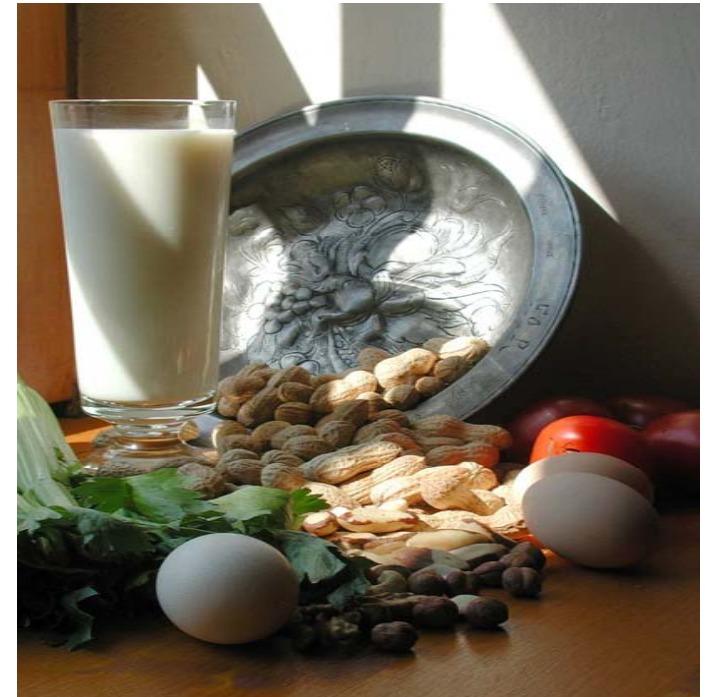
➤ Gluten/gliadin tests

➤ Milk allergens

- milk proteins

- whey proteins

by ELISA, LC-MS, 2D electrophoresis





The food allergen team

Arjon Van Hengel (Group leader)

Hubert Chassaigne (Protein analysis)

Jorgen Norgaard (LC-Q-TOF MS)

Linda Monaci (HPLC, focus on milk allergens)

Marcel Brohee (ELISA)

Antonio Gomez Galan (PCR)

Virginie Tregoat (Immunology, focus on milk allergens)





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