

# ***The control of Mycotoxins in Raw Materials***

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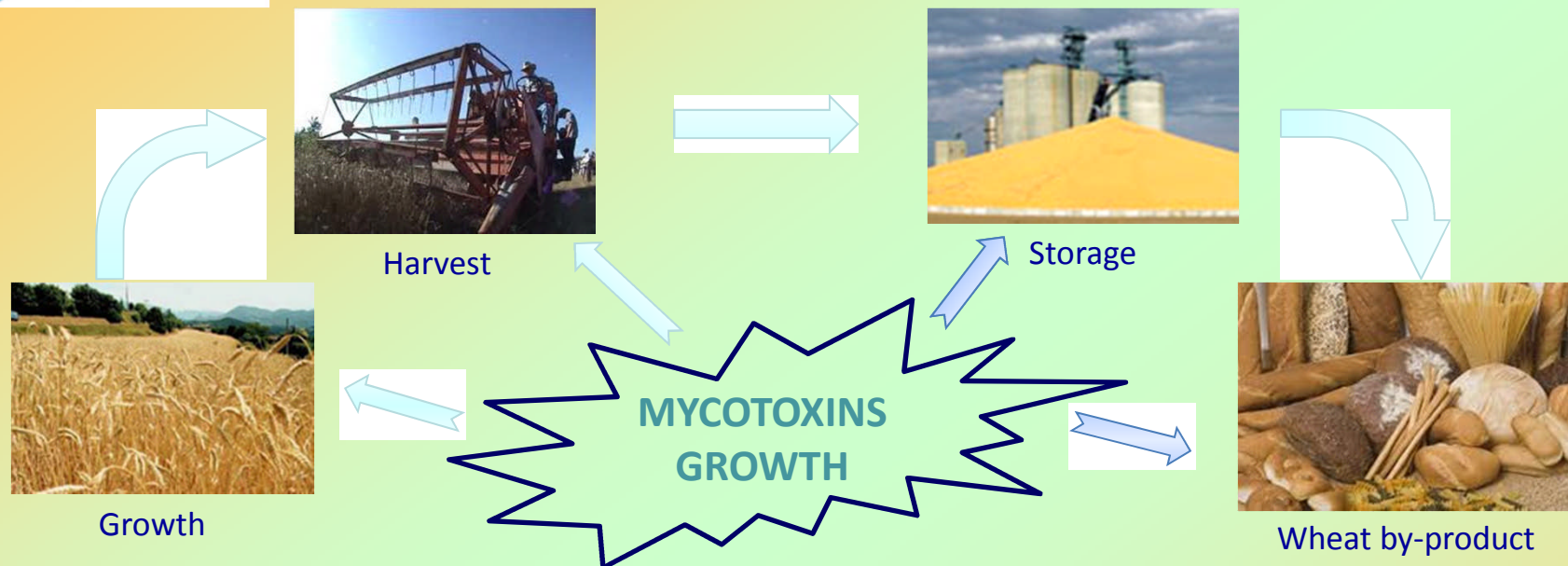
*Michele Suman*

**Tandem and High Resolution Mass Spectrometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012**

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## The story of the mycotoxins growth...

*From field... to table*



## The story of my presentation today...

1. *How to obtain reliable results in mycotoxin analysis*
2. *Tools for rapid detection & screening*
3. *Emerging analytical strategies*
4. *Chromatographic / Mass spectrometry confirmatory methods*
5. *Masked mycotoxins: a new scenario*

## Mycotoxins & their impact on cereals supply chain

Mycotoxins are **secondary metabolites produced by fungi** (more than 300 already identified) and represent **hazards to human and animal health** and also a relevant problem in the cereal supply chain at a worldwide level. Little is known about the effects of long-term, low-level exposure, especially regarding co-contamination.

Their **growth could happen since the pre-harvest** (cold-wet climatic regions) and also during transport/storage of raw-materials due to ineffective drying conditions. Heterogeneity of contamination and possible elevated levels in regional hot spots mean that **consistent sampling is difficult**.

**From an economic point of view, in agriculture** the decrease of plant products, the quality decay of seeds, and in stock-raising, the decrease of feed intake, problems of reproduction biology, and formation of mycotoxicosis **cause losses**.

Of all food and feed **safety related announcements, 60% refer to mycotoxin contamination (FAO statistics)**.

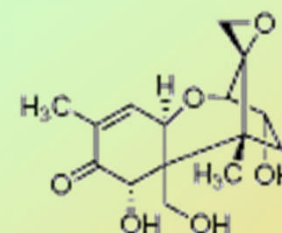
Today **more than 100 countries in the world regulate mycotoxin limits**, and distributors are responsible for values within those limits.

The **significance of quantitative analysis of Mycotoxin has been therefore increasing in the last years** both from producers' and distributors' side.

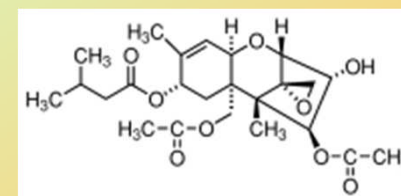
Since mycotoxins cause harm even in small concentrations, **it is important to provide analysis methods with very high sensitivity**.

# Mycotoxins of main concern in agricultural products \ food commodities

Aflatoxins (B1, B2, G1, G2)	<i>maize, spices, dried fruits and nuts</i>
Aflatoxins M	<i>milk and derivatives</i>
Ochratoxin A	<i>wheat, barley, maize, coffee, cocoa, dried fruit, wine, beer</i>
Deoxynivalenol	<i>wheat, maize, barley</i>
T2 \ HT2 Toxins	<i>wheat, maize, barley, rye</i>
Zearalenone	<i>maize, wheat</i>
Fumonisin (B1, B2, B3, B4)	<i>maize</i>
Penicillic Acid	<i>maize</i>
Moniliformin	<i>maize</i>
Ergot Alkaloids	<i>rye</i>
Patulin	<i>apples and derivatives</i>



Deoxynivalenol



T2 - Toxin



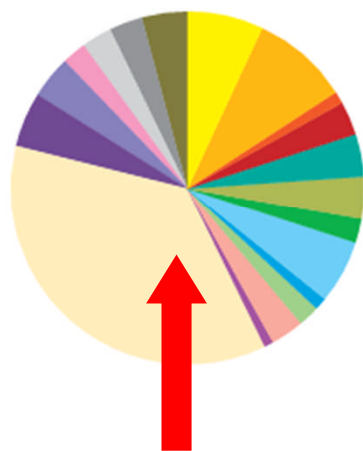


The Rapid Alert System  
for Food and Feed (RASFF)

## Annual Report 2010



### 2009 – BORDER REJECTIONS BY HAZARD CATEGORY



- (potentially) pathogenic micro-
- bad or insufficient controls
- biocontaminants
- composition
- food additives
- foreign bodies
- GMO/novel food
- heavy metals
- industrial contaminants
- labelling absent/incorrect
- microbiological contamination
- migration
- mycotoxins
- not determined/other
- organooleptic aspects
- packaging defective/incorrect
- parasitic infestation
- pesticide residues
- residues of veterinary medicinal products

### 2010 – TOP 10 NUMBER OF NOTIFICATIONS

#### By origin

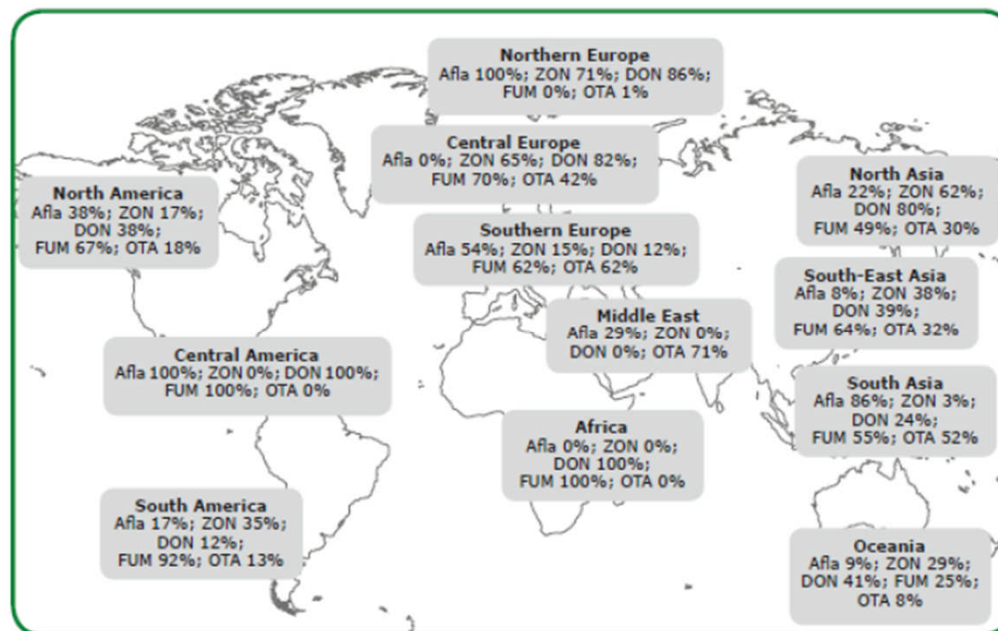
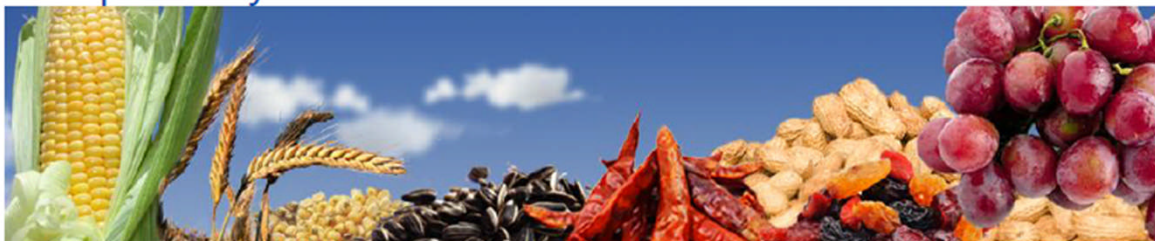
hazard	product category	country	notifications
aflatoxins	herbs and spices	India	96
aflatoxins	nuts, nut products and seeds	Argentina	95
aflatoxins	nuts, nut products and seeds	China	78
aflatoxins	fruit and vegetables	Turkey	58
aflatoxins	nuts, nut products and seeds	Iran	56
aflatoxins	nuts, nut products and seeds	Turkey	50
aflatoxins	nuts, nut products and seeds	The United States	49
unauthorised genetically modified	cereals and bakery products	China	46
mercury	fish and fish products	Spain	41
migration of chromium	food contact materials	China	35

#### By notifying country

hazard	product category	country	notifications
aflatoxins	nuts, nut products and seeds	Netherlands	139
aflatoxins	nuts, nut products and seeds	Germany	87
aflatoxins	herbs and spices	United Kingdom	69
mercury	fish and fish products	Italy	52
migration of chromium	food contact materials	Italy	43
parasitic infestation with Anisakis	fish and fish products	Italy	41
aflatoxins	nuts, nut products and seeds	Spain	35
aflatoxins	nuts, nut products and seeds	Greece	30
aflatoxins	nuts, nut products and seeds	United Kingdom	29
aflatoxins	nuts, nut products and seeds	Italy	29

rometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012

## European Mycotoxins Awareness Network



\* % refers to percentage of positive samples (>LOD)

Figure 1 gives an overview on the distribution of mycotoxins amongst different world regions.

From the 804 survey samples analysed, 31 %, 48 %, 62 %, 63 % and 32 % tested positive for contamination with Afla, ZON, DON, FUM and OTA, respectively. Mycotoxins are a ubiquitous problem as 89 % of the analysed samples show the presence of, at least, one mycotoxin. The presence of more than one mycotoxin in 53 % of the samples raises the attention to the problem of synergistic effects caused by multiple mycotoxins in animal feeds.

...often more than one  
single mycotoxin  
involved...

EMAN BULLETIN  
Autumn 2011

safety - Workshop Rome October, 11-12<sup>th</sup> 2012  
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## Mycotoxins analysis – main steps

### How to obtain reliable results ?

#### HOW TO SAMPLE A BIG LOT ?

- **Take** incremental samples in truck, cargo, in silo
- **Agregate** incremental samples
- **Divide** into sub-samples
- **Keep** samples
- **Send** samples to labs for analysis

**Sampling  
uncertainty**

+

#### HOW TO DETECT AND ANALYSE ?

- **Rapid detection** tools for **traders**
- **Standardized analysis** methods for accredited **labs**

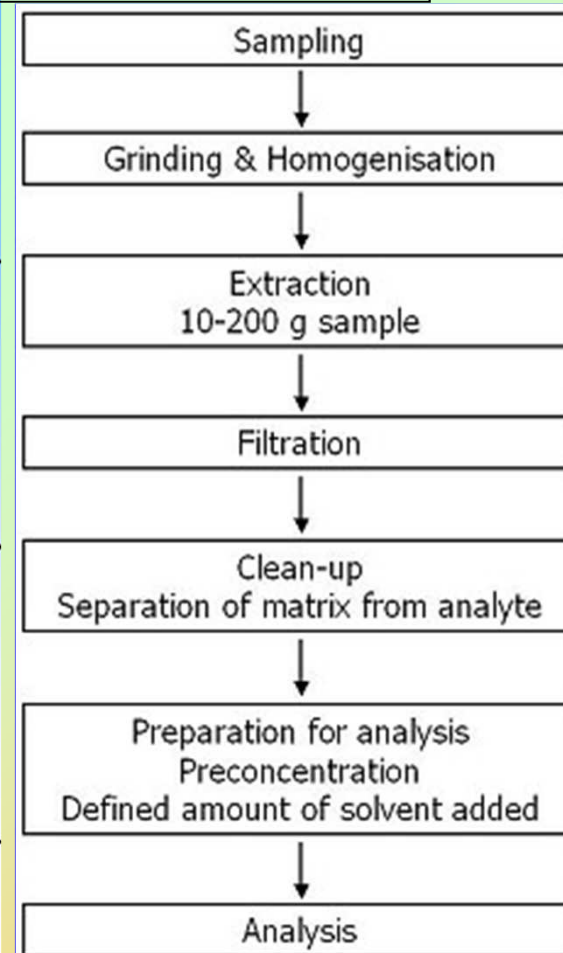
**Analysis  
uncertainty**

=

#### HOW TO INTERPRET ANALYTICAL RESULTS Trade to first-processing companies

**Total  
uncertainty**

Source: S. Picardat – 6<sup>th</sup> Fusarium Forum, Bruxelles 2009





# 1° Issue: Harmonize the complicate starting point: ...sampling!!



COMMISSION REGULATION (EC) No 1881/2006  
of 19 December 2006  
setting maximum levels for certain contaminants in foodstuffs  
(Text with EEA relevance)

COMMISSION REGULATION (EC) No 1126/2007  
of 28 September 2007  
amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in  
foodstuffs as regards *Fusarium* toxins in maize and maize products  
(Text with EEA relevance)

COMMISSION REGULATION (EC) No 401/2006  
of 23 February 2006  
laying down the methods of sampling and analysis for the official control of the levels of  
mycotoxins in foodstuffs  
(Text with EEA relevance)

## EU Legislative Requirements



## Sampling protocol projects

copa\*cogeca  
european farmers european agri-cooperatives

	Official controls (CE/401/2006)	IWA Seattle	CEN pr24333.2	AFNOR XP V03-777
<b>Project leader</b>	DGSANCO	AOCS, ICC, AACC, ANSI	CEN TC338 (DE, FR, UK)	AFNOR
<b>Publication</b>	<b>23/02/2006</b>	Mid 2009 ?	Summer 2009	<b>June 2008</b>
<b>Scope</b>	Regulated mycotoxins	technological and safety criteria,	technological and safety criteria	technological and safety criteria
<b>Number of samples</b>	-	<b>Project based on pr24333.2</b>	<b>pr24333.2 (uncertainty: 8%)</b>	<b>Less than pr24333.2 (uncertainty: 15%)</b>
<b>Statistical model</b>	<b>NO</b>	<b>YES for technological and safety criteria NO for GMO</b>	<b>YES for technological and safety criteria</b>	<b>YES for technological and safety criteria</b>
<b>Products</b>	<b>Food products</b>	<b>All grains and derived products</b>	Cereals and derived products	Cereals and derived products
<b>Transport</b>	Road, Railway, Cargo	Road, Railway, Cargo	Road, Railway, Cargo	Road, Railway, Cargo



## SOME OF THEM....:

### *Trichothecenes*

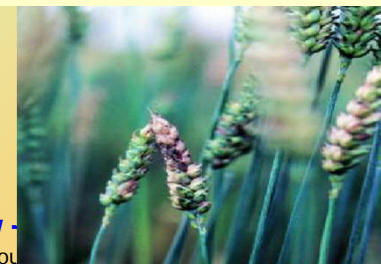
- A major class of *Fusarium* toxins are the Trichothecenes. Over 150 Trichothecenes are known, of which **deoxynivalenol (DON) is the most well-known**. Other trichothecenes of major significance include acetyl DON, nivalenol (NIV), **T-2** toxin and **HT-2** toxin.
- **DON is commonly present in wheat**. DON leads for example to undesirable **effects on the immune system**, growth retardation in children and feed refusal by pigs. It determines also **neurotoxic effects**.

### *Zearalenone*

- A **mycoestrogen**, Zearalenone has attracted attention because environmental estrogens have the potential to disrupt sex steroid hormone functions. **Genotoxicity** is also a reported concern; furthermore, occasional outbreaks of Zearalenone mycotoxicosis in livestock are known to cause **infertility**.
- This toxin is **found almost entirely in grains, in highly variable amounts** ranging from a few to thousands of ng/g.

### *Fumonisin*

- These mycotoxins are **mainly produced by the maize pathogens** *Fusarium verticillioides* and *Fusarium proliferatum*. Maize containing foods are in fact the major fumonisin concern for food industry.
- Fumonisin are highly water-soluble and are **extremely stable to a variety of heat/chemical processing operations**. They are **associated with increased incidence of esophageal cancers** (IARC, 1993), present **immunosuppressive effects** and may be a risk factor in neural-tube and **related birth defects**.

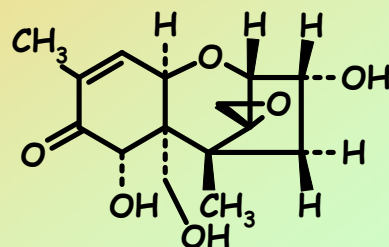


## FUSARIUM MYCOTOXINS

Main contaminants of cereals (wheat, maize, others)  
(very different chemical structures...)

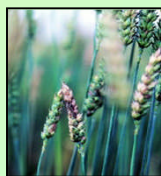
### Trichothecenes

(*F. graminearum*, *F. culmorum*)

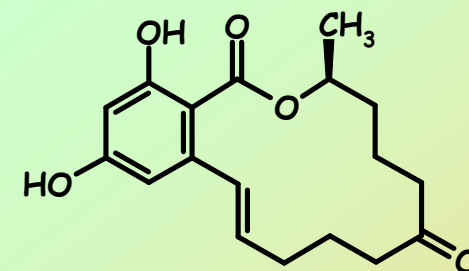


DON

Immunosuppression

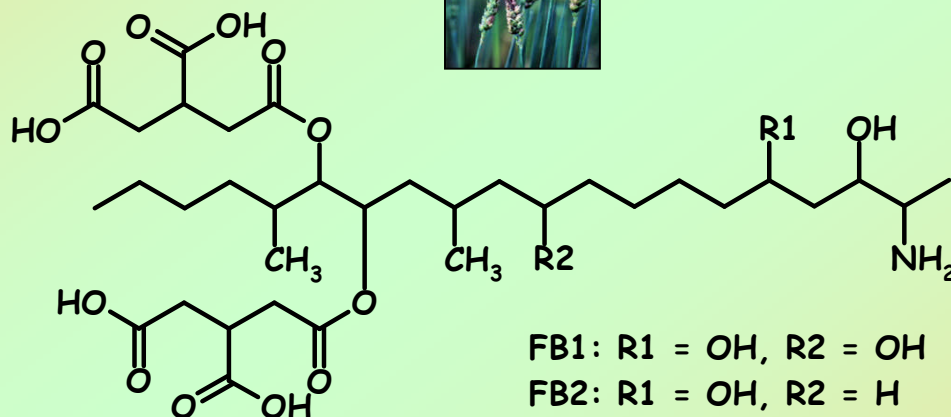


### Zearalenone



ZON

Oestrogenic activity



FB1: R1 = OH, R2 = OH  
FB2: R1 = OH, R2 = H  
FB3: R1 = H, R2 = OH

### Fumonisin

(*F. verticillioides*, *F. proliferatum*)

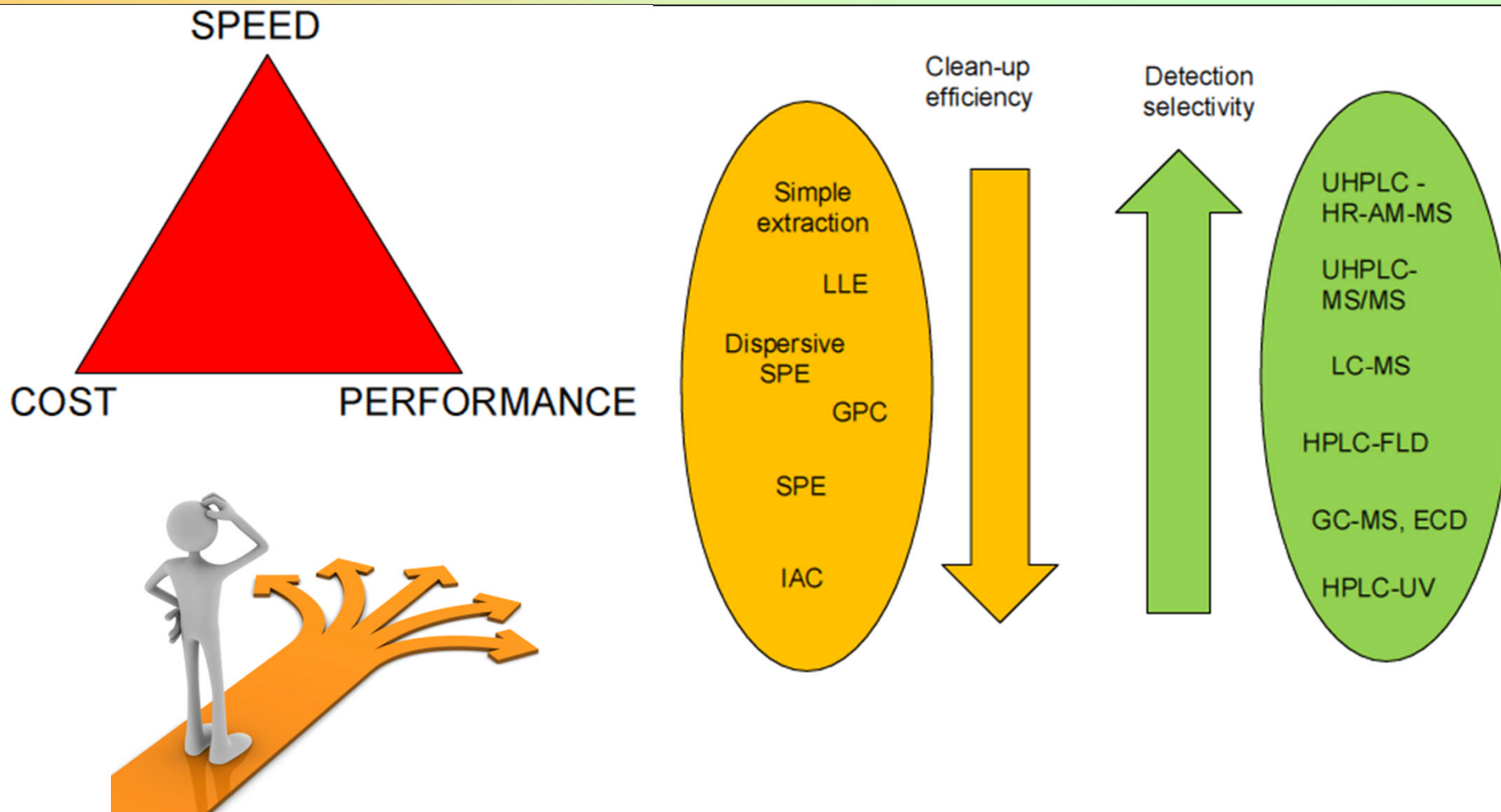
FB1: class 2B carcinogen (IARC)

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# Analytical Strategies – Directions...



## ***Extraction Step – some examples***

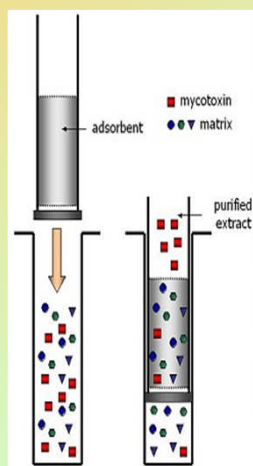
Toxin	Food	Extraction Solvent
Aflatoxins	Maize, Peanuts Milk powder	acetone:water (85:15) CH <sub>3</sub> Cl:water (91:9) acetone:water (70:30)
Ochratoxins	Barley	CH <sub>3</sub> Cl + 0.1M H <sub>3</sub> PO <sub>4</sub>
Patulin	Apple juice	ethyl acetate
Trichothecenes	Cereals	MeOH:water (90:10)

**Methods AOAC : Association of Official Analytical Chemists**



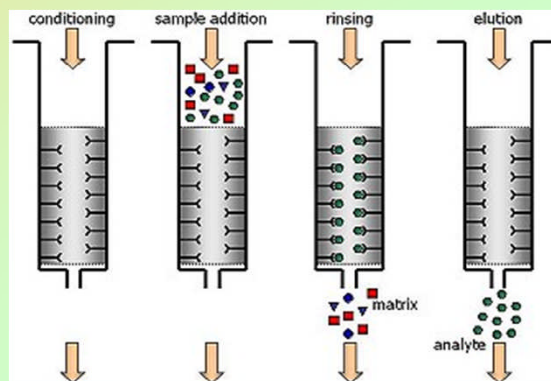
# Samples clean up: find the right compromise!

## Chemical Clean up



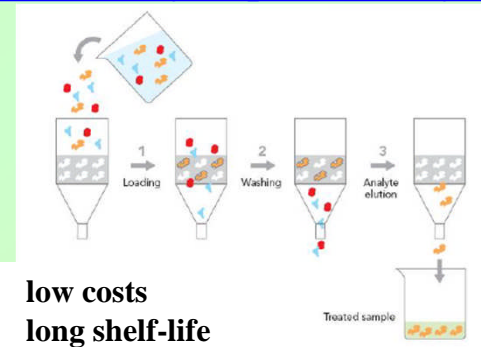
- easy and quick
- low selectivity
- chemical stability
- low costs

## Immunoaffinity Clean up



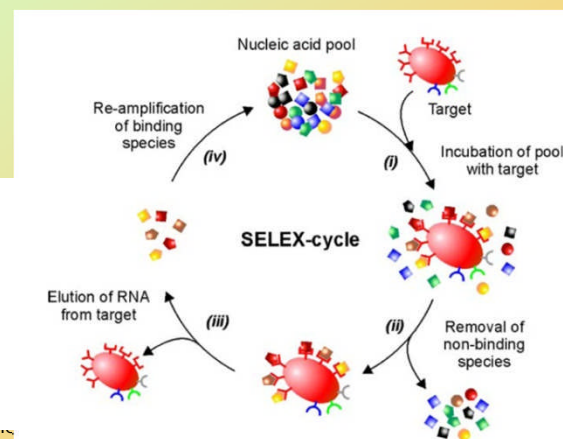
- high selectivity
- cleaner extracts
- variability batch to batch
- expensive

## Molecularly Imprinted Polymers



- low costs
- long shelf-life
- binding/rebinding
- low selectivity

## Aptamers



- high affinity/specificity
- in vitro selection
- broad range of applications, including biosensors, affinity columns, LFD,...

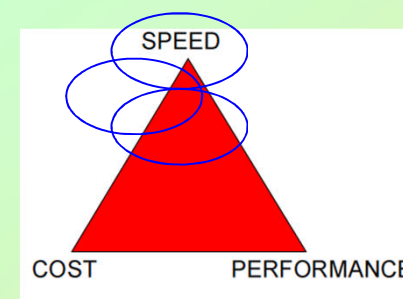
Examples

Tandem and High Resolution

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# ANALYTICAL TECHNIQUES ON MYCOTOXINS ISSUE



**Rapid Screening solutions**

*Michele Suman*

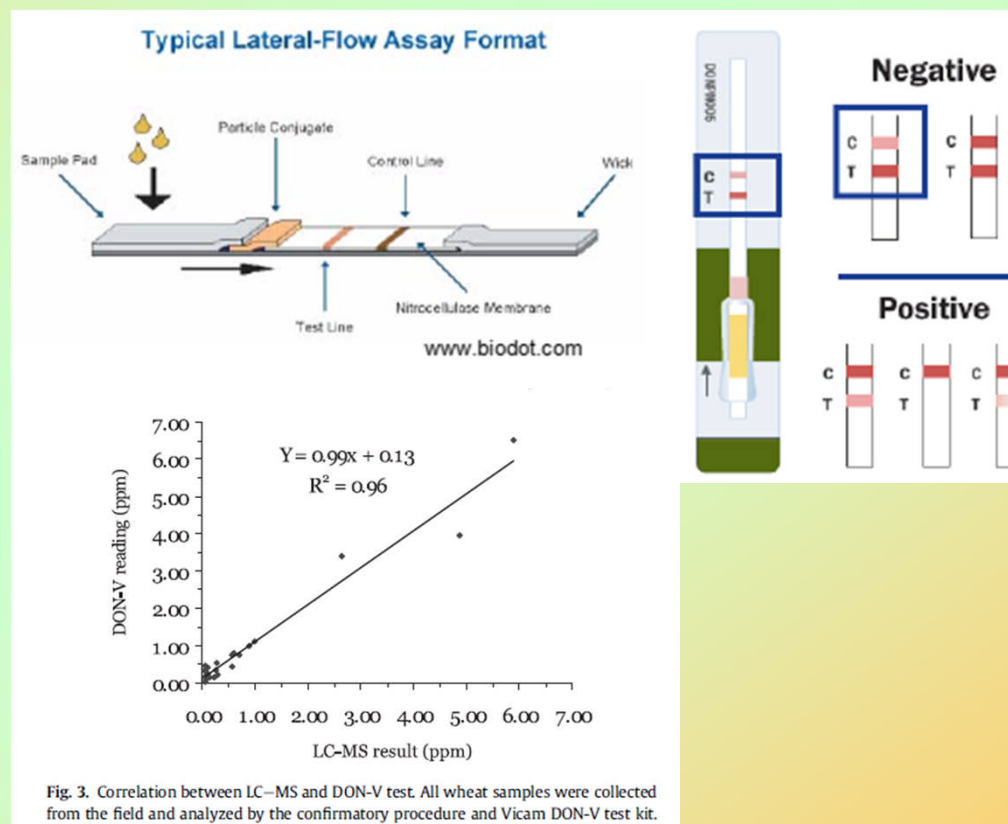
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## Rapid Screening Solutions: focus on Lateral Flow Devices development!

- antibody-colloidal gold particle complex mixed with sample extract
- migration onto a nitrocellulose membrane
- mycotoxin-protein conjugate coated on test zone to capture free antibody-colloidal gold
- photometric reflectance reader for colour reaction

- They are studied for having a fast answer “on-site” (attractive also in the places where there is no laboratory setting available) of the contamination
- Possibly portable, cost-effective,...
- No expensive equipment\high trained personnel required
- ❖ Analytical determination for “semiquantitative” proposals: yes/no answer at a certain cut off level.
- ❖ Work is going on in the quantitative screening direction...looking for adequate levels of accuracy, repeatability and reproducibility...



Development and practical application in the cereal food industry of a rapid and quantitative lateral flow immunoassay for deoxynivalenol

J. Liu<sup>a</sup>, S. Zanardi<sup>b</sup>, S. Powers<sup>a</sup>, M. Suman<sup>b,\*</sup>

Food Control 26 (2012) 88–91

<sup>a</sup> VICAM A Waters Business, 34 Maple St., Milford, MA 01757, USA

<sup>b</sup> Barilla G. R. E.lli SpA, Food Research Labs, via Mantova 166, 43122 Parma, Italy

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Tandem and High

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2012  
pany.

## Multi-mycotoxin dipsticks: Protocol of analysis



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



Methanol/water  
Blending  
(for all mycotoxins)

Dilution  
different  
dilution factors



Incubation at 40°C  
Migration  
*Optimized conditions*

Dipstick reader (Readsensor)



**Total analysis time:  
30 min**



Angelo Visconti, Veronica Lattanzio, Annalisa De Girolamo,  
Vincenzo Lippolis, Michelangelo Pascale  
Institute of Sciences of Food Production (ISPA)  
National Research Council of Italy (CNR)

6<sup>th</sup> April 2011, CTICC, Cape Town, South Africa



MYCORED AFRICA  
2011 CONFERENCE

MYCORED





## Multi-mycotoxin dipsticks: Analysis of Naturally Contaminated Maize Samples

Achieved cut off levels in cereals, cereal foods, maize feed

CUT OFF levels (µg/kg) (fixed at target levels corresponding to 80% of European MRL)

	ZEA	T-2 +HT-2	DON	FB <sub>1</sub> +FB <sub>2</sub>
WHEAT	80	400	1400	-
OATS	80	400	1400	-
MAIZE	280	400	1400	3200
MAIZE FEED	2400	400	9600	5000
WHEAT based BREAKFAST CEREALS	40	80	400	-
MAIZE based BREAKFAST CEREALS	80	80	400	640

Sample	ZEA		T-2 +HT-2		DON		FB <sub>1</sub> +FB <sub>2</sub>	
	dipstick	LCMSMS µg/kg	dipstick	LCMSMS µg/kg	dipstick	LCMSMS µg/kg	dipstick	LCMSMS µg/kg
1	NEG	n.d.	NEG	n.d.	NEG	n.d.	NEG	725
2	NEG	n.d.	NEG	n.d.	POS	24200	POS	8150
3	POS	420	POS	392	NEG	298	NEG	725



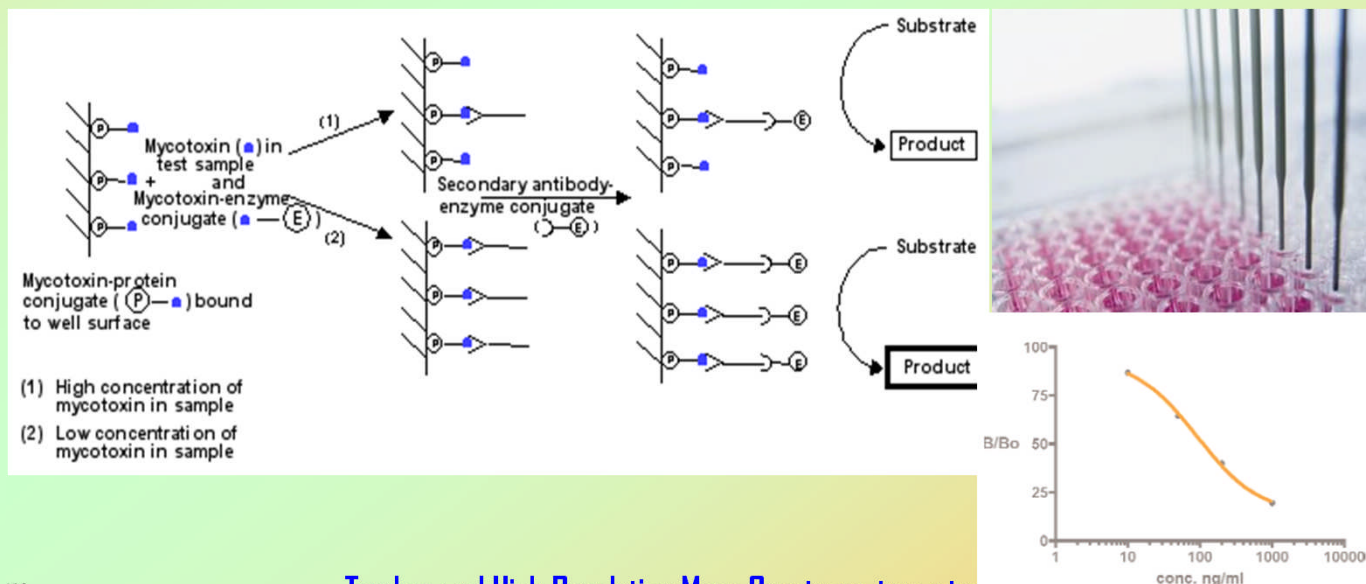
...still a lot of work to be done for achieving the goal of the «multi»-lfd solutions...

## Enzyme-Linked Immunosorbent Assay - ELISA

The common methods of analysis for mycotoxins are based on the use of ELISA (Enzyme-Linked Immunosorbent Assay) techniques, mainly adopted for **rapid and relatively cheap screening**, exploiting also generally **ready to use reagents**.

The specific antibody is linked to a solid phase: sample extract is added, then the enzymatic conjugated constituted from the **mycotoxins tied to a peroxidase** is added in excess and goes to occupy the remained free bounding sites. The detection happens by indirect way, in fact the addition of the substrate and the **chromogen determine the appearance of a colourful compound**.

The currently tests in commerce generally have an **incubation time of 20-60 min** and comprise the simultaneous analysis of tenths of samples; the technique is fast but can be subject to **crossed reactivity**.



## ANALYTICAL TECHNIQUES ON MYCOTOXINS ISSUE



**Emerging new strategies**

*Michele Suman*

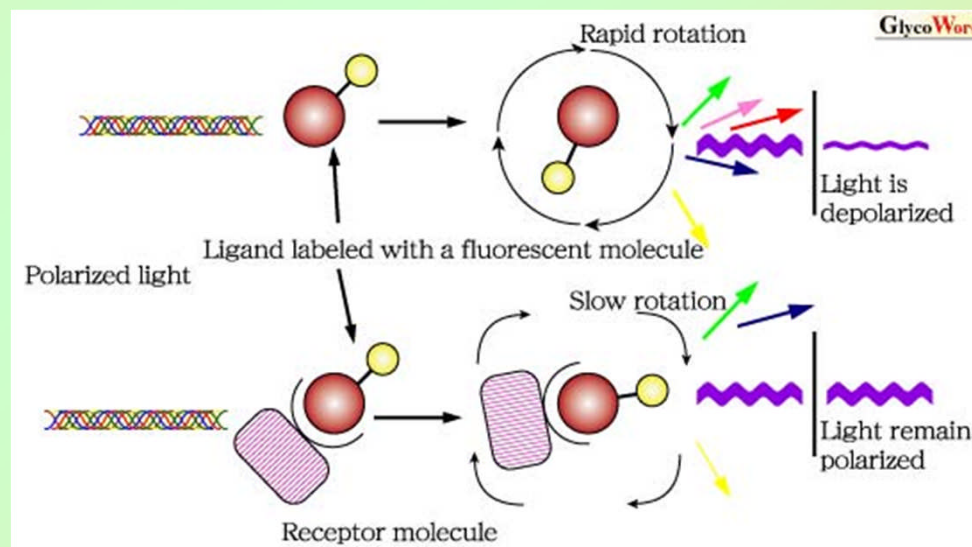
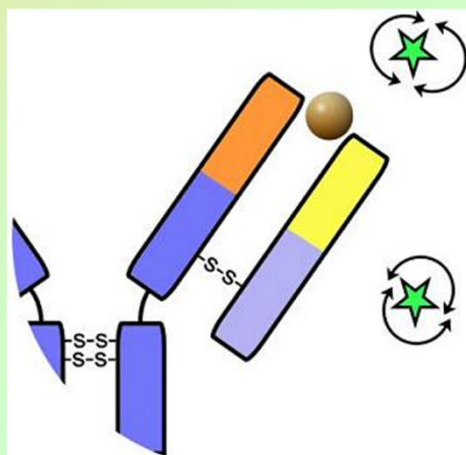
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## FP (Fluorescence Polarisation immunoassay format)

The **toxin is marked with a fluorotrope**. Marked toxin and free toxin (extracted from the sample) compete with antibodies for the specific binding sites. If the antibody bind marked toxin, the increase of the mass of the complex cause **change of the polarity of the incident radiation**. The measured change of polarization is therefore inversely proportional to the free toxin concentration in the sample.

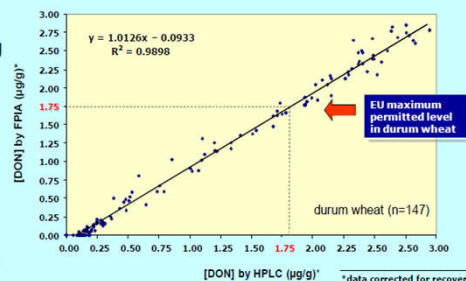


Source: Chris Maragos, *Toxins* 2009, 1(2), 196-207; doi:[10.3390/toxins1020196](https://doi.org/10.3390/toxins1020196)



## FPIA – DON in wheat and derivative products

- ✓ **applicability:** durum wheat, common wheat, semolina, pasta
- ✓ **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., J. Food Prot., 2006, 69, 2712-2719

## Automated FPIA - DON in wheat and derivative products\*



- ✓ The automated FP system has been developed by assembling a FP reader with an autosampler assisted by a PC through a specific software for data handling.

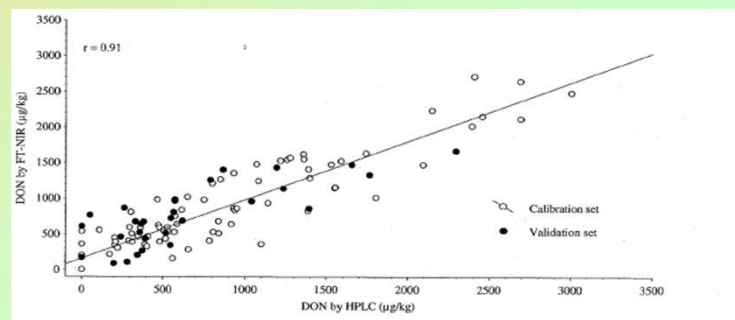
### ADVANTAGES

- Fully automated
- Easy-to-use
- Good precision (<5%)
- Useful and practical alternative to HPLC
- More convenient than HPLC for routine analyses due to higher throughput  
(15 samples / 3 h vs. 1 sample / 3 h)

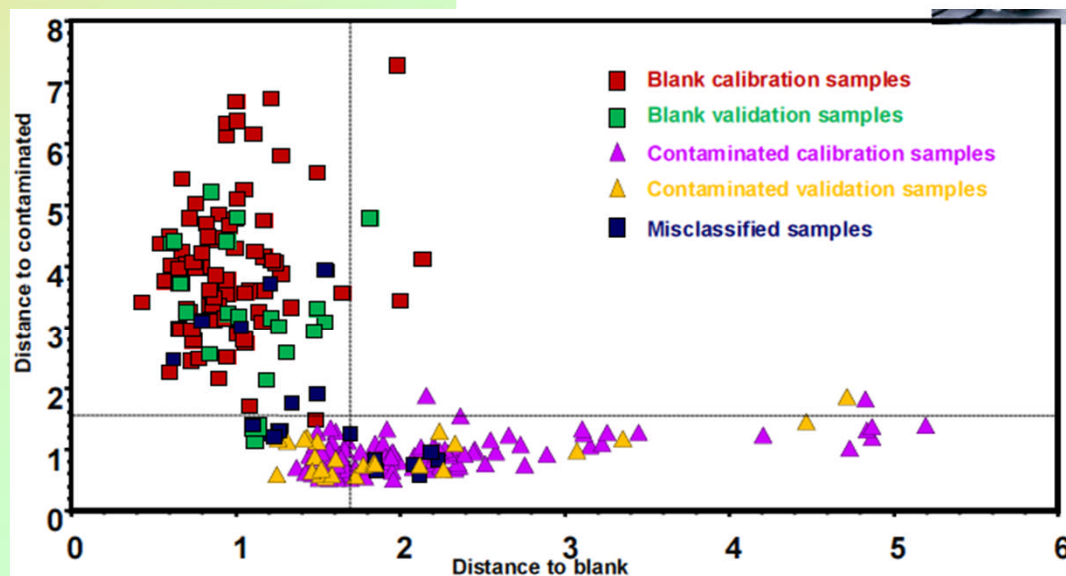
\* European Patent Application No. 1882938A2. Visconti A., Pascale M., Lippolis V., Ranieri R., Silvestri M. e D'Alessandro A.

### Near-infrared Spectroscopy - (NIR / FT-NIR)

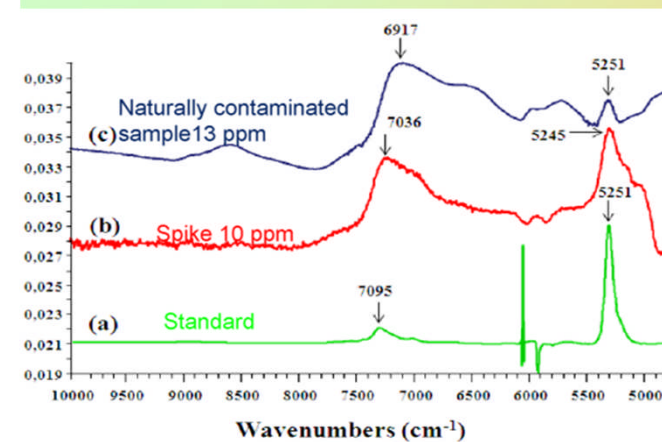
Fourier transform near-infrared spectroscopy (FT-NIR) was used for **rapid and non-destructive analysis** of deoxynivalenol (DON) in durum and common wheat. The relevance of using ground wheat samples with a homogeneous particle size distribution to minimize measurement variations and avoid DON segregation.



FT-NIR semi-quantitative model to discriminate between blank and naturally contaminated wheat samples at around 300 µg/kg



De Girolamo, A., Lippolis, V., Nordkvist, E. and Visconti, A.: Rapid and non-invasive analysis of deoxynivalenol in durum and common wheat by Fourier-Transform Near Infrared (FT-NIR) spectroscopy, Food Additives & Contaminants: Part A, 26:6, 907 — 917, (2009)



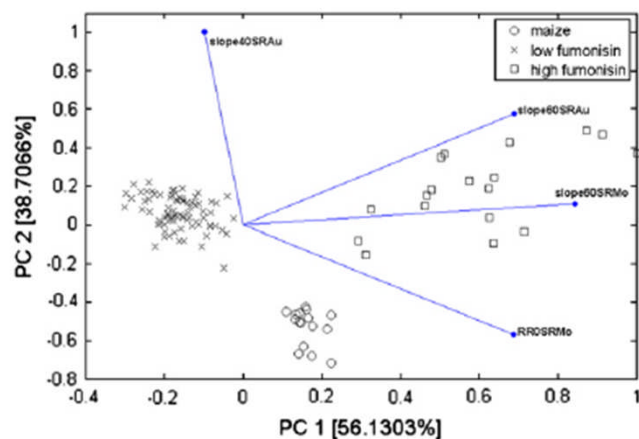
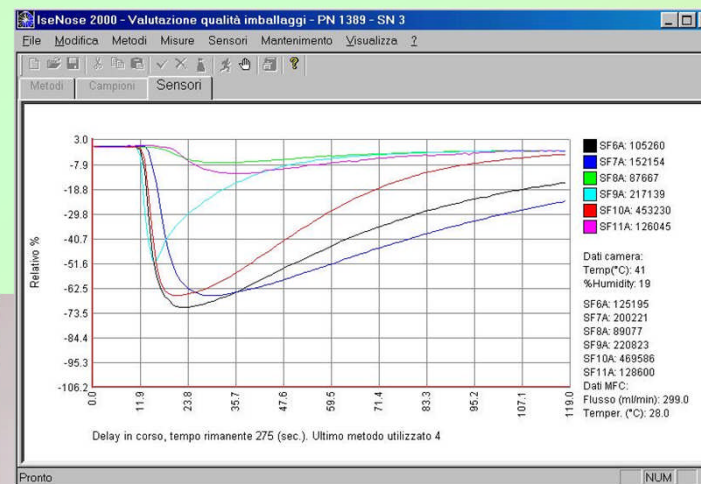
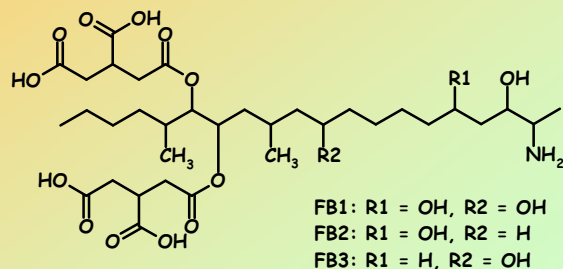
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## Electronic Nose for prediction of high or low contamination levels

Electronic nose, based on metal oxide semiconductor chemical sensors arrays, demonstrate ability to diagnose fungal contamination and to detect \predict high or low fumonisins content in inoculated maize cultures.



**Fig. 4.** PCA biplot of the first two principal components PC1 and PC2 (scores and loadings were scaled to fit on the same plot) obtained by selecting only four features: R/ROSRMo, Slope60SRMo, Slope60SRAu and Slope40SRAu. The samples are labelled with their fumonisin content. Clusters corresponding to the fumonisin content are separated on the PC1. The sterile maize class (maize) is well separated from all the other samples on both PCs.



Source: E. Gobbi, M. Falasconi, E. Torelli, G. Sberveglieri; *Food Research International* 2011, Vol. 44, pp. 992–999

Michele Suman

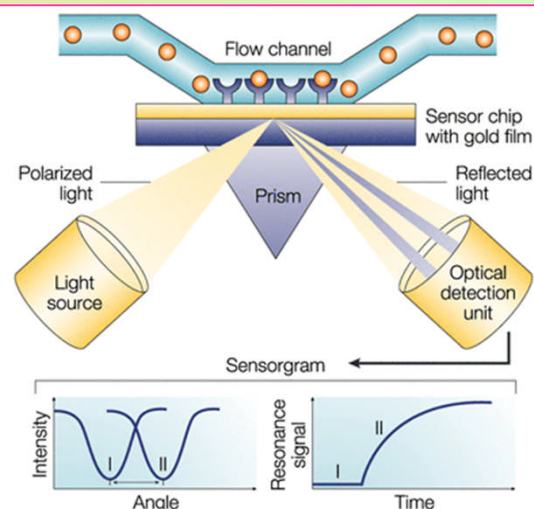
**Tandem and High Resolution Mass Spectrometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012**

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## SPR (Surface Plasmon Resonance-based immunoassays)

It uses **antigen (protein+toxin) in a solid phase** that is connected with an optical measurement device. Specific antibodies are before incubated with the sample to be analyzed, then they are injected on the solid surface. If the sample is not contaminated, the antibodies incubated, remained free, are tied to the antigenic substrate causing a **change of the optical characteristics** of the matrix analyzed. The optical signal is therefore inversely proportional to the toxin concentration in the sample. It is a relatively new technique, less sensitive regarding ELISA, but probably more robust.

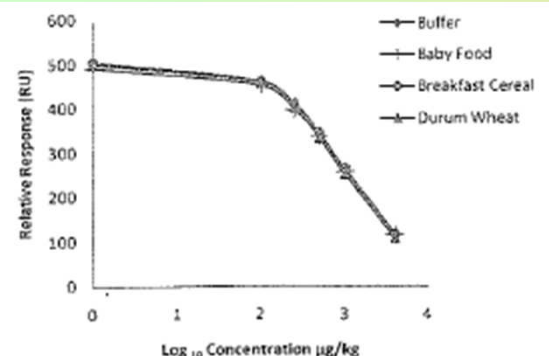


Nature Reviews | Drug Discovery

Source: Matthew A. Cooper *Nature Reviews Drug Discovery* 1, 515-528 (July 2002)

### Rapid Surface Plasmon Resonance Immunoassay for the Determination of Deoxynivalenol in Wheat, Wheat Products, and Maize-Based Baby Food

- assay has been proven to offer sensitive, accurate and reliable results
- It seems to be potentially applicable also to other cereal matrixes
- no clean-up in the extraction phase

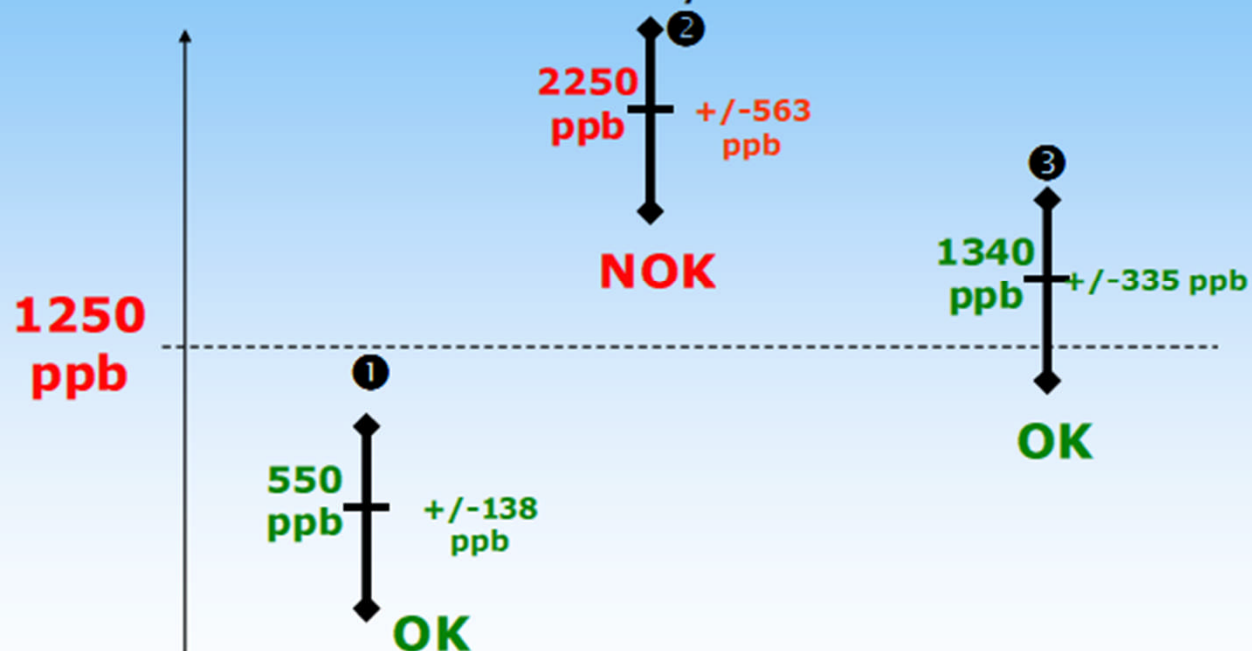


Source: J. Meneely et al.  
*J. Agric. Food Chem*  
58, 8936-8941 (2010)



## Uncertainty about result analysis

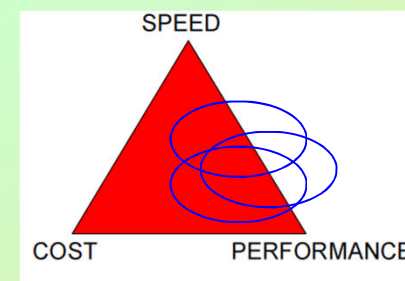
**Example** : DON results on wheat for food chain  
with uncertainty rate = 25%



**Results interpretation according to  
Reg. CE/401/2006 Annex II.4.4**

15

## ***ANALYTICAL TECHNIQUES ON MYCOTOXINS ISSUE***



**Consolidated solutions**

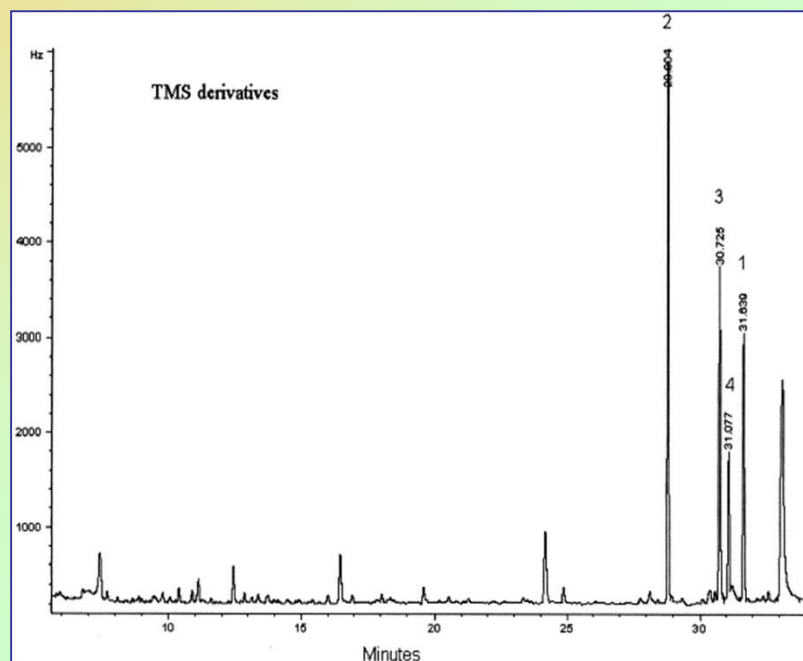
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## Gas chromatographic analysis

The Gas chromatographic analysis has had **relatively little diffusion** in the mycotoxins research due to the fact that most of these molecules is not volatile. However applications exists for **not fluorescent mycotoxins** like Type A and B Trichothecenes, working especially with Electron Capture (ECD) and Mass Analyzer (MS) detectors: the analysis is **highly sensitive but need immunoaffinity/SPE clean-up and a pre-derivatization approach**, mainly with formation of trimethylsilyl- and fluoroacil-derivatives.



**Total ion chromatogram  
TMS-derivatives:**  
(1) DON; (2) 3-ac-DON; (3)  
DAS; (4) FUS-X

LOD:

DON → 5 µg/kg



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## HPLC-UV and HPLC-FLD Techniques

RP-HPLC-UV and RP-HPLC-FLD are **widely diffused approaches** for **Fusarium mycotoxins** determinations, as they guarantee a **great sensibility and accuracy (with respect to ELISA)**, allowing moreover the **analysis automation**. For example, the type B Trichothecenes can be revealed via UV exploiting the double bond on the C8.

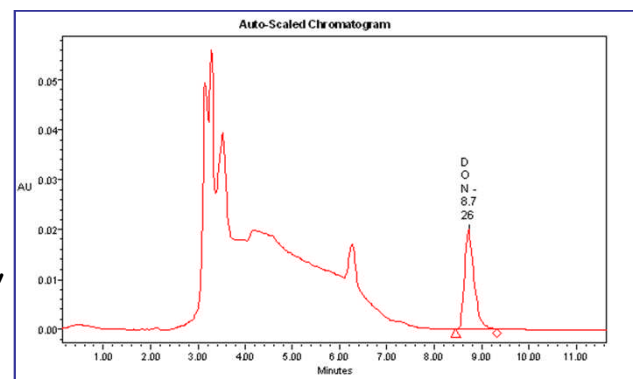
The same analysis cannot be lead on the type A for absence of adequate chromogenic groups; consequently it is necessary to act with a **pre- or post-column derivatization** in order to form adducts detectable by fluorescence (derivatization agents commonly adopted are anthracene, coumarin-carbonyl chlorides, acetyl acetate\ammonium acetate).

### Trichothecenes B: method HPLC-UV

Trichothecenes B: present a chromophore group

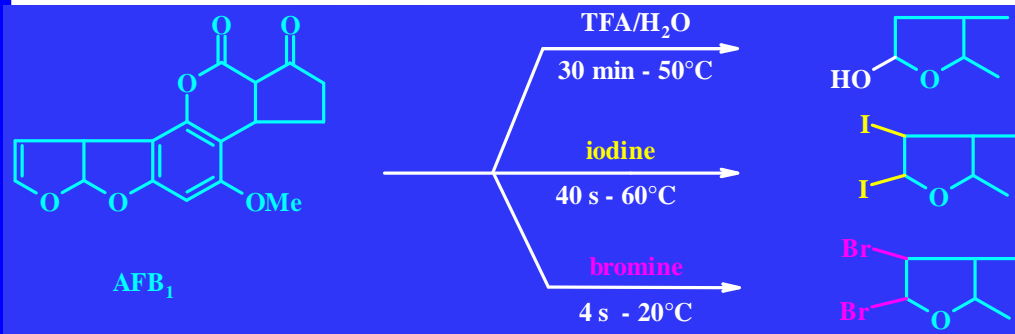
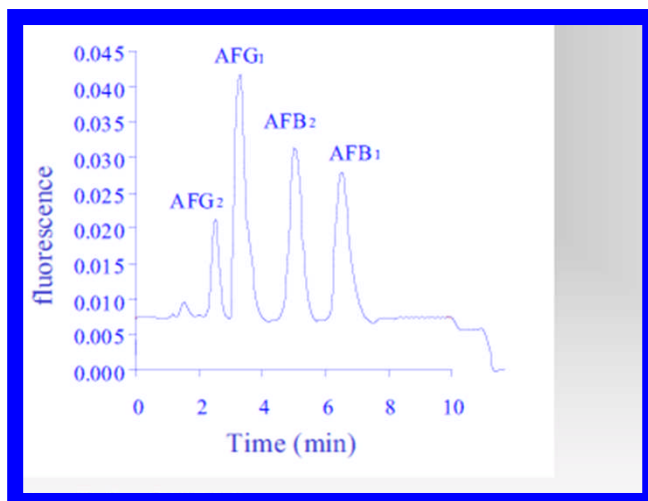
LOQ ~ 100 µg/kg

Relatively easy procedure but with low specificity,  
unless using dedicated IAC clean up



Durum wheat flour spiked up to reach a level of 700 µg/kg DON

### Aflatoxins: method based on HPLC-FLD



Aflatoxins: pre and post column derivatization for improving fluorescence properties



## Ultra performance\fast liquid chromatography UPLC / UHPLC

Small particle size (e.g. 1.7  $\mu\text{m}$ )

Shorter narrow-bore columns (e.g. 50 mm x 2.1 mm)

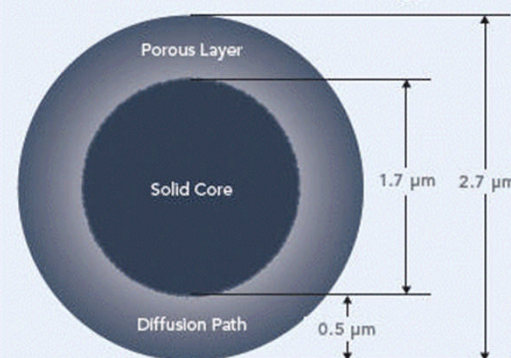
Higher pressures than conventional columns (up to 600 bar)

**Short analysis times (1-3 min)**

**No loss in column efficiency**

**Improved sensitivity**

FIGURE 4: Fused-Core Particle Technology



Fused-Core particle technology was developed by Jack Kirkland to produce UHPLC columns that could be effectively used with either UHPLC or conventional HPLC equipment. As the name implies, Fused-Core particles are manufactured by fusing a porous silica layer onto a solid silica particle.

Y. Ren et al. / J. Chromatogr. A 1143 (2007) 48–64

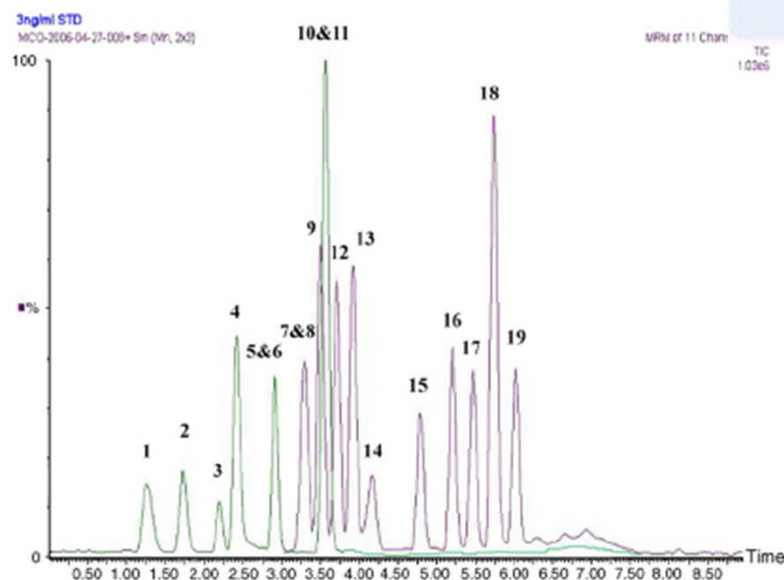


Fig. 4. The total ion chromatogram (TIC) of 17 mycotoxins plus zearalanone (IS) positive and negative ions: (1) NIV; (2) DON; (3) FX; (4) OTA; (5) 3-ADON; (6) 15-ADON; (7) ATG<sub>2</sub>; (8) ATM<sub>1</sub>; (9) ATG<sub>1</sub>; (10) ZON; (11) ZAN<sup>-</sup> (IS); (12) ATB<sub>2</sub>; (13) ATB<sub>1</sub>; (14) CTN; (15) HT-2; (16) T-2; (17) ZAN<sup>+</sup> (IS); (18) SMC; (19) VCG. The UPLC conditions were described in Section 2.4.

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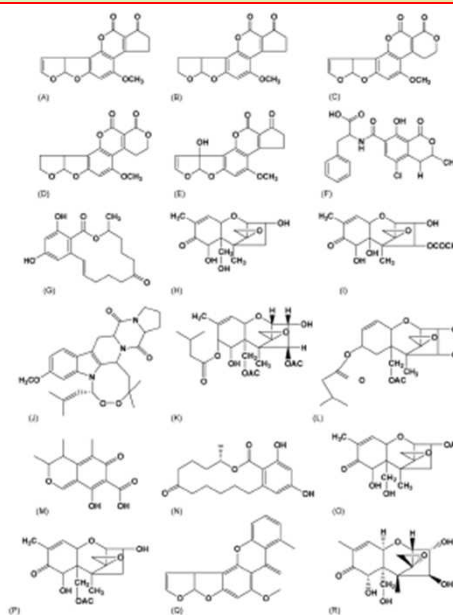
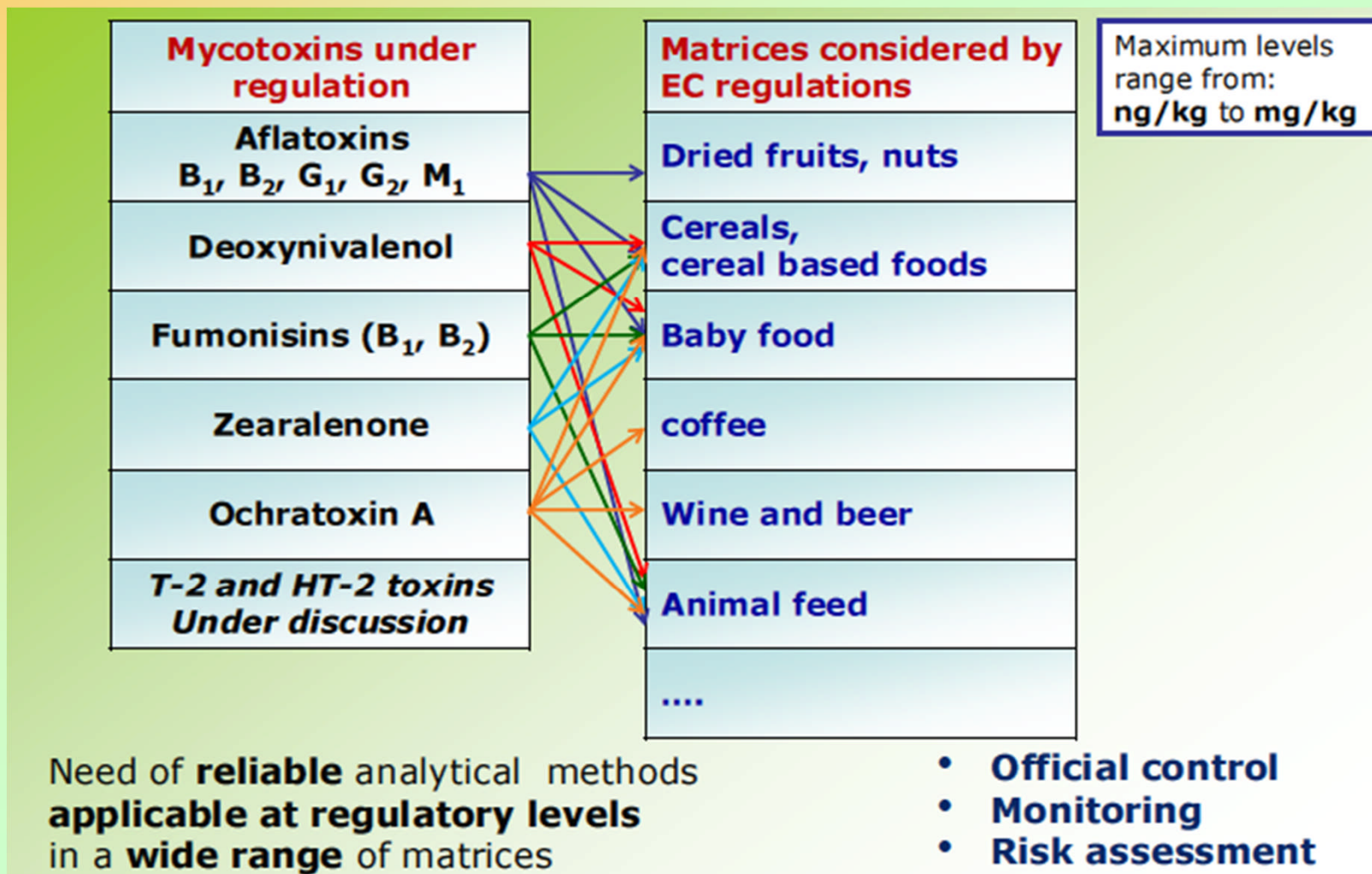
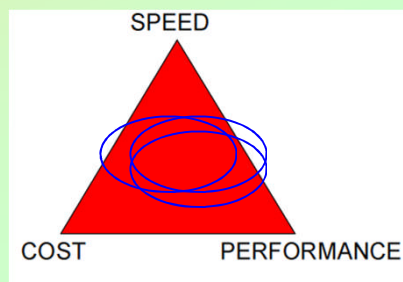


Fig. 4. Chemical structure of agaperone in the present study: (A) ACB; (B) ACB; (C) ACB; (D) ACB; (E) ACB; (F) ACB; (G) ACB; (H) ACB; (I) ACB; (J) ACB; (K) ACB; (L) ACB; (M) ACB; (N) ACB; (O) ACB; (P) ACB; (Q) ACB; (R) ACB; (S) ACB.

# EC regulations 1881/2006 and 1126/2007 Maximum permitted levels of mycotoxins



## ANALYTICAL TECHNIQUES ON MYCOTOXINS ISSUE



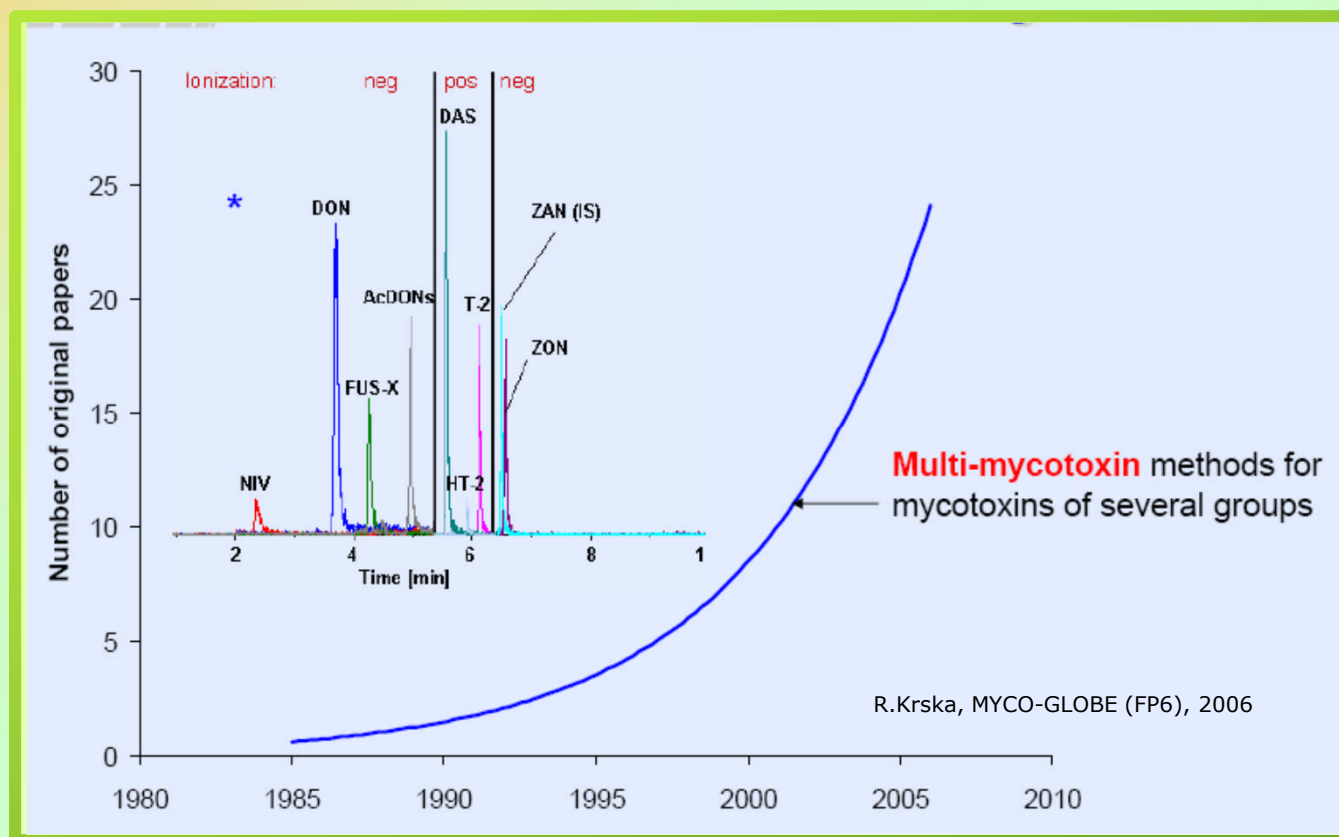
**Accurate-sensitive solutions**

*Michele Suman*

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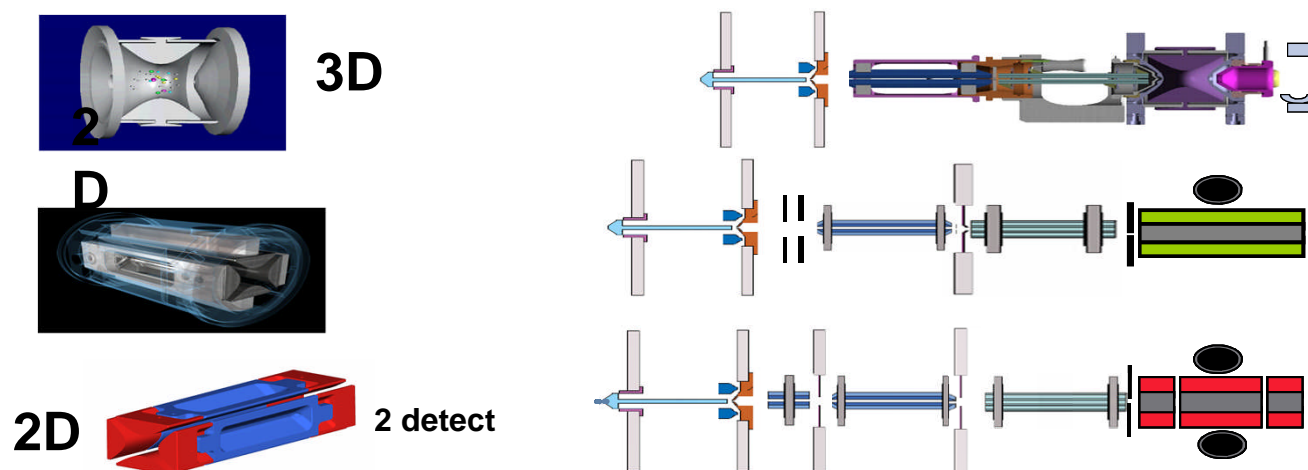
## Trend: Multi-micotoxin methods using LC-MS(MS)





## HPLC-MS<sup>n</sup> ION TRAP MASS SPECTROMETRY

- Ion trap mass spectrometry has recently undergone very rapid development and is emerging as interesting technique due to the possibilities to extend high mass/charge measurements and mass range, allow **MS<sup>n</sup> experiment** together with **selective ion manipulation techniques**.
- With respect to triple quadrupoles limitations occur in dynamic range, accurate mass measurement and a lower quantitative precision.
- **Quadrupole ion trap (3D)**, thanks to **relatively low cost, easy coupling with ion sources (ESI or APCI)**, small size, limited pressure requirements and **experimental flexibility** permit to reach satisfying compromises in terms of number and quality of mass spectrometry analytical experiments.
- Furthermore, the overall sensitivity and quantitative analysis performance (linearity and reproducibility) of **recent linear ion trap (2D) instruments** can be considered close to that of common triple quadrupoles.



## Fumonisin: ION TRAP LC/MS<sup>n</sup> approach:

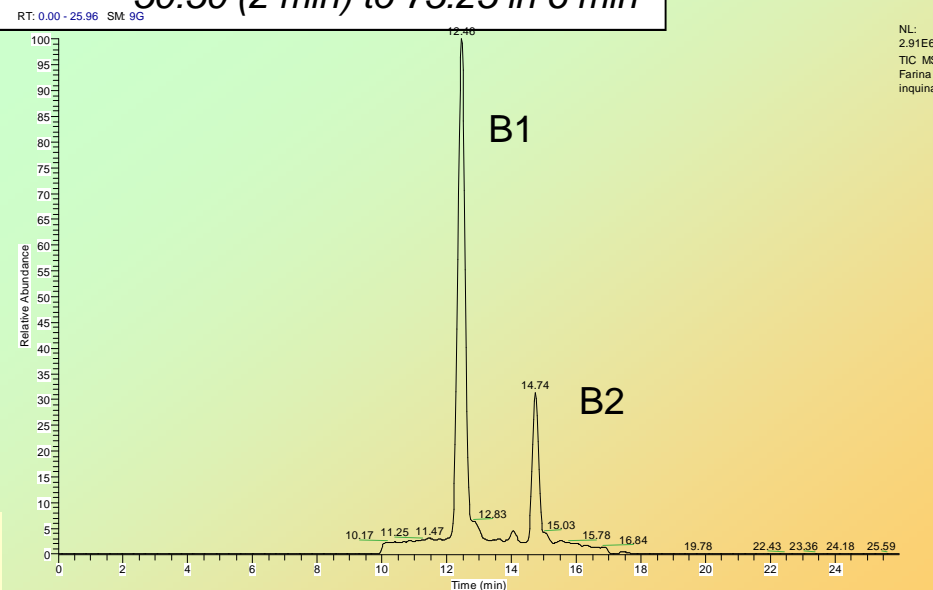
- 1. Sampling: 25g
- 2. Extraction with: 100 mL of MeOH: HCl (0.1M) = 3:1 (v/v)
- 3. Homogenization: *Ultraturrax* blending 2 minutes
- 4. Centrifugation: 3000 rpm 10 minutes
- 5. Filtration: 3 mL on syringe filter
- 6. LC-MS analysis
- Column: Synergi 4 $\mu$ m Hydro-RP 80 $^{\circ}$ , 150mm x 2.00mm,
- Mobile phase: MeOH:Formic acid 0.1% 50:50 (2 min) to 75:25 in 6 min



Mycotoxin	Parent ion m/z	Fragments monitored m/z
FB1	722	704, 528
FB2	706	688, 512

MS/MS parameters for detection of fumonisins:  
Fragmentation pattern characterized by losses  
of water and/or tricarballic acid molecules

Source: Suman, M. ; **XIIth IUPAC International Symposium on  
Mycotoxins and Phycotoxins**  
Istanbul – Turkey, May 21 – 25th , 2007



Chromatogram obtained from a Fumonisin naturally contaminated

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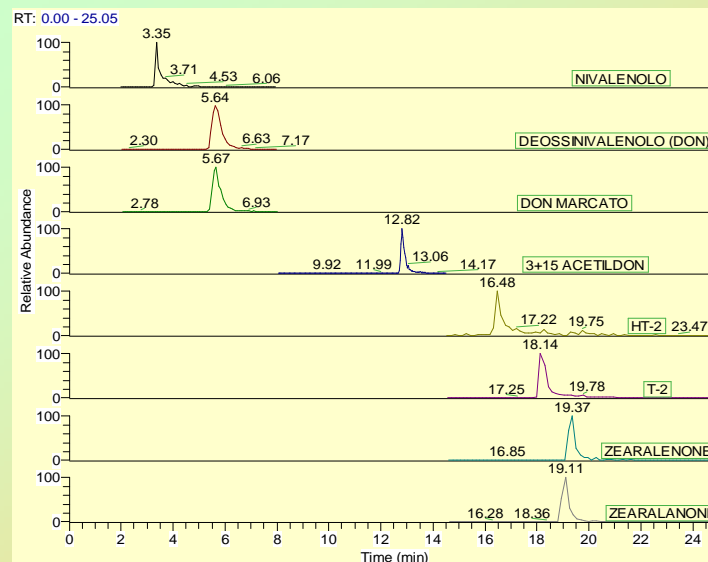
## Trichothecenes and ZON: ION TRAP LC/MS<sup>n</sup> approach:

- 1. Sampling: 20g
- 2. Extraction: 100 mL of a mixture of acetonitrile/water (84:16, v/v)
- 3. Homogenization: Ultraturrax blending 3 minutes
- 4. Filtration: 6 mL
- 5. Addition to a vial containing internal standards: ZAN + (<sup>13</sup>C<sub>15</sub>)-DON
- 6. Evaporation to dryness: under a nitrogen stream
- 7. Clean up: through MycoSep® 226 column.
- 8. Final preconcentration and LC-MS<sup>n</sup> analysis

Zearalanone (ZAN), which does not occur in nature, was used as internal standard for quantification of Zearalenone (ZON).

An isotope-labelled (<sup>13</sup>C<sub>15</sub>)-DON internal standard was used for the determination of the other trichothecenes and in particular for DON to efficiently correct for losses during sample preparation as well as matrix effects and ion-suppression effects in the ESI source.

Mycotoxin	Parent ion m/z	Fragments monitored m/z	Normalized Collision Energy %
[NIV+CH <sub>3</sub> COO] <sup>-</sup>	371	281, 311	24
[DON+CH <sub>3</sub> COO] <sup>-</sup>	355	295, 265	24
[( <sup>13</sup> C <sub>15</sub> )-DON +CH <sub>3</sub> COO] <sup>-</sup>	370	310, 290	24
[FUS-X+CH <sub>3</sub> COO] <sup>-</sup>	413	353, 187	20
[ADONs+CH <sub>3</sub> COO] <sup>-</sup>	397	337, 173	24
[DAS+NH <sub>4</sub> ] <sup>+</sup>	384	349, 307	20
[HT-2+NH <sub>4</sub> ] <sup>+</sup>	442	381	24
[T-2+NH <sub>4</sub> ] <sup>+</sup>	484	423	24
[ZON-H] <sup>+</sup>	317	299, 273, 203, 161	34
[ZAN-H] <sup>+</sup>	319	301, 275, 205, 163	34



Source: M. Suman, D. Catellani; *World Mycotoxins Journal* 2008, Vol-1(3), pp. 255-262.

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## HPLC-MS<sup>n</sup> TRIPLE QUADRUPOLE MASS SPECTROMETRY

Liquid Chromatography hyphenated to Mass spectrometry (LC-MS, **mainly Ion Traps and Triple Quadrupole instruments**) is nowadays the most flexible and effectiveness (**high sensitivity and selectivity**) technique used in order to determine mycotoxins in **many different matrixes**.

### Pros

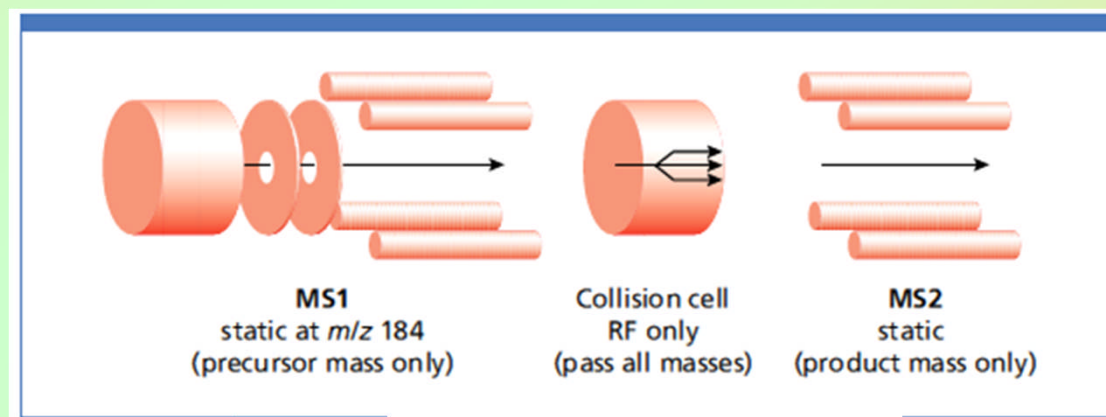
- unit resolution tandem MS experiments
- relatively low price within MS instruments
- easy operations
- high sensitivity in MRM experiments
- allows parent ion scanning & neutral loss studies

### Cons

- limited to MS<sup>2</sup> experiments

### Applications

- general tandem MS instrument
- sensitive detection needs (MRM)



Lemière

GUIDE TO LC-MS - December 2001

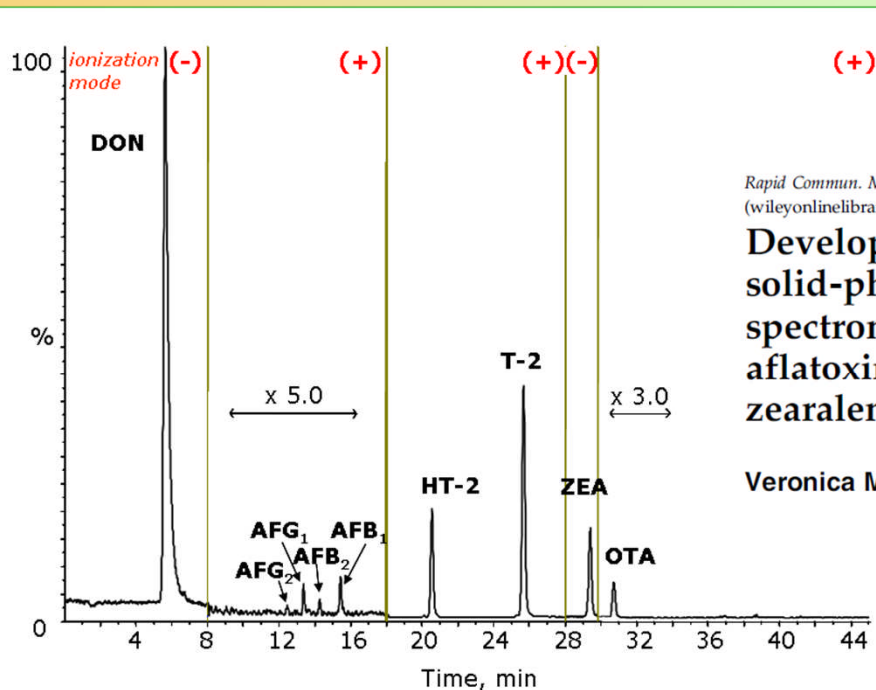
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## *SPE\LC-MS/MS to simultaneously quantify AFLA, OTA, DON, ZEA, T2-HT2 in cereal based foods*



Rapid Commun. Mass Spectrom. 2011, 25, 1869–1880  
(wileyonlinelibrary.com) DOI: 10.1002/rcm.5047

**Development and in-house validation of a robust and sensitive solid-phase extraction liquid chromatography/tandem mass spectrometry method for the quantitative determination of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>, ochratoxin A, deoxynivalenol, zearalenone, T-2 and HT-2 toxins in cereal-based foods**

Veronica M. T. Lattanzio<sup>1\*</sup>, Stefania Della Gatta<sup>1</sup>, Michele Suman<sup>2</sup> and Angelo Visconti<sup>1</sup>

Electrospray ionization triple quadrupole tandem mass spectrometry (MRM mode) [<sup>13</sup>C-labelled mycotoxins as IS]

V.M.T. Lattanzio, S. Della Gatta, M. Suman, A. Visconti, *Rapid Commun. Mass Spectrom.* 2011, 25, 1869-1880



**Wheat flour  
Barley flour  
Oat flour**



**Wheat based crisp bread  
Rye based crisp bread**

## Sample



*Extraction*

acetonitrile/water  
(84/16)



*Filtration (Whatmann N°4)  
Evaporation  
Reconstitution in  
methanol/water (10/90)*

**Clean-up with  
OASIS® HLB**

(polymeric SPE cartridges)

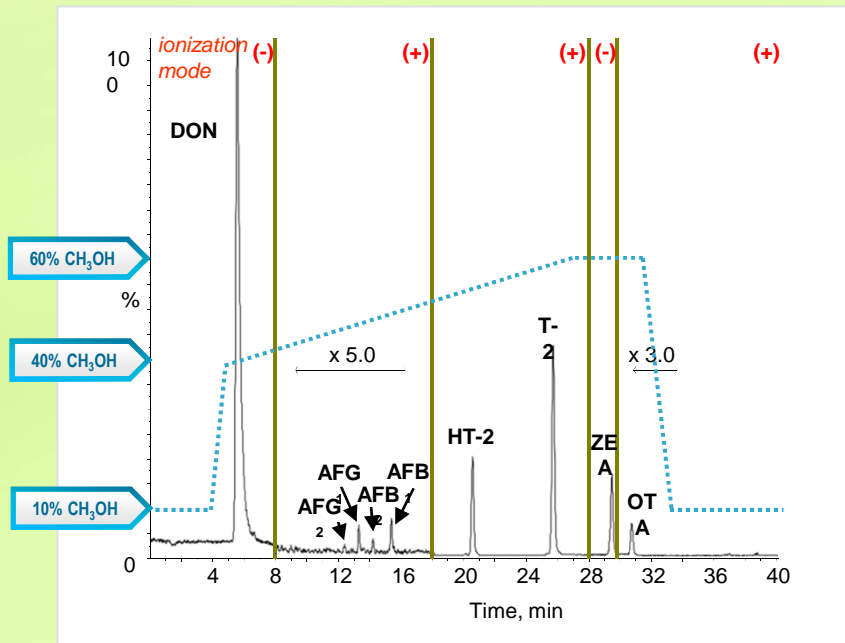


## LC-ESI-MS/MS analysis

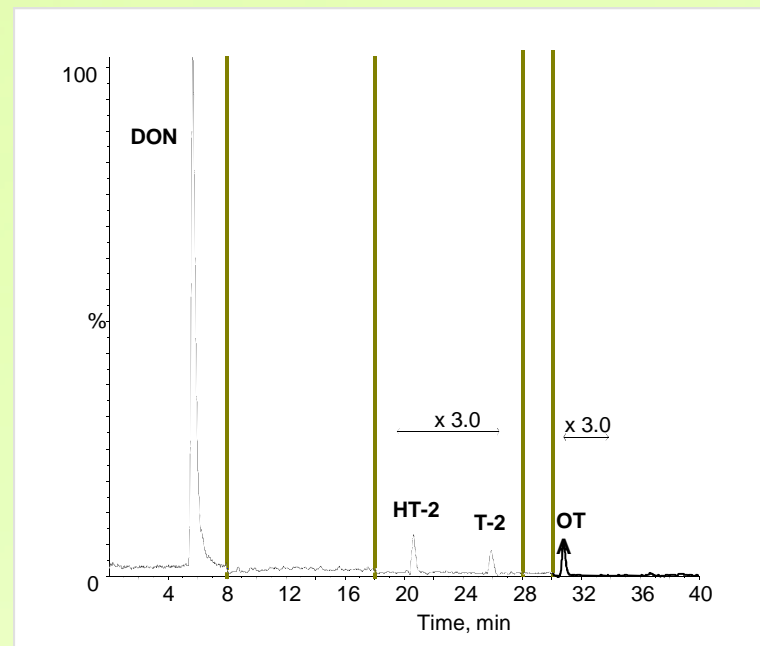
**Multiple Reaction Monitoring detection**

1 quantifier transition – 1 qualifier transition

**<sup>13</sup>C-labelled mycotoxins as IS**



**Total ion chromatogram (sum of MRM transitions) of a wheat based crisp bread sample extract spiked with: 750 µg/kg DON; 1 µg/kg AFG<sub>2</sub>, AFB<sub>2</sub>; 3 µg/kg AFG<sub>1</sub>; 5 µg/kg AFB<sub>1</sub>; 50 µg/kg HT-2, T-2; 75 µg/kg ZEA; 3 µg/kg OTA.**



**Total ion chromatogram (sum of MRM transitions) of wheat flour sample naturally contaminated with 898 µg/kg DON; 9 µg/kg HT-2; 2 µg/kg T-2; 6 µg/kg OTA.**

**Column:** Kinetex C18 (100 mm × 2.10 mm, 2.6 µm) – **Instrument:** QTrapMSMS System Applied Biosystems  
**Mobile phase:** Methanol/water containing 0.5% acetic acid,  
 1mM ammonium acetate; gradient elution; flow rate 200µL/min, without splitting.  
**Injection volume:** 20 µL (equivalent to 100 mg matrix).



# Recoveries, % (RSDr, %)



Method recoveries and repeatability were evaluated at contamination levels encompassing the EU maximum permitted levels for each considered mycotoxin.

MATRIX	DON	AFB <sub>1</sub>	AFB <sub>2</sub>	AFG <sub>1</sub>	AFG <sub>2</sub>	HT-2	T-2	ZEA	OTA
Level 1	300	2	0.4	1.2	0.4	20	20	30	1.2
Level 2	750	5	1	3	1	50	50	75	3
Level 3	1500	10	2	6	2	100	100	150	6

Mean recoveries (three spiking levels) ranged from 91 to 103 % for deoxynivalenol, from 76 to 104% for aflatoxins, from 87 to 106% for T-2 and HT-2 toxins, from 85 to 93% for zearalenone, from 78 to 100% for ochratoxin A. Relative standard deviations were less than 11%.

MATRIX	DON	AFG <sub>2</sub>	AFG <sub>1</sub>	AFB <sub>2</sub>	AFB <sub>1</sub>	HT-2	T-2	ZEA	OTA
Barley flour	103 (5)	98 (9)	100 (7)	101 (7)	102 (3)	106 (5)	104 (3)	92 (8)	94 (4)
Durum wheat flour	96 (2)	91 (8)	90 (7)	93 (6)	90 (6)	98 (3)	97 (2)	85 (6)	81 (8)
Oat flour	101 (4)	85 (5)	104 (5)	96 (8)	103 (5)	101 (5)	98 (4)	93 (11)	94 (7)
Wheat based crisp bread	100 (2)	102 (6)	104 (5)	94 (10)	102 (10)	105 (2)	103 (4)	92 (9)	100 (4)
Rye based crisp bread	91 (4)	100 (8)	88 (4)	94 (9)	76 (3)	94 (4)	87 (2)	80 (5)	78 (2)

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Based on the obtained detection limits, the method allows to assess, with a single analysis, compliance of the tested cereal foods with the EU maximum permitted levels for OTA, DON, and ZEA, while providing quantitative data on aflatoxin contamination at levels slightly above the EU regulated levels.

- ✓ In barley flour, wheat flour and wheat based crisp bread LODs lower than  $1.1 \mu\text{g}/\text{kg}$  were obtained for all toxins detected in **positive ion mode**, whereas higher LODs were obtained for ZEA (from  $2.2$  to  $4.0 \mu\text{g}/\text{kg}$ ) and DON (from  $3.9$  to  $29.0 \mu\text{g}/\text{kg}$ ) that were detected in negative ion mode thus suffering higher baseline noise.

MATRIX	DON	AFG <sub>2</sub>	AFG <sub>1</sub>	AFB <sub>2</sub>	AFB <sub>1</sub>	HT-2	T-2	ZEA	OTA
Barley flour	10.3	0.2	0.7	0.3	0.5	1.1	0.5	4.0	0.3
Durum wheat flour	3.9	0.1	0.2	0.3	0.3	0.3	0.2	2.8	0.1
Oat flour	40.2	1.3	0.6	0.2	0.3	2.4	1.2	3.5	0.2
Wheat based crisp bread	29.0	0.4	0.7	0.4	0.5	0.5	0.5	2.2	0.1
Rye based crisp bread	59.2	1.9	2.6	1.1	1.1	1.7	0.9	5.8	0.4

	DON	AFs totali	AFB <sub>1</sub>	HT-2	T-2	ZEA	OTA
Maximum permitted levels ( $\mu\text{g}/\text{kg}$ )	750	4	2	Under discussion		75	3

Analysis of reference materials for

**aflatoxins,  
deoxynivalenol,  
zearalenone**

demonstrated the trueness of the results obtained by the proposed method.



	Assigned value µg/kg	Satisfactory range µg/kg	Level of <sup>13</sup> C-IS addition µg/kg	Results obtained by the proposed method µg/kg
<b>DON in wheat flour FAPAS T2256</b>	774	517 - 1032	700	748 ± 112*
<b>ZEA in breakfast cereals FAPAS T2257</b>	69.5	38.9 - 100.1	100	48.6 ± 10*
<b>AFs in maize FAPAS T04148</b>	AFB <sub>1</sub> 5.07	AFB <sub>1</sub> 2.84 - 7.30	AFB <sub>1</sub> 5.0	AFB <sub>1</sub> 5.3 ± 1.2*
	AFB <sub>2</sub> 1.06	AFB <sub>2</sub> 0.60 - 1.53	AFB <sub>2</sub> 2.0	AFB <sub>2</sub> 1.1 ± 0.5*
	AFG <sub>1</sub> 2.97	AFG <sub>1</sub> 1.66 - 4.27	AFG <sub>1</sub> 5.0	AFG <sub>1</sub> 2.8 ± 0.7*
	AFG <sub>2</sub> 1.25	AFG <sub>2</sub> 0.70 - 1.80	AFG <sub>2</sub> 2.0	AFG <sub>2</sub> 1.2 ± 0.5*

\* Maximum standard uncertainty calculated according to 401/2006/EC



## LC-HRMS methods for multi-mycotoxin analysis in foods and related performances.

Reference	Target mycotoxins-matrix	Extraction solvent - cleanup	LOD range (µg/kg)	Recovery range (%)	Type of LC-MS detection	Mass accuracy*
Tanaka et al. 2006	13 mycotoxins (including DON, ZEA, HT-2, T-2, AFs) in corn, wheat, cornflakes, biscuits.	Acetonitrile/water (85:15) Multisep #226	Wheat: 0.1-3.8 Corn: 0.1-4.9	Wheat: 71-132 Corn: 81-133	LC-APCI-TOFMS	≤ 2.5 ppm
Zachariasova et al. 2010a	11 mycotoxins (including DON, HT-2, T-2, ZEA, FBs) in maize, wheat, barley	Water 0.1% formic acid/acetonitrile (QuEChERS)	Maize: 5-50	Maize: 43-120	UPLC-ESI-TOF-MS	≤ 9.6 ppm
		Water 0.1% formic acid/acetonitrile (1:1) Direct injection	Maize: 10-30	Not tested	<b>UPLC-APCI-Orbitrap™ MS</b>	≤ 5 ppm
Vaclavik et al. 2010	12 mycotoxins (including DON, de-epoxyDON, acetyl-DON, nivalenol, ZEA) in wheat and maize	Water/acetonitrile (3:4) QuEChERS	Maize/Wheat: 80-100	Maize/Wheat: 95-118	<b>DART-Orbitrap™ MS</b>	≤ 3.8 ppm
Zachariasova et al. 2010b	32 mycotoxins (including DON, HT-2, T-2, ZEA, AFs, OTA) in beer	Acetonitrile	0.5 – 60 µg/L	86-124	<b>UPLC-APCI-Orbitrap™ MS</b>	≤ 5 ppm (confirmatory ion, in opposite polarity)
Herebian et al. 2009	32 mycotoxins (including DON, HT-2, T-2, ZEA, FBs, AFs, OTA) in wheat and maize	Acetonitrile/water/acetic acid, (79:20:1) Direct injection	Maize: 0.4-2000 Wheat: 0.4-200	Maize: 68-152 Wheat: 87-131	<b>LC-ESI-LTQ-Orbitrap™</b>	≤ 1 ppm

## HPLC-MS<sup>n</sup> | Multiresidual analysis by accurate MS screening TOF & QTOF MASS SPECTROMETRY

The **natural coexistence of several mycotoxins** impose to optimize the global cost of these analysis through the development of multiresidual analytical strategies.

The **variability of physicochemical properties** of the screened residues determines difficulties to get high recoveries by simple sample preparation and mass accuracy could be not always sufficient to separate analytes from coeluting species.

Multi-mycotoxin analysis in food is a real future challenge: in this contest, the new frontier is the **full scan MS screening** strategy, in particular exploiting high performance Time of Flight (TOF) and Orbitrap<sup>TM</sup> technology instruments, possibly coupled to Ultra (High) Performance Liquid Chromatography (U-(H)PLC).



### Pros

- High scan rate (up to 20000 scan/s)
- High resolution especially in reflectron mode
- High speed electronics
- Virtually no limit on mass range
- High sensitivity

### Cons

- Strict demands on initial energy and spatial distribution of ions
- High performance electronics needed
- Relatively low resolution in linear mode
- High cost

### Applications

- High resolution measurements using reflectron
- High sensitivity measurements
- Detection of narrow / transient chromatographic signals
- Full scan MS screening Non.Targeted Analysis



# HPLC-MS<sup>n</sup> \ Multiresidual analysis by accurate MS screening ORBITRAP<sup>TM</sup> MASS SPECTROMETRY

## Pros \ Applications

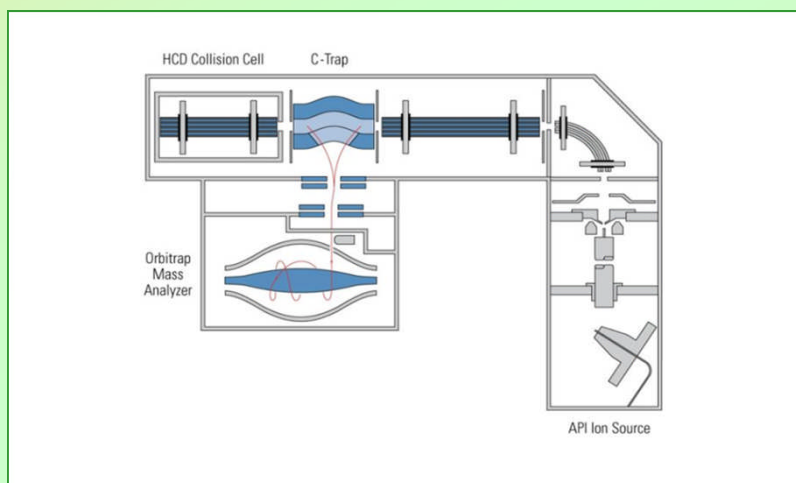
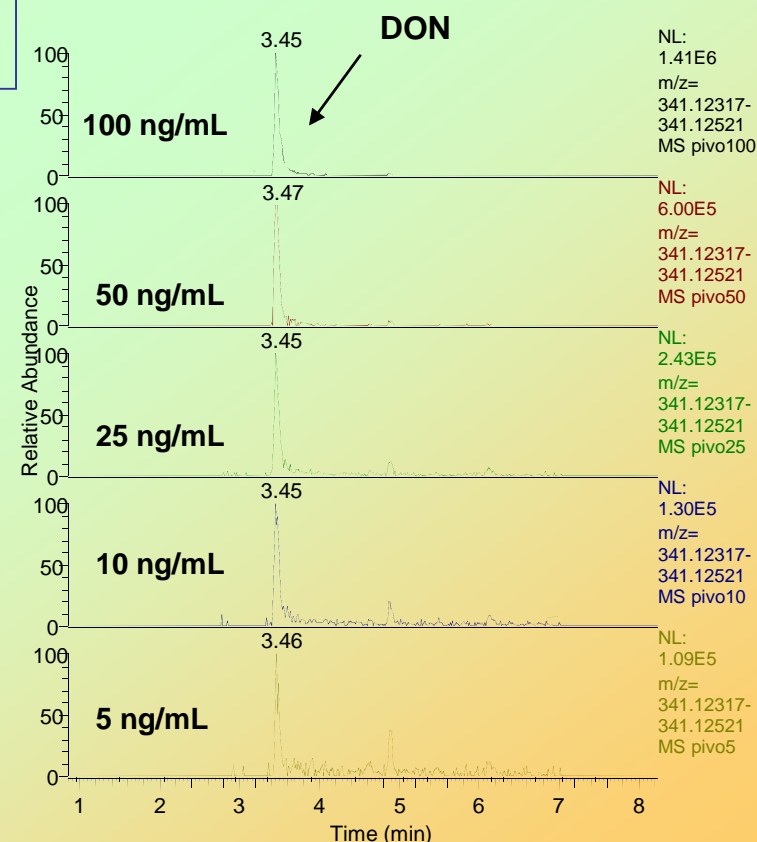
- mass accuracy and resolving power
- dynamic range
- positive and negative acquisition in one run
- quantitation and MS screening \ non targeted analysis capabilities
- post acquisition data mining
- stability and robustness
- simple instrument tuning

## Cons

- High performance electronics needed
- High cost

Source: M. Godula – EIS Food Safety Solutions Conference – 21 April 2010 – Paris

## UHPLC-Orbitrap DON in beer



Michele Suman

Tandem and High Resolution Mass Spectrometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012

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## Let's try the HRMS direction...

**Thermo**  
SCIENTIFIC

V.M.T. Lattanzio, S. Della Gatta, M. Godula, A. Visconti, *Food Add. Contam.* 2011, 1-14, DOI: 10.1080/19440049.2011.593192



**Wheat flour  
Barley flour  
Barley flour**



**Wheat based crisp bread  
Rye based crisp bread**

### Sample



*Extraction*

acetonitrile/water  
(84/16)



*Filtration (Whatmann N°4)*

*Evaporation*

*Reconstitution in methanol/water (10/90)*

**Purificazione con  
OASIS® HLB**  
(polimeric SPE cartridges)

**LC-HRMS analysis**

Michele Suman

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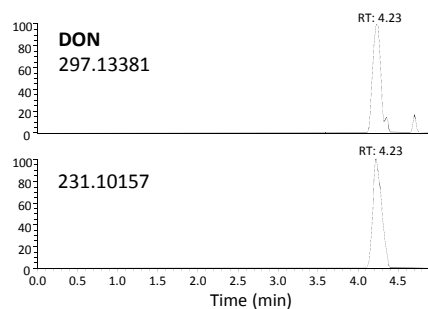
Workshop Rome October, 11-12<sup>th</sup> 2012

## MS/MS detection HRMS detection legislation requirements

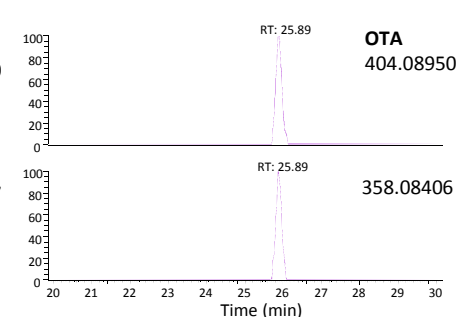
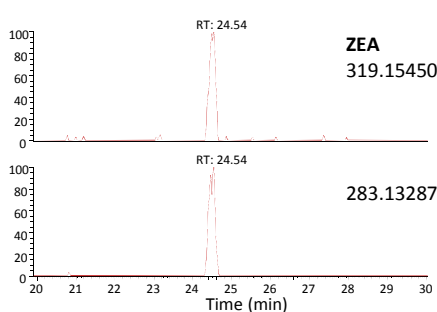
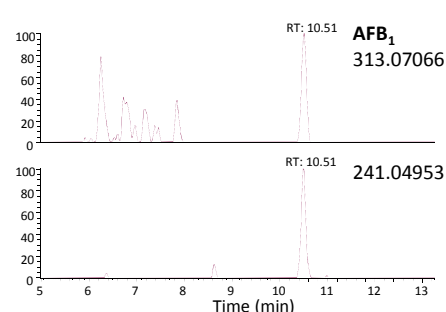
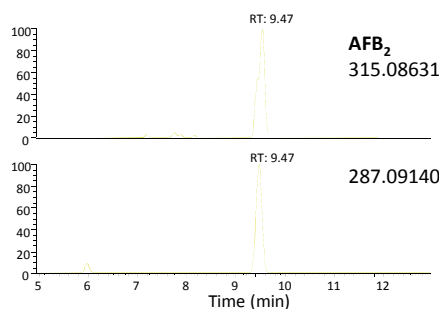
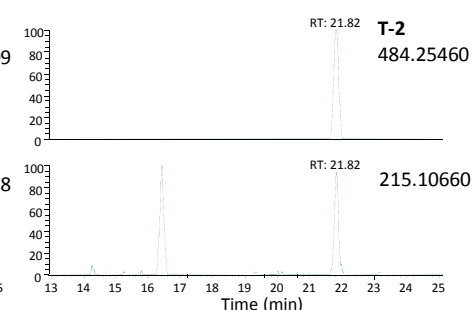
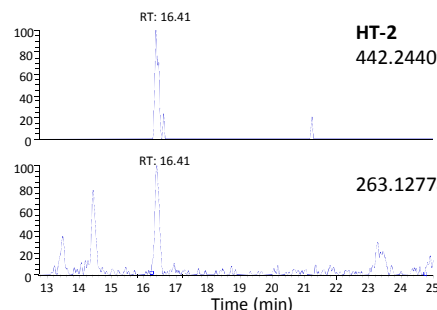
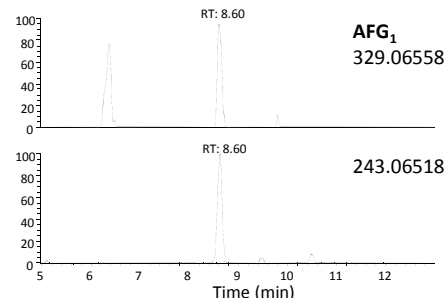
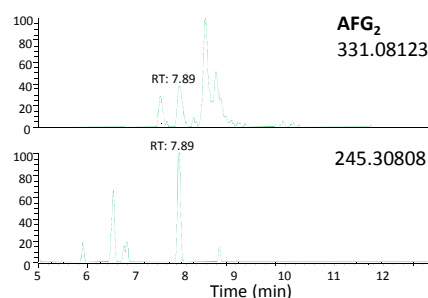
*EC performance criteria (2002/657/EC)  
Doc No Sanco/10684/2009\**

MS/MS	HCD-HRMS
1 precursor ion 2 daughters ions	2 ions Mass accuracy < 5ppm*
<b>example: AFB<sub>1</sub></b>	
313.0 – 241.1	313.07066
313.0 – 213.4	241.04953

# LC-HCD-HRMS chromatogram of a spiked wheat flour extract



100.000 FWHM  
Tolerance: 10 ppm



Spiking levels: 25 µg/kg DON, AFG<sub>1</sub>, AFB<sub>1</sub>, T-2, HT-2, ZEA, OTA and 6.2 µg/kg AFG<sub>2</sub>, AFB<sub>2</sub>

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# Comparison of MS/MS and HCD-HRMS approaches

## RECOVERIES and REPEATABILITY

*EC acceptance criteria (401/2006/EC)*

		Recoveries, % (RSDr %)								
		DON	AFG <sub>2</sub>	AFG <sub>1</sub>	AFB <sub>2</sub>	AFB <sub>1</sub>	HT-2	T-2	ZEA	OTA
Spiking levels (µg/kg)		300	0.4	1.2	0.4	2	20	20	30	1.2
Wheat flour	HCD-HRMS	102 (5)	90 (8)	89 (0)	95 (2)	81 (6)	104 (4)	98 (6)	76 (6)	97 (9)
	MRM	95 (2)	n.d.	82 (4)	84 (6)	89 (4)	95 (4)	92 (4)	95 (9)	74 (7)
Crisp bread (wheat)	HCD-HRMS	104 (0)	102 (5)	104 (4)	80 (2)	102 (2)	105 (1)	103(1)	85 (1)	93 (2)
	MRM	100 (0)	n.d.	106 (5)	85 (10)	102 (6)	107 (2)	108 (6)	84 (5)	101 (3)
Crisp bread (rye)	HCD-HRMS	105 (1)	93 (2)	95 (6)	93 (8)	87 (4)	100 (3)	95 (3)	101 (9)	74 (13)
	MRM	95 (3)	91 (7)	79 (2)	85 (7)	77 (3)	97 (2)	91 (3)	96 (7)	82 (2)



# Comparison of MS/MS and HCD-HRMS approaches

## DETECTION LIMITS

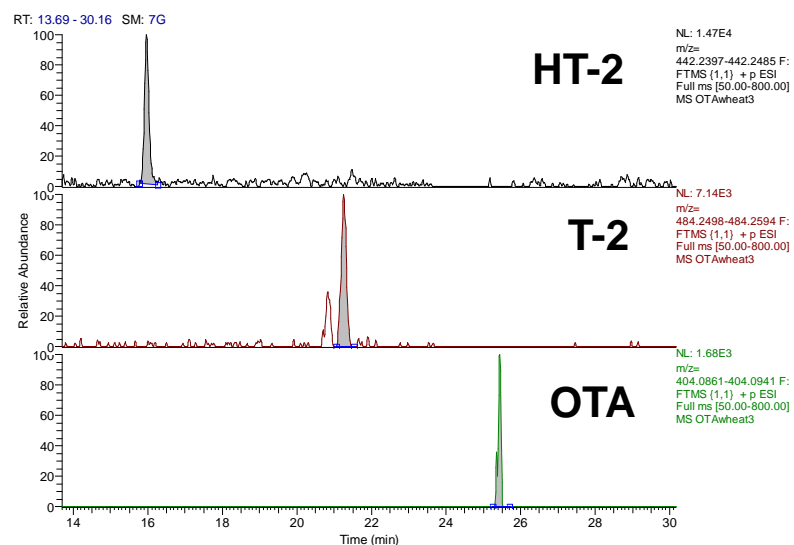
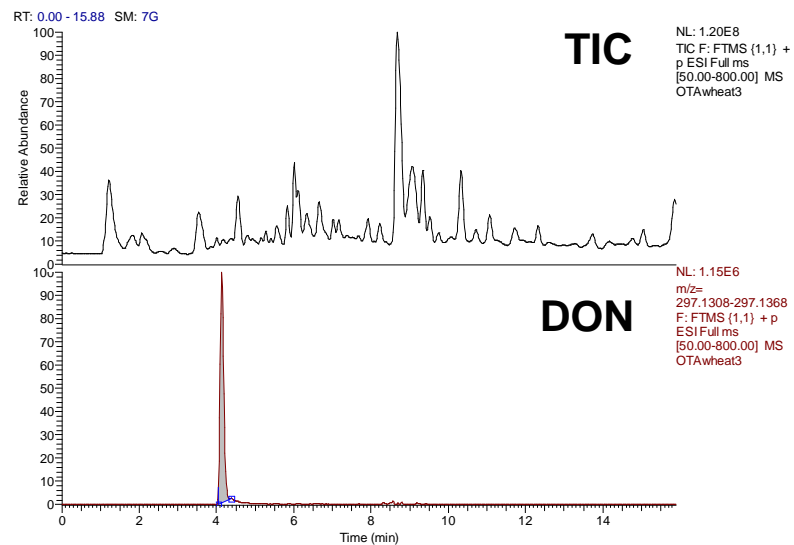
*EC maximum permitted levels (1881/2006/EC)*

	LOD ( $\mu\text{g/kg}$ )					
	Wheat Flour		Barley Flour		Crisp bread (rye)	
	HCD-HRMS	MRM	HCD-HRMS	MRM	HCD-HRMS	MRM
<b>DON</b>	1.6	3.9	1.8	10.3	2.3	59.2
<b>AFG<sub>2</sub></b>	1.5	0.1	0.5	0.2	0.5	1.9
<b>AFG<sub>1</sub></b>	0.6	0.2	1.1	0.7	1.2	2.6
<b>AFB<sub>2</sub></b>	0.7	0.3	0.5	0.3	0.5	1.1
<b>AFB<sub>1</sub></b>	1.0	0.3	1.0	0.5	1.6	1.1
<b>HT-2</b>	1.7	0.3	2.5	1.1	1.7	1.7
<b>T-2</b>	1.0	0.2	0.5	0.5	1.6	0.9
<b>ZEA</b>	1.0	2.8	1.4	4.0	2.3	5.8
<b>OTA</b>	1.4	0.1	1.9	0.3	2.9	0.4

Michele Suman

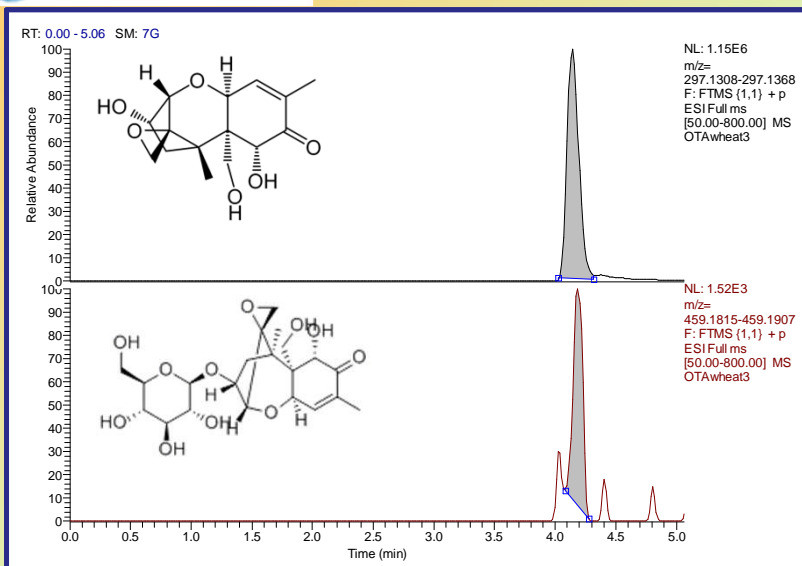
Tandem and High Resolution Mass Spectrometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012

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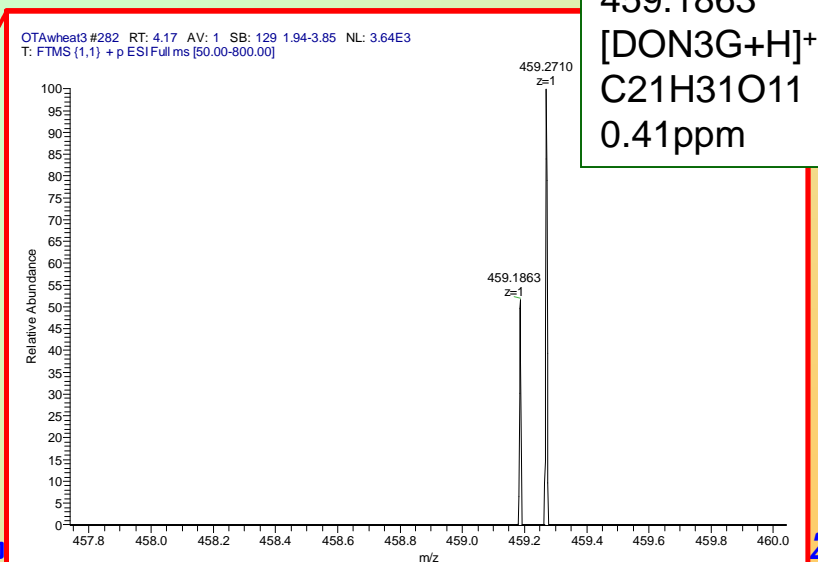
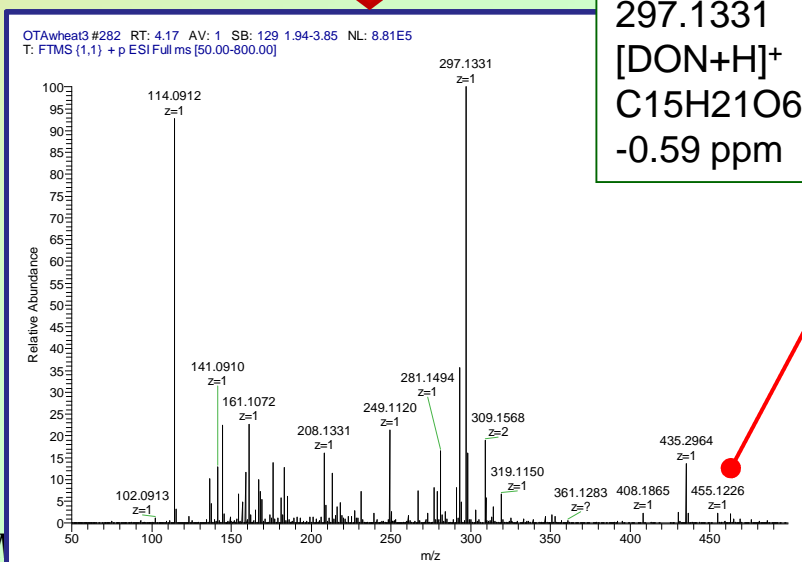
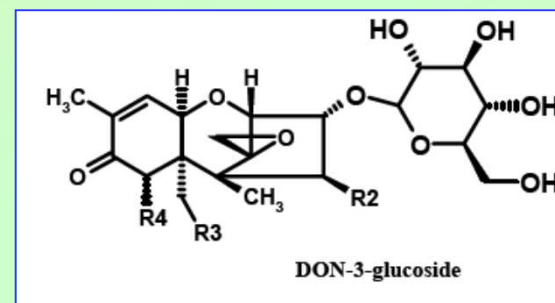


Soft wheat flour  
naturally contaminated with:  
898 µg/kg DON;  
9 µg/kg HT-2;  
2 µg/kg T-2;  
6 µg/kg OTA

100.000 FWHM  
Tolerance 10 ppm



## Deoxynivalenol-3-glucoside







# COMPARISON



	<b>SPE MRM</b>	<b>SPE HCD-HRMS</b>
<b>Detection approach</b>	<b>Targeted</b>	<b>Untargeted (full scan)</b>
<b>Compliance with 2002/657/EC Doc No Sanco/10684/2009</b>	<b>Yes 1 precursor ion 2 daughters ions</b>	<b>Yes 2 ions Mass accuracy &lt; 5ppm</b>
<b>LOD µg/kg (range)</b>	<b>0.1-59.2</b>	<b>0.5-2.9</b>
<b>Recoveries % (range)</b>	<b>70-114</b>	<b>74-105</b>
<b>Repeatability % (range)</b>	<b>0-14</b>	<b>0-13</b>
<b>Matrix effects (SSE% range)</b>	<b>70%-106%</b>	<b>67%-100%</b>
<b>Time of analysis (sample preparation and LC-MS analysis)</b>	<b>3 H</b>	
<b>Clean-up device</b>	<b>3 €/column</b>	

## Let's expand HRMS potentialities...

### Research article

Journal of  
MASS  
SPECTROMETRY

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# Targeted screening of pesticides, veterinary drugs and mycotoxins in bakery ingredients and food commodities by liquid chromatography-high-resolution single-stage Orbitrap mass spectrometry<sup>†</sup>

Emiliano De Dominicis,<sup>a</sup> Italo Commissati<sup>a</sup> and Michele Suman<sup>b,\*</sup>

Multiscreening residues analysis  
of pesticides, antibiotics and toxins by  
LC/MS Exactive  
(Orbitrap Technology)

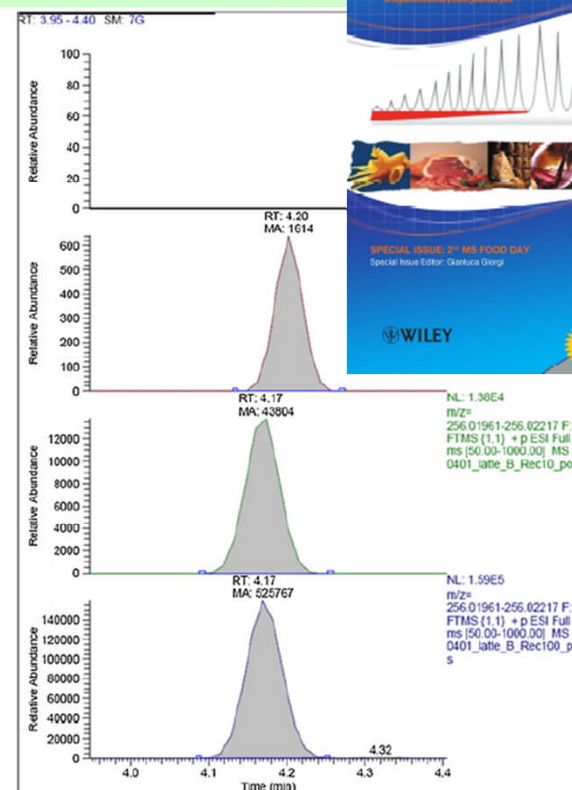


Figure 4. LC-MS/MS Full Scan Exactive (Orbitrap); extracted ion  $m/z$  256.02089 for sulfathiazole in: a) a milk sample, b) a milk sample spiked at  $1 \mu\text{g kg}^{-1}$ , c) a milk sample spiked at  $10 \mu\text{g kg}^{-1}$  and d) a milk sample spiked at  $100 \mu\text{g kg}^{-1}$ .

## Choice of molecules

### Pesticides

carbendazim
carbaryl
desethylterbuthylazine
simazine
pymetrozine
dodine
metoxuron
prometryn
oxycarboxin
(D,L)-metalaxyl
piperonyl butoxide
azoxystrobin
tebuconazole
pirimiphos methyl
malathion
tricyclazole

### Toxins

aflatoxin B1
aflatoxin B2
aflatoxin G1
aflatoxin G2
ochratoxin A
deoxynivalenol
toxin T2
toxin HT2
zearalenon
fumonisin B1
fumonisin B2
aflatoxin M1

### Antibiotics

abamectin
tetracycline
chlortetracycline
oxitetracycline
chloramphenicol
thiabendazole
sulfathiazole
sulfadimethoxine

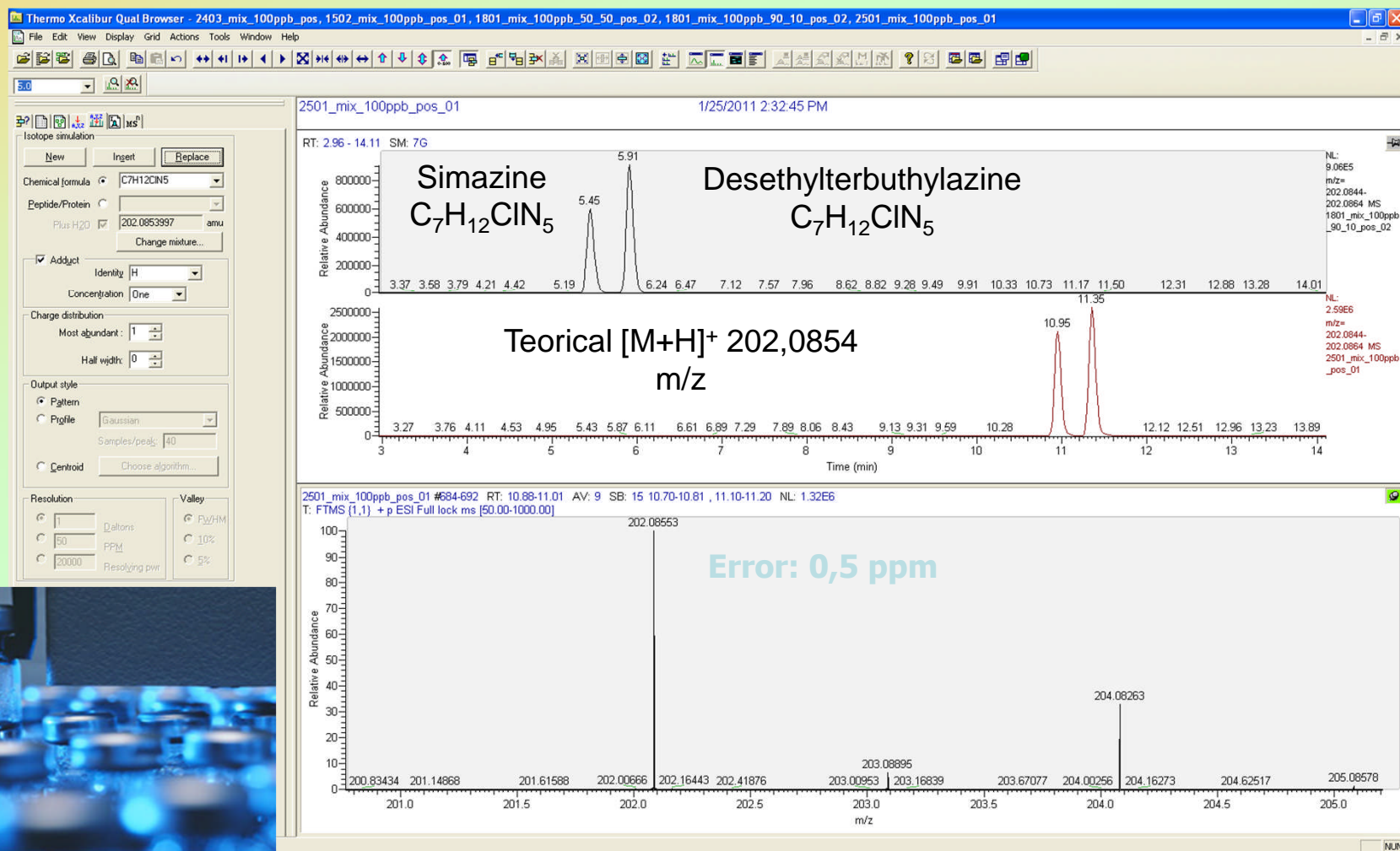
## Choice of matrices

Raw materials: milk, flour

Finished product: bakery product

## Instrumental Method Development

- MS analysis (source parameters: temperatures, voltages, gas flows, etc.)
- LC analysis (column, mobile phases and buffers, flow, gradient, etc.)





## Sample Preparation - Sheet Flow



Sample

1<sup>st</sup> extraction/ L/L rip. Vortex

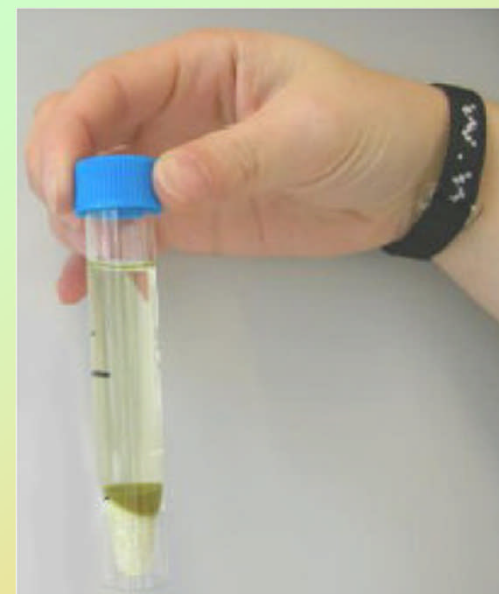
$(\text{CH}_3\text{CN}_{(\text{CH}_3\text{COOH } 1\%)} / \text{H}_2\text{O} / \text{MgSO}_4 / \text{CH}_3\text{COONa})$

2<sup>nd</sup> purification D-SPE

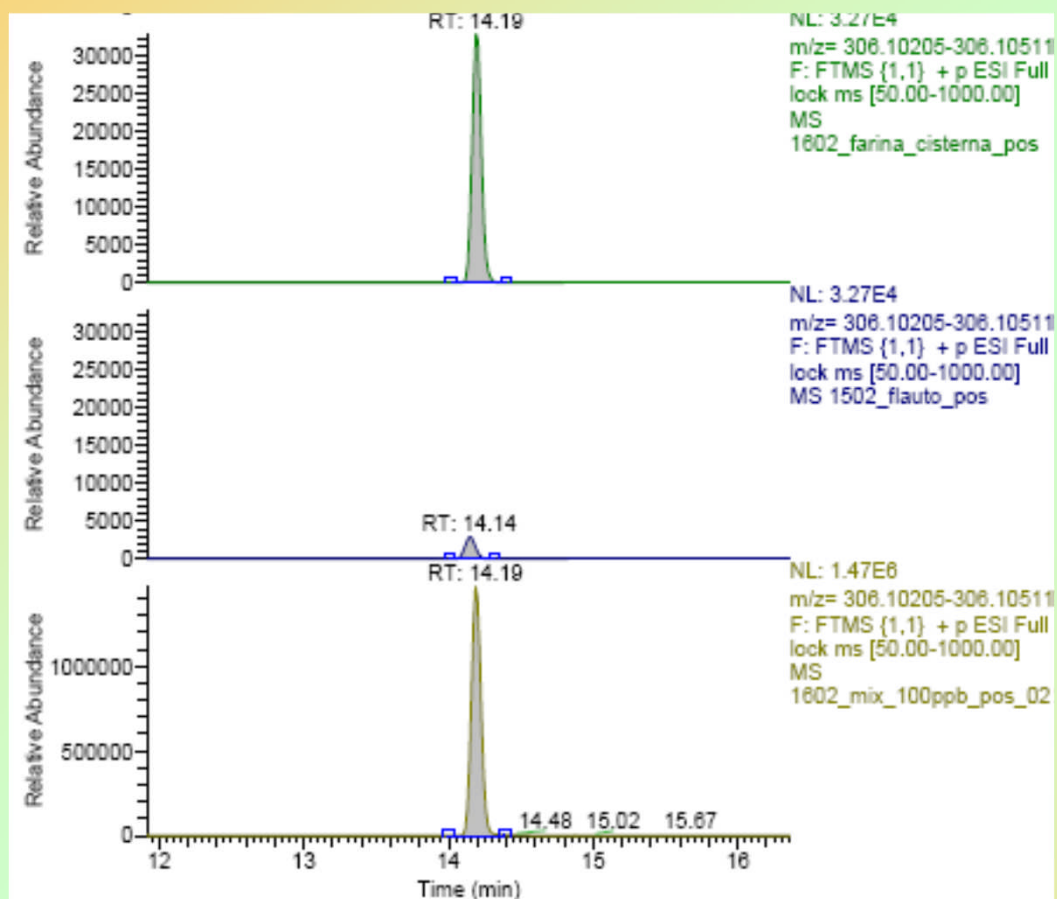
$(\text{PSA-C18-MgSO}_4)$

**Solvent Change (Dry N<sub>2</sub> 30 ° C)**

**Inj. Exactive Orbitrap LC-MS  
(Resolution set: FWHM 100.000)**



Full scan results obtained on finished product: pirimifos methyl



Pirimifos methyl occurred in Flour

Pirimifos methyl detected  
in Bakery Product

Pirimifos methyl: reference material

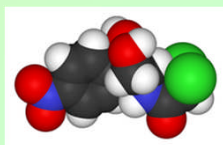
## Manufacturing minicake “blank” and “black” (industrial and pilot plant production lines)

Spiking of the minicake with different contaminants, exploiting a combined use of industrial and pilot plant production lines

Minicake final weight: 35g

Aroma solution weight: 1.5g

- Minicake sandwich sides produced on the industrial line
- Milk cream produced on the industrial line
- Spiking of the contaminants through opportune solutions directly obtained with the standard liquid aroma solution used in the production line
- Application of the aroma-contaminants solution via spray-drying off-line on the separated sandwiches exploiting pilot plant production lines
- Application of the milk cream aliquot off-line on the separated sandwiches exploiting pilot plant production lines
- Combination of the two sandwiches (cream in the middle in this way) exploiting pilot plant production lines
- Final packaging into the standard polypropylene film



## Results obtained on minicakes “blank” and “black”: chloramphenicol (CAP) with clean up MYCOSEP-226 and LXQ

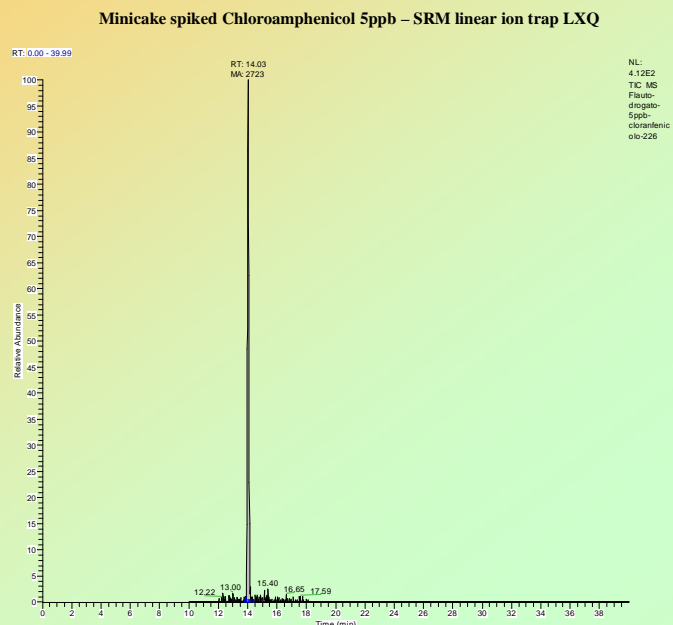
### Extraction and clean-up protocol with MYCOSEP-226

Sample (10 g)

1<sup>st</sup> extraction ACN/water 84/16 (100 ml)

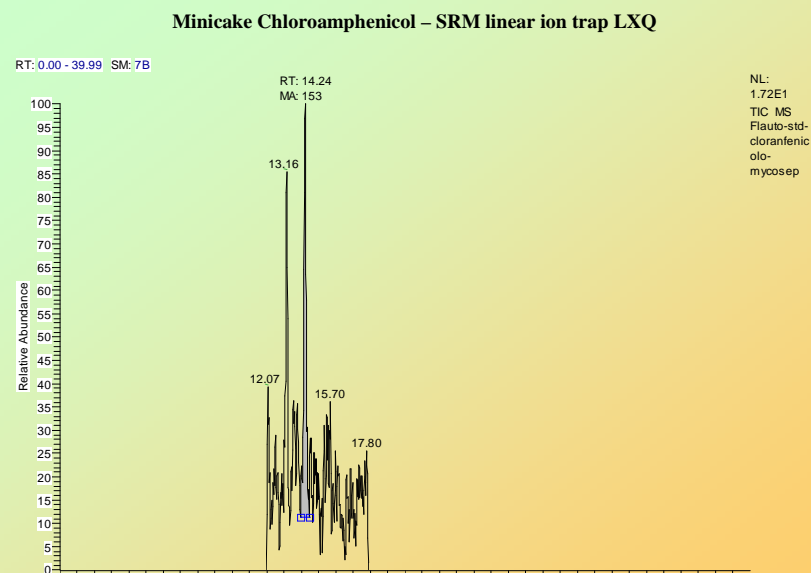
2<sup>nd</sup> purification Mycosep (+CH<sub>3</sub>COOH)

3<sup>rd</sup> Concentration/Dry with N<sub>2</sub>/Change solvent  
(2 ml of extract to 0,2 ml of MetOH/waters 60/40 0,5% CH<sub>3</sub>COOH)



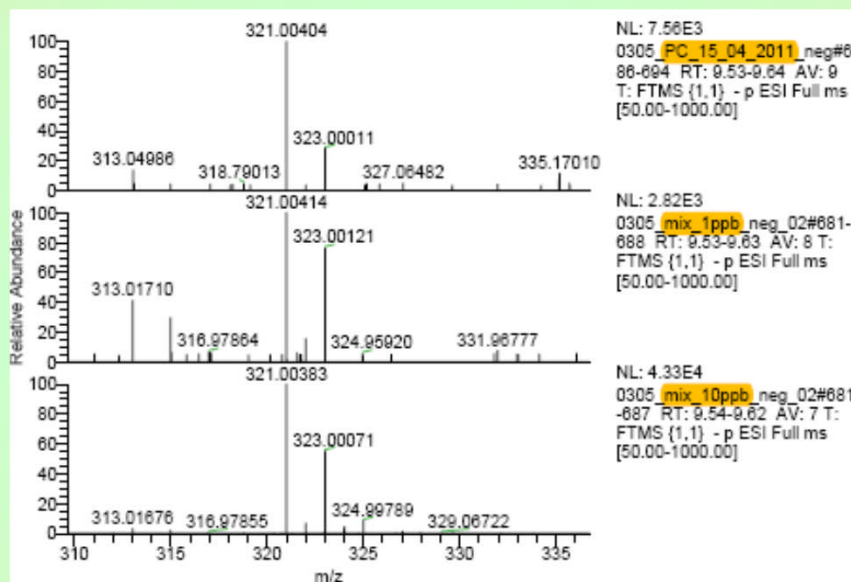
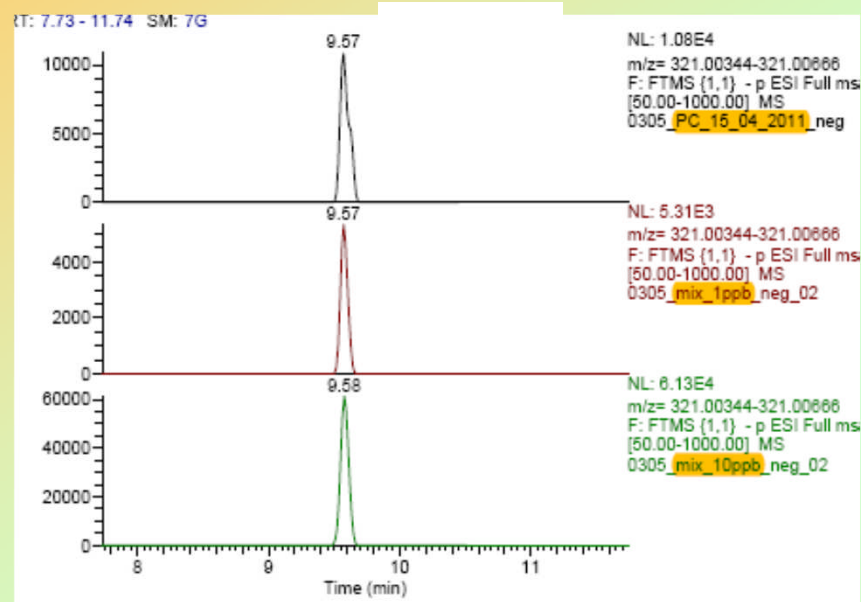
CAP reference material 5  
ng/ml in matrix

Sample PC spiked with CAP:  
CAP: DETECTED?  
very low signal...





## Results obtained on minicakes “blank” and “black”: chloramphenicol (CAP) by LC/MS Exactive (Orbitrap Technology)



Sample spiked with CAP  
**DETECTED** by m/z 321,00404  
and  
**CONFIRMED** by m/z 323,00011

## Full scan results obtained on minicakes “blank” and “black”: pesticides...

	Sample: Unknown 1		Sample: Unknown 2		Sample: Unknown 3		Sample: Unknown 4		Sample: Unknown 5	
	Teorical	Results	Teorical	Results	Teorical	Results	Teorical	Results	Teorical	Results
Carbendazim		< LOD		< LOD		< LOD		< LOD		< LOD
Carbaril		< LOD		< LOD		< LOD		< LOD		< LOD
Desetilterbutilazina		< LOD		< LOD		< LOD		< LOD		< LOD
Simazina		< LOD		< LOD		< LOD		< LOD		< LOD
Pimetrozina		< LOD		< LOD		< LOD		< LOD		< LOD
Dodina		< LOD		< LOD		< LOD		< LOD		< LOD
Metoxuron		< LOD		< LOD		< LOD		< LOD		< LOD
Prometrina		< LOD		< LOD		< LOD		< LOD		< LOD
Oxicarboxin		< LOD		< LOD		< LOD		< LOD		< LOD
(D,L)-Metalaxil		< LOD		< LOD		< LOD		< LOD		< LOD
Piperonil butossido		< LOD		< LOD		< LOD		< LOD		< LOD
Azoxistrobina		< LOD		< LOD		< LOD		< LOD		< LOD
Tebuconazolo		< LOD		< LOD		< LOD		< LOD		< LOD
Pirimifos metile	occurred	detected	occurred	detected	added	detected	added	detected	occurred	detected
Malathion		< LOD		< LOD		< LOD		< LOD		< LOD
Triciclazolo		< LOD		< LOD		< LOD		< LOD		< LOD

Pyrimiphos Methyl correctly detected in the sample codified PC (spiked concentration: 5 µg/Kg) even if also the not spiked sample shows a little presence (few ppb again) in terms of natural contamination

## ... toxins and antibiotics

	Sample W1: Unknow 1		Sample D: Unknow 2		Sample P: Unknow 3		Sample PC: Unknow 4		Sample W: Unknow 5	
	Teorical	Results	Teorical	Results	Teorical	Results	Teorical	Results	Teorical	Results
Aflatossina B1		< LOD		< LOD		< LOD		< LOD		< LOD
Aflatossina B2		< LOD		< LOD		< LOD		< LOD		< LOD
Aflatossina G1		< LOD		< LOD		< LOD		< LOD		< LOD
Aflatossina G2		< LOD		< LOD		< LOD		< LOD		< LOD
Ocratossina A		< LOD		< LOD		< LOD		< LOD		< LOD
Deossinivalenol	occurred	detected	added	detected	occurred	detected	occurred	detected	occurred	detected
Tossina T2		< LOD	added	detected		< LOD		< LOD		< LOD
Tossina HT2		< LOD	added	detected		< LOD		< LOD		< LOD
Zearalenone		< LOD	added	< LOD		< LOD		< LOD		< LOD
....										
....										
Cloramfenicolo		< LOD		< LOD		< LOD	added	detected		< LOD
Tiabendazolo		< LOD		< LOD		< LOD		< LOD		< LOD
Sulfatiazolo		< LOD		< LOD		< LOD		< LOD		< LOD
Sulfadimetossina		< LOD		< LOD		< LOD		< LOD		< LOD

Deoxynivalenol correctly detected in the sample codified D (spiked concentration: 20 µg/Kg) even if also the not spiked sample shows a little presence (few tenths of ppb again) in terms of natural contamination

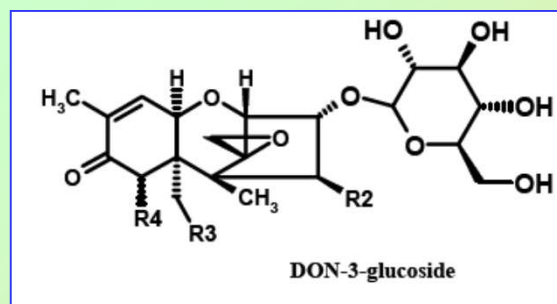
T2 and HT2 toxins correctly detected in the sample codified D (spiked concentration: 10 µg/Kg)

ZEA not detected even if present (spiked concentration: 10ppb) because for this specific molecule the cut-off was higher, 10-100 µg/Kg and not 1-10 µg/Kg

Chloroamphenicol correctly detected in the sample codified PC (spiked concentration: 5 µg/Kg)

## CONJUGATED OR “MASKED” MYCOTOXINS

- In recent years it emerged that, many structurally related compounds, generated by **plant metabolism**, can co-exist together with the native toxins: for example, plants are able to convert the relatively apolar trichothecenes in more polar derivatives **via conjugation** with sugars, amino acids or sulphate groups, in order to compartmentalize them in vacuoles
- Food processing, especially heating or fermentation steps, can potentially alter mycotoxins: **mechanical or thermal energy** during the transformation process may cause modification, inducing **reactions with macromolecular components** such as sugars, proteins or lipids as well as **release of the native form**
- **DON-3β-glucopyranoside** (DON-3G) was firstly identified in cereals by comparing the fragmentation pattern with a chemically-synthesized standard. LC-ESI-MS<sup>2</sup> analysis of naturally contaminated wheat and maize samples showed DON-3G to be the major form of masked DON. It was also recently found in barley, malt and beer and currently the authors are still focusing their work on it.
- Masked mycotoxins are not easy to be extracted/cleaned up. There also a lack of adequate analytical standard up to now. They **can escape routine analytical methods** but they can **potentially be released after ingestion by hydrolysis** in the gastrointestinal tract: preliminary results indicate that DON-3G is resistant to the acid conditions in the stomach of mammals and to most enzymes, while several bacteria in the intestinal tract of humans are able to cleave it into DON.





# Hidden fumonisins: analytical approach



**"Traditional"**  
approach

Sample

**Hydrolysis**  
approach

Extraction:  $\text{H}_2\text{O}:\text{CH}_3\text{OH}$  (30:70, v/v)

LC-ESI-MS/MS

**FREE FUMONISINS**

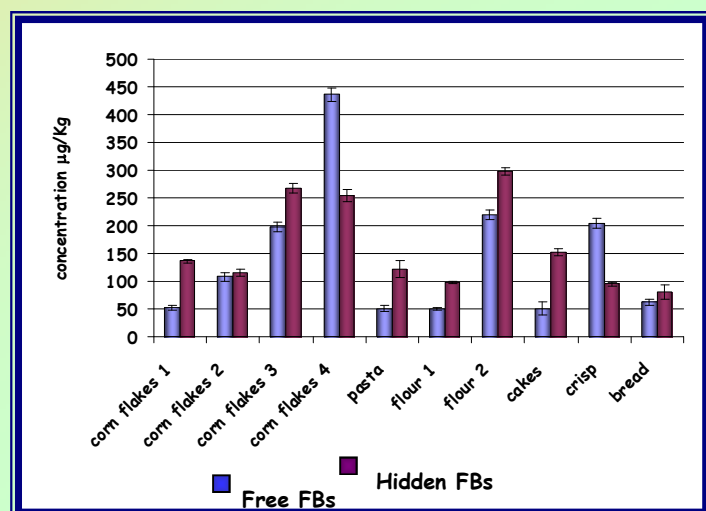
Alkaline hydrolysis  
(KOH 2 N, 25°C, 60 min)

Extraction with  $\text{CH}_3\text{CN}$

LC-ESI-MS/MS

**TOTAL FUMONISINS**  
(as HFBs)

**HIDDEN FUMONISINS = TOTAL - FREE**



*Source:*

Dall'Asta, Galaverna, Mangia, Sforza, Dossena, Marchelli, *Mol. Nutr. Food Res.* 2009, 53, 492

and High Resolution Mass Spectrometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012

## DEOXYNIVALENOL-3-GLUCOSIDE :



Food and Agriculture Organization  
of the United Nations

JECFA/72/SC

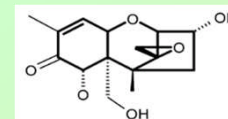


World Health  
Organization

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES  
Seventy-second meeting  
Rome, 16–25 February 2010

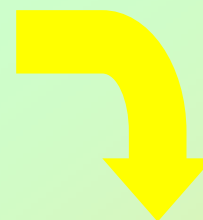
SUMMARY AND CONCLUSIONS  
Issued 9 March 2010

DEOXYNIVALENOL (DON)  
EU Legislation



Bread (including bakery products), pastry,  
biscuits, minicakes and breakfast cereals

500  
µg/kg



### 1.3 Deoxynivalenol (DON)

As 3-acetyl-deoxynivalenol (3-Ac-DON) is converted to deoxynivalenol (DON) in vivo and therefore contributes to the total DON-induced toxicity, the Committee decided to convert the provisional maximum tolerable daily intake (PMTDI) for DON to a group PTMDI of 1 µg/kg bw for DON and its acetylated derivatives (3-Ac-DON and 15-Ac-DON). In this regard, the

Committee considered the toxicity of the acetylated derivatives equal to that of DON. The Committee concluded that, at this time, there was insufficient information to include DON-3-glucoside in the group PMTDI.



## DON & DON-3G quantification by Ion-Trap LC-MS\MS

### DON-3-glucoside analytical method

#### Sample preparation:

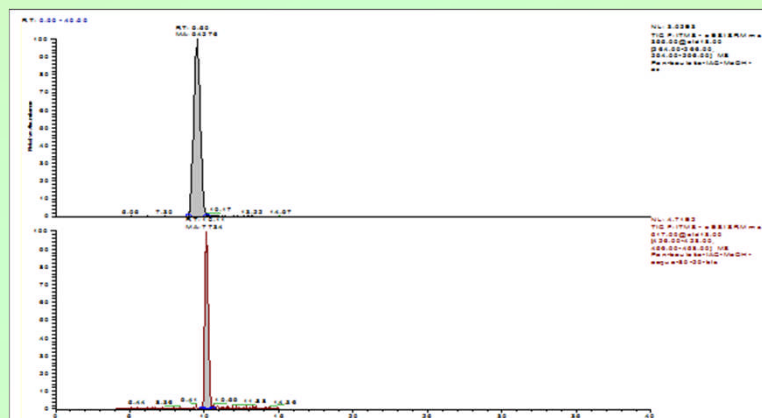
- Extraction with methanol/water (80:20, v/v)
- Clean up through immunoaffinity column (DON Neocolumn 8340, Neogen, USA)

#### LC Parameters:

- Kinetex C18 column (2.6  $\mu$ m; 100 Å; 150 mm 2.10 mm; Phenomenex, USA)
- Linear binary gradient: 0.5% CH<sub>3</sub>COOH water solution (eluant A) and CH<sub>3</sub>OH (eluant B).
- Flow rate 0.2 ml/min, temperature 30° C, inj. vol. 5  $\mu$ l. Total run 35 minutes.

#### MS Parameters:

- Linear Ion Trap LXQ mass spectrometer (Thermo Finnigan).
- Electrospray ionization (ESI) experiments.
- Multiple Reaction Monitoring (MRM) experiments executed.



extracted ion chromatogram of DON (9.50 min) and DON-3G (10.1 min) from a naturally contaminated bread extract.

The method was in-house validated on a bread matrix:

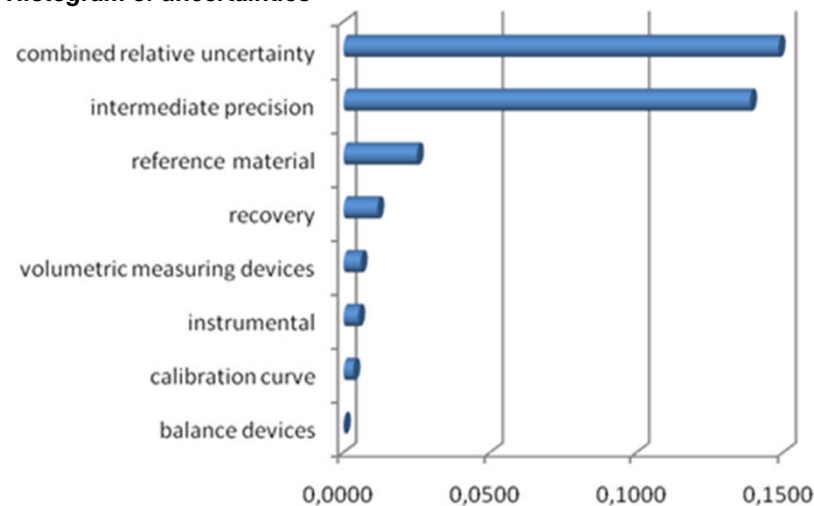
- matrix-matched linearity ( $r^2 > 0.99$ ) established range of 10 - 200  $\mu$ g kg<sup>-1</sup>
  - trueness expressed as recovery was close to 90%
  - good intermediate precision (overall RSD < 8%)
- adequate detection\ quantitation limits (4 and 11  $\mu$ g kg<sup>-1</sup>, respectively)
  - expanded uncertainty equal to 29%.

M. Suman, E. Bergamini, D. Catellani, A. Manzitti, 2012

**Food Chemistry IN PRESS**

<http://dx.doi.org/10.1016/j.foodchem.2012.06.085>

### Histogram of uncertainties



Michele Suman

Tandem and High Resolution Mass Sp

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# SUMMARIZING...

## Reliable results on mycotoxins

- sampling
- sample preparation
- sample clean up
- analytical detection & method
- analytical results interpretation

## LFD Methods

- Fast answer “on-site” ♥
- Semiquantitative proposals ♠
- Matrix effects \ cross reactivity ♠

## ELISA Methods

- Relatively rapid & cheap screening ♥
- Quantitative results ♥
- Matrix effects \ cross reactivity ♠
- Handling expertise ♠
- Poor validation ♠

## HPLC Methods

- Sensitivity and accuracy ♥
- Analysis automation ♥
- Versatility & robustness ♥
- Clean up protocols ♠
- Derivatization in some cases ♠
- Trained staff ♠

## LC-MS Methods ☺ ☺

- High sensitivity and selectivity ♥
- Mass accuracy & resolving power ♥
- Reduced sample preparation needs ♥
- High cost & high performance electronics ♠
- Full scan MS screening \ Non Targeted Analysis ♥
- Experimental flexibility & multiresidual approaches ♥
- Parent ion scanning, MRM, neutral loss studies ♥
- Trained staff ♠
- Matrix effects \ Ion suppression ♠

## Other future method perspectives

- FPIA \ SPR \ FTNIR for future rapid screening and \ or quantitation
- Universal extraction + HRMS conditions for multi-known \ novel toxins detection \ evaluation + post data acquisition

## Other future issues on masked mycotoxins

- Plant metabolism & processing effects
- Toxicological implications
- Dedicated analytical strategies



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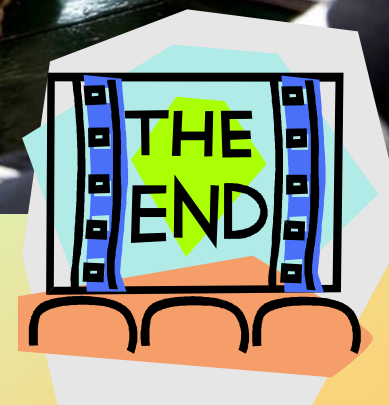
**Tandem and High Resolution Mass Spectrometry: a tool for food safety – Workshop Rome October, 11-12<sup>th</sup> 2012**

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**Thank you**



*Michele Suman*

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