



Detection of allergens in food : new developments, analytical tools, and matrix effects

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1. CONSUMER CHOICE:

Allergens are, unlike GMO, a Health Issue for the consumer

2. QA/QC

Allergens are a liability issue for the producer

3. EU REGULATIONS

EU Legislation

2000/13/EC

6.5.2000

EN

Official Journal of the European Communities

L 109/29

**DIRECTIVE 2000/13/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL
of 20 March 2000
on the approximation of the laws of the Member States relating to the labelling, presentation and
advertising of foodstuffs**

EU Legislation

Key Points 2000/13/EC:

General rules for labelling and advertising of Foodstuffs, incl.

- **25% rule** for ingredients (no labelling if ingredient < 25%)
- Irradiated foodstuffs
- Alcoholic beverages
-

But: labelling of ALLERGENIC COMPOUNDS not regulated

EU Legislation

New Regulation 2003/89/EC

25.11.2003

EN

Official Journal of the European Union

L 308/15

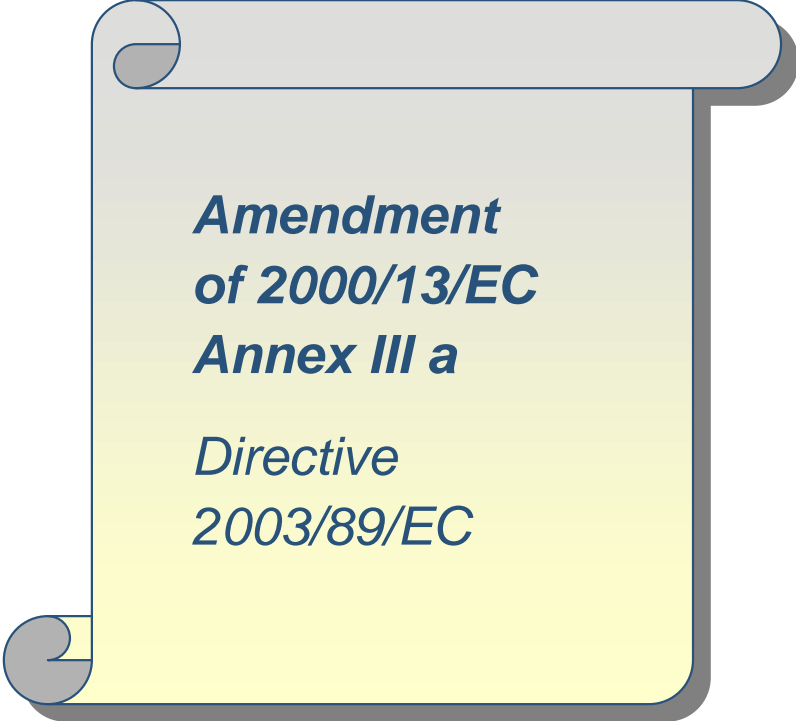
**DIRECTIVE 2003/89/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL
of 10 November 2003**

amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs

EU Legislation

The new regulation will cover:

- Cereals containing gluten and products thereof
- Crustaceans and products thereof
- Eggs and products thereof
- Fish and products thereof
- Peanuts and products thereof
- Soybeans and products thereof
- Milk and dairy products (including lactose)
- Nuts and nut products
- Sesame seeds and products thereof
- Celery and products thereof
- Mustard and products thereof
- Sulphite at concentrations of at least 10 mg/kg

A graphic of a rolled-up scroll with a yellow-to-white gradient background and a grey border. The text is centered on the scroll.

*Amendment
of 2000/13/EC
Annex III a*

*Directive
2003/89/EC*

Four main reasons for non-declared ingredients :

- Intentional substitution for higher priced products
- Accidental cross-contamination during processing
- Wrong assumption : (e.g. allergen is no longer present after heat treatment)
- False negative test results → matrix effects

***How can Allergens
be detected?***

Most important detection methods

<i>Method</i>	<i>Type</i>	<i>Pro</i>	<i>Con</i>
<i>ELISA (plate & dipstick)</i>	<i>Immuno / Protein</i>	<i>Cheap (?), fast, high-throughput high sensitivity</i>	<i>High variability, only single target</i>
<i>PCR (standard, real-time)</i>	<i>DNA</i>	<i>Fast, specificity, multi-screening potential, high-throughput</i>	<i>Indirect measurement (DNA marker)</i>
<i>MS</i>	<i>Protein / Peptide</i>	<i>High-throughput</i>	<i>Labour intensive, not ready for routine analysis</i>

The choice of the test method depends on the matrix

How to avoid choosing the wrong method ? eurofins

Eurofins offers a customised, 3-step **validation plan**, exactly matching the customers' requirements :

1) Consulting:

Analysis of the customers' products

Identification of key control points & target products

Elaboration of the most appropriate analytical strategy (DNA and/or protein-based tests)

2) Setup of a customer-specific validation plan:

Preparation of spiked validation samples, based on the customers' target products

Precise determination of product-specific detection limits (LoD) of the methods

Examples :

- Determination of the detection limit in terms of DNA target copies
- Determination of the detection limit in terms of mg species /kg matrix (« ppm »)
- Determination of the detection limit in terms of ppm allergen

3) Choice of the best performing and fully validated test method

Is the PCR a suitable tool for detection of allergens ?

Fields of application:

- **Quality control of raw materials**
 - cross contamination

- **HACCP**
 - i.e. monitoring of the efficacy of cleaning procedures, e.g. testing of swabs and rinsing water etc

- **Quality control of finished products**
 - PCR often allows for detection of allergic plant or animal species in processed foods

The Multi-Aller-Gene[©] - PCR Screening + Species ID

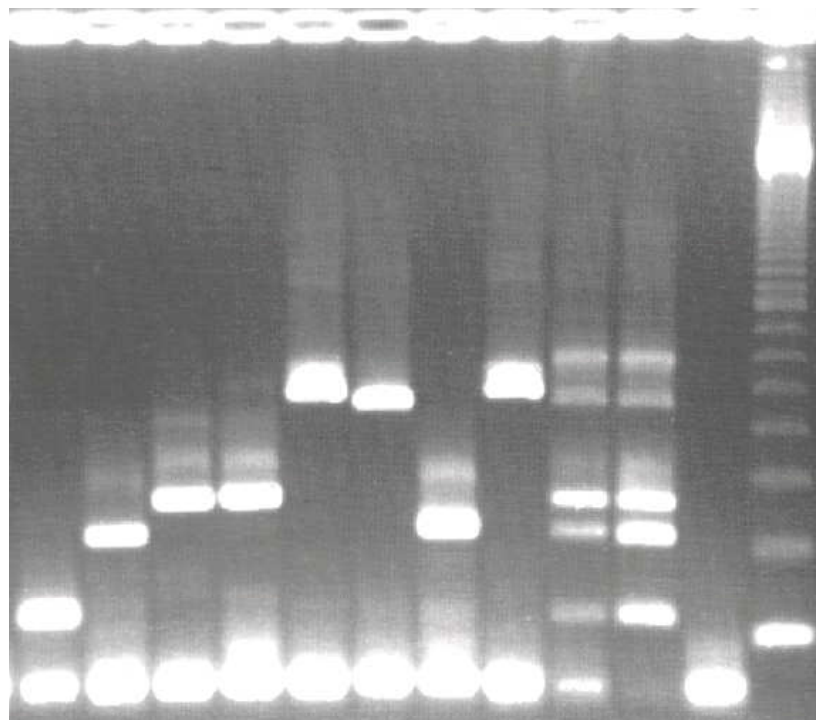
- DNA extraction from raw materials and finished products
 - Simultaneous DNA amplification of plant species causing food allergies:
 - wheat, barley, rye, oat, peanut, hazelnut, walnut & pecan nut, sesame, celery, soya, and mustard
 - 1st level of specificity:
 - Species-specific PCR primers
 - 2nd level of specificity:
 - Detection of species-specific PCR products by specific hybridisation probes
 - 3rd level of specificity:
 - Species identification by means of the melting curve technology
- ⇒ Test validated on raw materials & finished products
- ⇒ PCR offers highest specificity & sensitivity
- ⇒ Fast method → Short TAT

PCR Allergen Detection Assays – new developments

Example: Detection of gluten containing cereals by melting curve analysis

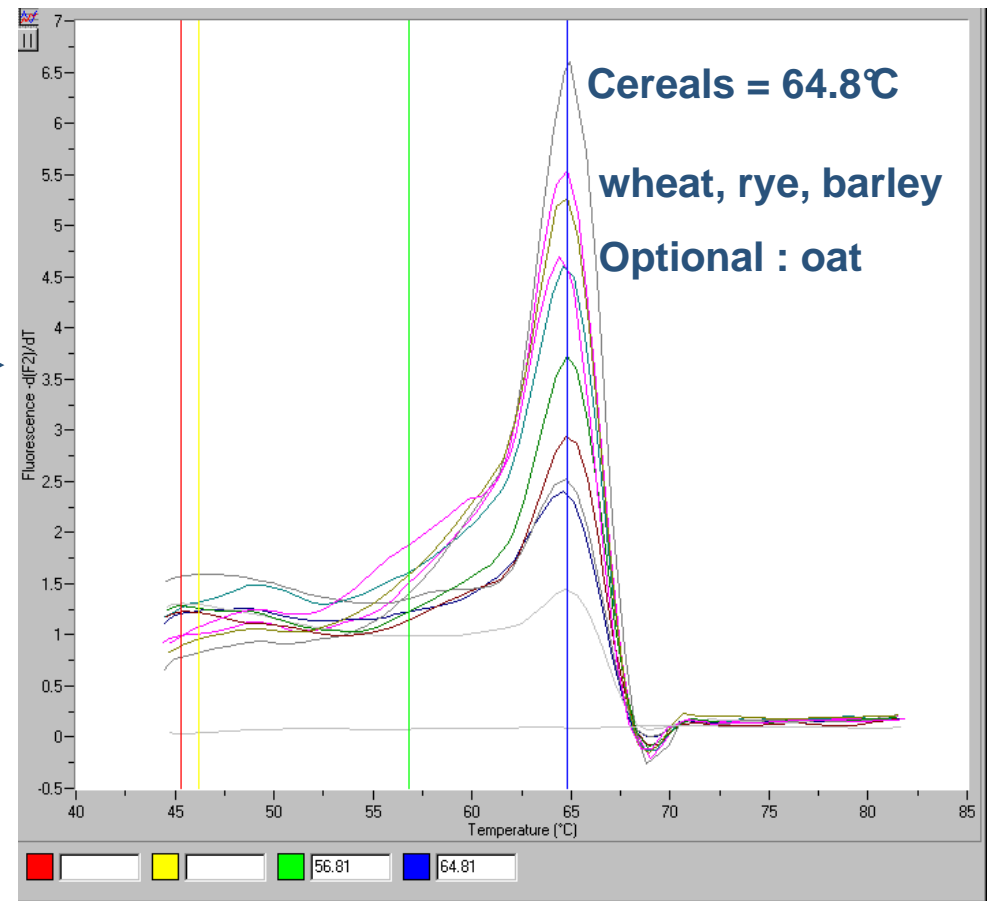
Identification of cereals within less than **15 min**

1 2 3 4 5 6 7 8 9 10 11 12



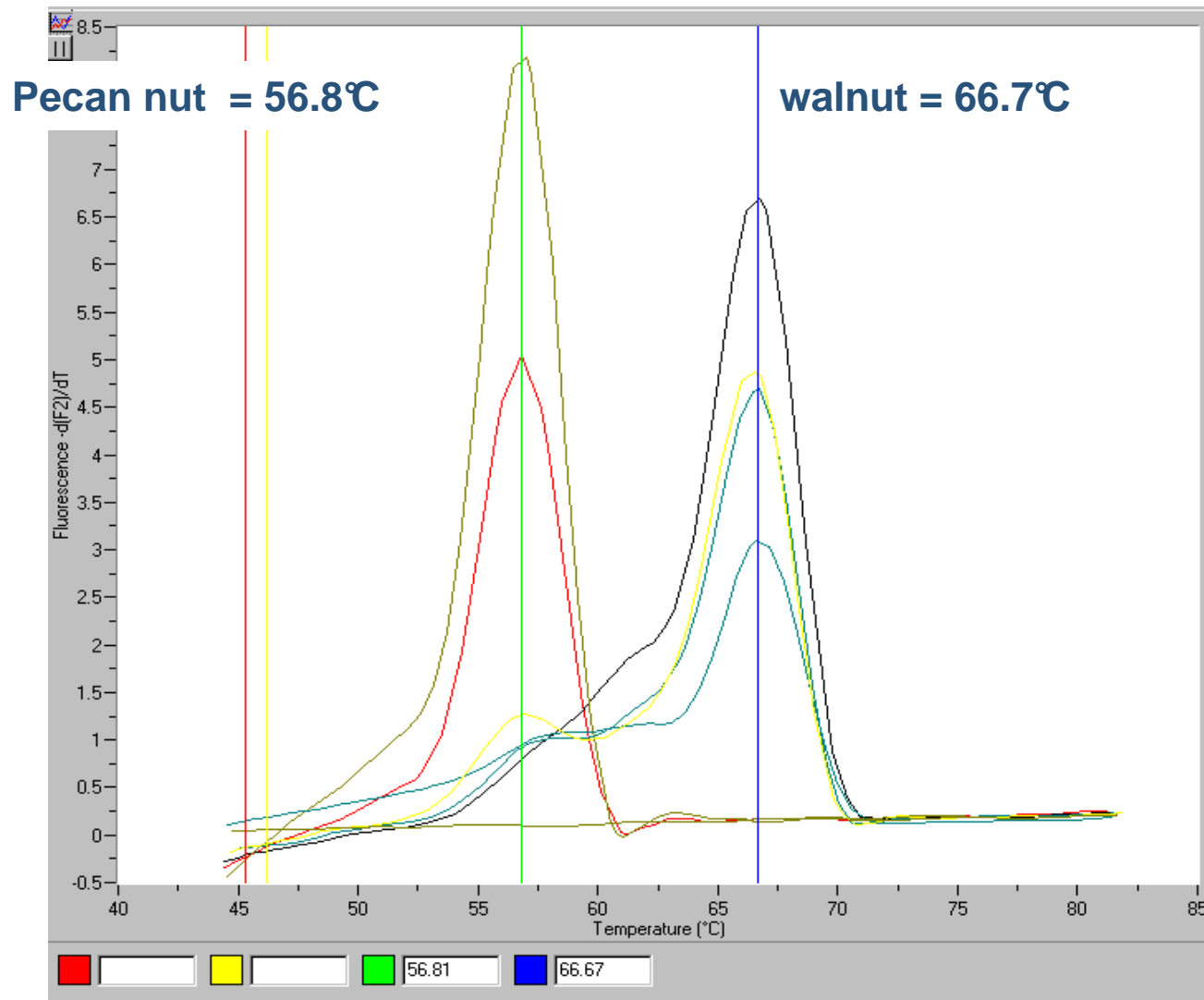
Simplex PCR

Multiplex PCR



PCR Allergen Detection Assays – new developments

Co-Detection of walnut and pecan nut



Pecan nut and walnut are co-amplified but can be easily distinguished by different melting curve temperatures

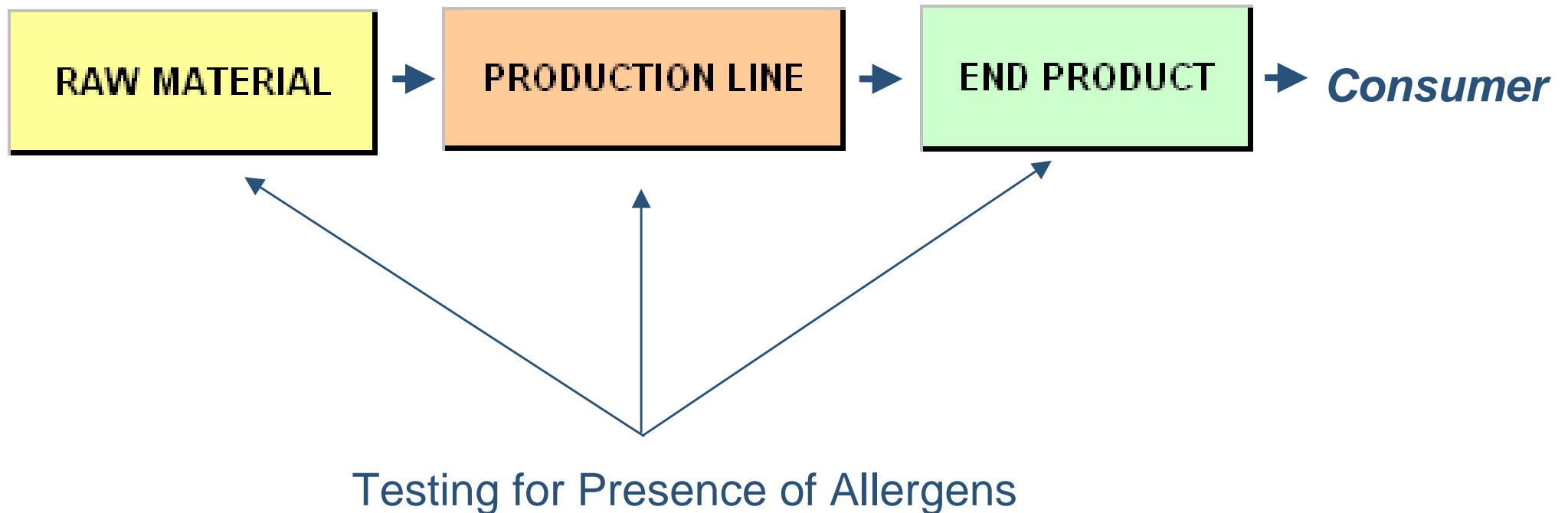
Numerous validation studies have been successfully carried out for customers from the food industry, including the following products :

Flours, chocolate products, desserts, dairy products, salty biscuits, sweets, rinsing water, ...

***How to setup a HACCP
concept for allergens,
on the basis of validated
test methods ?***

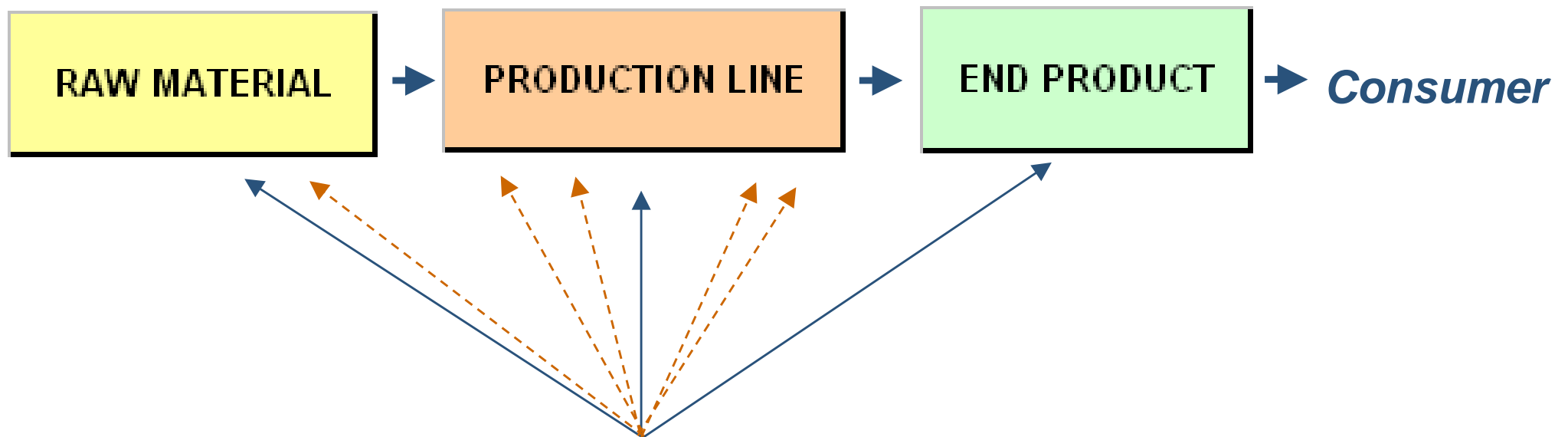
How to set up a HACCP concept including allergen detection ?

Main Control Points (simplified)



How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT



- 1) How to identify the critical steps and key control points ?
- 2) How to identify the right products to be tested ?

How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part I Control of Raw Materials

RAW MATERIAL



Examples :

Gluten free cereals (e.g. corn)

Cocoa beans

Fruits & fruit preparations

Vegetables & vegetable preparations

RISK (examples)

Presence of wheat, barley, oat, rye ?

Presence of tree nuts ?

Presence of soybean or mustard ?

Recommended test :

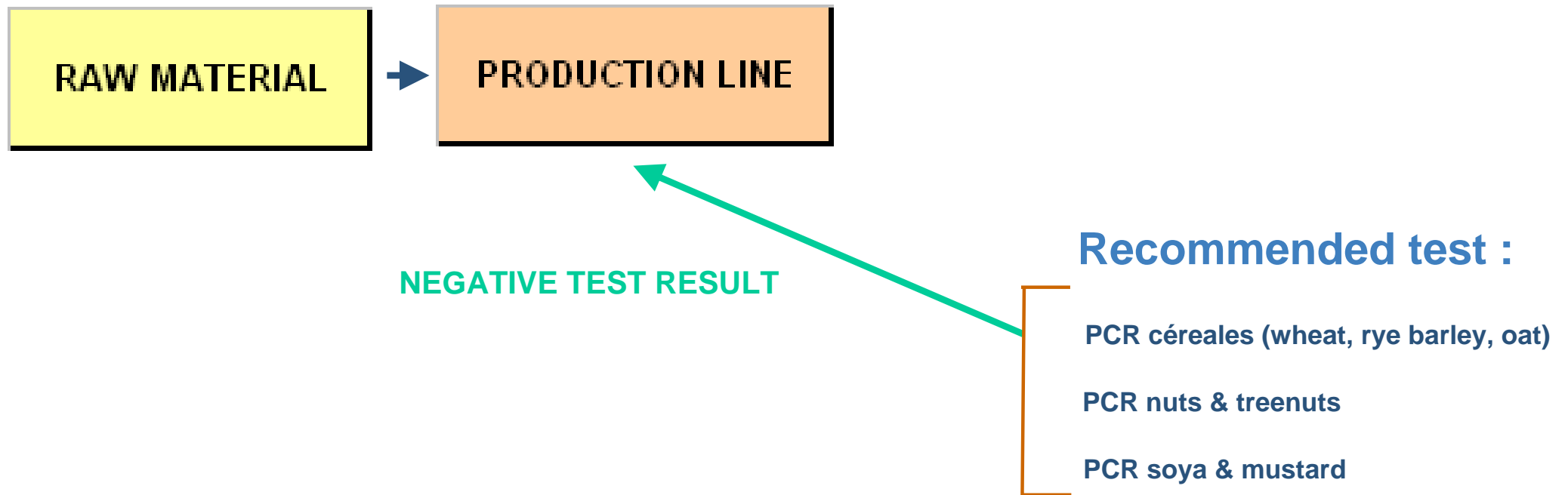
PCR céréales (wheat, rye, barley, oat)

PCR nuts & tree nuts

PCR soya & mustard

How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part I Control of Raw Materials



How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part II Control of the production line

PRODUCTION LINE



Examples :

Production of instant meals

Production of chocolate

Production of baby food with fruits or vegetables

RISK:

Cross contamination WITHIN the production site,
(e.g. traces of allergens still present after cleaning
of the production line)

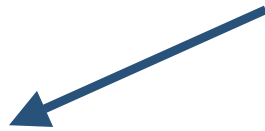
**Example : production of treenut-free chocolate
after production of chocolate with hazelnuts.**

How to verify absence of treenuts ?

How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part II Control of the production line

PRODUCTION LINE



Examples :

Production of instant meals
Production of chocolate
Production of baby food with fruits or vegetables

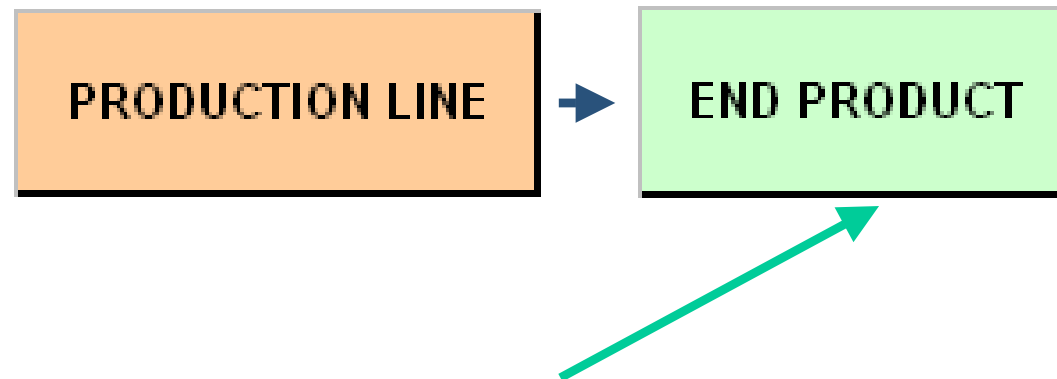
Analytical approach :

- testing of rinsing solutions
- testing of cleaning material (swabs)
- testing of the first products after cleaning of the production line

How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part II

Control of the production line



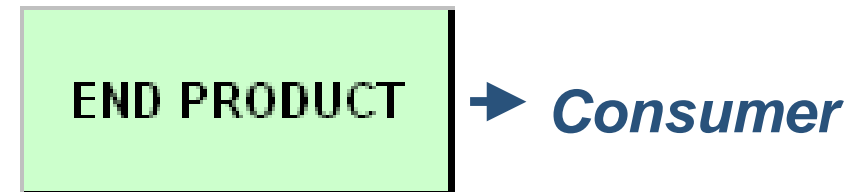
IF TEST RESULT NEGATIVE :

Cleaning procedure fit for purpose !

How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part III

Final control of the end product



Examples :

Instant meal
Hazelnut-free chocolate
Baby food with fruits or vegetables

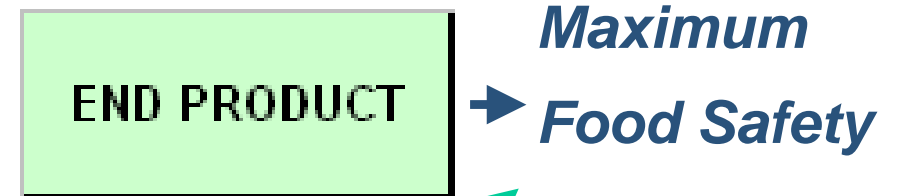
RISK:

Cross contamination after production,
e.g. during packaging, storage, or transport

How to set up a HACCP concept including allergen detection ?

RISK ASSESSMENT – Part III

Final control of the end product



IF RESULT NEGATIVE

Tests :

Only long term experience in allergen testing
can avoid false negative results and provide food safety

A Case Study



Team involved in the validation study :

- Stéphane Laruelle
- Alexandre Voirin

Eurofins Team involved in the validation study:

- Patricia Guzzardi (Analytical Service Manager)
- Brigitte Lefebvre (Lab technician)
- Andreas Pardigol

Validation study for the determination of the LOD



A validation study carried out by Eurofins and Yoplait

Aim of the study :

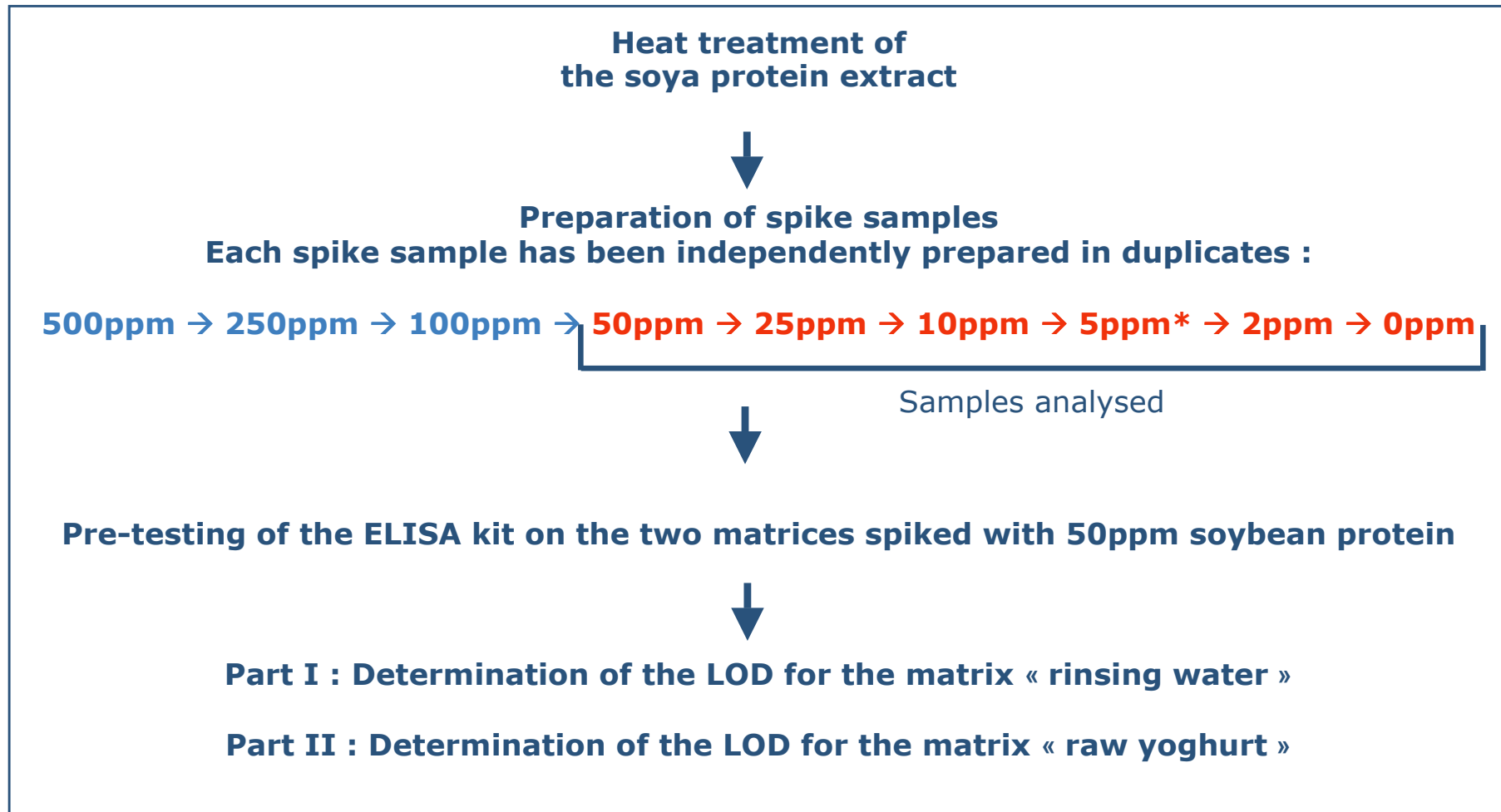
- Determination of matrix-dependent detection limits (LOD) of commercially available soybean proteins ELISA tests ⁽¹⁾ and the Eurofins “*Multi-Aller-Gene* © *Soya DNA Detection Assay*”
- The “true” detection limits of the methods have been validated on 2 key matrices systematically monitored for absence of soya traces during the production process :
“*Rinsing water*” and “*raw yoghurt*”

Approach :

- To determine matrix-specific detection limits of each test method, both matrices have been spiked with decreasing amounts of heat-treated soybean protein extracts
→ simulation of potential cross contamination before, during, or after the production process under most realistic conditions
- **Testing of spiked samples** → precise determination of the matrix-specific detection limits

⁽¹⁾ «ELISA Systems Soy Residue» (ESSRD-48) kit

Flow chart :



* additional matrix prepared in single replicates

Part I

Matrix : Rinsing water
Test : Soybean protein ELISA

Determination of the detection limit * in spiked rinsing water

* In the framework of the present study, the LOD has been defined as the lowest concentration of analyte resulting in repeatable detection of the analyte in the sample, i.e. measurement of a positive signal above the detection limit indicated by the kit manufacturer or determined during method validation.

Validation study for the determination of the LOD

1st part : feasibility study

Pre-testing of the ELISA kit on samples spiked with 50ppm (mg/l) soya protein extract

Sample	Spike level: Soya protein content	Replicates	Repeat measurements	Dilution factor	soya protein (ppm)	Mean (ppm)	SD	RSDr (%)
Rinsing water	50ppm	Sample 1	a	10	43.99	37.2	5.1	13.8
			b	10	34.39			
			a	20	32.34			
			b	20	38.22			
		Sample 2	a	10	40.39	48.13	9.6	20.0
			b	10	39.96			
			a	20	59.62			
			b	20	52.56			

Conclusion :

- The data obtained on different dilutions show satisfactory results in terms of quantification accuracy and precision (RSDr) of the ELISA test system
- No significant matrix effects observed.

Validation study for the determination of the LOD

2nd part : validation study

determination of the detection limit of the soybean protein ELISA test on spiked rinsing water samples

Sample	Spike level: Soya protein content	Replicates	Repeat measurements	Dilution factor	soya protein (ppm)	Mean (ppm)	Mean (ppm)	SD	RSDr (%)
Rinsing water	0 ppm	Sample 1	a	10	no signal	no signal			
			b	10	no signal				
		Sample 2	a	10	no signal				
			b	10	no signal				
	2 ppm	Sample 1	a	10	no signal	no signal	1.6		
			b	10	no signal				
		Sample 2	a	10	2.79				
			b	10	0.41				
	10 ppm	Sample 1	a	10	11.81	9.24	12.7	4.5	35.3
			b	10	6.68				
		Sample 2	a	10	16.04				
			b	10	16.12				
50 ppm	Sample 1	a	10	48.37	46.35	46.5	2.3	4.9	
		b	10	44.33					
	Sample 2	a	10	48.6					
		b	10	44.86					

Conclusion :

- The detection limit of the ELISA test for rinsing water is in between 2ppm (+/- results) and 10ppm (4/4 positive results)
- For a more precise determination of the LOD, the following tests have been carried out :
 - repeatability study on the sample spiked with 2ppm soya protein
 - re-testing of samples containing an intermediate concentration (→ preparation of a sample containing 5ppm soya protein)

Validation study for the determination of the LOD

Validation study :

Repeatability study on a sample spiked with 2ppm soya protein

Sample	Spike level: Soya protein content	Replicates	Repeat measurements	Dilution factor	soya protein (ppm)
Rinsing water	2 ppm	Sample 2	a	10	2.07
			b	10	no signal
			c	10	1.79
			d	10	no signal
			e	10	2.01
			f	10	no signal

Conclusion :

Confirmation of the first test results : signal detection at the 2ppm spike level is not repeatable. The “true (repeatable)” detection limit is > 2ppm

Validation study for the determination of the LOD

Validation study :

testing of samples containing an intermediate concentration (5ppm)

Sample	Spike level: Soya protein content	Repeat measurements	Dilution factor	soya protein (ppm)	Mean (ppm)
Rinsing water	5 ppm	a	10	4.43	4.5
		b	10	4.51	
	10 ppm	a	10	9.4	9.6
		b	10	9.81	

Conclusion :

The limit of detection is 5ppm

For the matrix “rinsing water”, a negative ELISA results indicates that the sample contains less than 5ppm of soya proteins.

Part II

Matrix : Raw yoghurt

Test : *Multi-Aller-Gene* © *Soya DNA Detection Assay*

Determination of the detection limit in spiked raw yoghurt

* In the framework of the present study, the LOD has been defined as the lowest concentration of analyte resulting in repeatable detection of the analyte in the sample, i.e. measurement of a positive signal above the detection limit indicated by the kit manufacturer or determined during method validation.

Validation study for the determination of the LOD

1st part : feasibility test

Pre-testing of the ELISA kit on samples spiked with 50ppm (mg/l) soya protein extract

Sample	Spike level: Soya protein content	Replicates	Repeat measurements	Dilution factor	soya protein (ppm)	Mean (ppm)	SD	RSDr (%)
Raw yoghurt	50ppm	Sample 1	a	11	19.3	9.8	9.5	97.5
			b	11	16.53			
			a	22	3.34			
			b	22	0			
		Sample 2	a	11	16.41	9.55	9.4	98.7
			b	11	18.82			
			a	22	0.15			
			b	22	2.82			

Conclusion :

- The data obtained demonstrate insufficient performance in terms of quantification accuracy and precision (RSDr) of the ELISA test system
 - Inconsistent data obtained for different dilutions of the protein extracts (matrix effects ??)
- The standard protocol supplied by the kit manufacturer did not lead to satisfactory results for this matrix

2nd part : optimisation of the ELISA test conditions :

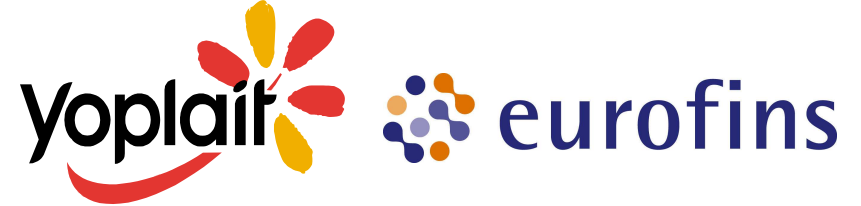
With regard to the results obtained, we conclude that this matrix negatively influences the ELISA test efficacy. To eliminate potential matrix effects, we have varied several testing parameters :

- **Extraction time & extraction volumes**
- **Dilution of protein extracts**
- **Incubation time of the immunological reaction**
- **Centrifugation parameters**

None of the tested parameters lead to satisfactory results.

Testing of the spiked raw yoghurt samples revealed a detection limit $> 10\text{ppm}$. Quantification of samples spiked with 25ppm and 50ppm lead to 2- to 3-fold underestimation of the expected results. We therefore concluded that the ELISA test exhibits a limited performance on this particular matrix.

Validation study for the determination of the LOD



The solution :

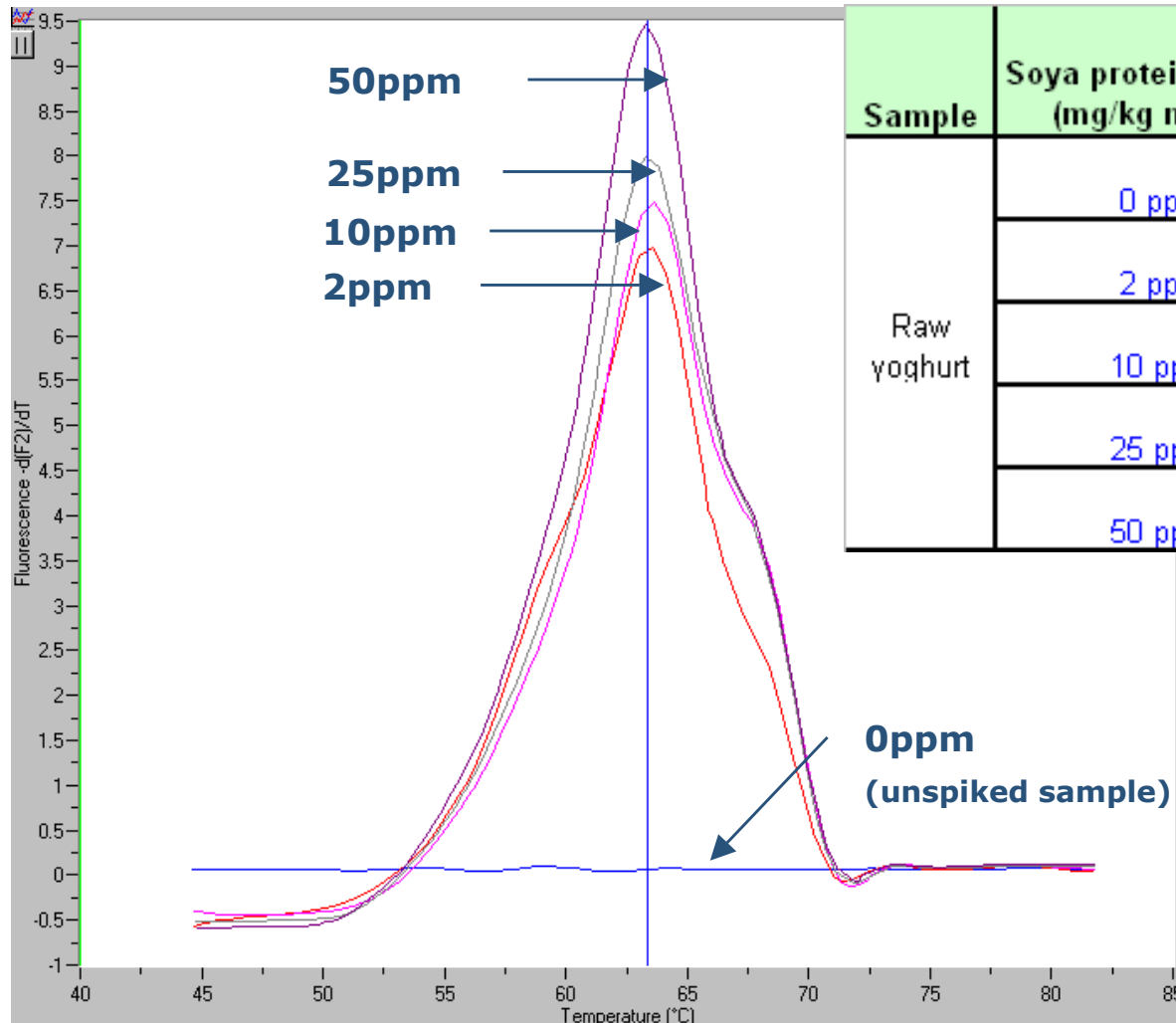
Due to the limited performance of the soybean protein ELISA test, we have changed the analytical tool :

The spike samples were re-analysed with the “*Multi-Aller-Gene* © *Soya DNA Detection Assay*” developed by Eurofins.

The test is based on a PCR system amplifying a soya-specific reference gene. Detection of soya PCR products is carried out by the melting curve technique.

Validation study for the determination of the LOD

PCR carried out on spiked raw yoghurt samples. Test results :



Sample	Soya protein content (mg/kg matrix)	Replicate	Detection of soybean DNA	Conclusion (presence / absence of soya proteins)
Raw yoghurt	0 ppm	a	no signal	negative
		b	no signal	
	2 ppm	a	+	positive
		b	+	
	10 ppm	a	+	positive
		b	+	
	25 ppm	a	+	positive
		b	+	
	50 ppm	a	+	positive
		b	+	

In contrast to the ELISA system, the PCR test exhibits excellent performance in spiked raw yoghurt samples. The PCR is able to detect soya DNA in samples spiked with 2ppm soya proteins

SUMMARY & CONCLUSIONS :

- Determination of matrix-specific method performance is a critical step in the setup of efficient HACCP concepts and analytical control plans to avoid false positive and, more importantly, false negative test results.
- The present validation study conducted on two key matrices of the production of dairy products has demonstrated significant differences in method performance, depending on the matrix
- For the two matrices *rinsing water* and *raw yoghurt*, the validation study allowed for the precise determination of matrix-specific performance parameters (LOD) of the most frequently applied allergen detection methods : ELISA & PCR

SUMMARY & CONCLUSIONS :

- Determination of matrix-specific method performance is a critical step in the setup of efficient HACCP concepts and analytical control plans to avoid false positive and, more importantly, false negative test results.
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MATRIX EFFECTS → Key message : don't trust – try out !