Food Safety and Mycotoxins: MycoRed Structure and Results

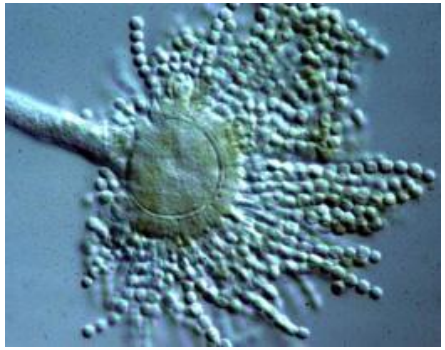
Michelangelo Pascale

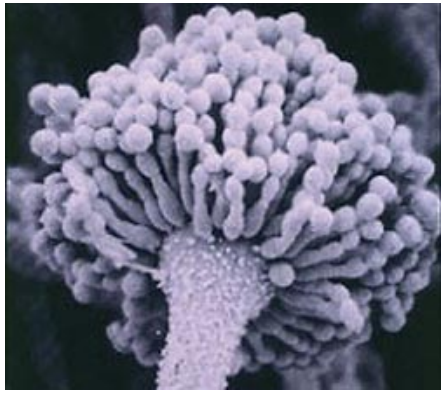
Institute of Sciences of Food Production (ISPA)
National Research Council of Italy (CNR), Bari, ITALY





WHAT ARE MYCOTOXINS?



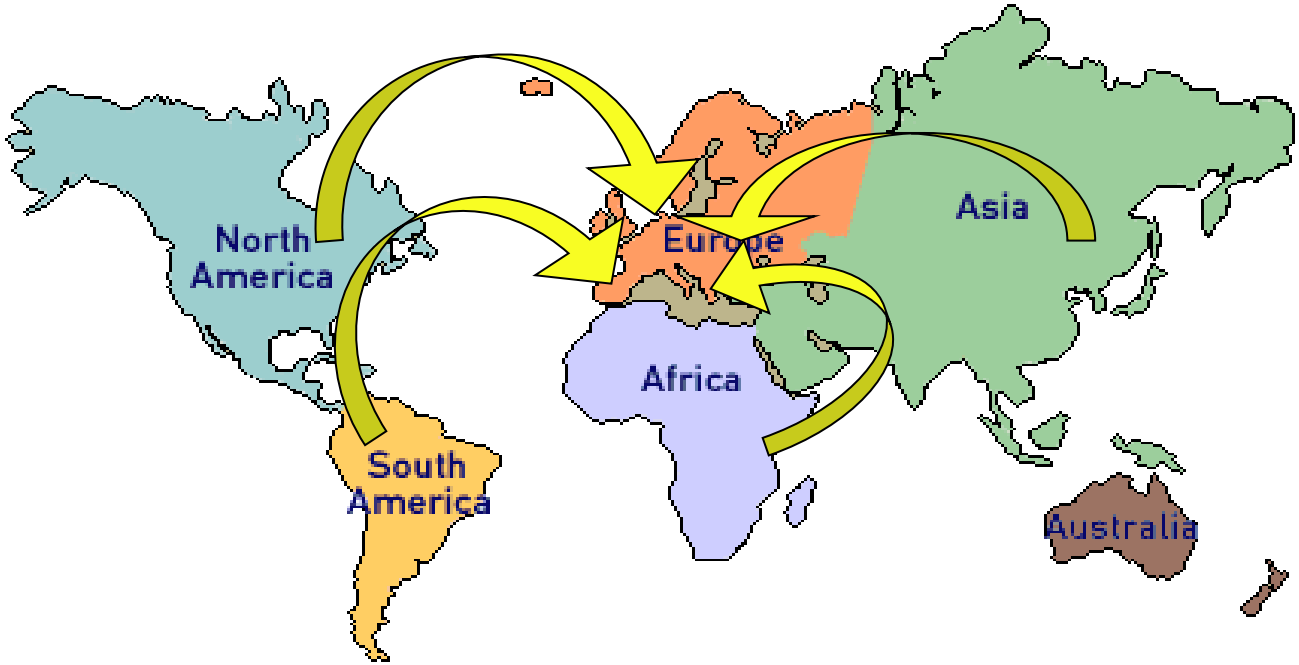


- Toxic secondary metabolites produced by moulds (mainly *Aspergillus*, *Penicillium, Fusarium*).
- Contamination occurs in the field and can increase during harvesting, drying and storage.
- More than 400 different mycotoxins are known about 10% of which occur in foods, being the main source for human exposure.
- Several fungal genera could produce the same mycotoxin / the same genus could produce different mycotoxins.

WHAT ARE MYCOTOXINS?

- Resistance to common thermal treatments.
- Cereals and cereal products, vegetables, dried fruit, spices, wine, beer, coffee, milk, cheeses, meat and eggs may be contaminated by mycotoxins.
- In the world, more than 25 % of foods are considered significantly contaminated by mycotoxins (source FAO).
- Contamination and severity of the problem vary from year to year and also from one geographic region to another.

Mycotoxin problems due to trade exchanges



Imported products with high risk of mycotoxin contamination:

- **maize** (fumonisins and aflatoxins) **from all continents**
- **cereals** (deoxynivalenol, ochratoxin A) **mostly from north and south America**
- **coffee** (ochratoxin A) **mostly South America & Africa**
- **pistachio nuts** (aflatoxins) **mostly from North Africa & Asia**
- **peanuts & other nuts** (aflatoxins) **mostly North, South America & Africa**
- **spices** (aflatoxins) **mostly from Asia & Africa**

... actions from EU to global level ....

Pre-harvest

FP5
RAFBCA
Risk Assessment of Fungal Biological Control Agents

FP5
FUCOMYR
Fusarium Resistant and Toxin Free Wheat

FP6
2E-BCAs in Crops
Enhancement of Biocontrol Agents

FP5
WINE-OCHRA -RISK
Ochratoxin risk Assessment and Management

FP5
RAMFIC
Fusarium Risk Assessment Models

Horizontal technologies

FP5
DETOX-FUNGI
Toxigenic fungi detection

FP5
MYCOTOX INCO-DEV
Mycotoxin Control in Latin American South cone

FP6
GOODFOOD
Quality monitoring in the food chain

FP7
CONffIDENCE
Inexpensive detection of Contaminants in the food chain

FP5
MYCOSENSE
Novel kit for rapid Mycotoxin Detection in food

Post-harvest

FP6
OTAPREV
Ochratoxin Prevention in Wheat

FP5
OCHRATOXIN A RISK ASSESSMENT

FP6
BIOCOP
Screening for contaminants in food

SAFE ORGANIC VEGETABLES
FP5
The Carrot Alternaria Toxin Model

FP5
CONTROL MYCOTOX FOOD
Mycotoxin prevention in cereals

Dissemination

FP6
MONIQA
Harmonizing methods in the food chain

FP5
COST 835
Network on mycotoxin and toxigenic fungi

FP6
MYCOGLOBE
Integration research on mycotoxins and toxigenic fungi

FP5
EMAN
European Mycotoxin Awareness Network

FP6
MYCONET
EU Network for identification emerging mycotoxin in wheat chain

FP7

MycoRed

H2020

SFS-13-2015
Biological contamination of crops and the food chain

Novel Integrated Strategies For Worldwide Mycotoxin Reduction in Food and Feed Chains (FP7-KBBE)

LARGE COLLABORATIVE PROJECT

Project Coordinator: Antonio F. Logrieco
(ISPA-CNR)

Budget:	**7189 M Euros**
Nº Partners:	**25**
Nº countries:	**17**
Personpower:	**1055**
Duration:	**48 months**
Period:	**2009-2013**

MycoRed Consortium

Project Coordinator: Antonio F. Logrieco

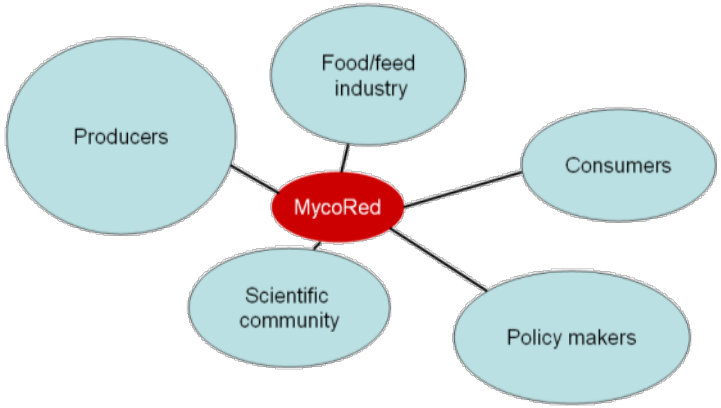
Research Centers	Universities	International Organizations	Companies
CNR (IT)	CRANFIELD (UK)	IITA (NG)	Romer (AT)
MRI (DE)	BOKU (AT)	CIMMYT (MX)	BF (AT)
PRI (NL)	DTU (DK)		MAT (IT)
CRC (HU)	UCSC (IT)		INC (ES)
INRA (FR)	UNRC (AR)		FEFANA (BE)
RIVM (NL)	UNIRoma1 (IT)		
TUBITAK MAM (TR)	DSA (IT)		
INBI (RU)	UdL (SP)		
NRC (EG)			
SAMRC (SA)			

The MycoRed project

The project addressed the problem of mycotoxins at global level, aiming to :

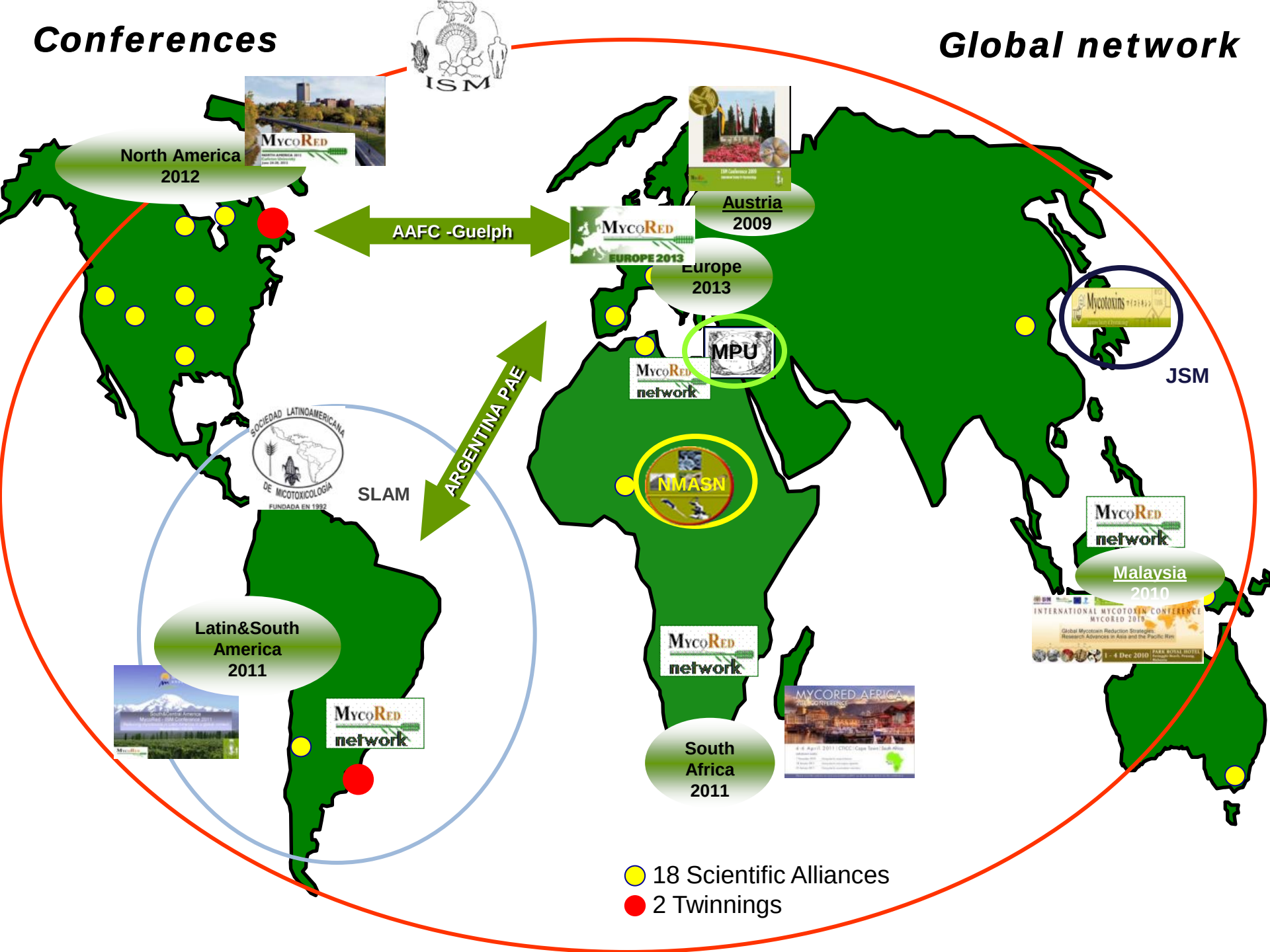
- develop novel solution-driven methodologies and handling procedures to reduce both pre- and post-harvest contamination in selected feed and food chains
- generate and disseminate information and education strategies to reduce mycotoxin risks worldwide
- create a wide interest among players and international network

High risk areas of Africa, Asia and Latin America have received major attention by cooperation with international agriculture and food organizations and by applying the results of all technical workpackages

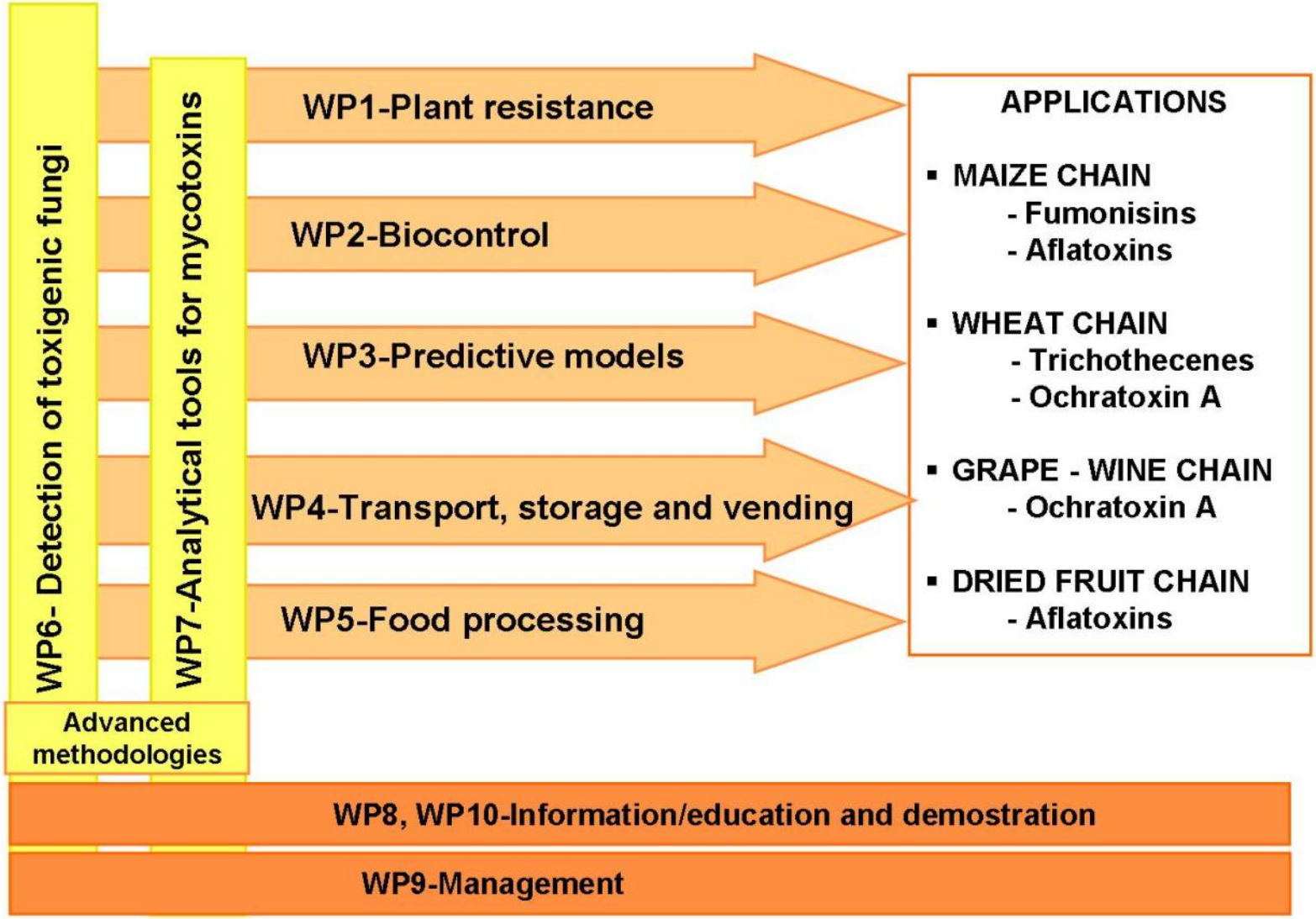


Conferences

Global network



Structure


WP6- Detection of toxigenic fungi
WP7-Analytical tools for mycotoxins
Advanced methodologies
WP1-Plant resistance
WP2-Biocontrol
WP3-Predictive models
WP4-Transport, storage and vending
WP5-Food processing
APPLICATIONS
MAIZE CHAIN
- Fumonisins
- Aflatoxins
WHEAT CHAIN
- Trichothecenes
- Ochratoxin A
GRAPE - WINE CHAIN
- Ochratoxin A
DRIED FRUIT CHAIN
- Aflatoxins
WP8, WP10-Information/education and demostration
WP9-Management

PRE-HARVEST (WP1)

Results FIELD

- Several <u>wheat</u> genotypes with low FHB incidence and DON content have been identified

- <u>In corn,</u> resistance to *F. graminearum*, *F. verticillioides* and *F. culmorum* have been detected in several hybrids

PRE-HARVEST (WP1)

Results FIELD

In **resistant maize** most of the genes provide a basal level of defence to the fungus <u>prior</u> to infection.

In **susceptible maize** most of the PR genes are induced <u>after</u> infection.

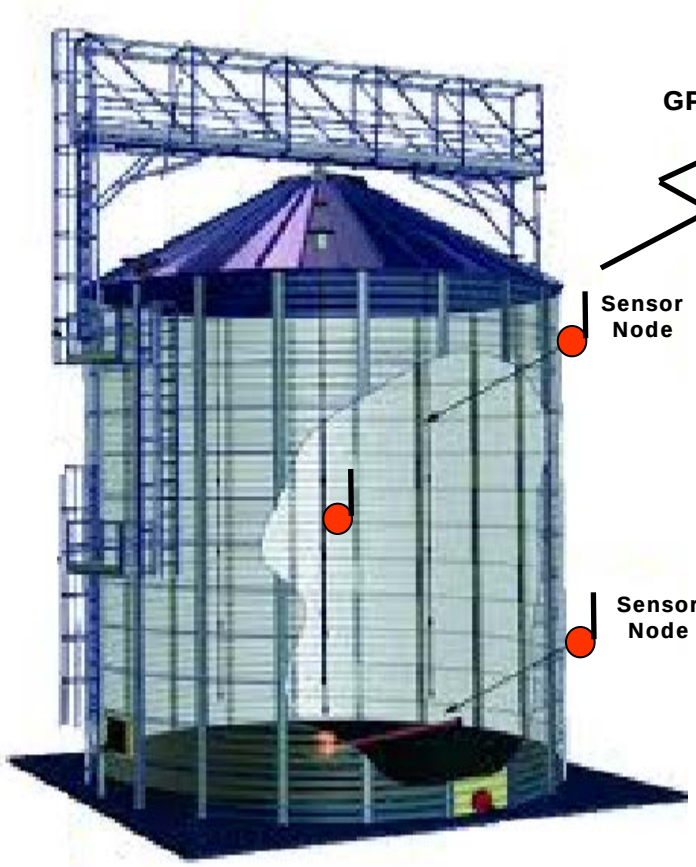
Infection with *Fusarium verticilloides*

Induction of about 80 early genes

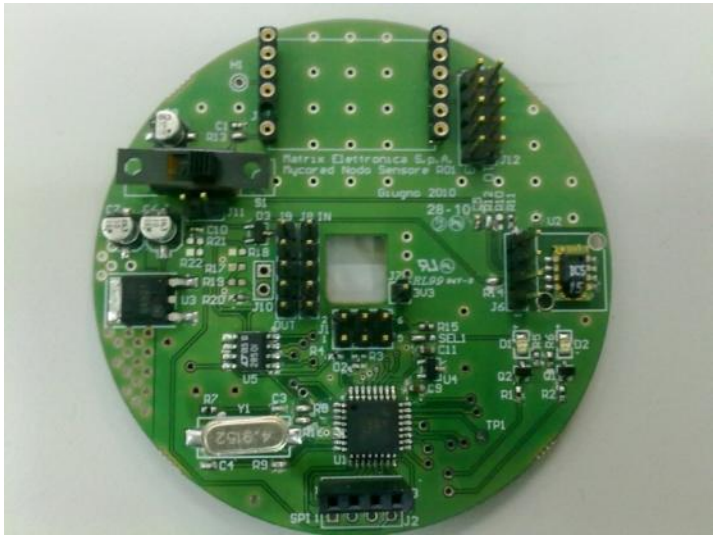
Induction of about 240 late genes

Results
STORAGE &
TRANSPORTATION

NOVEL POST-HARVEST AND STORAGE HANDLING PRACTICES (WP4)



Ambient intelligence system

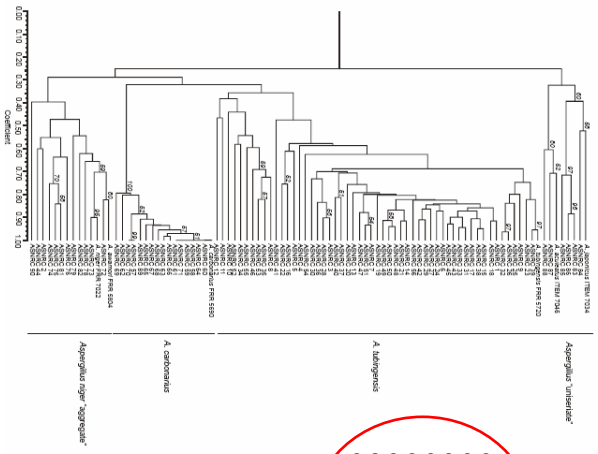


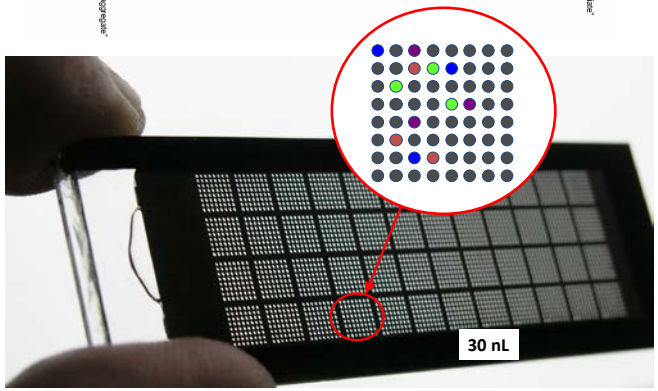
Sensor Node

A wireless sensor network device has been developed and used into pilot scale grain silos to monitor temperature, humidity and CO_2.

Results
RISK
ASSESSMENT

ADVANCED TECHNOLOGIES TO CONTROL TOXIGENIC FUNGI (WP6)





- **A large number of *Fusarium* species (+ 1000) were isolated from wheat kernels worldwide, showing intra and inter genetic variability and different toxigenic profiles.**

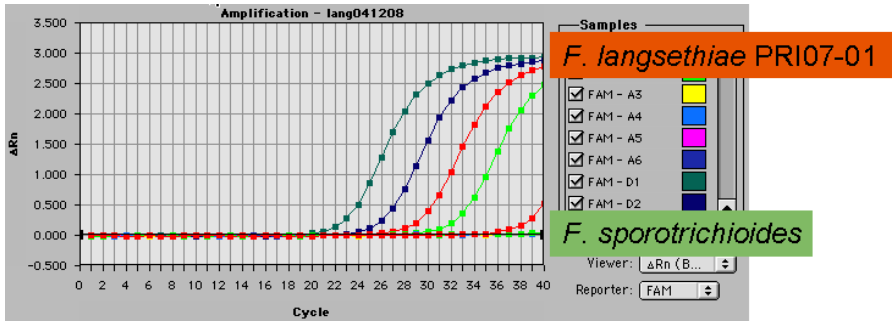
  All strains are deposited in ISPA-ITEM fungal Collection
  http://server.ispa.cnr.it/ITEM/Collection

- **Novel technologies (DNA arrays) to quantify *Aspergillus, Fusarium and Penicillium* from different commodities.**

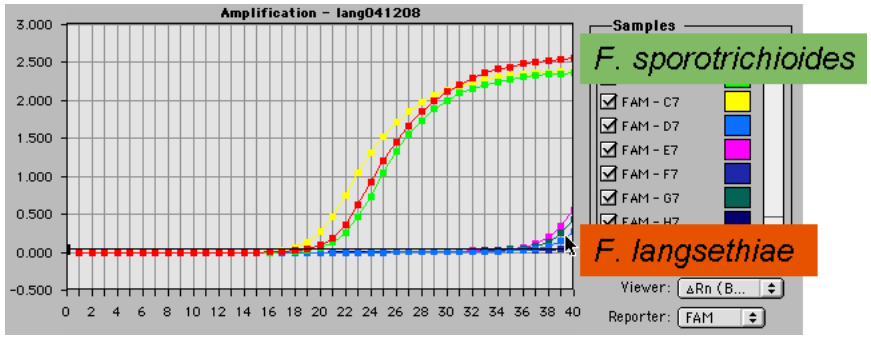
- **Novel approaches to control mycotoxigenic fungi by application of light at different wavelengths permitting a better control of fungal growth and toxin production.**

Results
RISK
ASSESSMENT

ADVANCED TECHNOLOGIES TO CONTROL TOXIGENIC FUNGI (WP6)



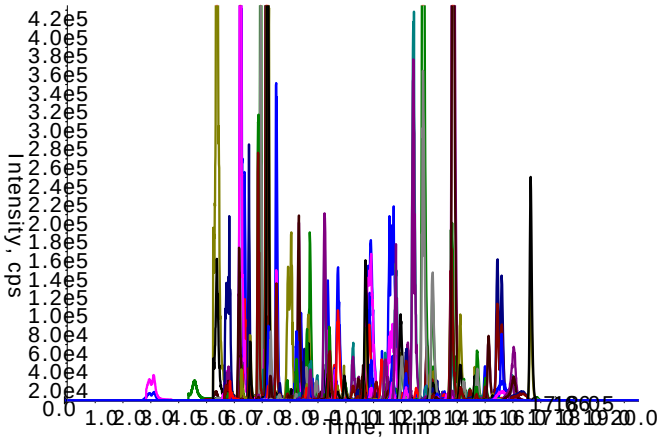
- Specific detection of ***F. langsethiae*** DNA with the *F. langsethiae* primer/probe combination.



- Specific detection of ***F. sporotrichioides*** using the corresponding primers and probe.

Results
RAPID
DETECTION

ADVANCED ANALYTICAL TOOLS FOR RAPID MYCOTOXIN DETECTION (WP7)



- Advanced quantitative analytical methods (LC-MS/MS) have been developed and validated for **rapid multi-mycotoxin** detection (250 metabolites) in all commodities addressed by MycoRed.
- A method for the determination of **masked species** of DON and ZEA has been developed.
- Two methods have been developed for the determination of **mycotoxin biomarkers** in urine.
- Rapid **test kits** (strip tests, FPIA) for the detection of DON, AFs and FUMs have been validated.

Rapid methods

Conventional method

EXTRACTION

↓

CLEAN-UP

↓

DETECTION
(GC, HPLC)

Time of analysis: 2 - 10 h

- Tedious sample preparation
  - Grinding of sample
  - Extraction
  - Clean-up
- Time consuming separation and detection
  - GC-ECD (MS)
  - HPLC-DAD (FD, MS)
- Expensive equipments and operation costs

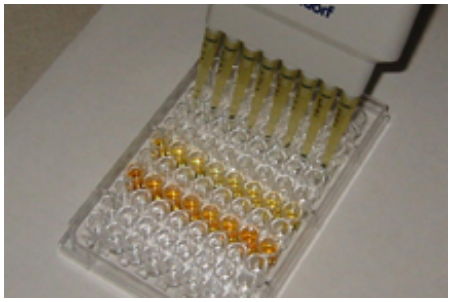
↓

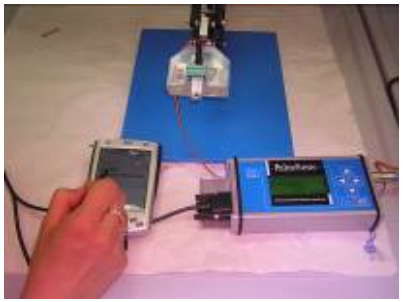
Growing demand for rapid and easy-to-perform methods

Time of analysis: 5 - 20 min

Rapid / Emerging methods for mycotoxin analysis

- **Immunoassays/immunosensors:**
  - Fast-Enzyme Linked Immunosorbent Assay (ELISA)
  - Flow-Through ImmunoAssays (FIA)
  - Lateral Flow Devices (LFD) or dipsticks
  - Fluorescence Polarization ImmunoAssays (FPIA)
  - Electrochemical Immunoassays (SPE)
  - Surface Plasmon Resonance-based sensors (SPR)
  - Multi Analyte Profiling technology (Luminex)
  - Biosensor arrays
- **Infrared spectroscopy** (NIR, MIR, FT-NIR)
- **Electronic nose** (MOS)
- **Methods using alternative receptor** (antibody fragments, peptides, molecularly imprinted polymers, aptamers)



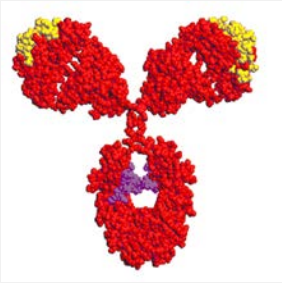



Fluorescence Polarization Immunoassay (FPIA)

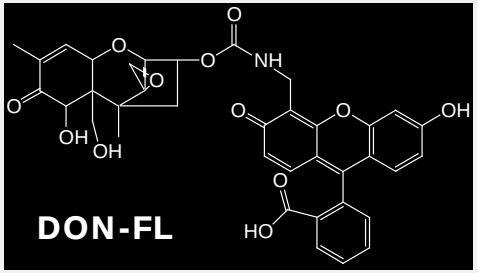
FPIA is a homogeneous competitive fluorescence immunoassay

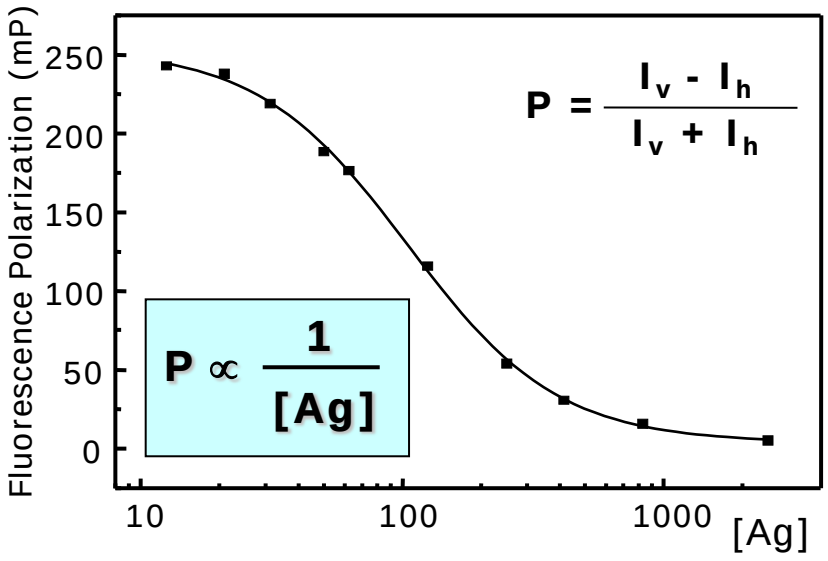
The assay is based on the competition in solution of free antigen (Ag) with a fluorescently tagged antigen (Ag-FL) for an Ag-specific monoclonal antibody.

Elements of an FPIA



- Specific antibody
- Tracer (fluorescent antigen)
- Instrument to measure fluorescence polarization





Assay parameters (i.e. incubation time, precision and sensitivity) depend upon the selection of the appropriate antibody-tracer combination.

FPIA – DON in wheat and derivative products

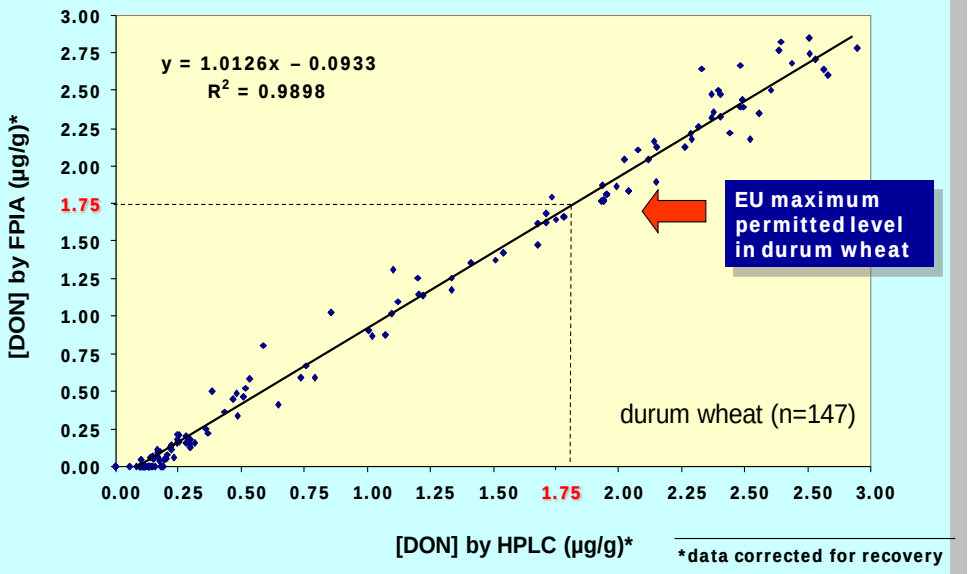
✓ **Applicability:** durum wheat, common wheat, semolina, pasta, bran and whole-wheat flour

✓ **Detection limit:** 0.08 µg/g

✓ **Accuracy:** 98-102%

✓ **Precision:** ≤ 4%

✓ **Time of analysis:** ≤ 10 min



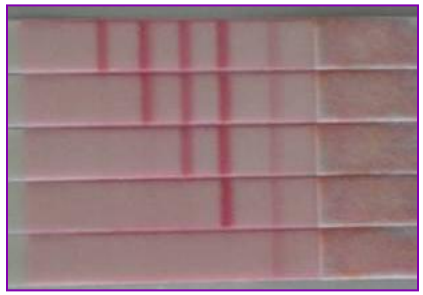
✓ **Linearity range:** 0.1 – 2 µg/g (for concentration >2 µg/g dilution of extract is required)

Lippolis V., Pascale M., Visconti A. (2006), J. Food Prot., 69, 2712-2719
Valenzano S., Lippolis V., Pascale M. et al., Food Analytical Methods, 2014, 7(4), 806-813

Multiplex dipstick - *Fusarium* toxins in cereals, cereal food, maize feed

wheat / oats / maize
maize feed
breakfast cereals

CTRL
FB1 + FB2
DON
T2 + HT2
ZEA



Negative sample
Positive ZEA
Positive ZEA / T2
Positive ZEA / T2 / DON
Positive ZEA / T2 / DON / FB1

Methanol / water extraction

Dilution with buffer

Incubation at 40°C, 10 min
Migration, 10 min

Dipstick reader
(*Readsensor*)

Total time of analysis: 30 min

Lattanzio et al., Analitica Chimica Acta 2012, 718:99-108

The commercial kit

unisensor

www.unisensor.be

MULTIPLEX: **6** mycotoxin analysed in **1** test

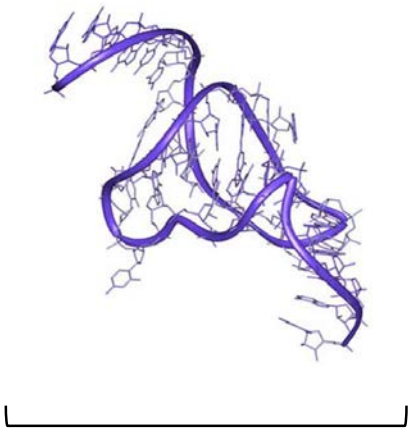
FAST: up to **8 samples** in **1 hour** (including sample preparation)

SENSITIVE: mycotoxin detection at levels close to EU regulatory limits

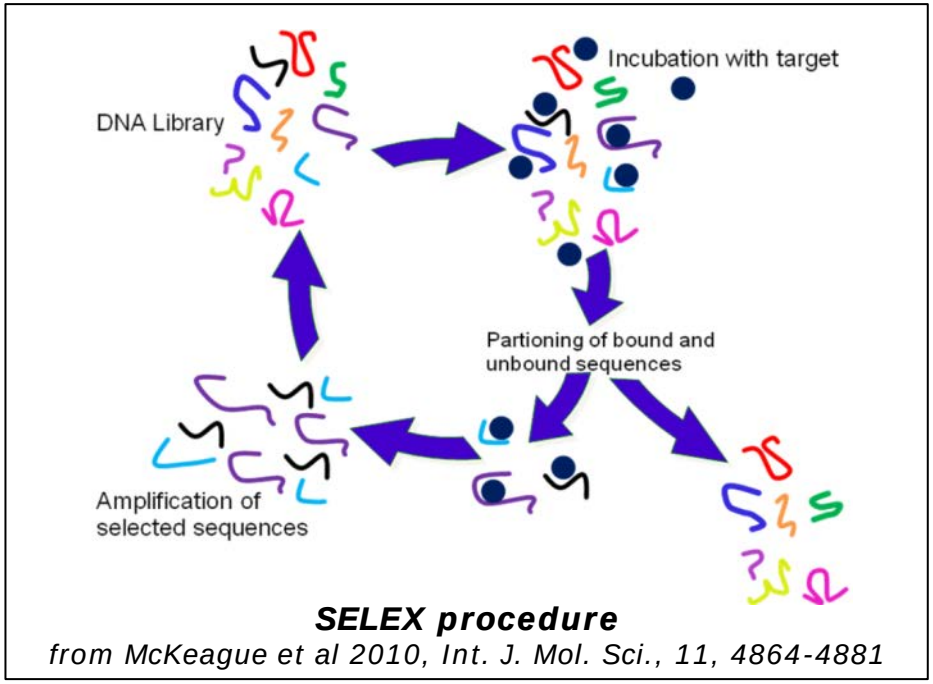
USER FRIENDLY: **5 min** for sample preparation, easily performed on site

Novel materials for mycotoxin analysis: Aptamers

Aptamer



~ 4 nm



SELEX procedure
from McKeague et al 2010, Int. J. Mol. Sci., 11, 4864-4881

- Aptamers are single-stranded oligonucleotides (DNA or RNA) that bind with high affinity and specificity to specific targets.
- Aptamers are produced by an *in vitro* selection process called SELEX (*Systematic Evolution of Ligands by Exponential)*.
- Aptamers, like antibodies, have potential in a broad range of applications including biosensors, affinity chromatography, lateral flow devices.
- Aptamers for OTA, FB_1, AFB_1 and ZEA have been produced.

DNA aptamer-SPE column clean-up / HPLC-FLD
OTA in wheat

Conjugation

OTA Aptamer

\+ Agarose based bead (diaminodipropylamine)

Incubation

Cross-linker (EDC)

Oligosorbent

DNA-aptamer SPE column

300 µL oligosorbent (24 nmol DNA)

Extraction *(60% methanol)*

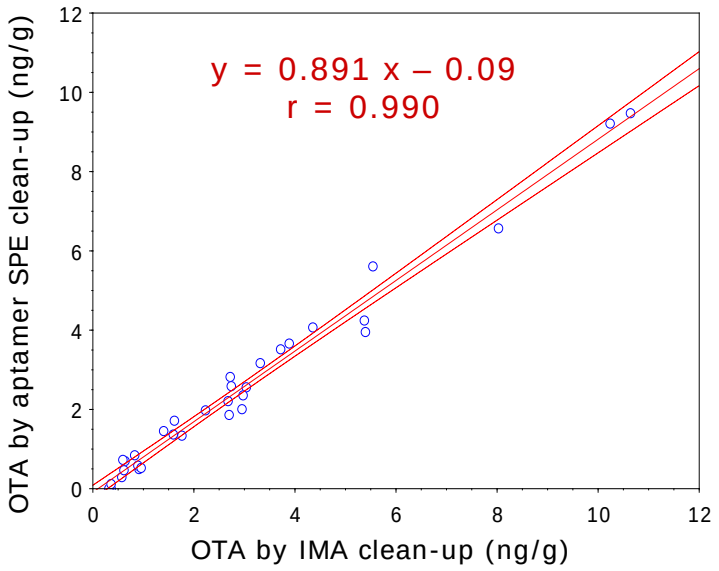
SPE purification

HPLC determination

Commercialization of columns (OTA-Sense®)

DNA aptamer-SPE column clean-up / HPLC-FLD
OTA in wheat

- ✓ LOQ: 0.08 µg/kg
- ✓ Accuracy: 74-88%
- ✓ Precision: ≤ 6%
- ✓ Linearity range: 0.08 – 50 µg/kg

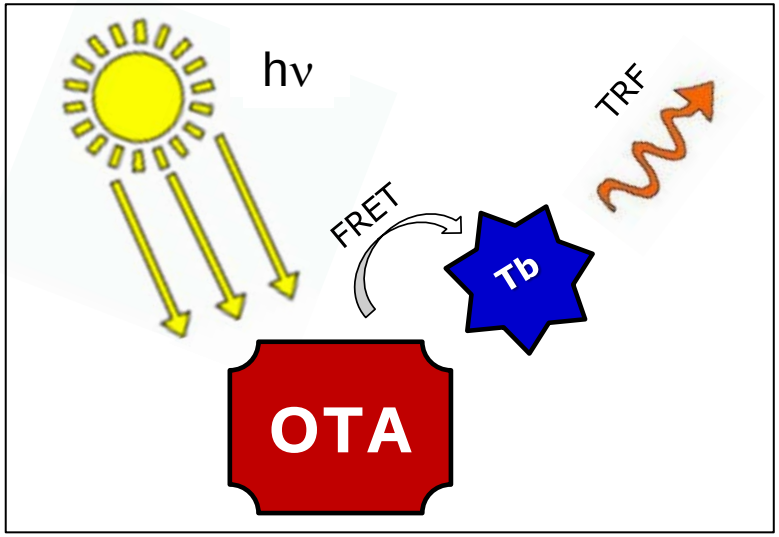


- ✓ Re-usability of aptamer –SPE columns: up to 5 times (recovery ≥97%)
- ✓ Validation: comparison with HPLC/IMA analysis (33 naturally contaminated wheat samples)

De Girolamo et al. 2011. *Food Chem.*, 127, 1378-1384.

DNA aptamer-SPE column clean-up / TR-FRET
OTA in wheat

Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET)



Extraction
(ACN:water)

OTA-Sense® purification

Dilution with terbium detection solution
Dilution with aptamer solution

TRF/FRET Determination
(λ_{ec} = 370 nm, λ_{em} = 540 nm)

DNA aptamer-SPE column clean-up / TR-FRET
OTA in wheat

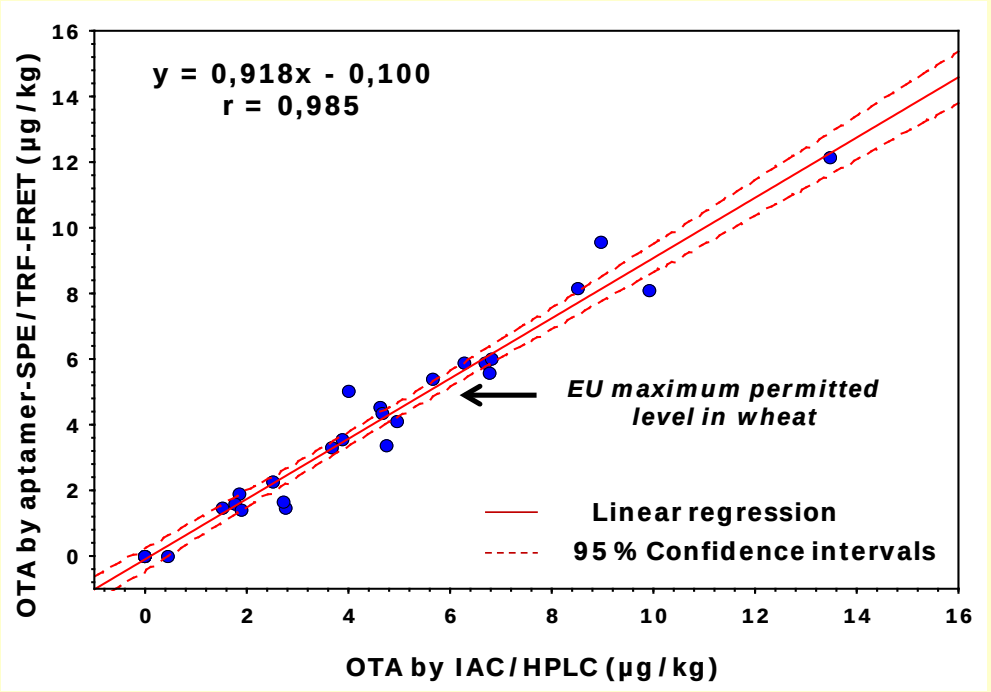
LOQ: 0.5 µg/kg

Accuracy: 72-81%

Precision: ≤ 6%

Linearity range: 1 - 25 µg/kg

Time of analysis: < 30 min



Validation: **comparison with HPLC / IMA analysis** (29 naturally contaminated wheat samples)

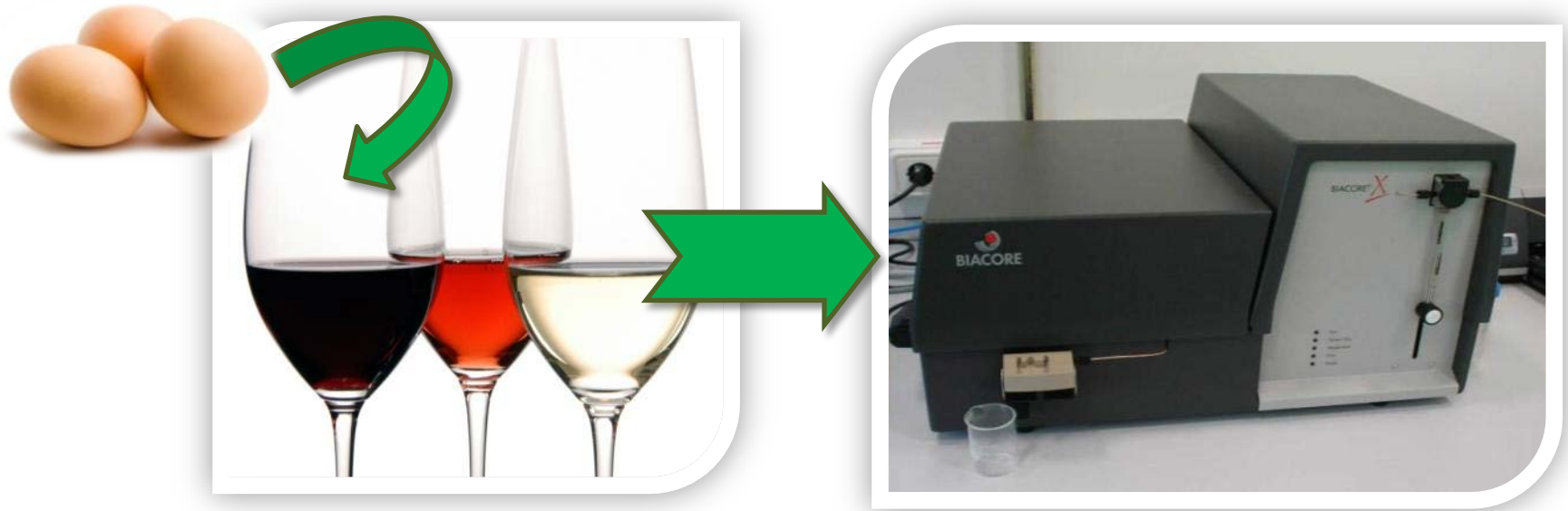
wheat test material (FAPAS T1976) found value **1.45 ± 0.03** µg/kg

(assigned value 2.10 µg/kg satisfactory range 1.18-3.03 µg/kg)

De Girolamo et al., 2012. Anal Bioanal Chem., 403, 2627

Rapid method based on antibody-recognition for food allergen detection

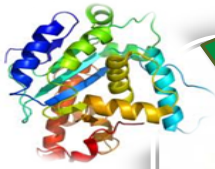
DEVELOPMENT OF A SURFACE PLASMON RESONANCE BASED BIOSENSOR FOR OVALBUMIN DETECTION IN WINES ALSO INTEGRATED WITH NANOTECHNOLOGIES



USE OF EGG POWDER TO PROMOTE WINE CLARIFICATION

R. Pilolli, A. Visconti, L. Monaci, 2014, J. Food Agric. Chem. (submitted)

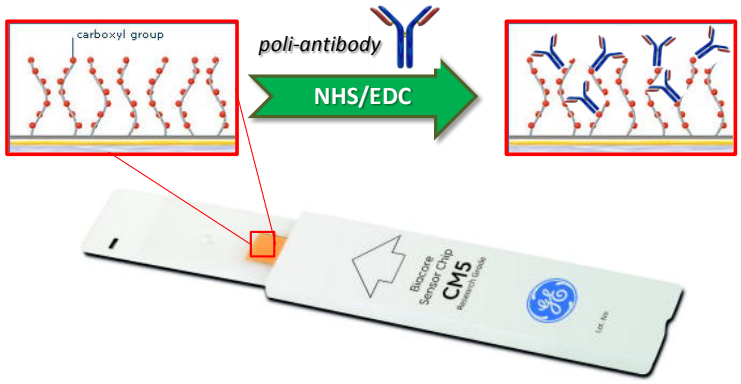
Surface Plasmon Resonance based biosensor for ovalbumin detection in wine



Ovalbumin

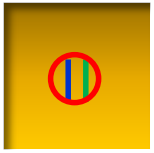
Fast pre-treatment

white wine spiked with ovalbumin

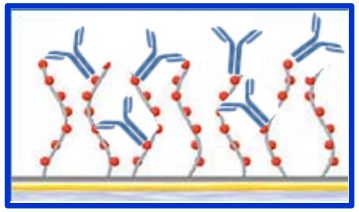


LIGAND: policlonal anti-OVA antibody
BINDING: Direct covalent binding was through an amine-coupling surface

2 FLOW CHANNELS

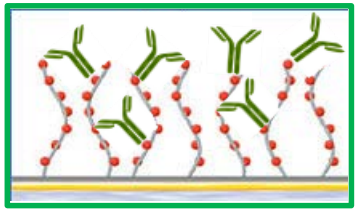


Sensing flow channel (FC_1)



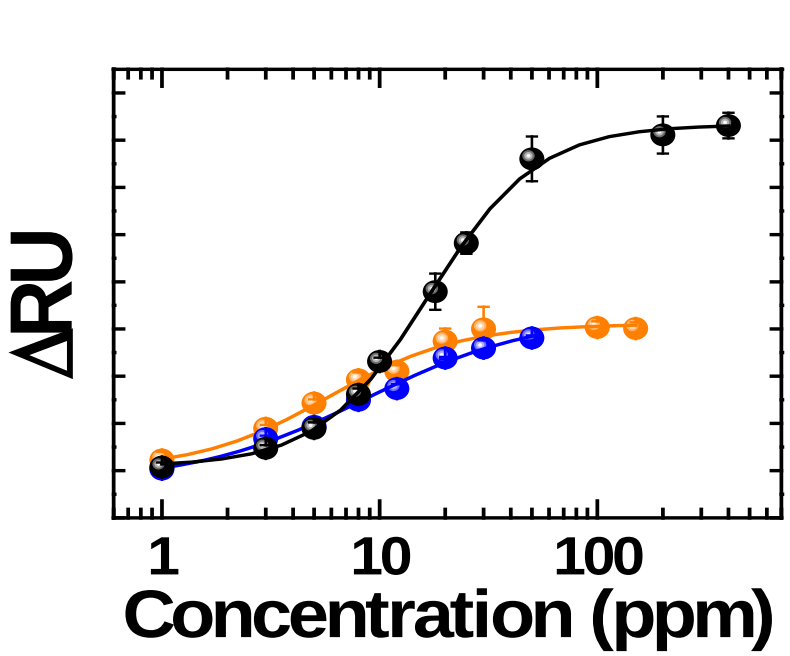
rabbit policlonal anti-OVA antibody

Reference flow channel (FC_2)



rabbit policlonal negative antibody

Assay sensitivity



- *Egg white standard solution*
- *Egg white spiked to Falanghina wine*
- *Egg white spiked to Pinot Grigio wine*

	EW standard solutions	White wine spiked with EW
LOD	0.7 µg/mL	1 µg/mL
LOQ	4 µg/mL	4 µg/mL

WORK IN PROGRESS .....

Mycotoxins

- ✓ Development of an **immunosensor based on electrochemical impedance spectroscopy** (EIS) for the determination of aflatoxin M1 in milk (in collaboration with CNR-NANO)
- ✓ Development of an **immunosensor based on EIS** for the determination of ochratoxin A (OTA) in meat products (in collaboration with CNR-NANO)

Aptamers

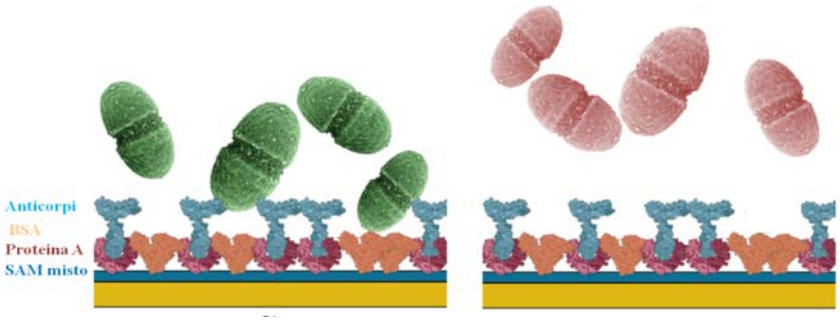
- ✓ Development of a **lateral flow devices** (LFD) for the determination of OTA in meat products.
- ✓ Selection of **aptamers for biogenic amines** (thyramine, histamine).

Allergens

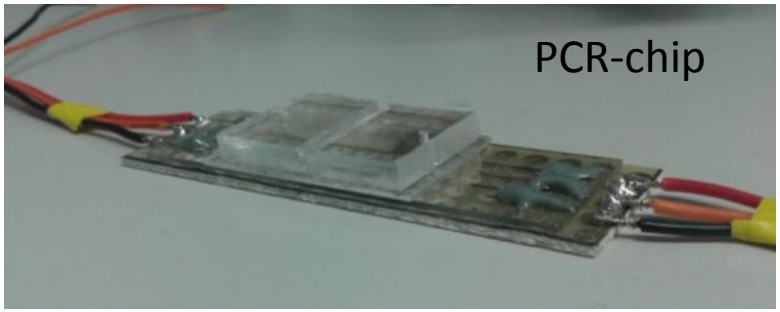
- ✓ Ovalbumin in wine: method transfer to a **miniaturized SPR portable device**.
- ✓ Coupling with **AuNPs to increase sensitivity** of the method.

WORK IN PROGRESS .....

Pathogens and microorganisms



- ✓ Development of an **immunosensor based on electrochemical impedance spectroscopy** (EIS) for the detection of pathogens (*Salmonella* spp., *Listeria monocytogenes)* in meat (in collaboration with CNR-NANO)



- ✓ Development of a **lab-on-a-chip** for the detection of pathogens (*Salmonella* spp., *Listeria monocytogenes, Campylobacte*r spp., *Staphylococcus aureus)* in meat (in collaboration with CNR-NANO)

- ✓ Development of a **lab-on-a-chip** for the detection of moulds and yeasts in dairy products (in collaboration with CNR-NANO)

Thanks for your attention

michelangelo.pascale@ispa.cnr.it