ANALYTICAL CHALLENGES IN DEVELOPING STRATEGIES FOR MYCOTOXIN PREVENTION AND CONTROL - ADVANCED AND RAPID METHODS FOR MULTI-TOXIN AND MULTI-BIOMARKER ANALYSIS

A. VISCONTI,
V.M.T. Lattanzio, M. Solfrizzo, V. Lippolis

National Research Council
Institute of Sciences of Food Production
CNR-ISPA, Bari

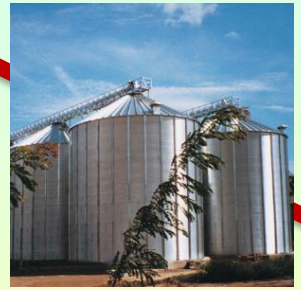
FUNGAL DEVELOPMENT AND MYCOTOXIN PRODUCTION IN FOOD

planting

harvest

logistic

logistic



storage

logistic

processing

distribution

Need to improve mycotoxin monitoring and prevention to minimize contamination at different critical steps of the food chain "from farm to fork"

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

Mycotoxins under regulation
Aflatoxins B_1, B_2, G_1, G_2, M_1
Deoxynivalenol
Fumonisins (B_1, B_2)
Zearalenone
Ochratoxin A
T-2 and HT-2 toxins *under discussion*

Matrices considered by EC regulations
Dried fruits, nuts
Cereals, cereal based foods
Baby food
coffee
Wine and beer
Animal feed
....

Maximum levels range from: **ng/kg** to **mg/kg**

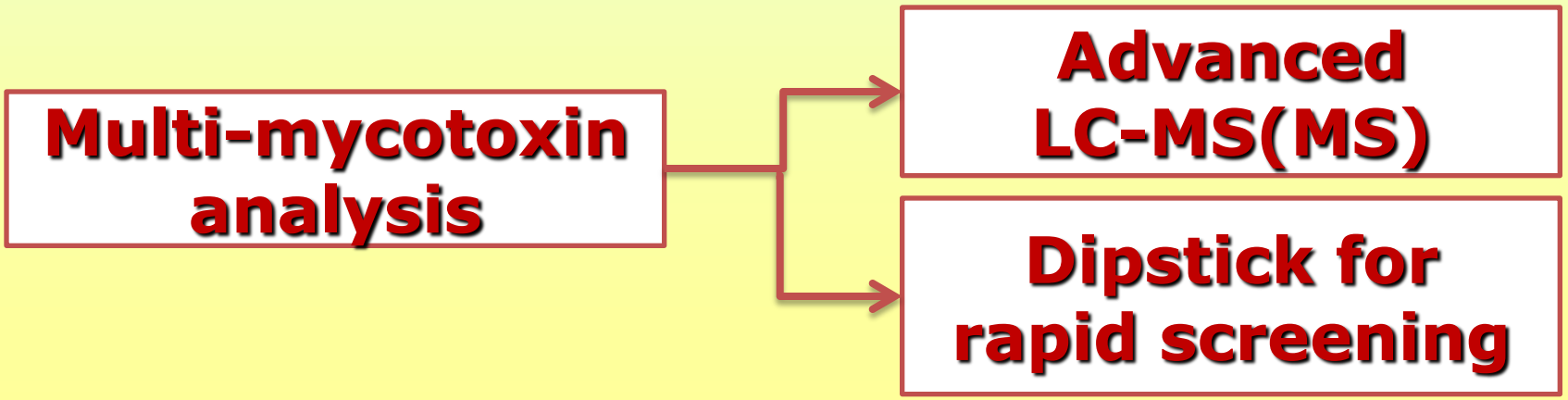
Need of **reliable** analytical methods **applicable at regulatory levels** in a **wide range** of matrices

PRESENTATION OUTLINE

✓**Multi-mycotoxin determination in food by LC-MS(MS)**
Tandem MS and high resolution MS approaches

✓**Multi-biomarker determination in human urine by LC-MS/MS**

✓**Multi-mycotoxin determination in food/feed by multiplex dipstick immunoassay**



Challenges in multi-mycotoxin method development for food matrices

- ⇨ **Sample preparation**
- ⇨ **Analyte detection**
- ⇨ **Method validation**
- ⇨ **Wide range of applicability**

HPLC
coupled with
Mass Spectrometry

Determination of aflatoxins, ochratoxin A and *Fusarium* toxins in cereal-based products by LC-MS/MS or LC-HRMS after SPE clean up

Wheat flour

Barley flour

Oat flour

Wheat based crisp bread
Rye based crisp bread

Breakfast cereals
Cereal biscuits

Sample

↓ *Extraction*

acetonitrile/water
(84/16)

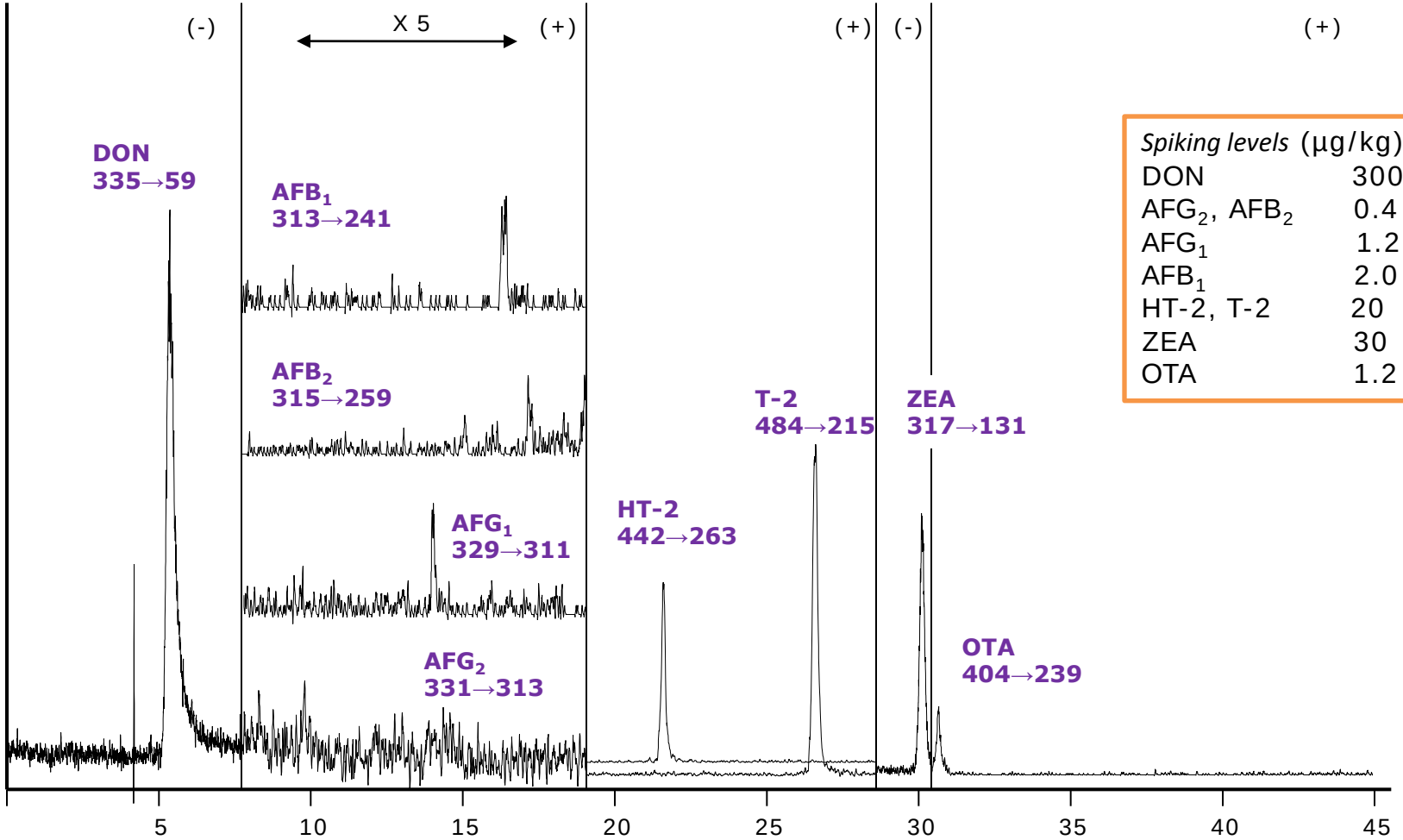
↓ *Filtration (Whatmann Nº4)*
Evaporation
Reconstitution with methanol/water (10/90)

Clean-up with
OASIS® HLB
(polimeric SPE cartridges)

LC-MS/MS analysis

LC-HRMS analysis

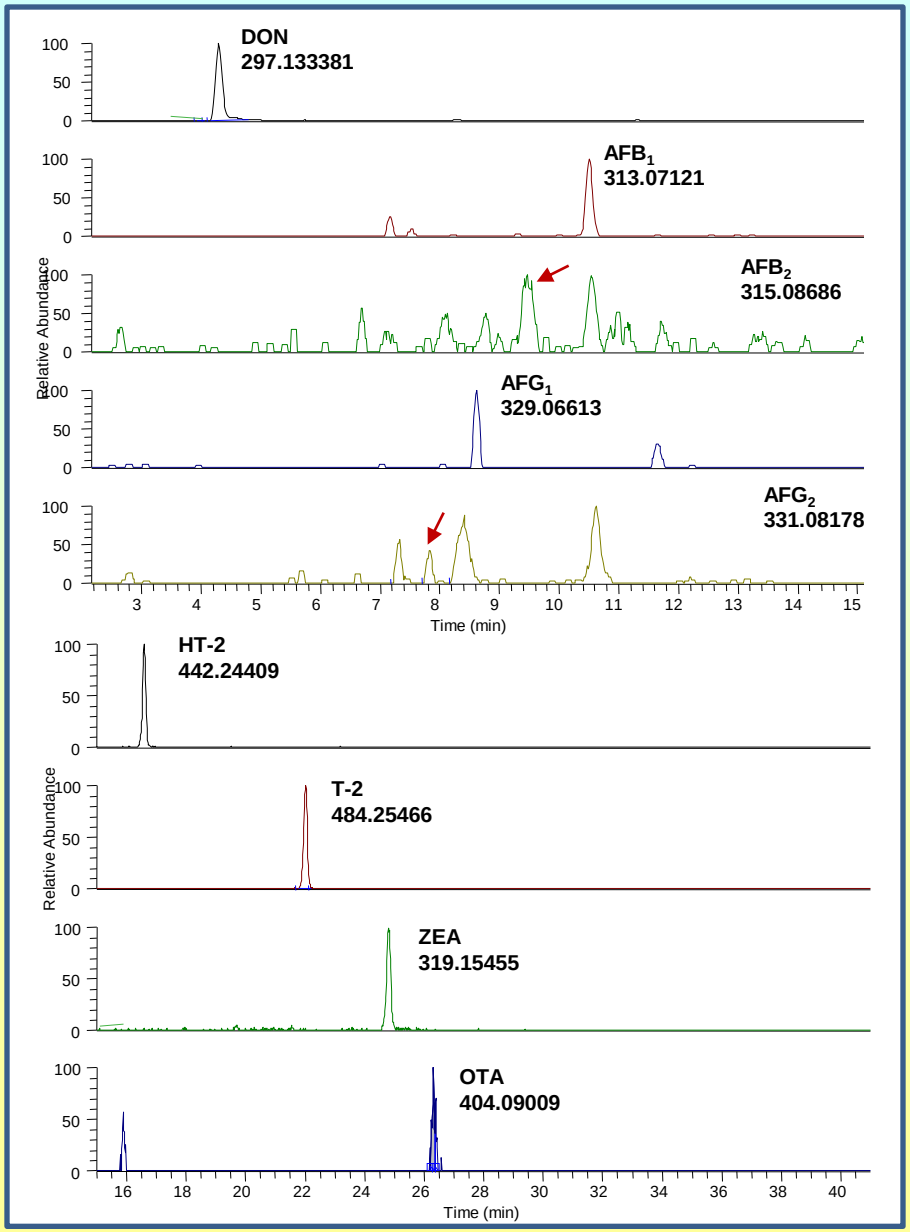
LC-MS/MS chromatogram of a spiked crisp bread extract



Spiking levels (µg/kg)	
DON	300
AFG_2, AFB_2	0.4
AFG_1	1.2
AFB_1	2.0
HT-2, T-2	20
ZEA	30
OTA	1.2

Column: Gemini RP18 (150 × 2.0 mm, 5 µm) Phenomenex
Flow: 200 µl/min
Column oven: 40 ºC
Solv A: H_2O, 0.5% acetic acid, 1mM $AcNH_4$
Solv B: CH_3OH, 0.5% acetic acid, 1mM $AcNH_4$
Injection volume: 20µl (100 mg sample)

LC-HRMS chromatogram of a spiked crisp bread extract



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Injection volume: 20µl (100 mg sample)

Methods performances and comparison between LC-MS/MS and LC-HRMS detection

RECOVERIES and REPEATABILITY

EC acceptance criteria (401/2006)

Recoveries, % (RSDr %) in WHEAT BASED CRISP BREAD									
Spiking level (µg/kg)	300	2	0.5	1.2	0.5	20	20	30	1.2
	DON	**AFG_2**	**AFG_1**	**AFB_2**	**AFB_1**	**HT-2**	**T-2**	**ZEA**	**OTA**
MS/MS	**100** (0)	**101** (6)	**106** (5)	**85** (10)	**102** (6)	**107** (2)	**108**(6)	**84** (5)	**101** (3)
HRMS	**104** (0)	**102** (5)	**104** (4)	**80** (2)	**102** (2)	**105** (1)	**103**(1)	**85** (1)	**93** (2)

Similar results in:
barley, wheat and oat flours, rye-based crisp bread

Methods performances and comparison between LC-MS/MS and LC-HRMS detection

DETECTION LIMITS

EC maximum permitted levels
(EC regulations 1881/2006 and 1126/2007)

LOD (µg/kg)		
	LC-HRMS	**LC-MS/MS**
DON	0.3 (+)	29.0 (-)
AFG_2	0.1	0.5
AFG_1	0.2	0.7
AFB_2	0.1	0.4
AFB_1	0.1	0.5
HT-2	0.3	0.5
T-2	0.3	0.5
ZEA	0.4	2.2
OTA	0.2	0.1

Methods performances and comparison between LC-MS/MS and LC-HRMS detection

MATRIX EFFECTS

Matrix effect SSE %		
	LC-HRMS	**LC-MS/MS**
DON	76	95
AFG_2	100	95
AFG_1	100	89
AFB_2	67	88
AFB_1	100	88
HT-2	90	95
T-2	94	103
ZEA	65	70
OTA	87	106

SSE = signal suppression/enhancement
= (slope of matrix calibration/slope of standard calibration)*100

*When **robust sample preparation** and **good chromatographic separation** are applied, **similar matrix effects** are obtained with different MS instrumentation and detection modes.*

Methods performances and comparison between LC-MS/MS and LC-HRMS detection

MS/MS detection HRMS detection legislation requirements

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

MS/MS	HRMS*
1 precursor ion 2 daughters ions	2 ions Mass accuracy < 5ppm
example: **AFB_1**	
313.0 – 241.1	313.07066
313.0 – 213.4	241.04953

* Frament ion obtaiend by collision cell induced fragmentation (HCD)

MoniQA

Towards harmonisation of analytical methods to monitor and control quality and safety in the food chain supply

Proficiency test for multi-mycotoxin methods based on LC-MS(MS)

***Aim of the study*: to obtain information on currently used LC-MS(MS) methodologies for multi-mycotoxin analysis and relevant performances.**

- ✓ **56 laboratories involved**
- ✓ **Distribution of materials ongoing (by Dec 15th, 2010)**
- ✓ **Results expected by February 2011.**

LC-MS/MS DETERMINATION OF MULTI-MYCOTOXIN BIOMARKER IN HUMAN AND ANIMAL URINE

✓ **A reliable indication of individual exposure to the major mycotoxins may be provided by a biomarker**

✓ **Potential markers include the parent compound or metabolite**

Mycotoxin	Urinary biomarker
aflatoxin B_1	aflatoxin M_1 (AFM_1)
ochratoxin A	ochratoxin A (OTA)
deoxynivalenol	deoxynivalenol (DON)
	de-epoxydeoxynivalenol (DOM-1)
zearalenone	alfa-zearalenol (α-ZOL)
	beta-zearalenol (β-ZOL)
fumonisin B_1	fumonisin B_1 (FB_1)

✓ **The response of DON, DOM-1, AFM_1, FB_1, α-ZOL, β-ZOL, OTA as biomarker of mycotoxin exposure has been demostrated in pig, rat and mouse.**

Challenges in multi-mycotoxin method development for biological fluids

Lack of suitable methods of analysis to detect simultaneously a range of chemically different metabolites at trace levels in biological fluids

⇨**Sensitivity** (up to pg/ml)

⇨**Sample preparation**

⇨ **Analyte detection**

⇨ **Method validation**

HPLC
coupled with
Mass Spectrometry

Sample preparation and analysis

Urine sample
Dilution with water

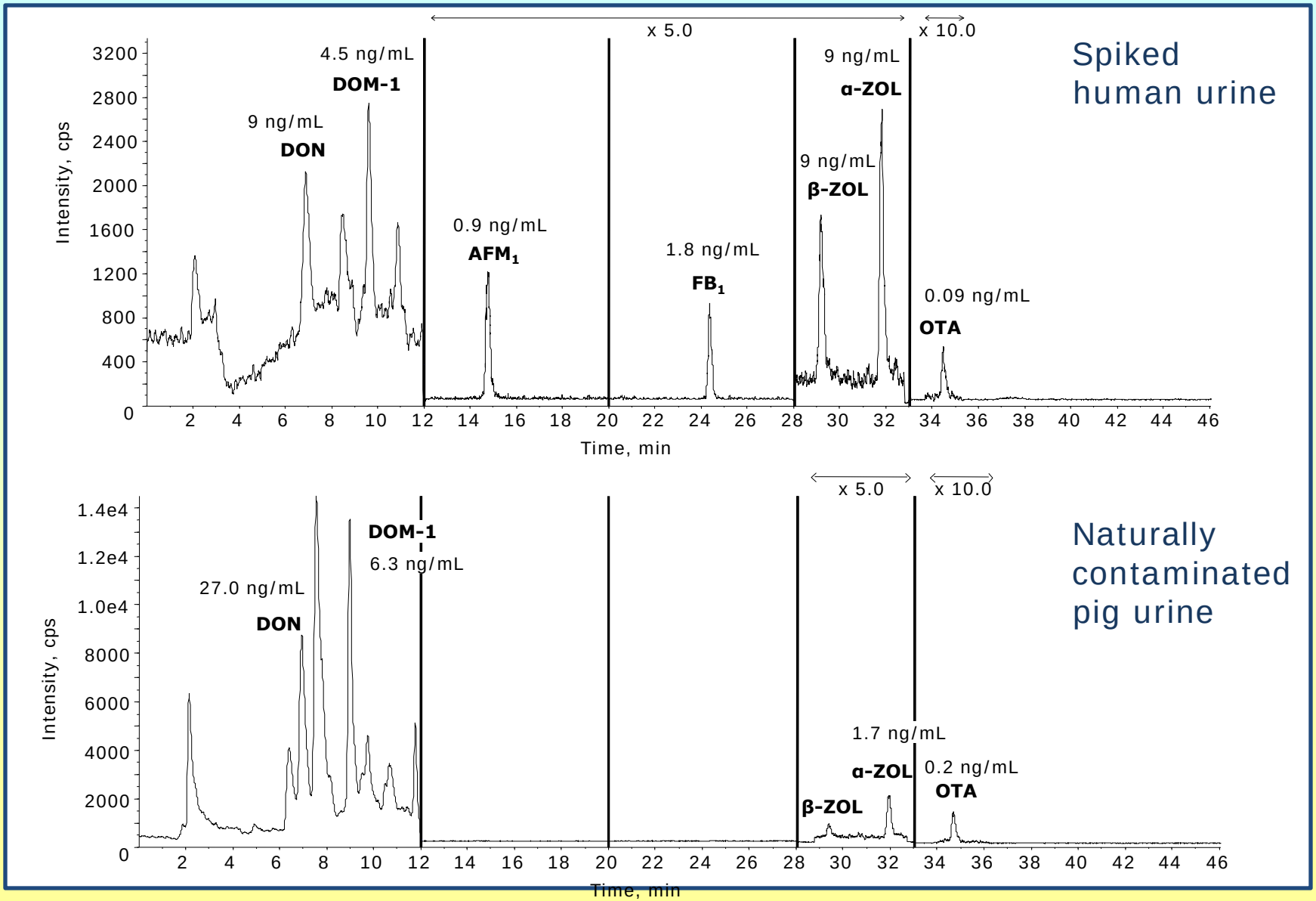
MYCO 6IN1 LC/MS/MS

Clean up
Immunoaffinity column + OASIS HLB column

LC-MS/MS analysis

DON
DOM-1
AFM_1
FB_1
α-ZOL, β-ZOL
OTA

MRM chromatograms of urine samples after IAC-SPE clean up



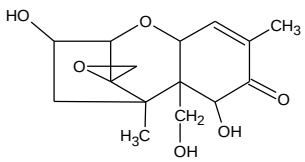
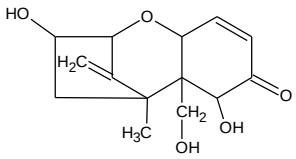
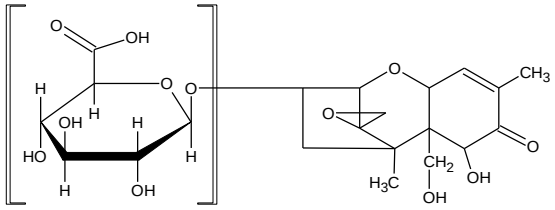
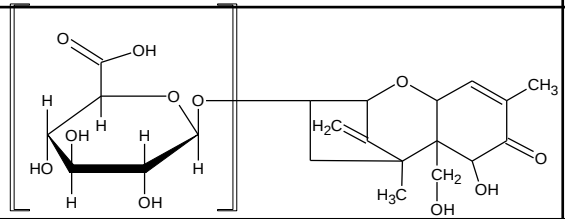
METHOD PERFORMANCES

Mean Recoveries, % (RSDr %) in Human Urine							
Spiking level range(ng/ml)	3-12			1.5-6.0	0.3-1.2	0.6-2.4	0.03-0.12
Mycotoxin	**DON**	**α-ZOL**	**β-ZOL**	**DOM-1**	**AFM_1**	**FB_1**	**OTA**
Recovery (RSDr)	**77** (13)	**72** (9)	**83** (18)	**78** (9)	**96** (8)	**62** (3)	**65** (8)

Limits of Detection (S/N = 3) in Human Urine (ng/ml)						
DON	**α-ZOL**	**β-ZOL**	**DOM-1**	**AFM_1**	**FB_1**	**OTA**
0.8	**0.8**	**2.2**	**0.8**	**0.06**	**0.1**	**0.02**

LC-MS/MS CHARACTERIZATION OF DON URINARY METABOLITE PROFILE IN HUMAN AND RATS

V.M.T. Lattanzio, M. Solfrizzo, A. De Girolamo, S. Chulze, A. Torres, A. Visconti, J. Chrom. B. XXX

	Molecular structure	Characteristic ions (m/z)		Presence in urine	
				Rat	Human
DON		**355.1 295.0 265.1 59.0**	*Negative ions*	**YES**	**YES**
DOM-1		**339.1 279.0 249.1 59.0**	*Negative ions*	**YES**	**NO**
DON glucuronide1		**490.2 297.2 249.2 231.1**	*Positive ions*	**YES**	**YES**
DON glucuronide2		**490.2 177.1 103.1 89.0**	*Positive ions*	**NO**	**YES**
DOM-1 glucuronide		**474.2 281.2 233.1 130.1**	*Positive ions*	**YES**	**YES**

the position of glucuronide moiety is indicative

Challenges in multi-mycotoxin method development
Rapid methods based on dipstick immunoassays

⇨**Antibody production and characterization**

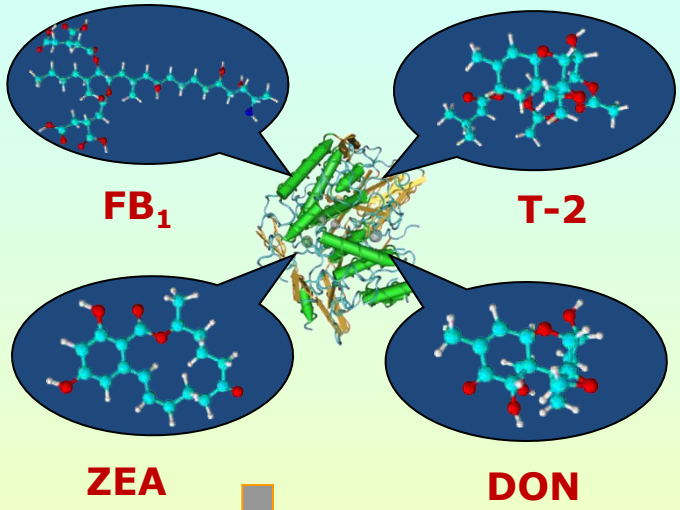
⇨**Multiplex dipstick design and assembly**

⇨**Development of simplified sample preparation protocols**

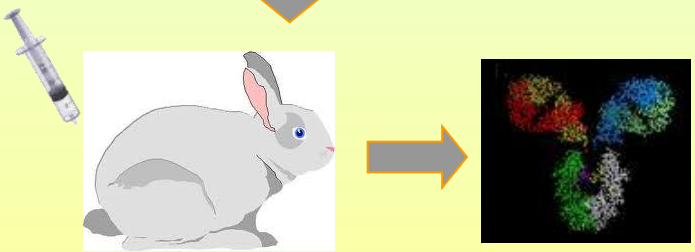
⇨**Method validation and application**

Antibody production and characterization

Polyclonal antibodies raised against **T-2/HT-2, DON, ZEA** and **FB_1/FB_2** have been produced on rabbits.



Immunogen synthesis



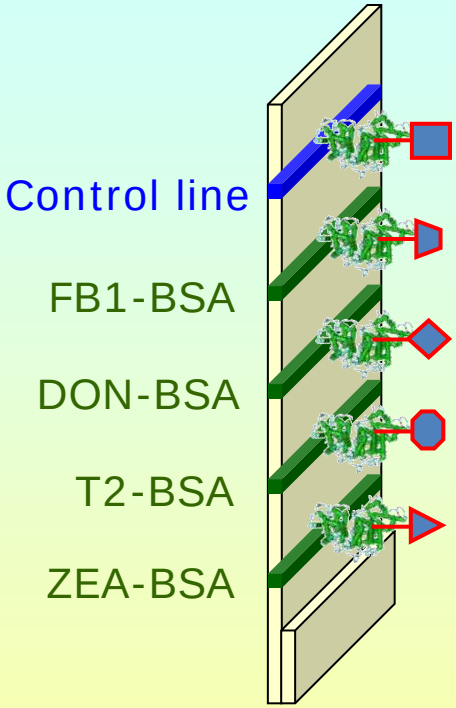
Rabbit immunization and antibody production

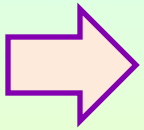
Selection of antibodies with suitable sensitivity

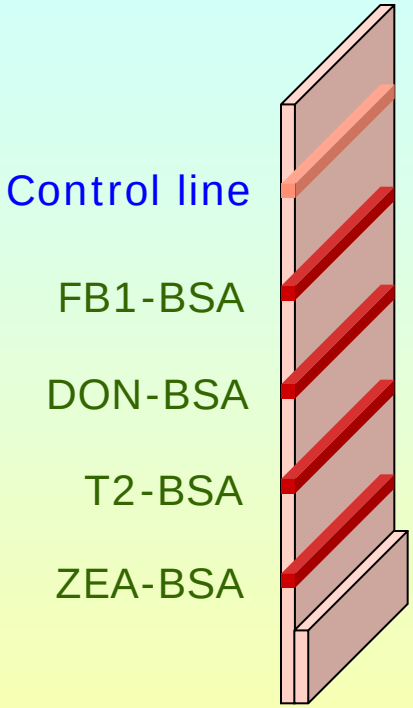
ELISA	FB_1	T-2	DON	ZEA
IC_{50} (ng/ml)	0.5	2.5	3	0.1

Multiplex dipstick design

Indirect competitive immunoassay

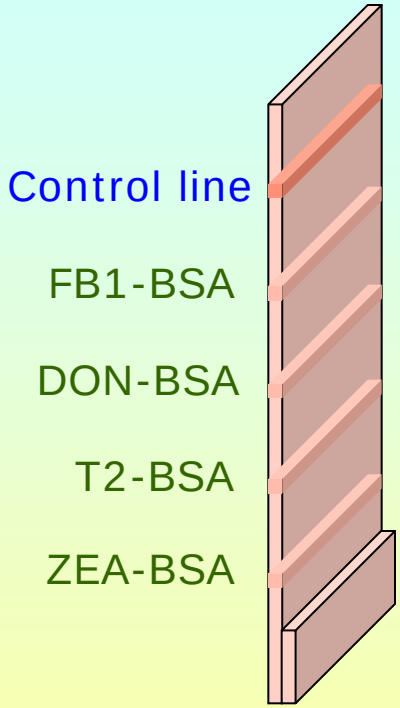






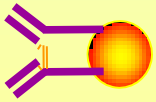
NEGATIVE sample

Test Lines darker than CTRL line



POSITIVE sample

Test Lines lighter than CTRL line



gold-labelled antibody

Materials chosen for method development

WHEAT

OAT

WHEAT BASED EXTRUDED SNACKS

Target toxins: DON, ZEA, T-2 and HT-2 toxins

MAIZE

MAIZE FEED

MAIZE BASED EXTRUDED SNACKS

Target toxins: DON, ZEA, FB_1, FB_2, T-2 and HT-2 toxins

Required cut off: 80% of EU maximum permitted levels

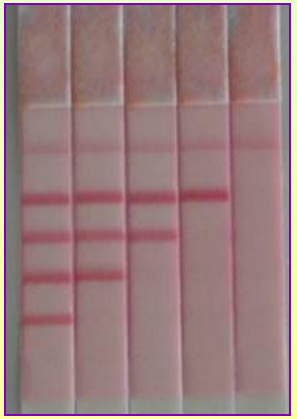
Sample preparation and analysis

Raw cereals
Breakfast cereals
Maize feed

Methanol/water
2 min blending

Dilution and analysis

Incubation at 40°C
Migration



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

METHOD PERFORMANCES

Extraction recoveries, % (RSDr %)				
	ZEA	**T-2 +HT-2**	**DON**	**FB_1+FB_2**
WHEAT	**103** (6)	**73** (7)	**97** (8)	-
OATS	**107** (9)	**73** (6)	**93** (4)	-
MAIZE	**105** (6)	**107** (4)	**105** (3)	**109** (3)

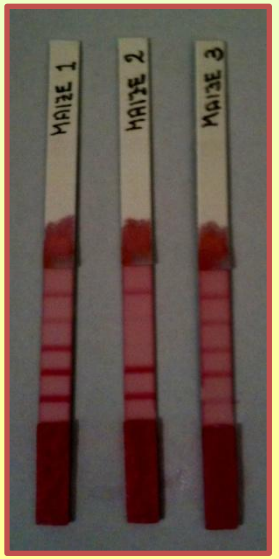
Recoveries were evaluated in triplicate at cut-off levels

CUT OFF levels (µg/kg)				
	ZEA	**T-2 +HT-2**	**DON**	**FB_1+FB_2**
WHEAT	**80**	**400**	**1400**	**-**
OATS	**80**	**400**	**1400**	**-**
MAIZE	**280**	**400**	**1400**	**3200**

Analysis of Naturally Contaminated Maize Samples

Sample	ZEA		T-2 +HT-2		DON		FB_1+FB_2	
	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*	**LOW POS**	*392*	**LOW POS**	*298*	**NEG**	*725*

Good agreement between dipstick and LC-MS/MS analysis.



CUT OFF levels (µg/kg)				
	ZEA	T-2 +HT-2	DON	FB_1+FB_2
MAIZE	280	400	1400	3200

CONCLUSIONS (I)

LC-MS(MS) MULTI-MYCOTOXIN DETERMINATION IN FOOD

Liquid chromatography coupled to tandem MS or high resolution MS provides a reliable tool for quantitation of mycotoxins in foods at regulatory levels.

Legislation requirements are fulfilled with respect to:

- **recoveries** (EC Regulation 401/2006)
- **repeatability** and **reproducibility** (EC Regulation 401/2006)
- **LOD** enabling to detect mycotoxins at regularory levels in the concerned matrices (EC regulations *1881/2006 and 1126/2007)*
- **mass spectrometry detection** *EC performance criteria (2002/657/EC) (Doc No Sanco/10684/2009)*

CONCLUSIONS (II)

LC-MS/MS DETERMINATION OF MYCOTOXIN BIOMARKERS IN HUMAN AND ANIMAL URINE

Advanced LC-MS/MS methodologies have been used for:

✓ **simultaneous determination of multi-mycotoxin biomarker in urine**

✓ **direct determination of mycotoxin metabolic profile at trace levels**

Methods based on high selective and sensitive LC-MS/MS are applicable to **monitoring programmes** providing a **complete and realistic framework of exposure levels and relevant metabolic routes.**

CONCLUSIONS (III)

MULTI-MYCOTOXIN DETERMINATION BY DIPSTICK IMMUNOASSAYS

✓**Multiplex dipstick immunoassays** for the determination of ZEA, T-2+HT-2, DON and FB_1+FB_2 in cereals, cereal-based food and maize feed have been developed, combining the concepts of "**multiplex**" and "**rapid**" detection.

✓The resulting immunoassay protocol is: **rapid, inexpensive,** and **easy-to-use.**

✓**Robustness and reliability of dipstick based methods should be demonstrated through interlaboratory studies.**

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THANK YOU
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