

Mycotoxins: Recent technical and analytical advances

Prof. Naresh Magan

*Applied Mycology Group, Cranfield Health, Cranfield
University, U.K.*

(n.magan@cranfield.ac.uk)

Content of talk

1. Drivers for mycotoxin research in the food chain
2. Utilisation of molecular approaches: understanding function for better control
3. Predictive modelling utilising ecological and environmental information
4. Development of analytical approaches for analysing individual and multiple mycotoxins
5. Conclusions and the future

1. What are the main drivers for research on mycotoxins in the food chain?

- Legislation - limits in Europe are to a large extent unified
- Risks to human and animal health
- Socio-economic aspects - Trade (WTO)
- QA of raw materials for processing (safety aspects)
- Consumer perceptions
- Surveillance impacts on cost to producers and ultimately consumers

Range of potency of carcinogens in test animals

<u>Compound</u>	<u>Dose</u>	<u>Relative potency</u>
Trichloroethylene	3	1
Carbon tetrachloride	0.02	150
Nitrosamines	0.005	6000
Sterigmatocystin	0.00003	100,000
Aflatoxin B1	0.000001	3,000,000

This is the reason why legislation has become important.

Pre-harvest

Field Preparation

Crop rotation

Ploughing

Soil fertilization

Crop development

Cultivar choice

Irrigation

Weed/pest/disease control

Post-harvest

On-farm storage

On-farm drying

HARVEST

Farm storage

Processing

Off-farm drying

Off-farm storage

Transport step

Used to identify CCPs



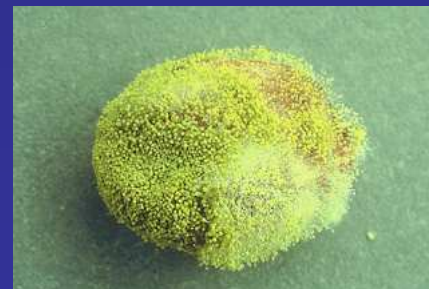
Utilisation of molecular approaches: understanding function for better control

In the last few years the whole genomes of specific mycotoxigenic fungi have become available:

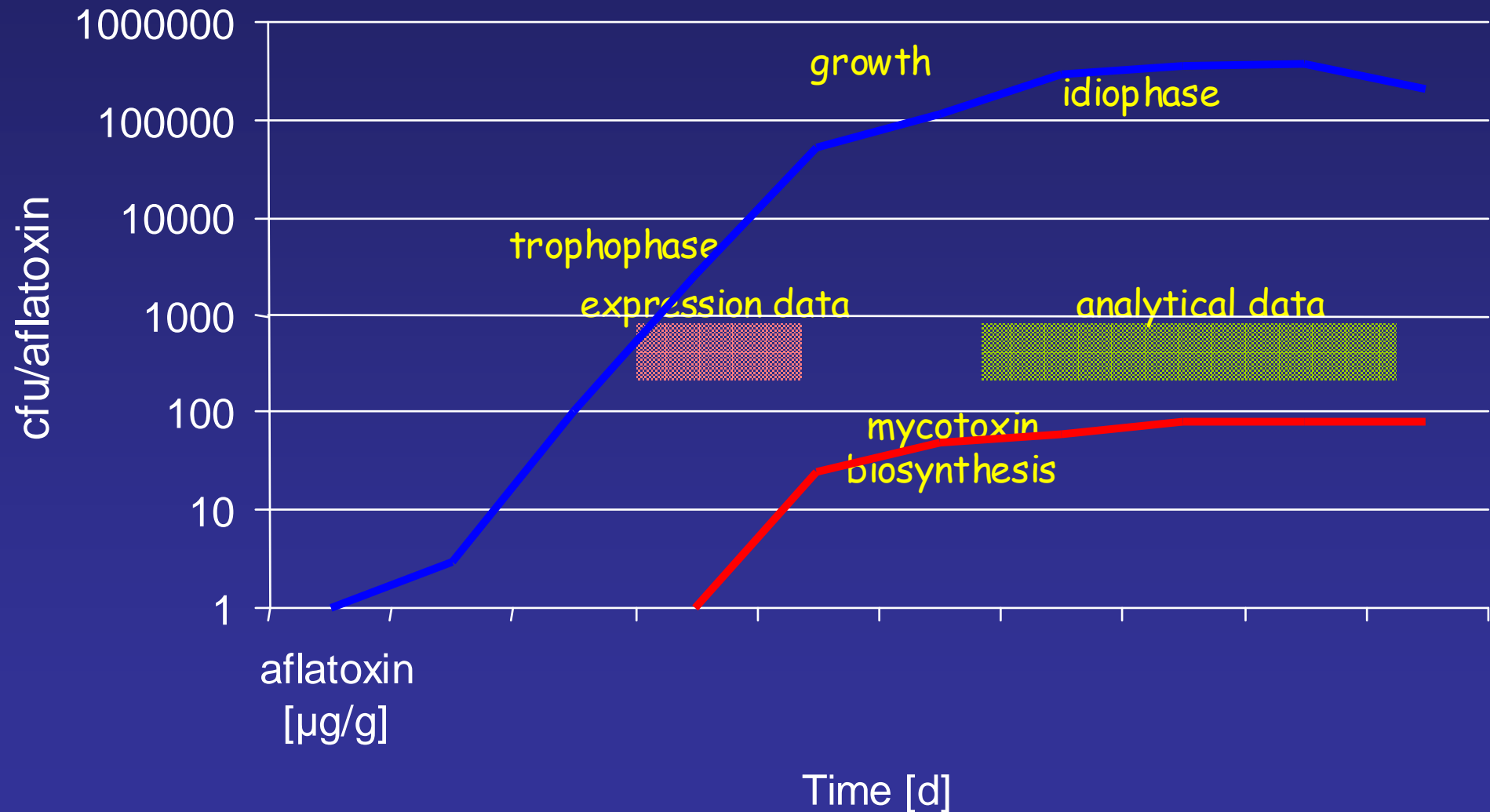
Fusarium graminearum (trichothecenes)

Fusarium verticillioides (fumonisins)

Aspergillus flavus (aflatoxins)

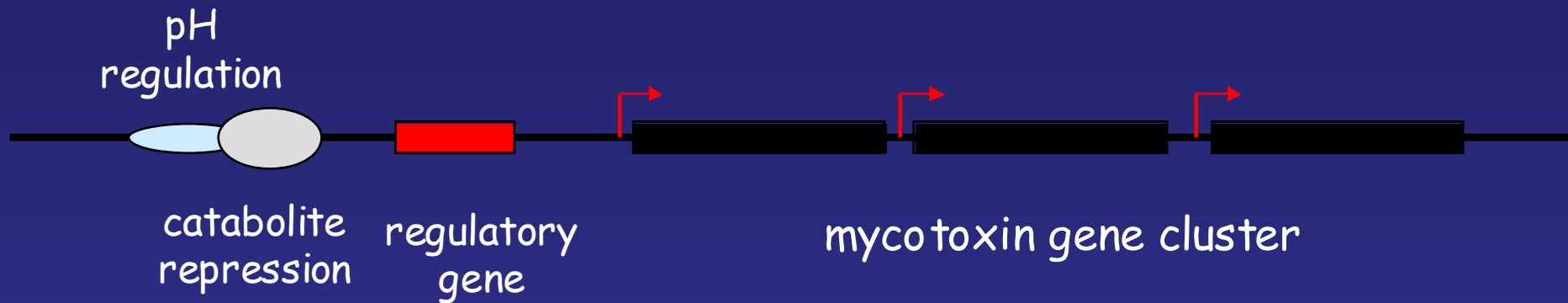


General correlation between growth and mycotoxin biosynthesis



— cfu/ml — aflatoxin [µg/g]

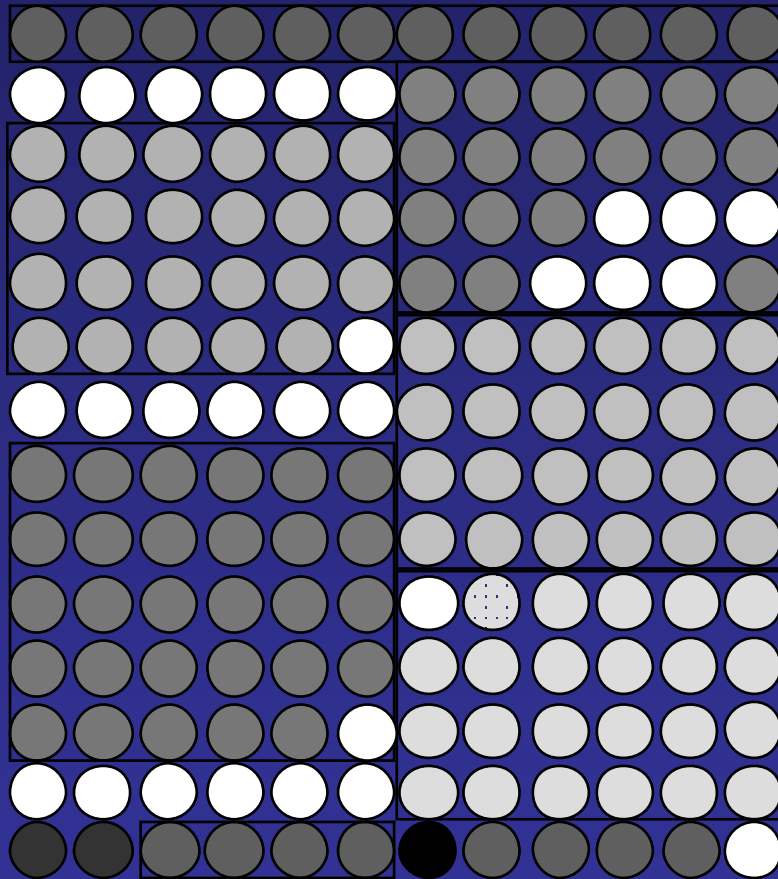
Influence on the activation of mycotoxin biosynthetic genes



- water activity (a_w)
- pH
- temperature
- nutritional substrate

Layout of the trichothecene sub-array

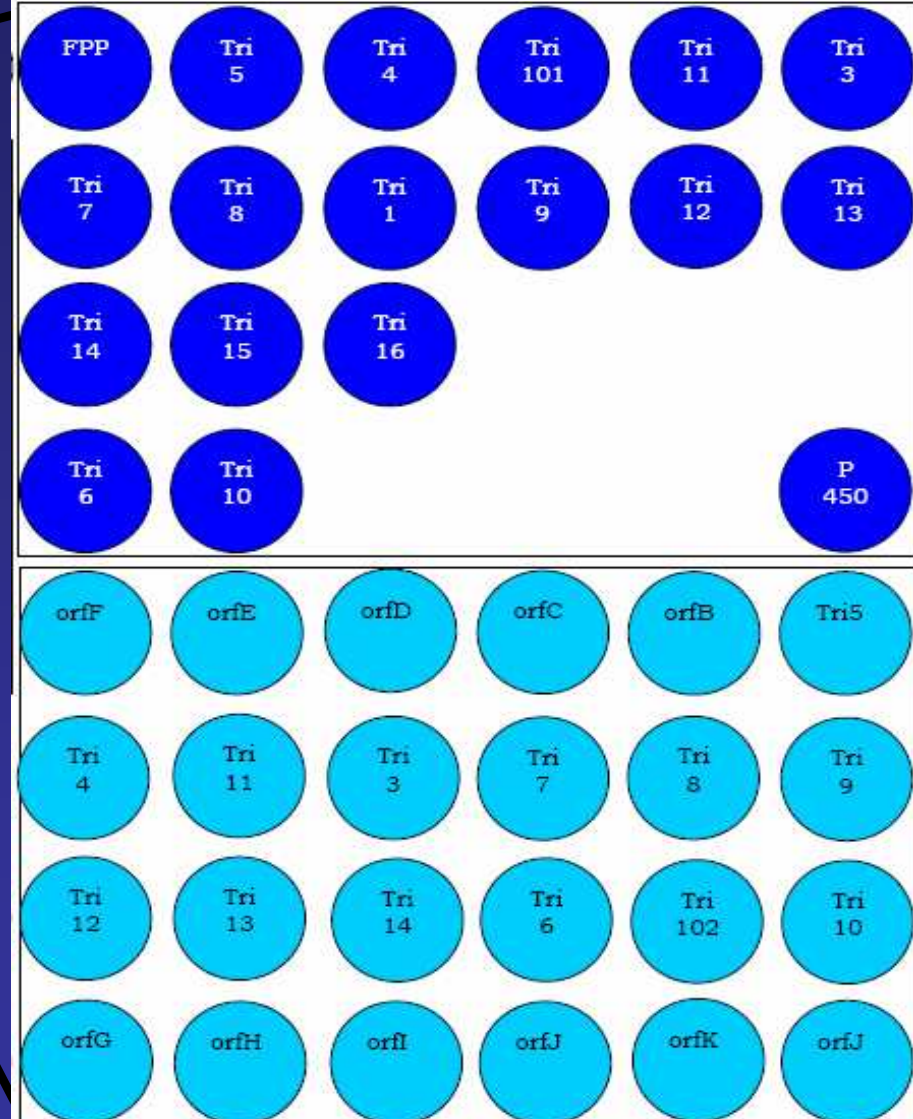
MycoChip



F. sporotrichioides

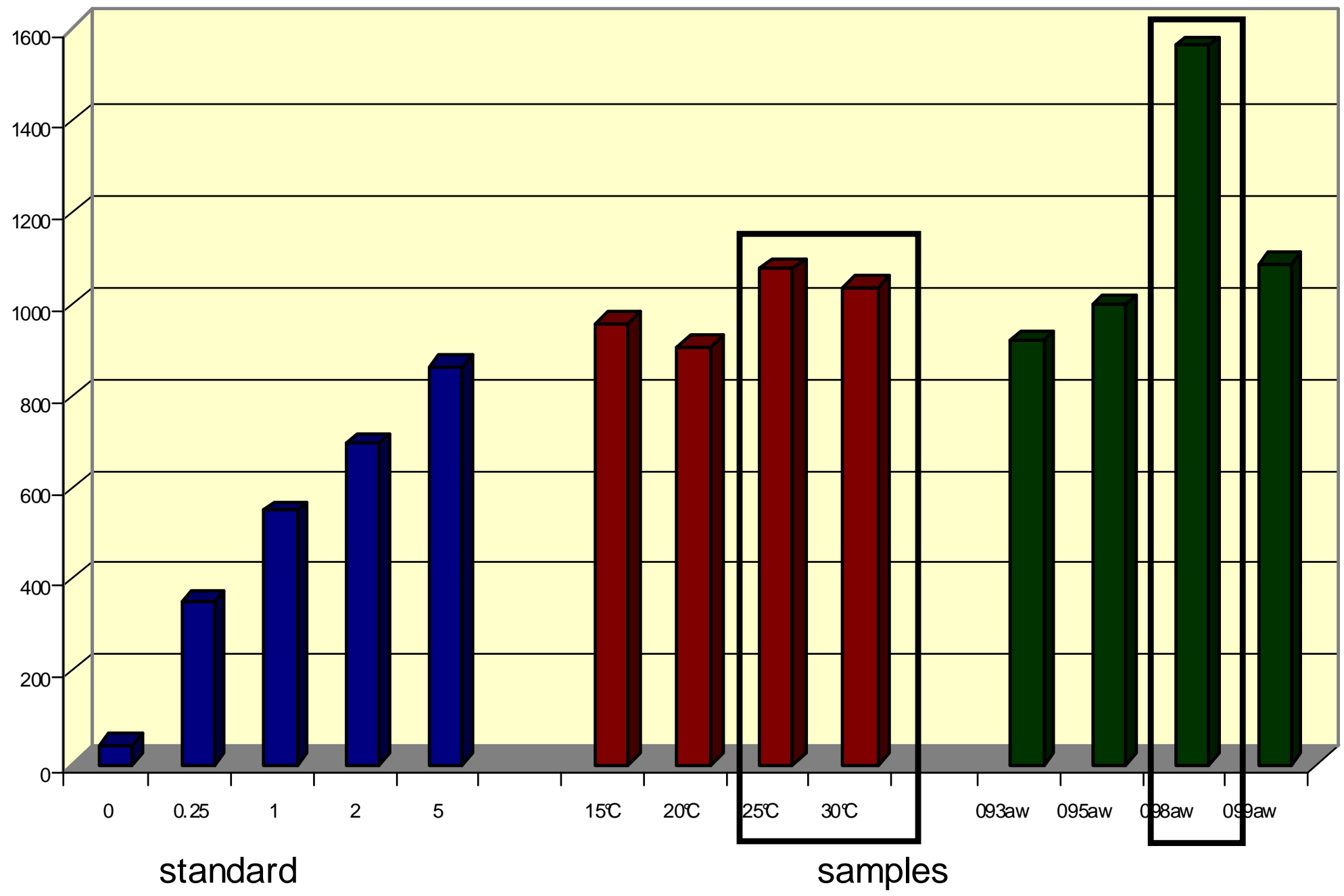
G. zeae

Trichothecene Subarray

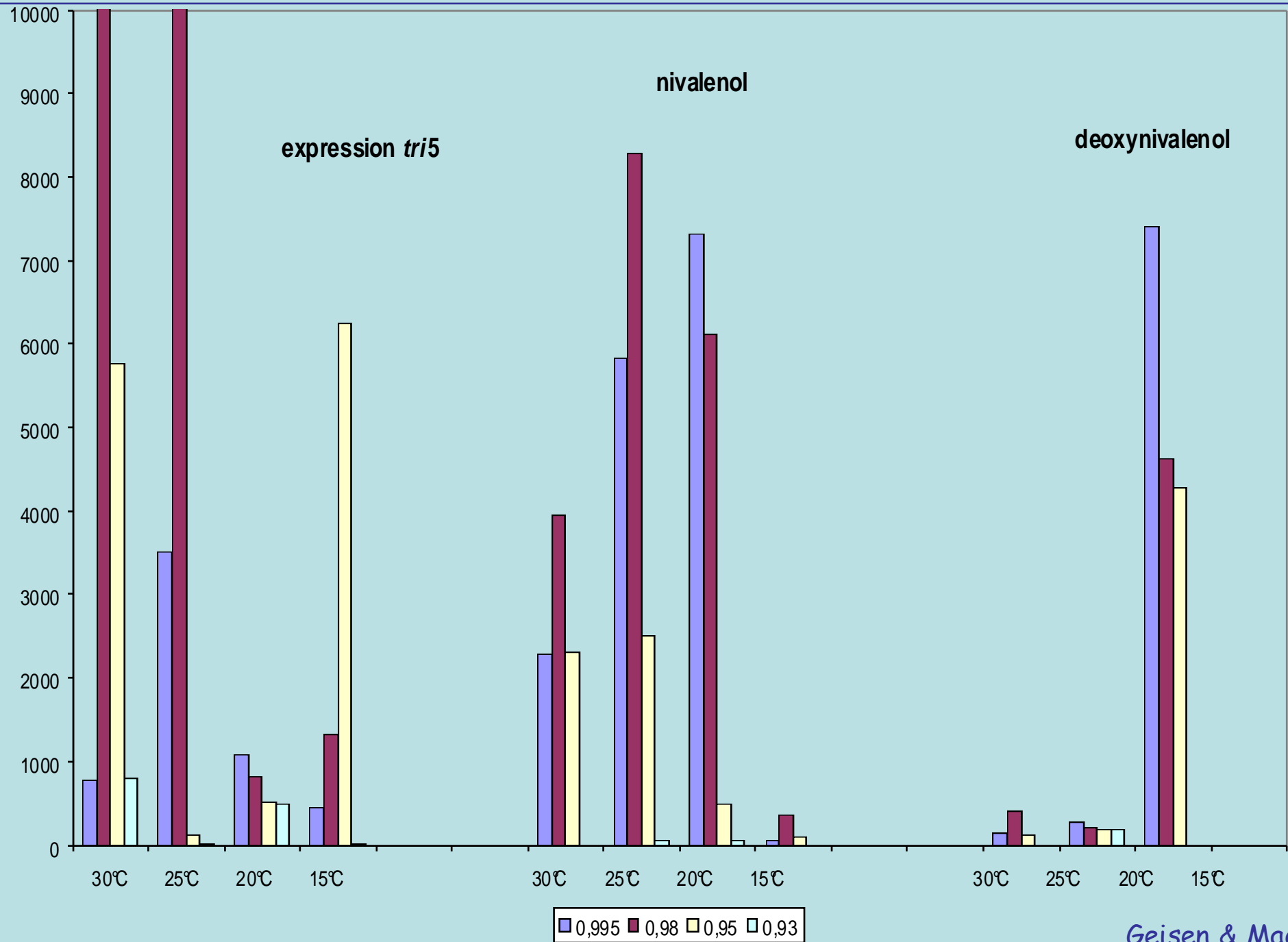


Ochratoxin genes; Aflatoxin genes, patulin genes

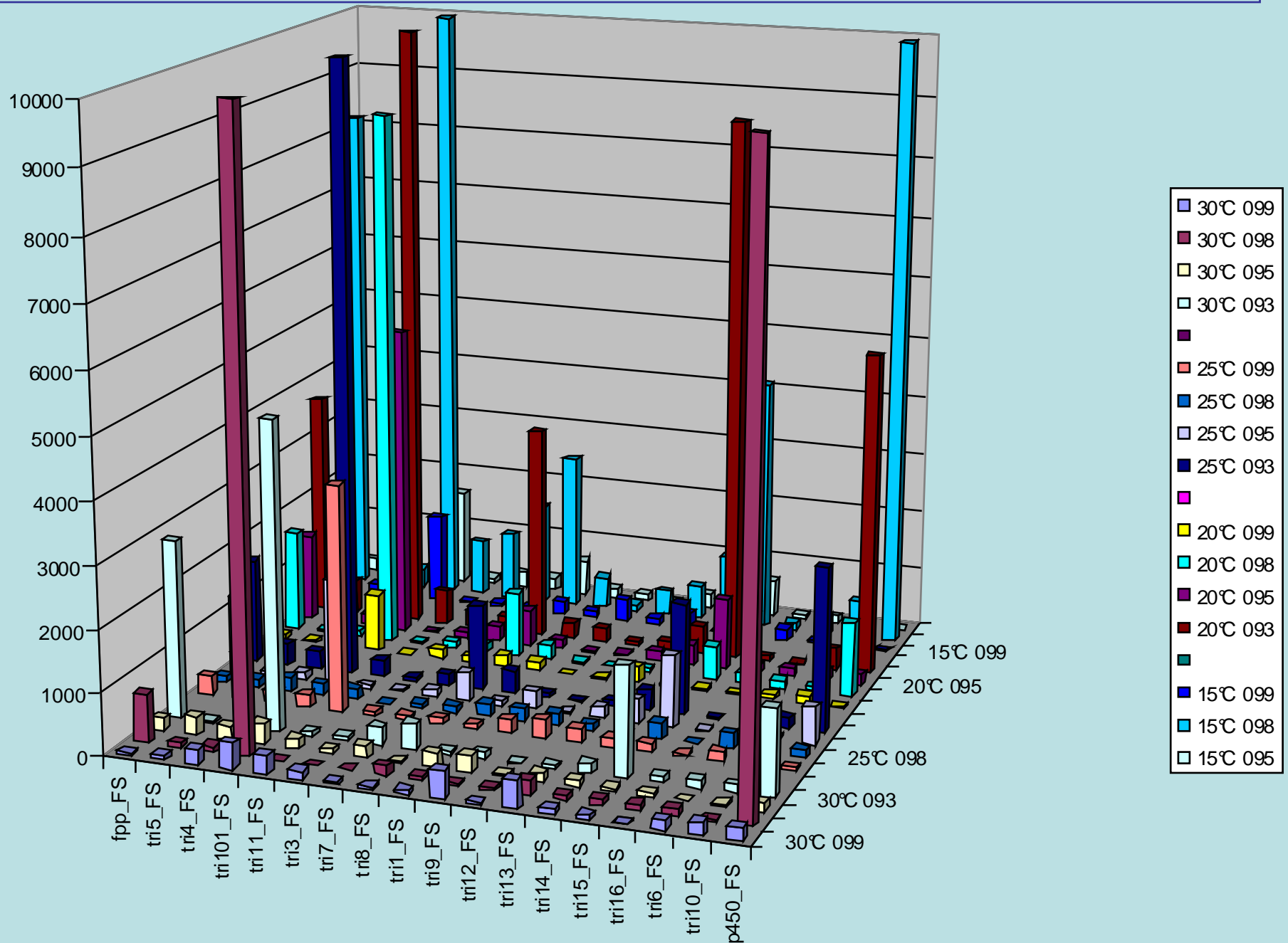
Trichothecene biosynthesis by *F. culmorum* under different single environmental factors



Correlation of certain genes of the cluster with trichothecene biosynthesis in *Fusarium culmorum*



Influence of external parameter on trichothecene cluster gene expression for *F. graminearum*



Advantages of using this approach?

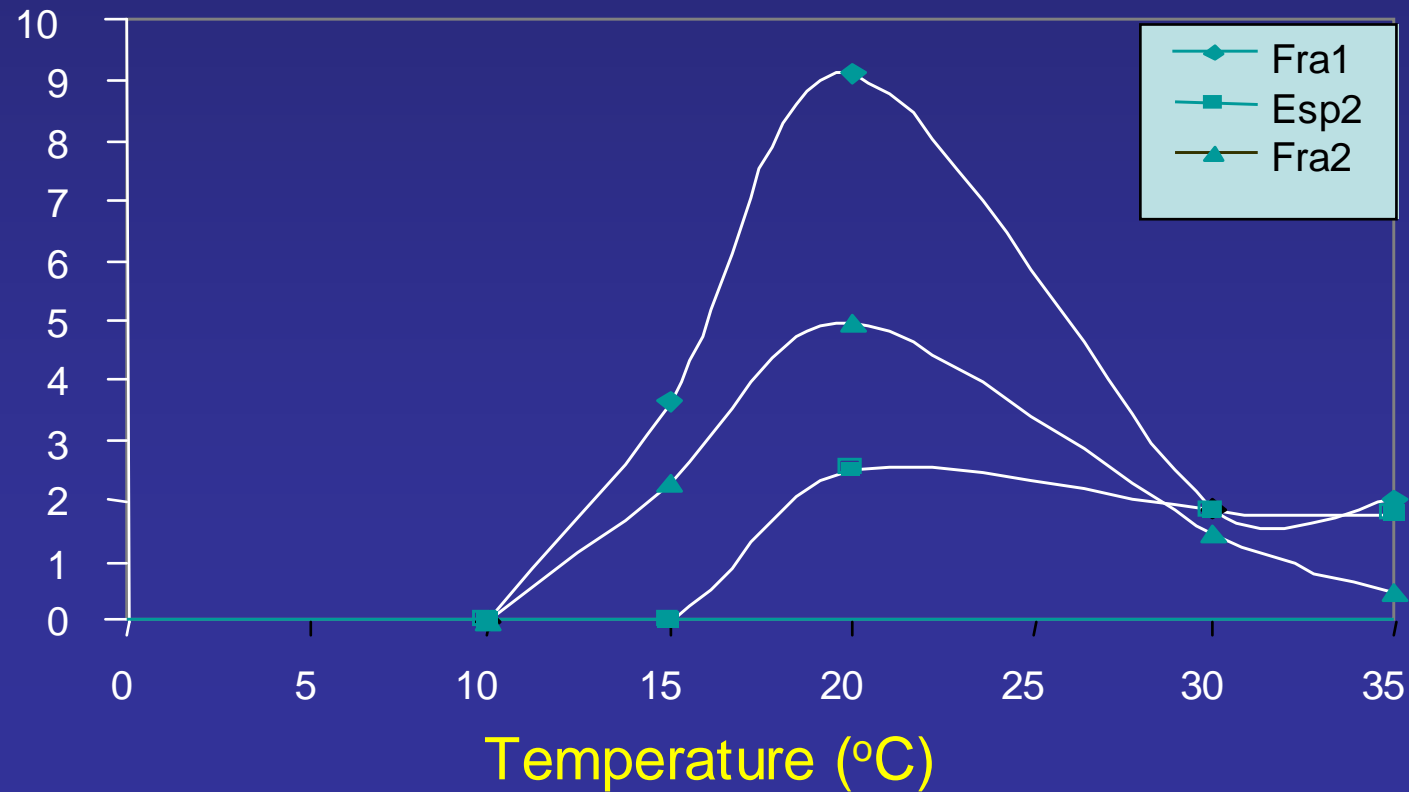
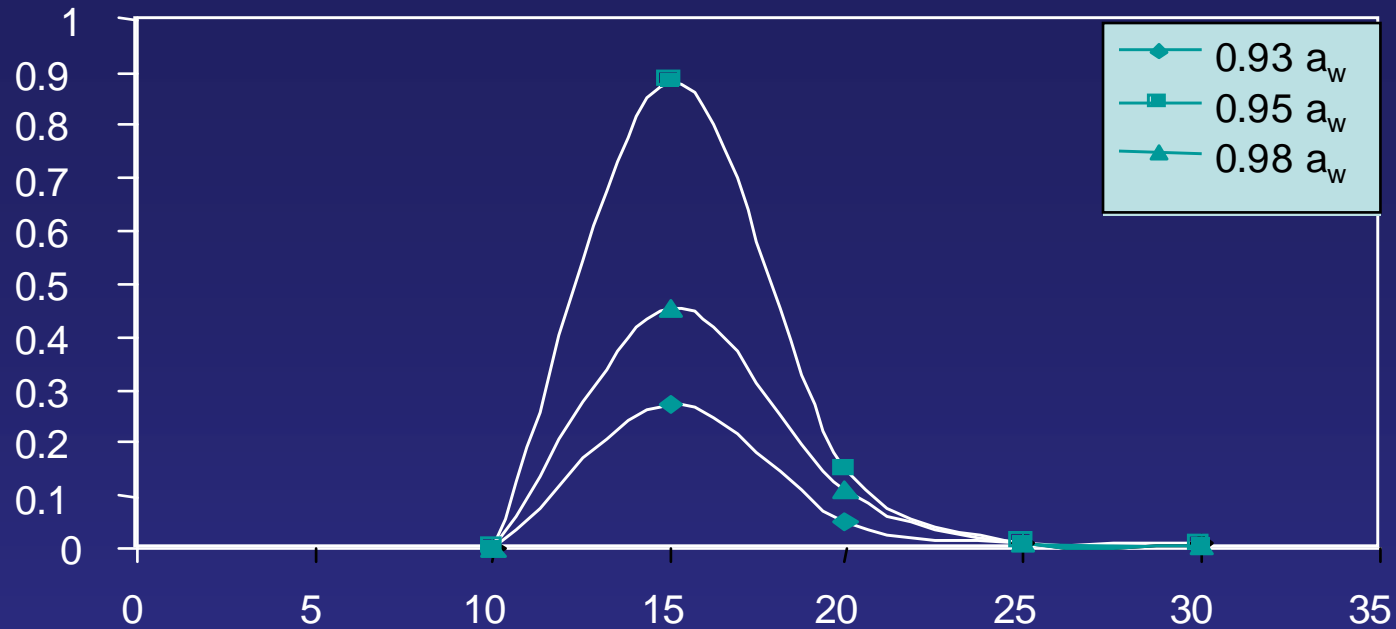
- ❑ Integrating mycotoxin gene expression with phenotypic toxin expression
- ❑ Growth of the mould not always optimum for mycotoxin production: thus more specific targeting of toxin genes under different simulated environmental conditions possible
- ❑ Targeting mycotoxin genes for novel control systems - much more specific
- ❑ Integrating molecular and phenotypic contamination levels to provide better targeted prevention systems

3. Predictive modelling utilising ecological and environmental information

Use data on:

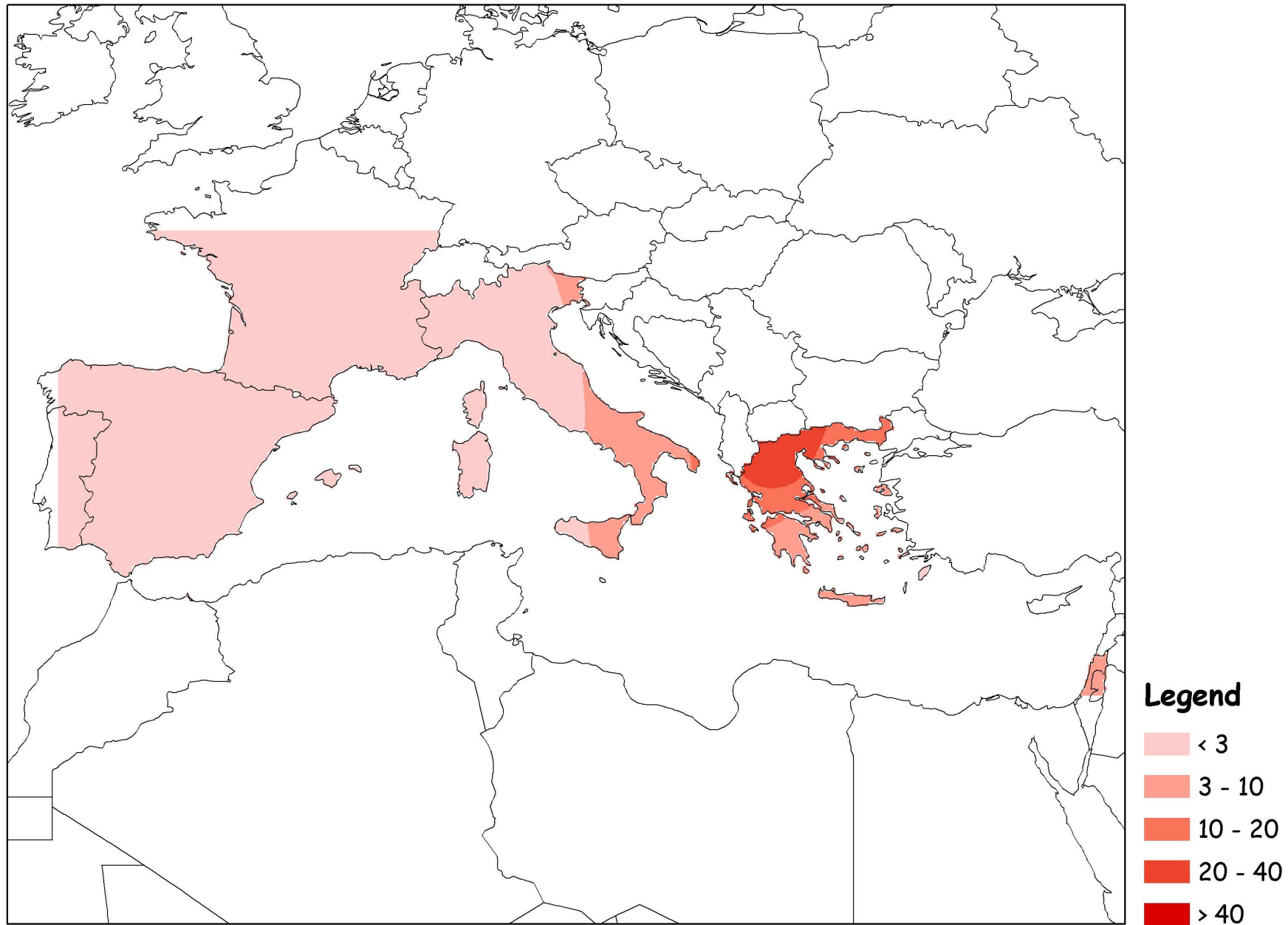
- (a) germination; growth and mycotoxin production
- (b) interactions between environmental factors and plant factors
- (c) used for both pre- and post-harvest prediction of toxins

Ochratoxin ($\mu\text{g g}^{-1}$)

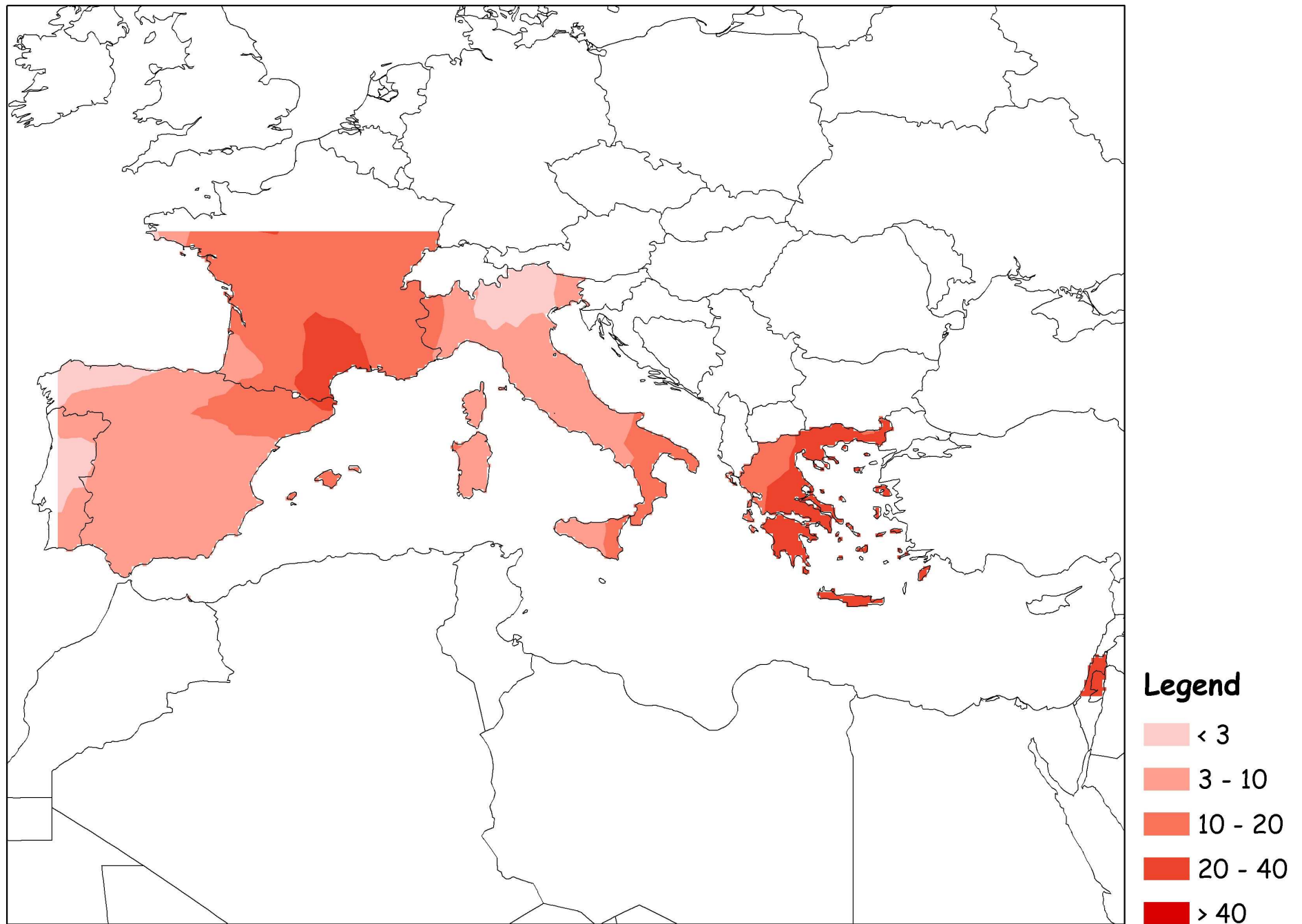


Temperature
x water
availability
effects on
OTA
production by
European
strains of
A. carbonarius





Incidence of *A. carbonarius* at early veraison in 2003
Similar data for 2004, 2005



Incidence of *A. carbonarius* at harvesting in 2003
Similar results in 2004, 2005

Battilani *et al.*, 2006



Numbers are maximum levels detected in ng/L

Battilani *et al.*, 2006



Development of DONcast

- DON data from +750 farm fields in Ontario, Canada (plus elsewhere) since 1996
- Agronomy and weather for each data point
- Diversity of all input variables is important
- Relationships identified (between input variables and DON)
 - Validated annually
- In addition to accuracy, it is equally important for models to be relatively simple in design, inexpensive to operate with input variables that are easily obtainable

Critical Timing of Weather Events Relative to Wheat Head Emergence

Inoculum Production

Infection at flowering

Fungal Growth

Critical Period

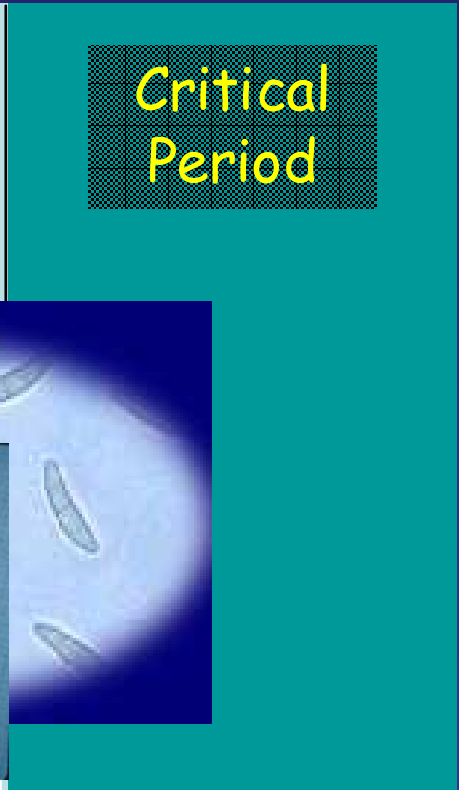
Critical Period

Critical Period

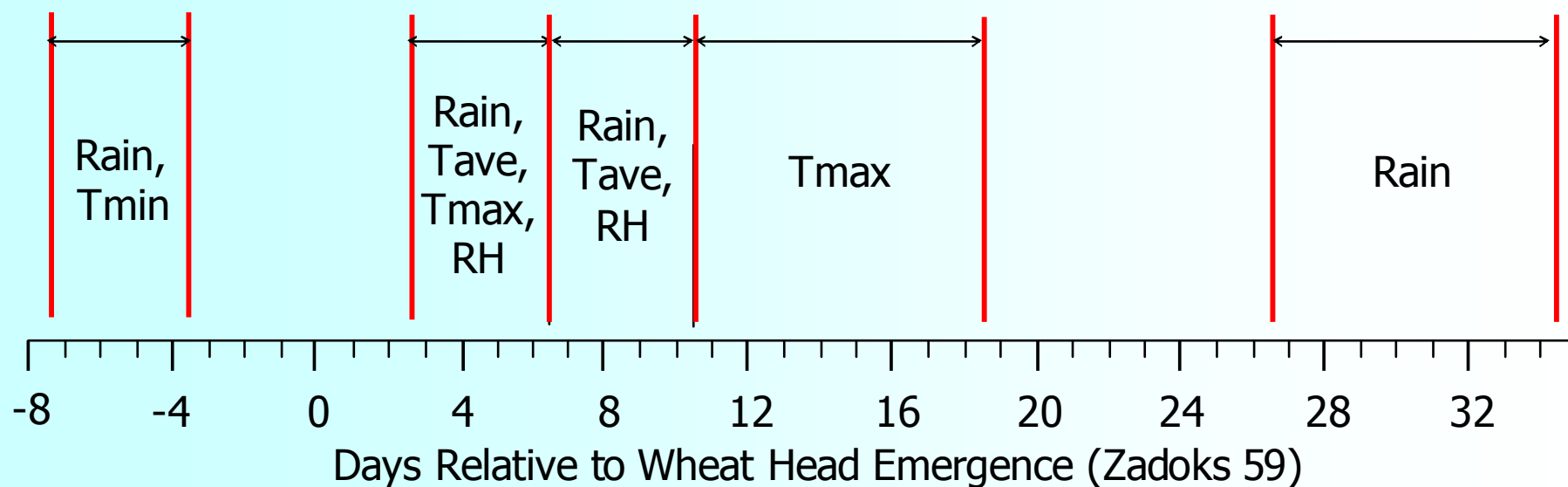
Day of Head Emergence

-7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11

Weather conditions

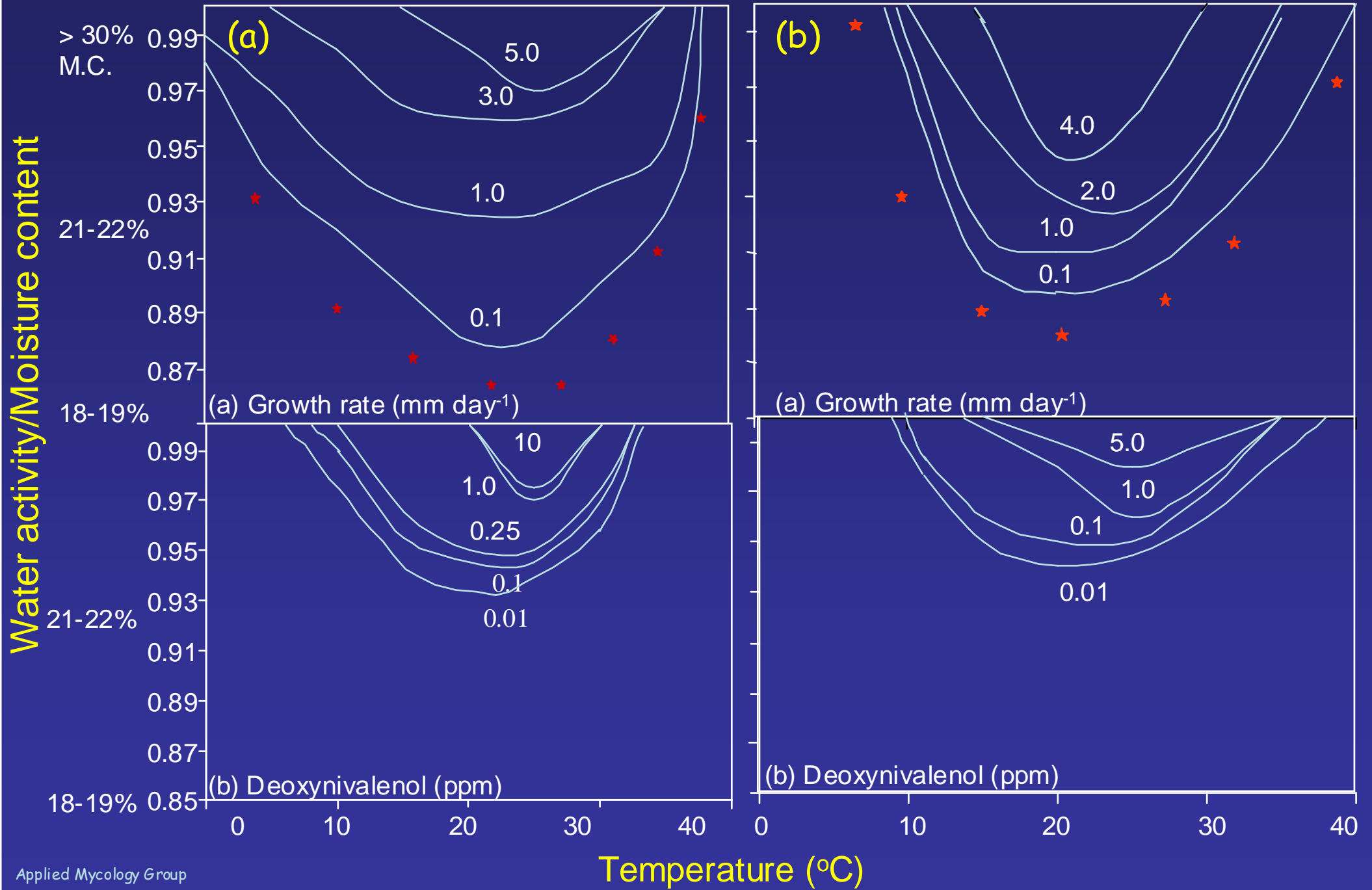


DONcast v2005 Critical Periods of Wx ~ from Heading to Harvest ~

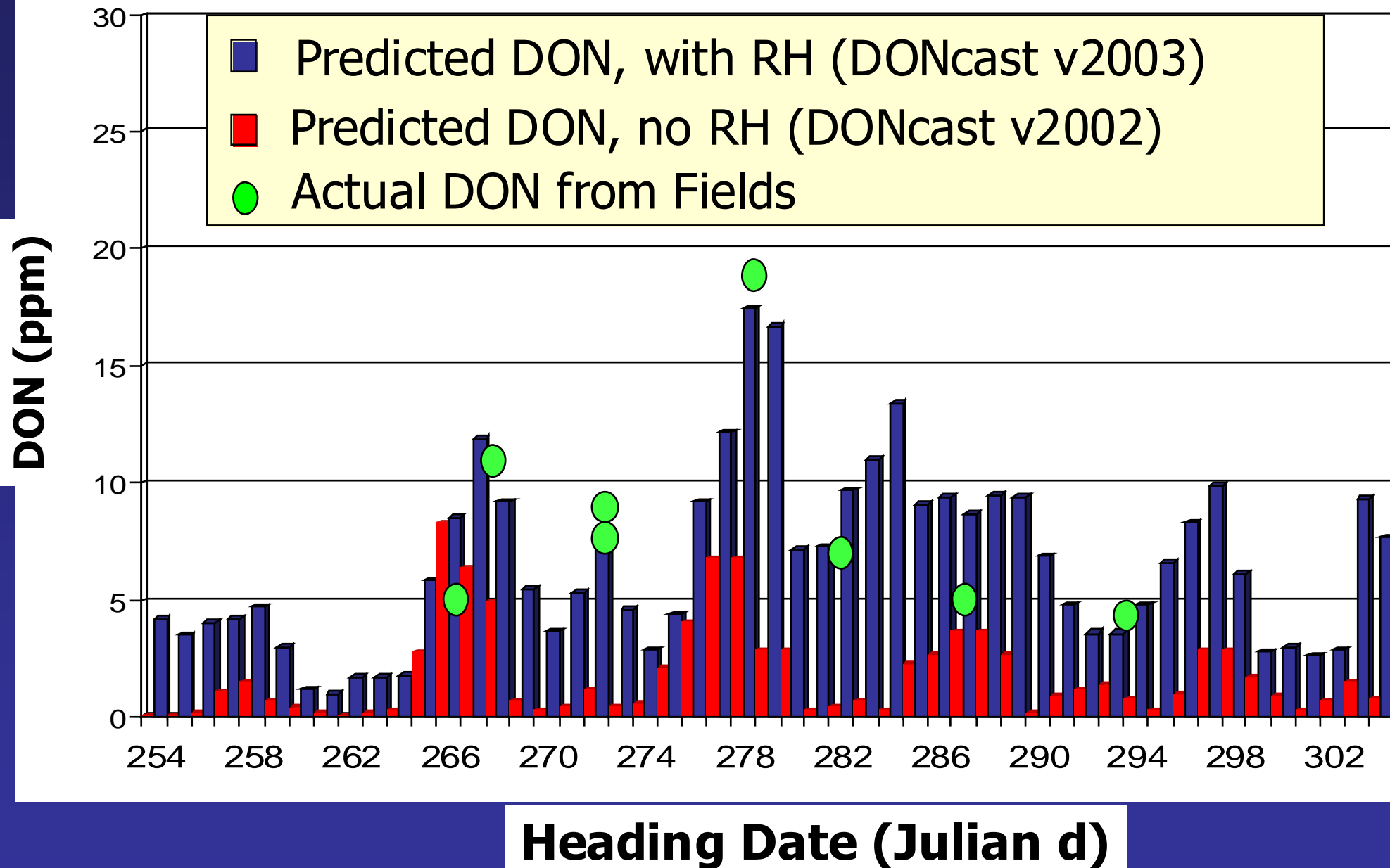


→ Heading ← Anthesis → Soft Dough → Harvest +/-

Comparison of profiles/limits for germination (*), growth (mm day⁻¹) and DON (ug g⁻¹) production: (a) *F.culmorum* (b) *F.graminearum* on wheat grain.



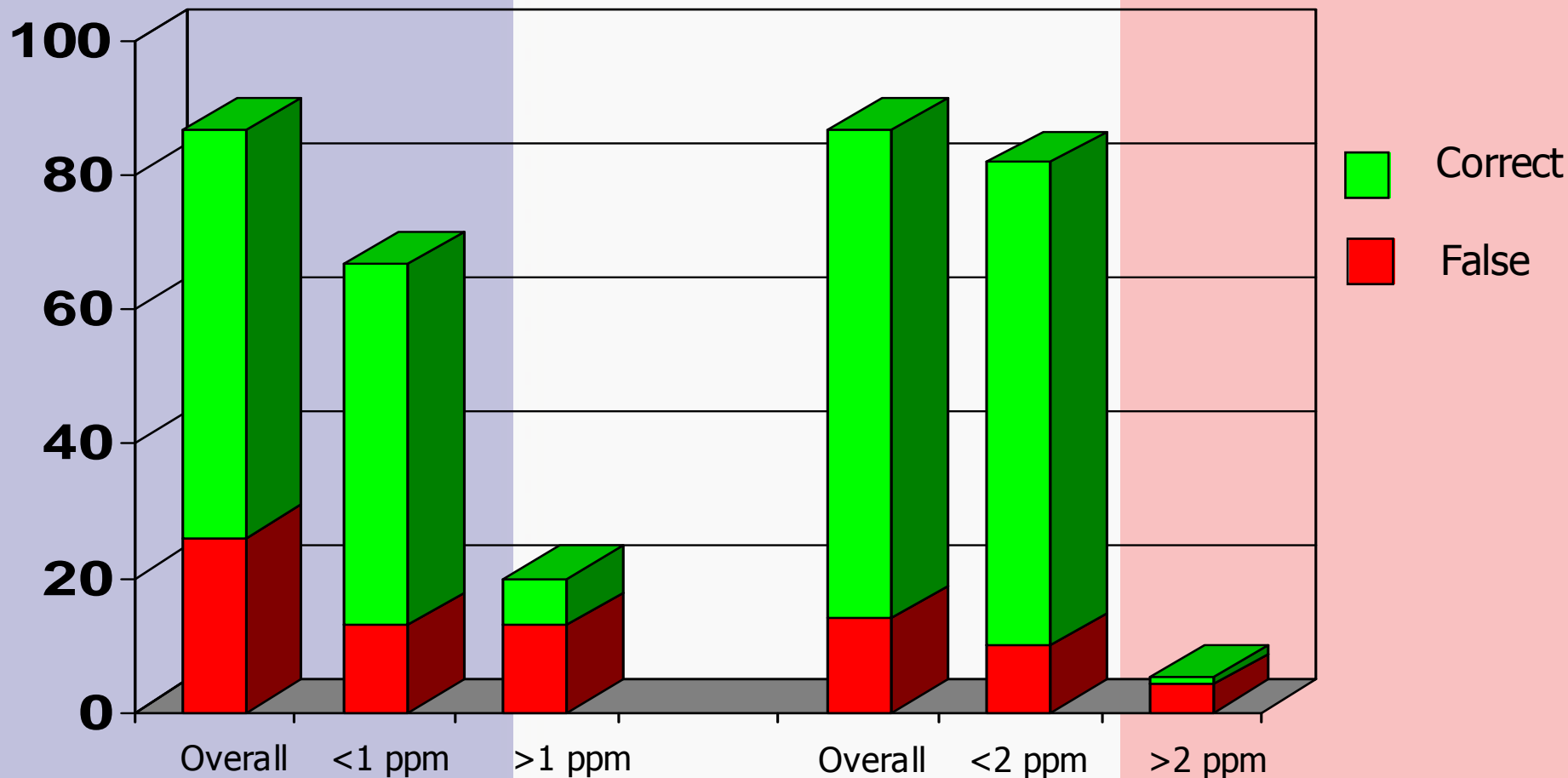
Daily DON Predictions for Salto, Uruguay, 2002



DONcast Validation in France, 2005

Predictions expected at heading using DONcast without rainfall parameter near maturity

No. Fields



Prediction Accuracy
Above/Below 1.0 ppm

Prediction Accuracy
Above/Below 2.0 ppm

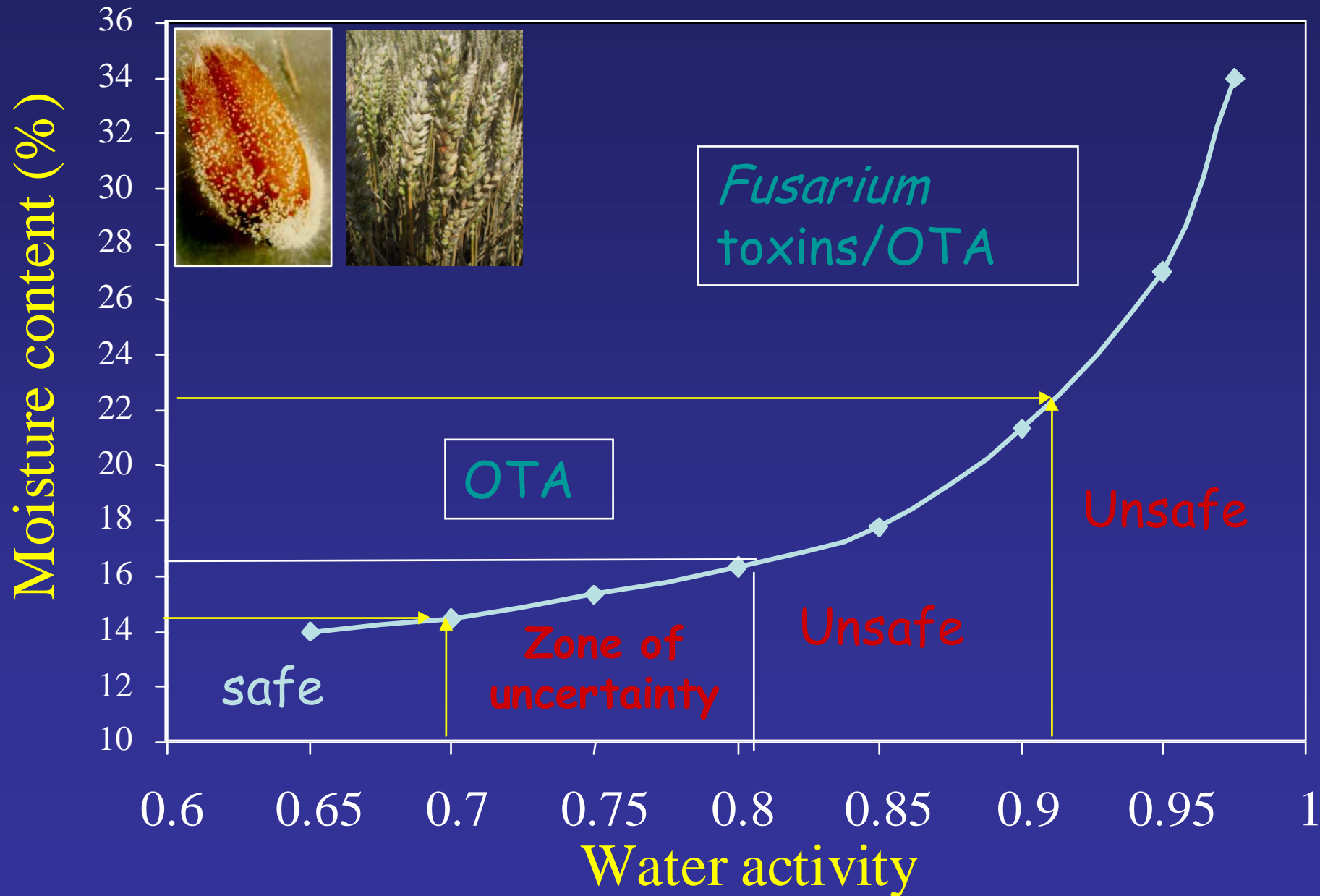
Hooker and Schafsma,
University of Guelph

Hooker and Schafsma,
University of Guelph

Uses for DONcast

- fungicide spray decisions at flowering
- retrospective estimates - health and supply risk
- producers making marketing decisions
- buyers for grain-sourcing decisions
- regulators to drive cost-effective monitoring

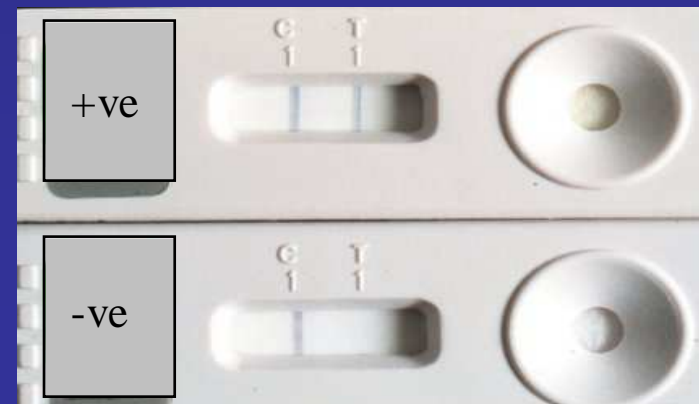
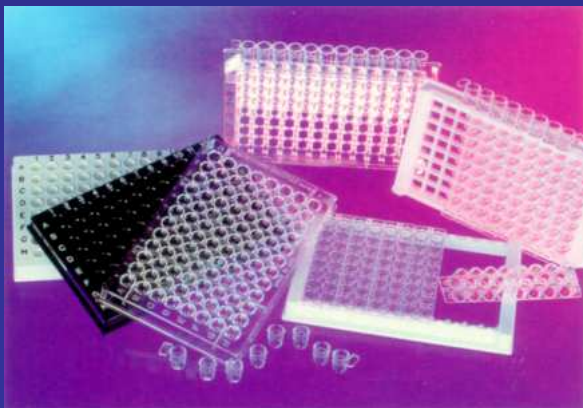
Post-harvest factors for QA - grain moisture content history



4. Development of analytical approaches for analysing mycotoxins

- Relevant/reliable detection systems - with real time/on-line applications for a range of mycotoxins in the food/feed chain required
- Appropriate technology for stake-holders in food chain; farmers to central surveillance labs to meet legislative requirements and monitoring of CCPs

lateral flow devices for multimycotoxin detection are now being developed



4. Development of analytical approaches for analysing mycotoxins

Interest in the development and application of Affinity Sensors is growing rapidly

- ❑ Increasing demands for simple sensitive and rapid analytical tools for decentralised analysis.
- ❑ Advances in hybridoma technology.
- ❑ Advances in synthetic receptor development.
- ❑ Developments in transduction methodologies.
- ❑ On - site testing for Risk management.

Affinity Sensors

Sensing Receptors

- Antibodies
 - Polyclonal
 - Monoclonal
 - Recombinant
- Cell receptors
- Single stranded DNA
- Natural and synthetic receptors
 - Molecularly Imprinted Polymers
 - Combinatorial chemistry
 - Phage display technology

Transducers

- Optical
- Electrochemical
- Thermometric
- Piezoelectric
- Magnetic

Peptide Receptors design: Ochratoxin/Aflatoxin M1

Amino acid	Binding energy (kcal mol ⁻¹)
Phenylalanine	-33.52
Proline	-32.10
Valine	-30.93
Isoleucine	-30.37
Leucine	-28.94
Cysteine	-28.67
Tyrosine	-27.29
Methionine	-26.33
Threonine	-25.55
Tryptophan	-22.71
Alanine	-21.87
Glutamate	-20.63
Aspartate	-19.86
Asparagine	-13.27
Lysine	-11.72
Histidine	-10.06

Amino acids
interacting
with high score

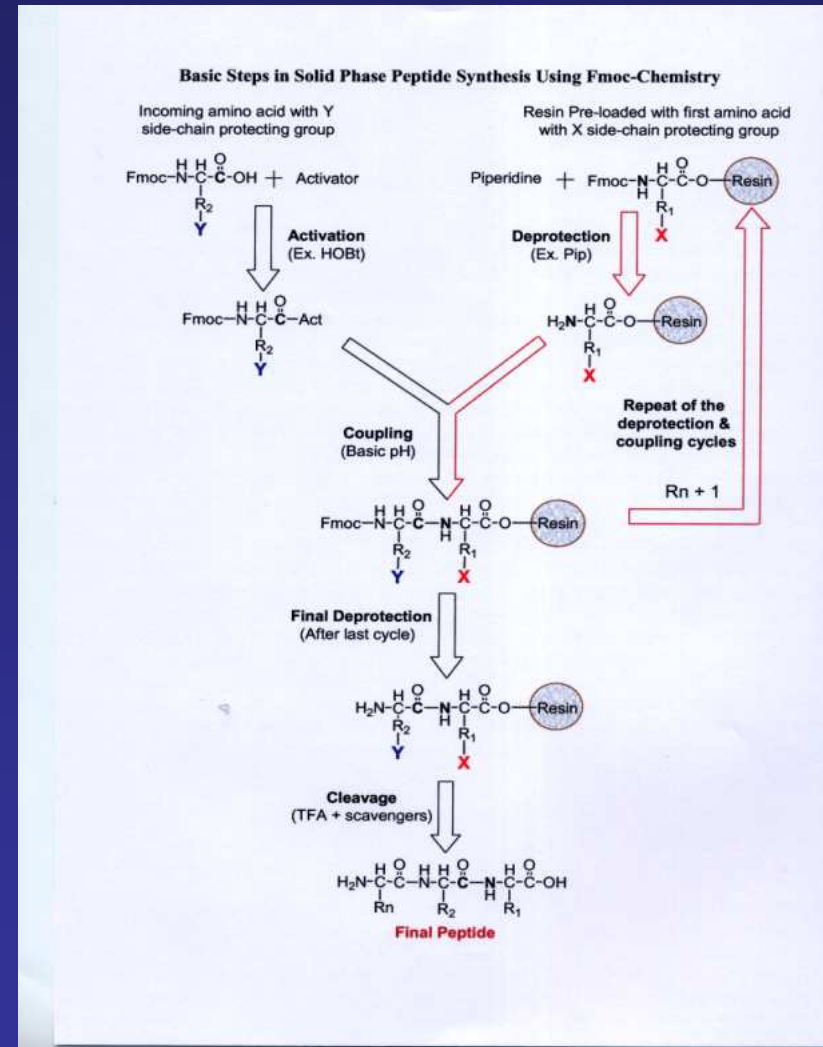
Amino acid sequence	Binding energy (kcal mol ⁻¹)
PSIVE	-46.45
IGA	-44.55
IGAP	-44.54
CSIVE	-42.13
IGAPA	-37.47
CGPAGI	-31.85
SPAGI	-31.56

De novo designed lead
peptide sequences
using computational
design tool

Modified peptide
sequences using
receptor-ligand
dynamic
simulations

Receptor Design

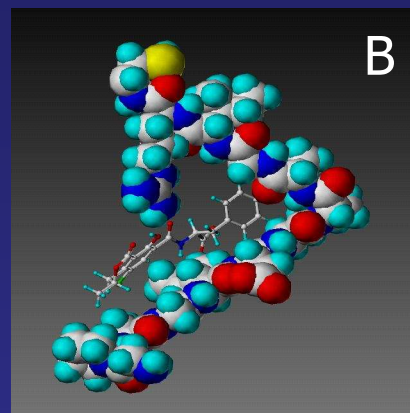
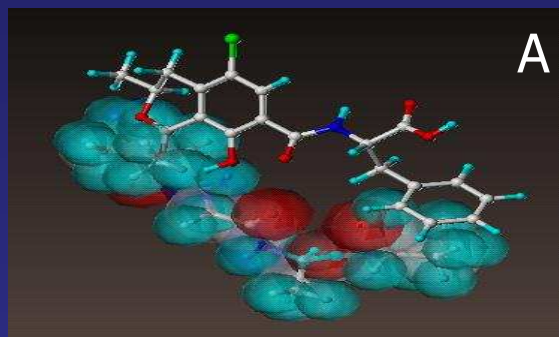
- The receptor design is constructed for the target analyte
- The receptors were then synthesized using solid phase chemistry
- The synthesized receptors were then screened for their affinity and specificity for the target analyte



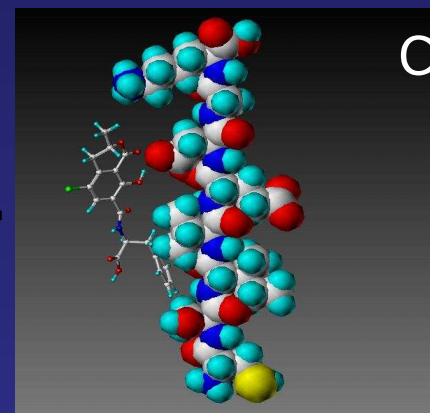
Solid Phase Peptide Synthesis Procedure

Peptide Receptors design:

Ochratoxin A

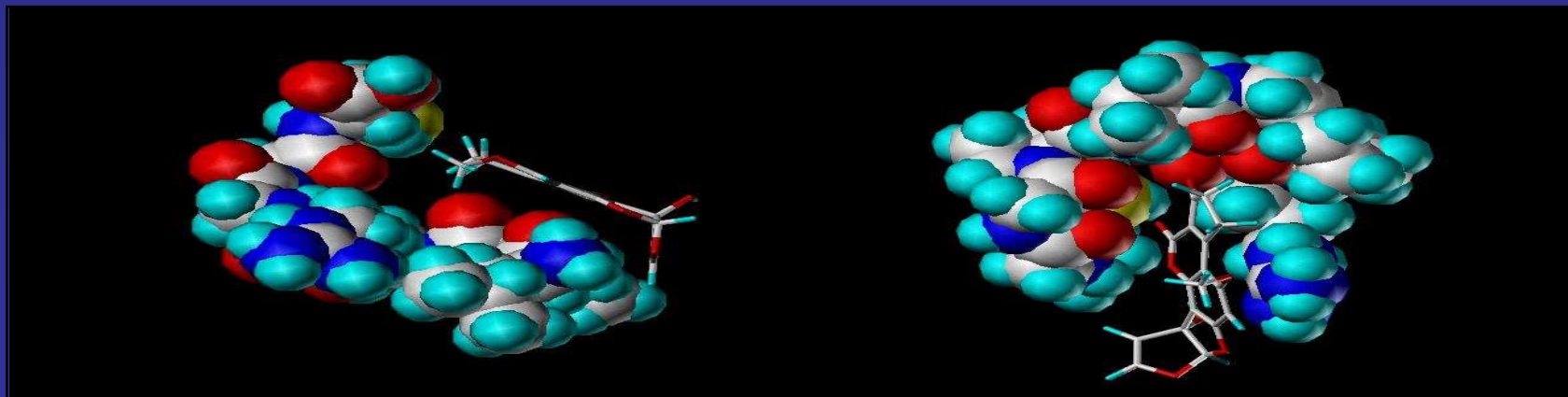


13-peptide



octapeptide

Aflatoxin M₁

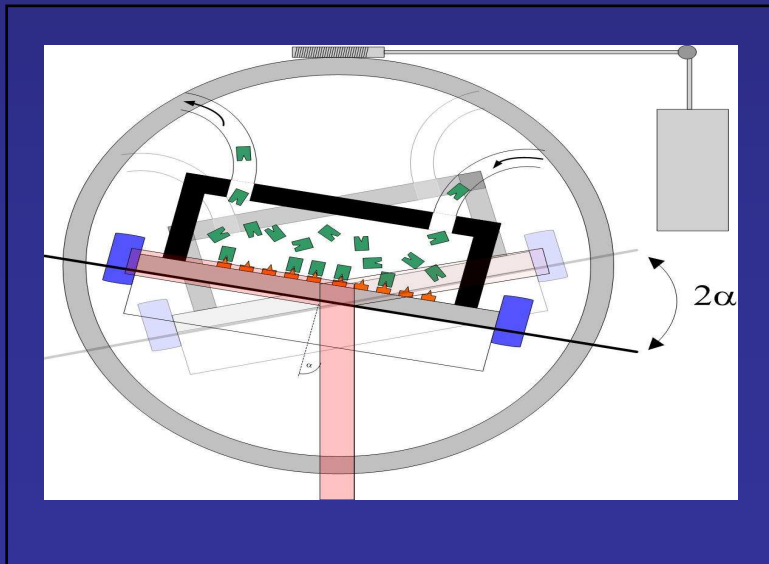
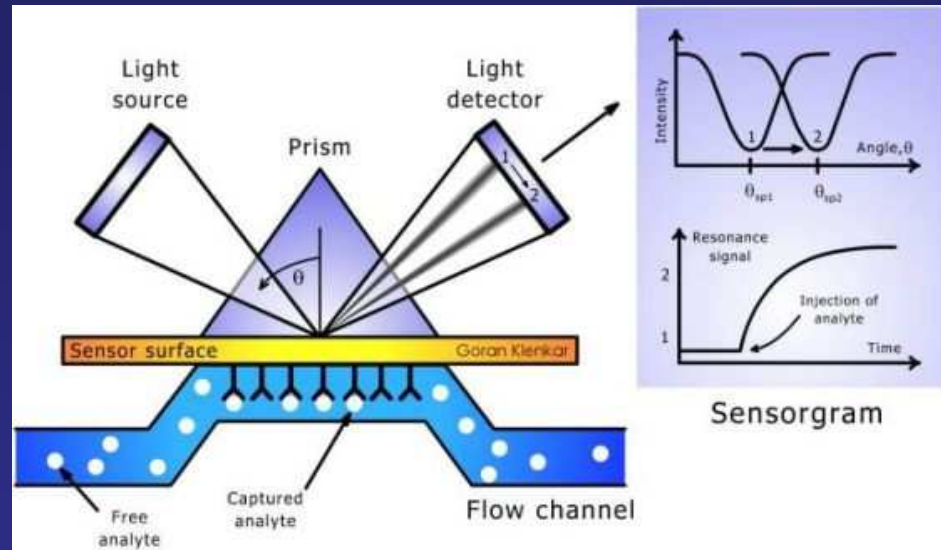


heptapeptide

nonapeptide

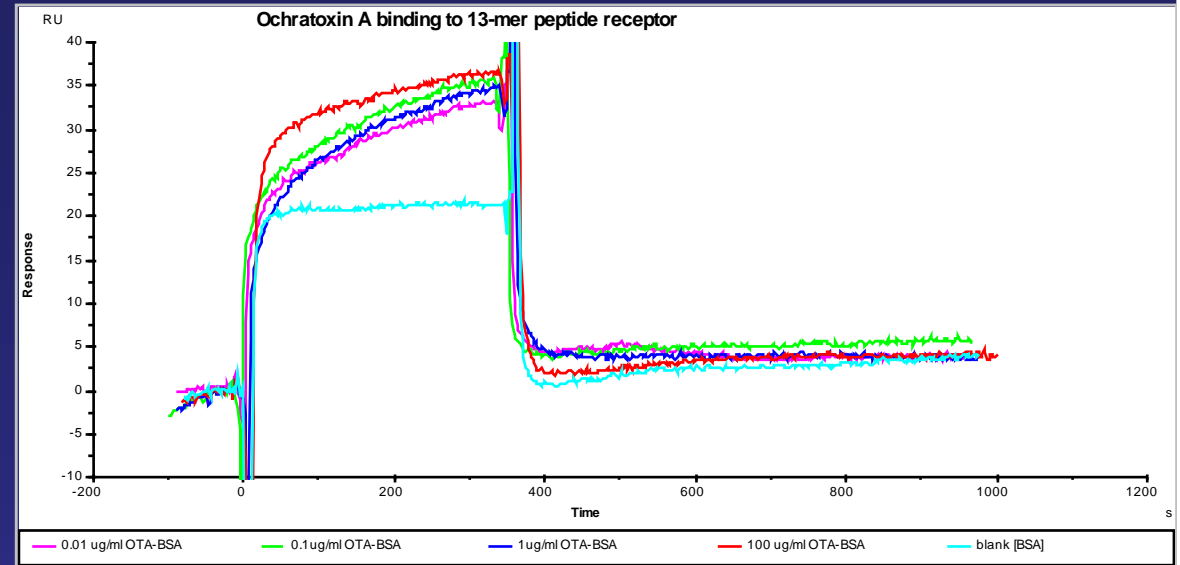
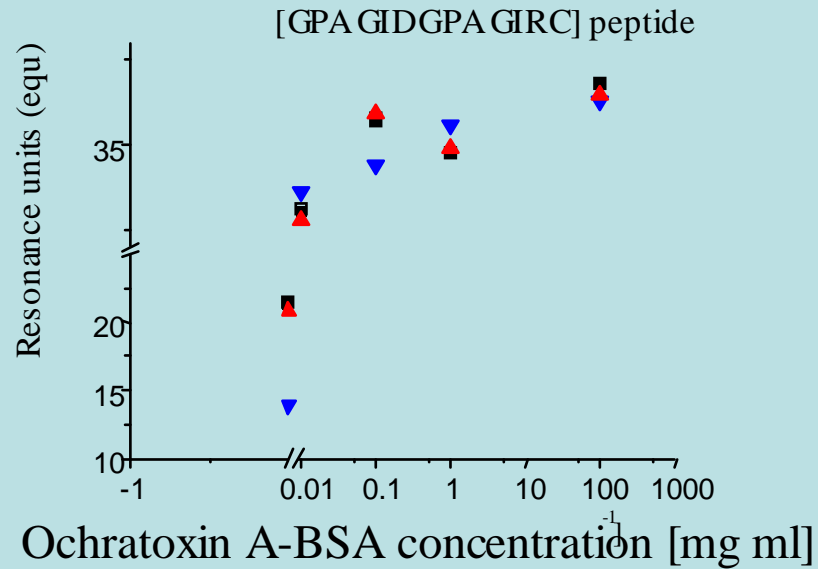
Affinity and Kinetics

- Biacore™ uses surface plasmon resonance (SPR) as detection principle was used to screen the receptors.

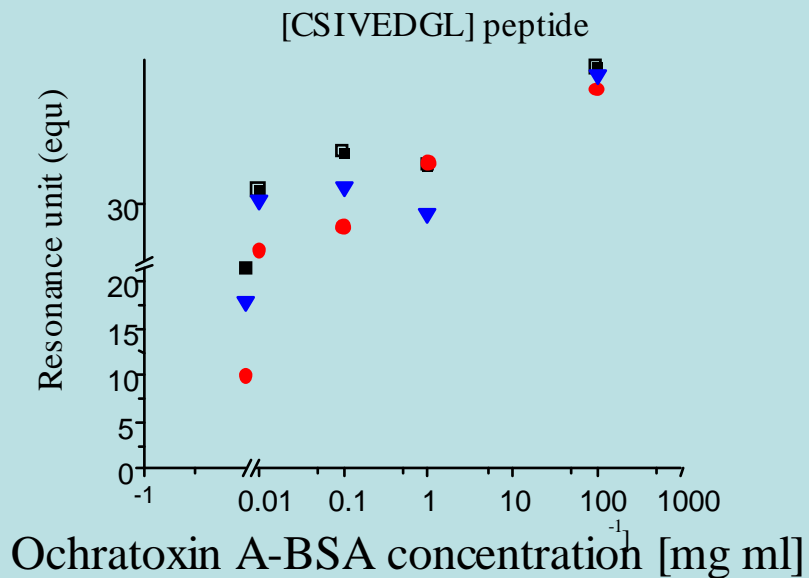


- Screening peptide receptors was carried out using optical waveguide lightmode spectroscopy (OWLS).

Ochratoxin A Receptor



Verification of selective binding to both 13-mer (top) and octapeptide (bottom) to ochratoxin A.

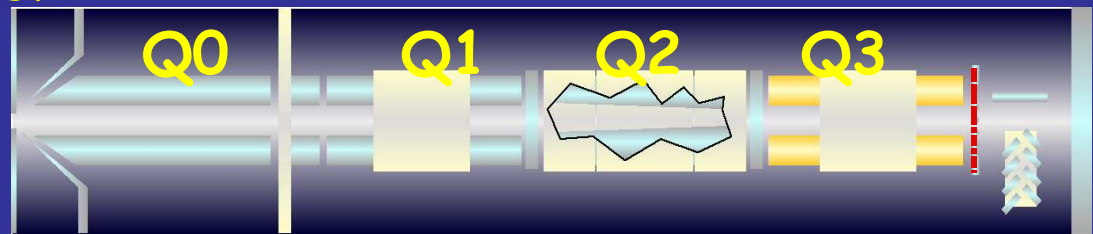


Immobilized component	Soluble component	Kinetic rates	Affinity constants
13-peptide	Ochratoxin A-BSA	k_a [M ⁻¹ s ⁻¹] = $10^4 \times 10^5$ k_d [s ⁻¹] = 1.4×10^2	K_A [M ⁻¹] = 7.40×10^6 K_D [M] = 4.34×10^7
Octapeptide	Ochratoxin A-BSA	k_a [M ⁻¹ s ⁻¹] = 1×10^6 k_d [s ⁻¹] = 1.3×10^2	K_A [M ⁻¹] = 1.49×10^6 K_D [M] = 6.69×10^5

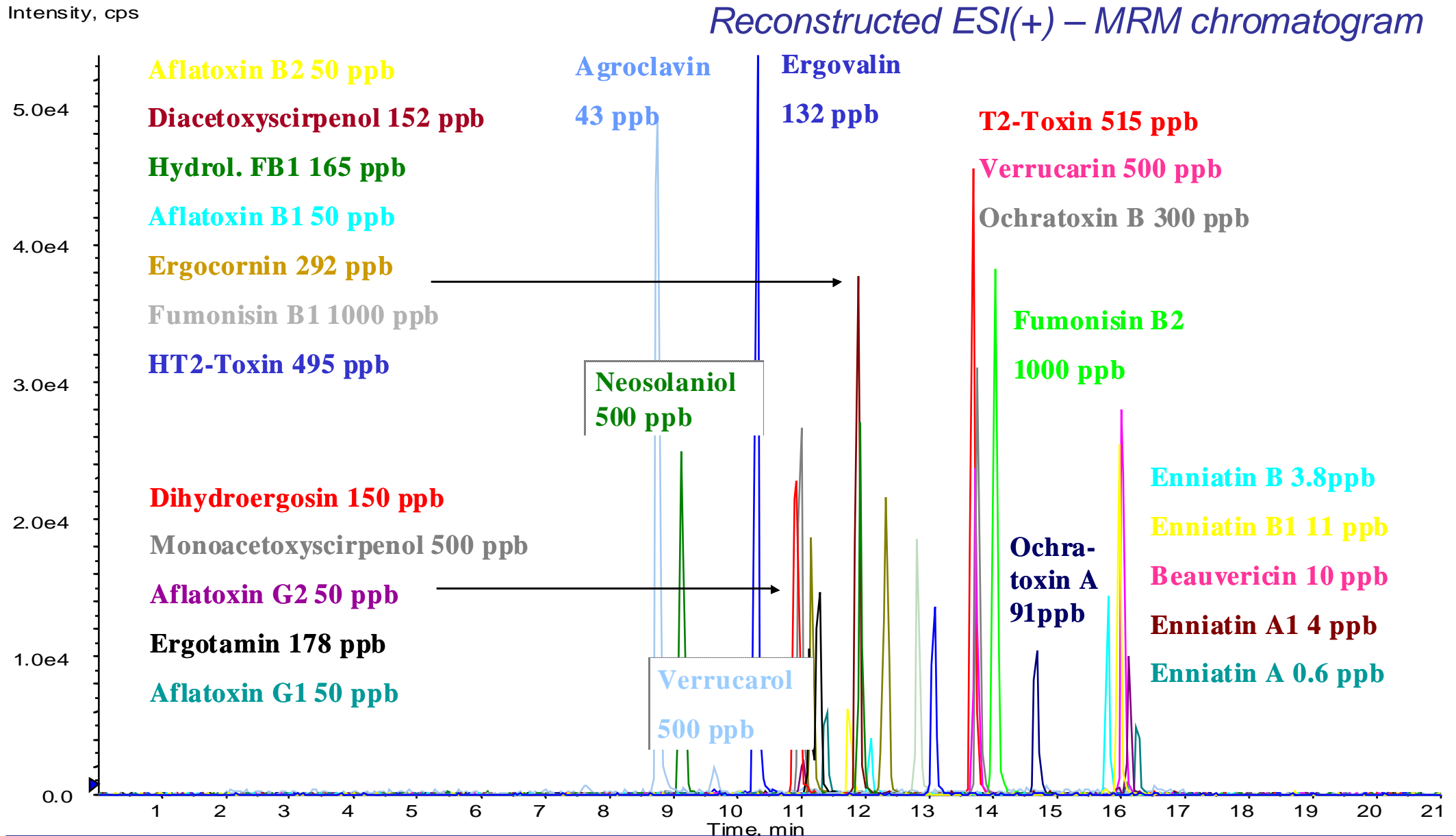
- *Peptides show good affinity for ochratoxin A*

Development of a multiresidue method for the determination of 39 mycotoxins in wheat and maize using the Q-Trap 4000 LC/MS-MS

- **Goal:**
- **Screening** of wide range of analytes necessary for evaluation of potential health hazard and synergistic effects of different mycotoxins
- **One method** for various analytical questions
- **Challenges:**
 - Wide range of relevant concentrations:
2 (AFB1) - 1500 (DON) $\mu\text{g}/\text{kg}$ \Rightarrow linearity, sensitivity
 - Chemical diversity of the 40 investigated analytes
 - Careful optimization of extraction solvent and mobile phase
 - Direct injection of (diluted) crude extracts: Signal suppression due to matrix effects?

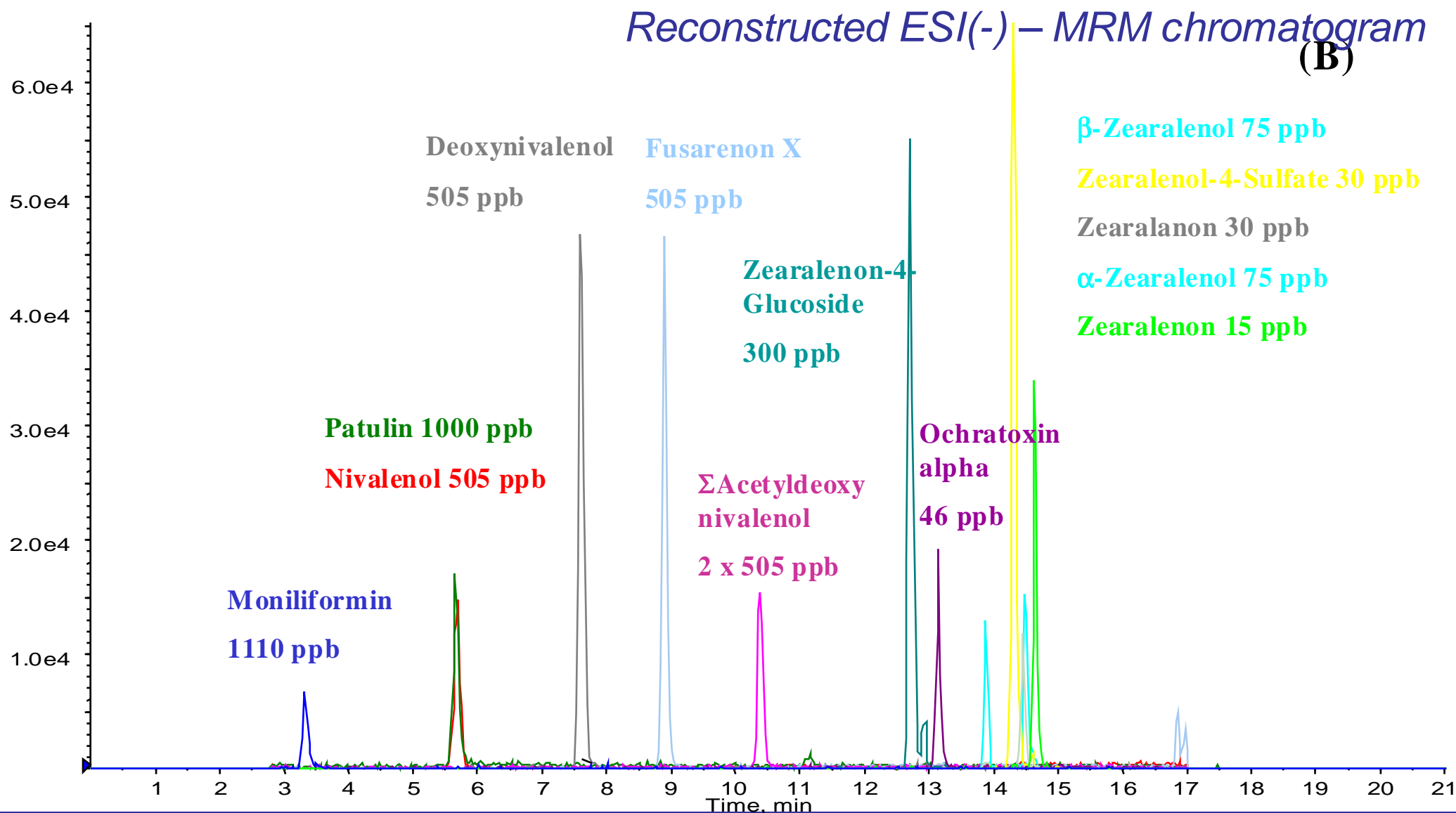


Simultaneous determination of 39 mycotoxins in wheat by LC-MS/MS without any clean-up - part 1



Simultaneous determination of 39 mycotoxins in wheat by LC-MS/MS without any clean-up - part 2

Intensity, cps



Conclusions and the future

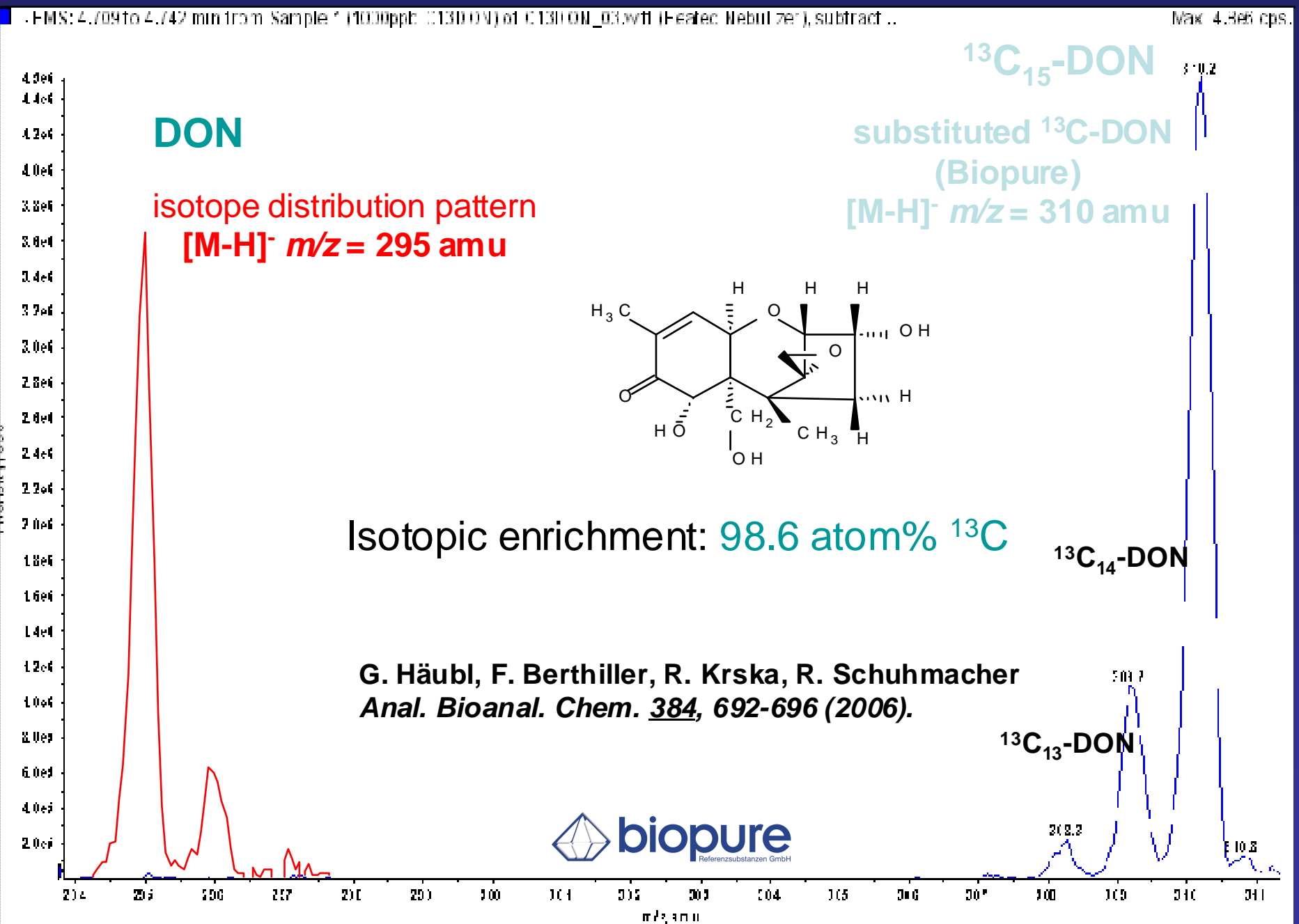
- Effective exploitation of genomic and metabolomic approaches for better targeting of control of phenotypic toxin contamination
- Predictive modelling approaches when combined with regional GIS information systems may provide effective tools for better risk assessment and hot spots for mycotoxin contamination identified
- Analytical methods for multiple mycotoxin assays may provide systems for better surveillance
- New LFDs for multimycotoxin analyses and development of synthetic peptides may be useful in the whole food chain
- Prevention strategies are dependent now on the effective integration of different types of data to minimise exposure to mycotoxins
- Sampling remains an important issue - which still needs attention through the whole food chain (what is a representative samples?)

Acknowledgements

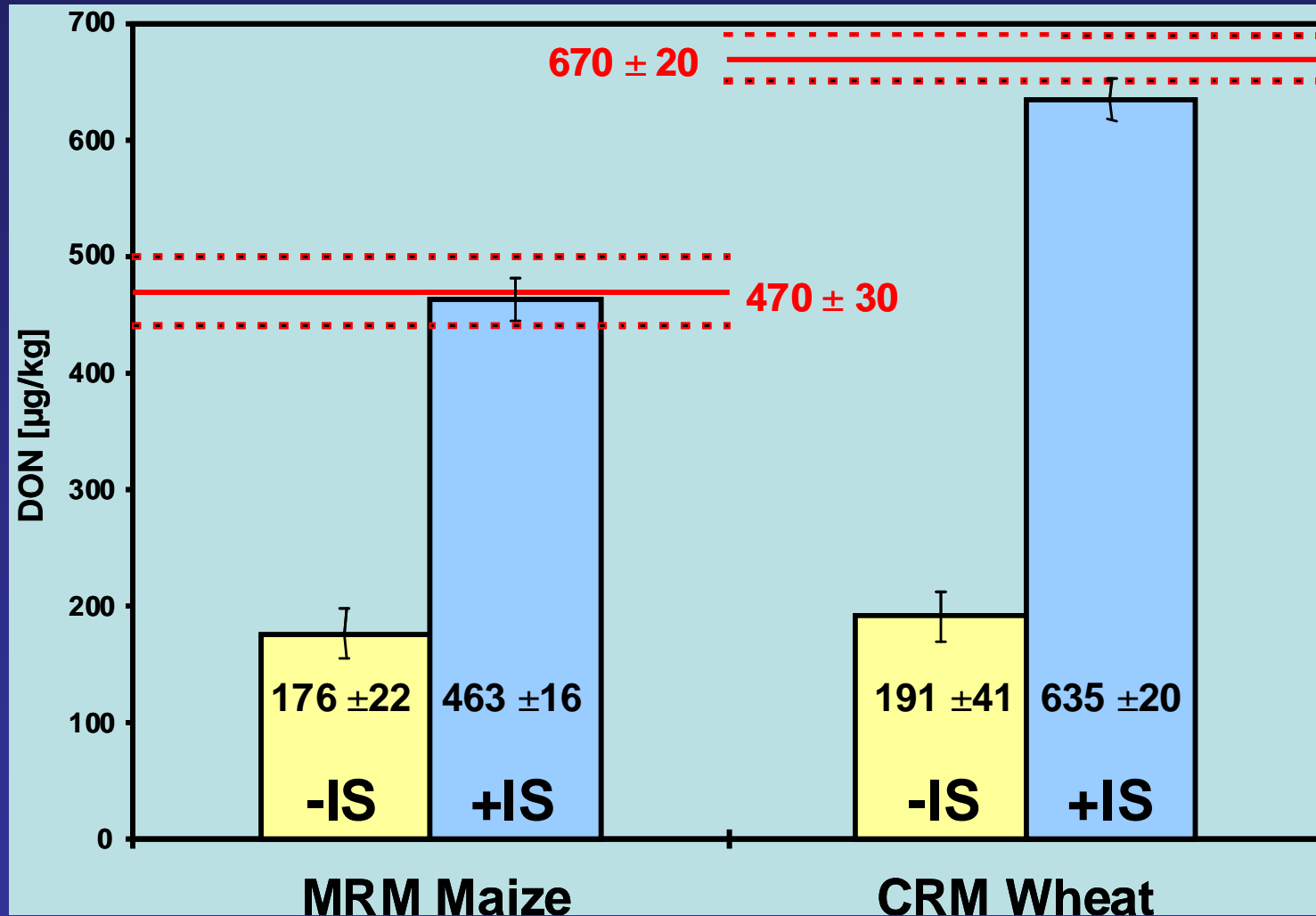
- Prof. R. Krska
- Dr. A. Schaafsma
- Dr. Sam Tothill
- Dr R. Geisen
- Dr. Maite Gonzalez

- Applied Mycology Group

Use of fully $^{13}\text{C}_{15}$ isotope substituted DON as Internal Standard to improve accuracy



Quantification of DON using LC-MS/MS with C13-DON as Internal Standard without clean-up



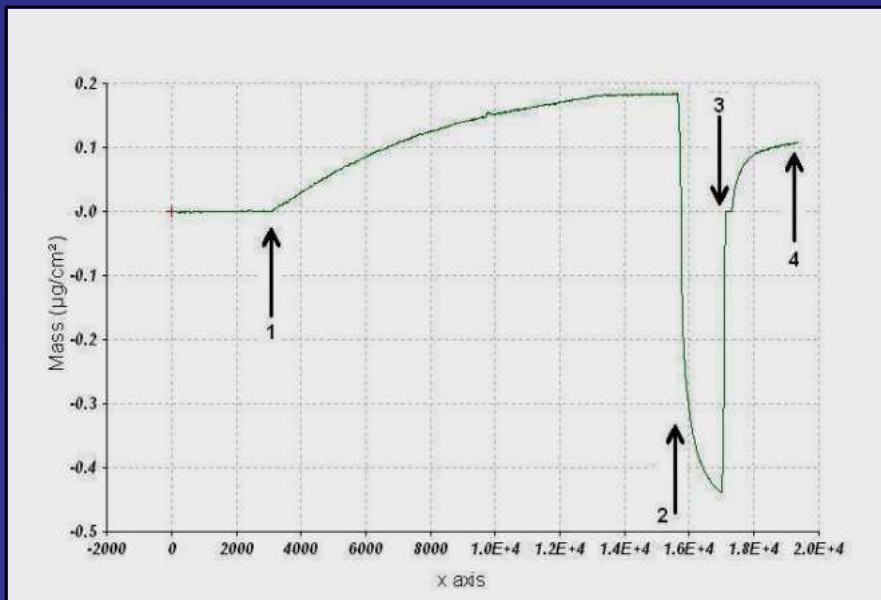
Centre for Analytical Chemistry
© Rudolf Krška IFA, Tulin



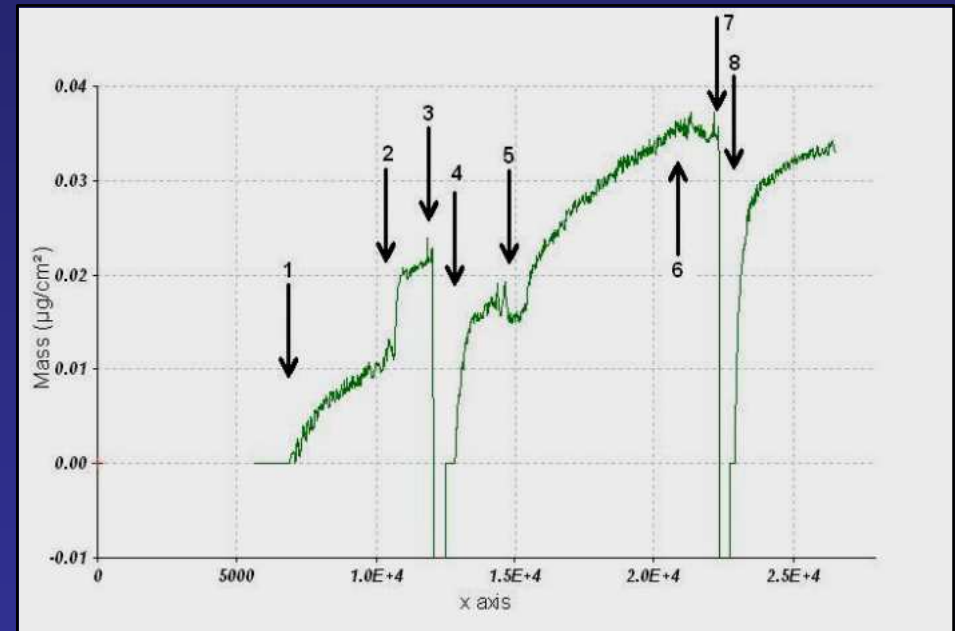
Ion suppression => Importance of internal standards for less sophisticated interfaces and complex food stuffs- ideally C13 labelled toxins

Aflatoxin M₁ Receptor

anti-aflatoxin M₁ antibody



peptide binds to BSA-aflatoxin M₁



The peptides were shown to have affinity and specificity to the aflatoxin M₁ toxin.