RANDOX
FOOD DIAGNOSTICS

Mycotoxins – Qualitative, Quantitative and Confirmatory Methods

Dr. Liberty Sibanda

Topics

Mycotoxin regulations – EU, USA & Codex

Sample preparation approaches

Different testing technologies

- Lateral flow test
- ELISA
- Microarray
- HPLC
- LC-MS/MS

Mold Contamination

Aflatoxins $B_{1,2}$, $G_{1,2}$

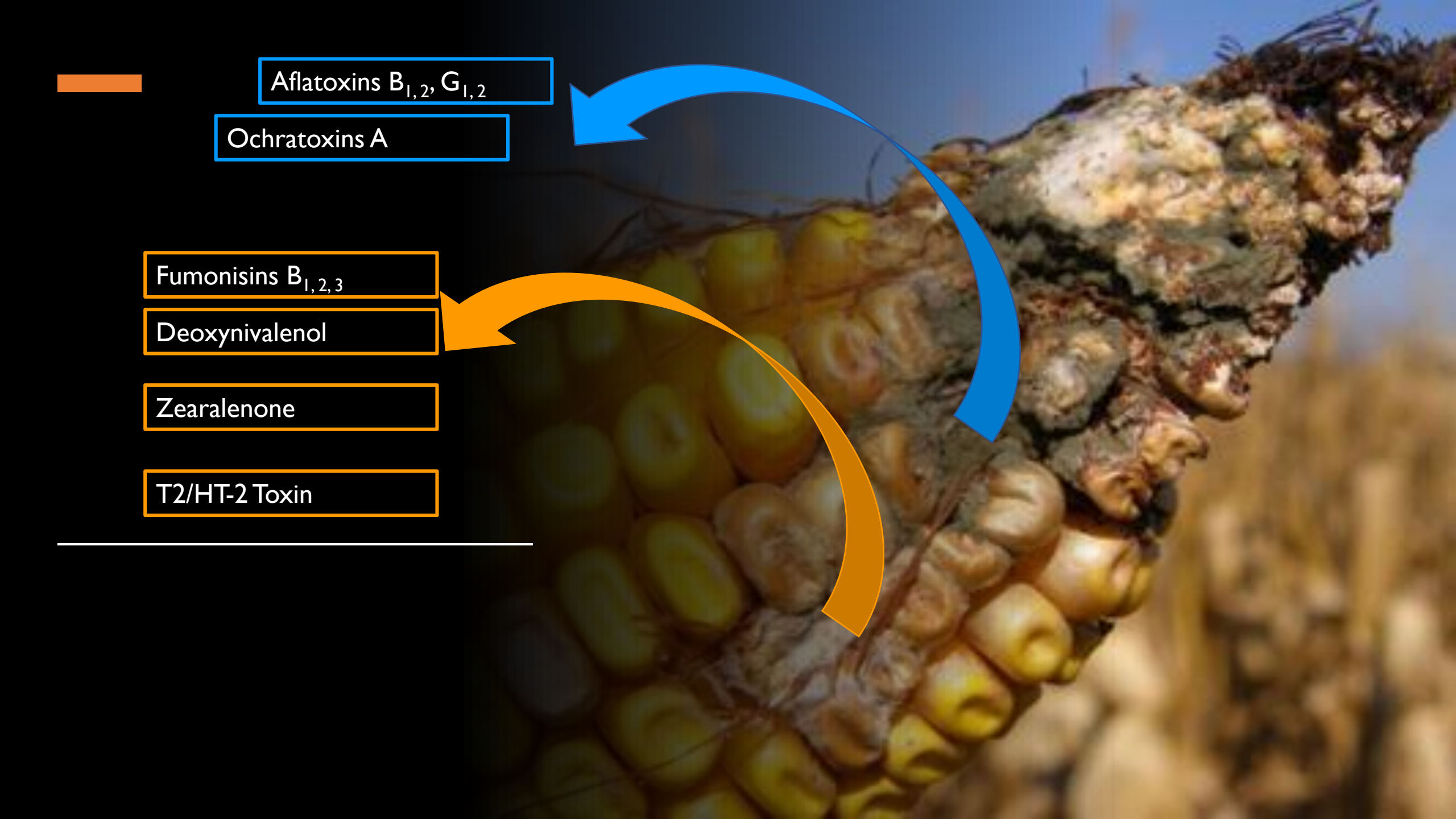
Ochratoxins A

Fumonisins $B_{1,2,3}$

Deoxynivalenol

Zearalenone

T2/HT-2 Toxin



Mycotoxin MRLS for Corn

Mycotoxin	Commission Regulation 1881/2006, as amended (ppb)	USA (ppb)	Codex (ppb)
Aflatoxin B_1	2		10
Total Aflatoxin $B_{1,2,}$ $G_{1,2}$	4	20	20
Ochratoxin A	3	10	5
Fumonisin $B_{1,2,3}$	1,000	2,000	2,000
Deoxynivalenol	750	1,000	2,000
Zearalenone	100		
	Commission Recommendation 2013/165/EU		
T-2/HT-2 Toxin	100		

Mycotoxin MRLS for Wheat

Mycotoxin	Commission Regulation 1881/2006, as amended (ppb)	USA (ppb)	Codex (ppb)
Aflatoxin B_1	2		
Total Aflatoxin $B_{1,2}$, $G_{1,2}$	4	20	
Ochratoxin A	5		5
Fumonisin $B_{1,2,3}$			
Deoxynivalenol	1,250		2000
Zearalenone	100		
	Commission Recommendation 2013/165/EU		
T-2/HT-2 Toxin	100		

Mycotoxin MRLS for Nuts

Mycotoxin	Commission Regulation 1881/2006, as amended (ppb)	USA (ppb)	Codex (ppb)
Aflatoxin B_1	5		
Total Aflatoxin $B_{1,2,}$ $G_{1,2}$	10	20	10
Ochratoxin A	5	-	5
Fumonisin $B_{1,2,3}$	-	-	-
Deoxynivalenol	-	-	-
Zearalenone	-	-	-
T-2 Toxin	-	-	-
HT-2 Toxin	-	-	-

Mycotoxin MRLS for Feed

Mycotoxin	Commission Regulation EC 574/2011 (ppb)	USA (ppb)	Codex (ppb)
Aflatoxin B_1	20	300	
Total Aflatoxin $B_{1,2}$, $G_{1,2}$			20
	Commission Recommendation EC No 576/2006		
Ochratoxin A	250		
Fumonisin $B_{1,2,3}$	60,000	60,000	
Deoxynivalenol	8,000	10,000	
Zearalenone	2,000		
	Commission Recommendation 2013/165/EU		
T-2/HT-2 Toxin	500		

IMMUNOASSAYS

Sample Preparation

Homogenised ground (5 – 25 g)

Extracted by shaking with:

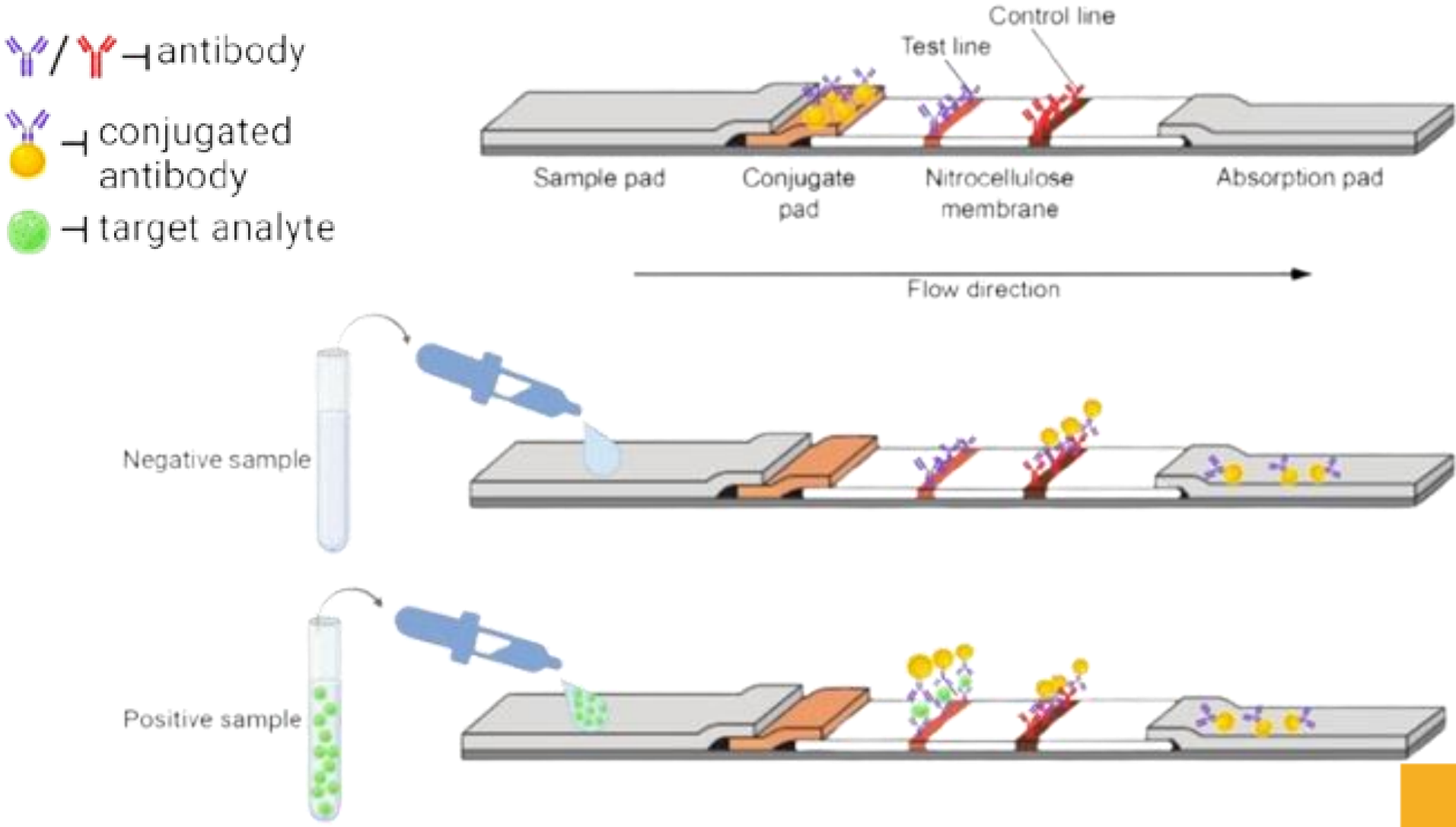
- Water
- MeOH/H_2O (80/20; v/v)
- ACN/MeOH/H_2O (50/40/10;v/v/v)

The sample is centrifuged OR filtered

Supernatant is diluted with either H_2O or Wash Buffer

Lateral Flow Dipstick

Current Testing Strategies



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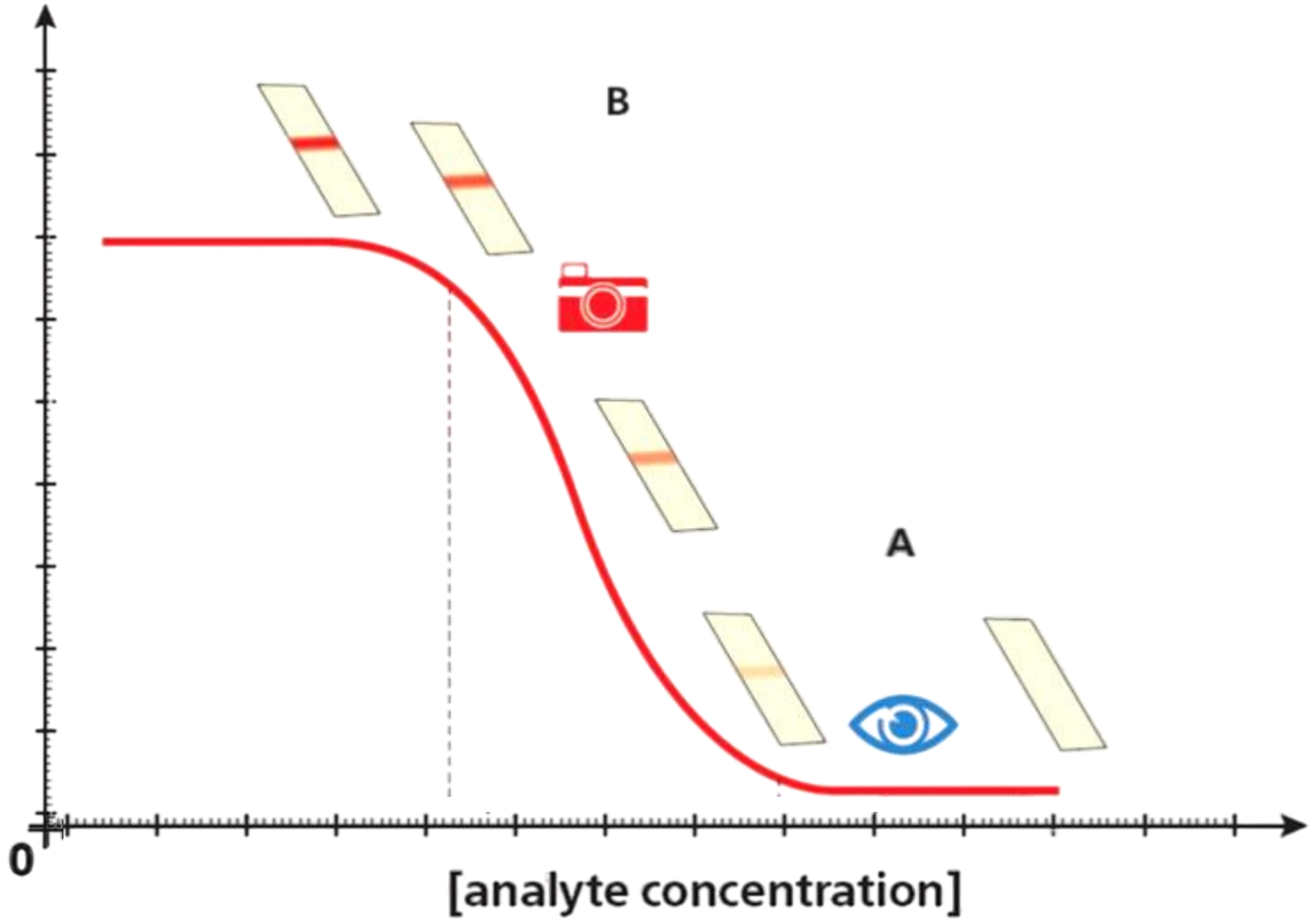
Negative | Positive | Negative

Control Line

Test Line



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Performance Data of Lateral Flow Test

	TIME	LOD
Aflatoxin B_1	6 mins	2 ppb
Ochratoxin A	5 mins	1.1 ppb
Fumonisins	3 mins	300 ppb
T2 Toxin	5 mins	50 ppb
Zearalenone	5 mins	36 ppb
Deoxynivalenol	3 mins	300 ppb

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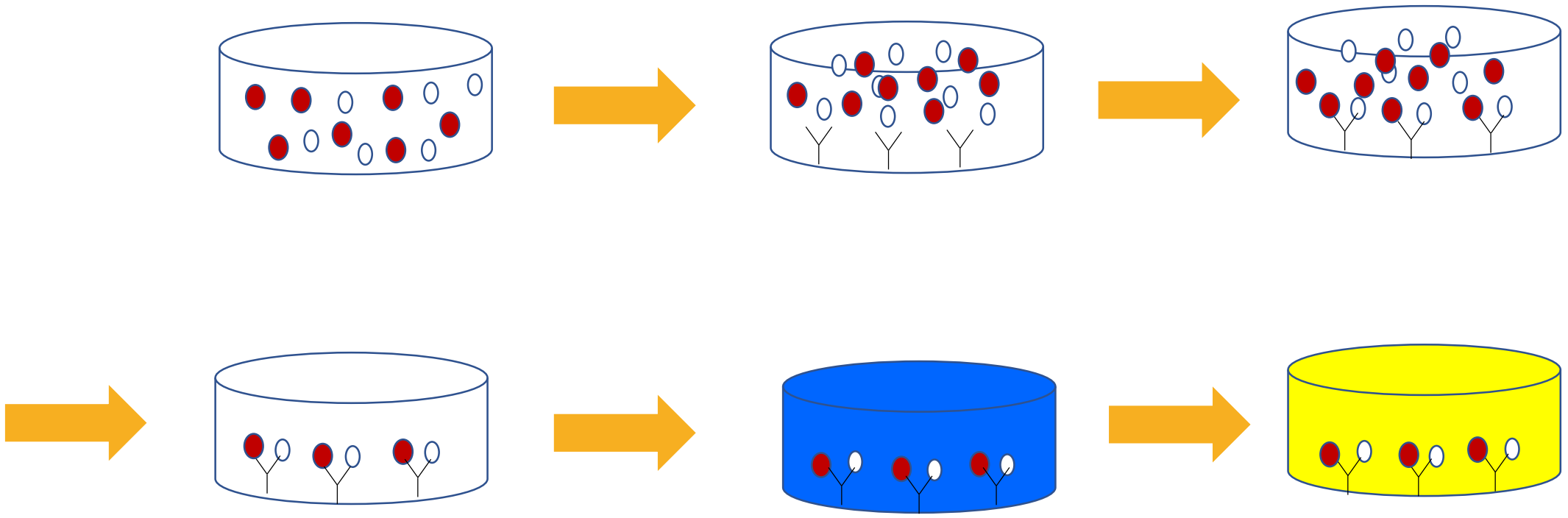
Enzyme-Linked Immunosorbent Assay (ELISA)

Mycotoxin-Enzyme Conjugate

Mycotoxin

S Substrate

Anti-Mycotoxin Antibodies



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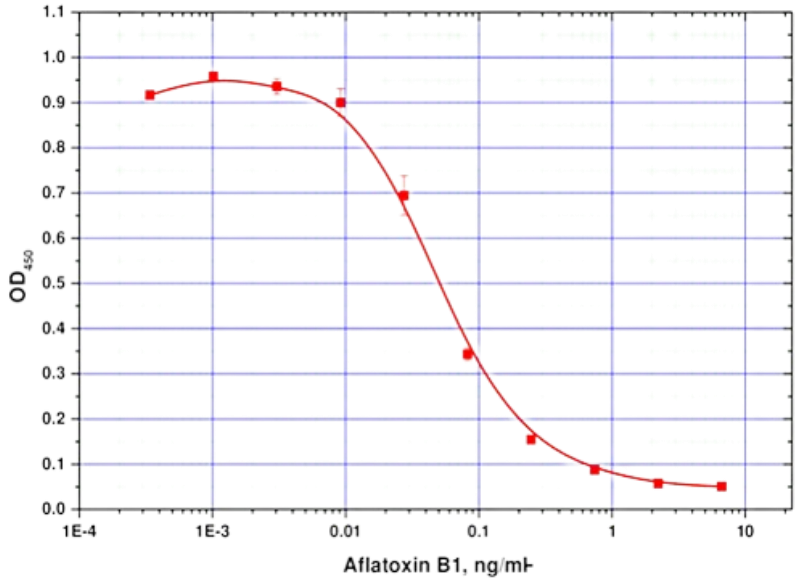
Sample Extraction:

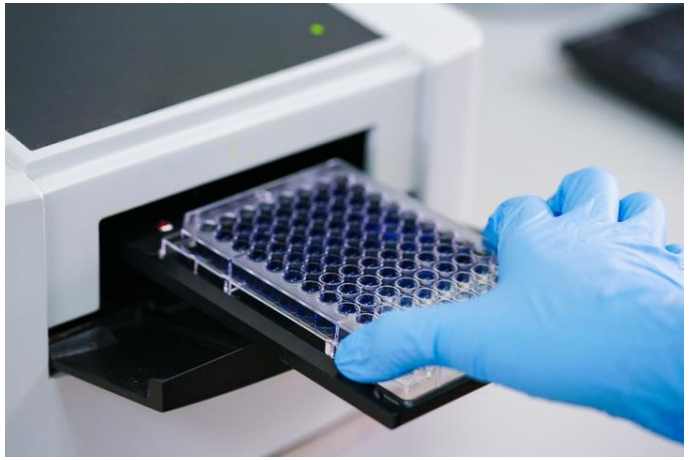
- Weighing
- Shaking with extraction solution
- Centrifugation or filter

Assay:

- Add standards + samples + enzyme conjugate
- Incubate
- Wash
- Add substrate

- Stop reaction and read





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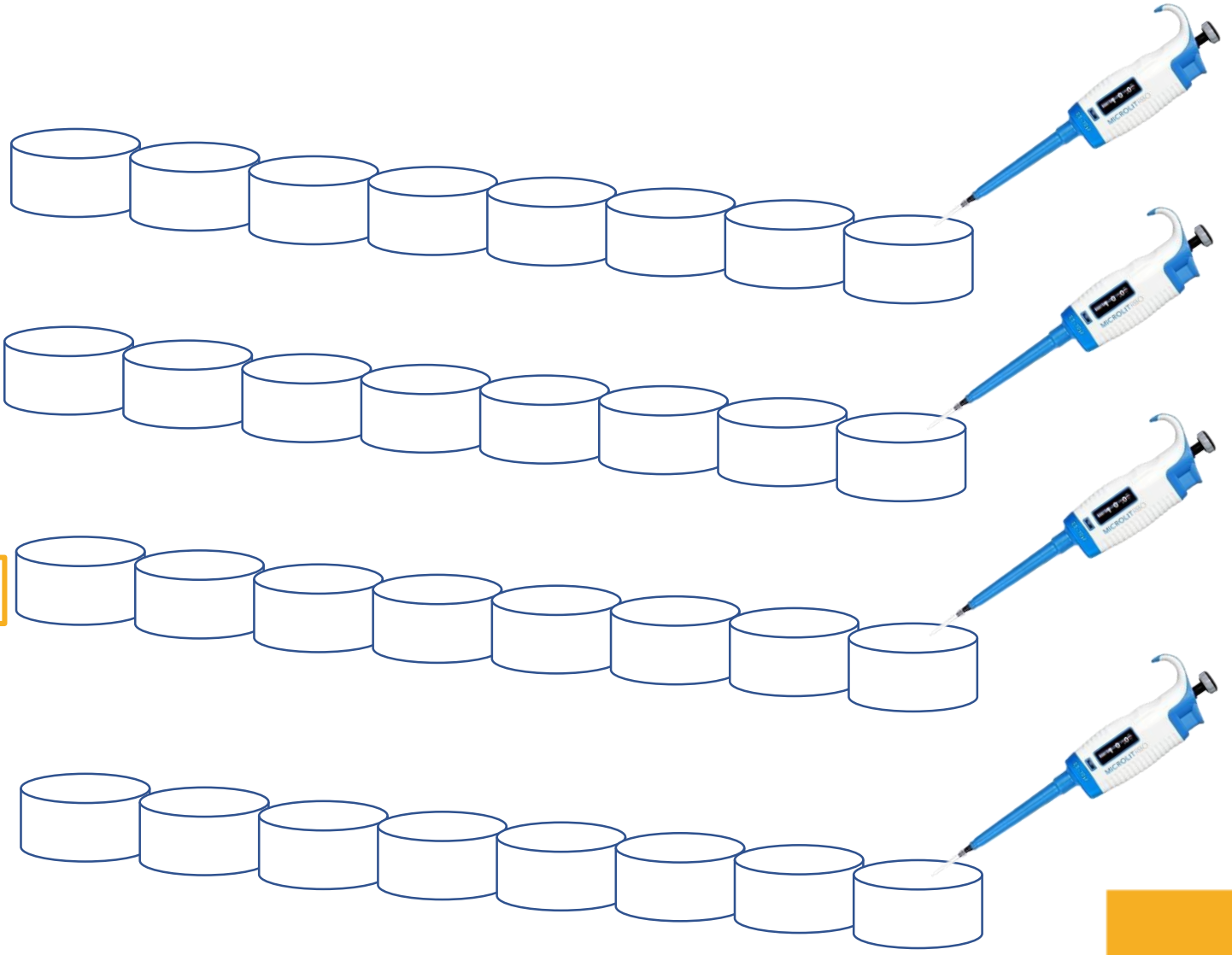
AFLATOXIN B_1

OCHRATOXIN A

DEOXYNIVALENOL

FUMONISINS



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Performance Data of ELISA

	TIME	LOD	LOQ	RECOVERY
Aflatoxin B_1 FAST	5 mins	1.5 ppb	5 ppb	97%
Aflatoxin Total FAST	10 mins	0.6 ppb	2.1 ppb	101%
Ochratoxin A FAST	15 mins	0.8 ppb	2 ppb	100%
Fumonisins FAST	20 mins	15 ppb	50 ppb	105%
T2 Toxin FAST	20 mins	13 ppb	17.5 ppb	97%
Zearalenone FAST	20 mins	5 ppb	10 ppb	99%
Zearalenone	45 mins	0.04 ppb	7.6 ppb	102%
Aflatoxin B_1	140 mins	0.005 ppb	0.058 ppb	98%
Deoxynivalenol	60 mins	0.5 ppb	2 ppb	106%

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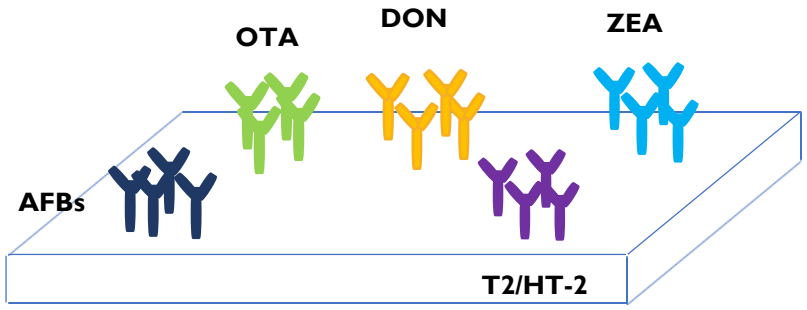
Biochip Array Technology (BAT) for Simultaneous Detection of up to 9 Mycotoxins in a Single Sample
High Throughput Quantitative Detection

Biochip Array Technology

- Biochip is a ceramic platform
- Mabs are spotted on specific locations
- Competitive immunoassay (mycotoxin in sample competes with mycotoxin conjugated to HRP enzyme
- Chemiluminescence signal generation



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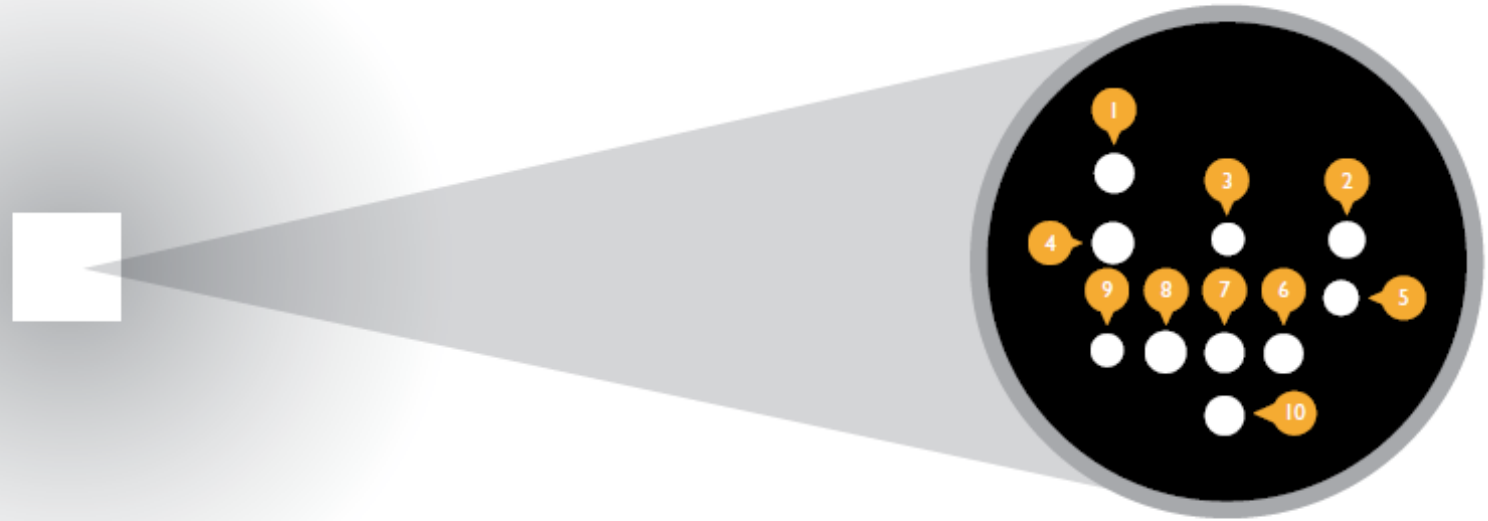
Biochip Array Technology –Biochip layout eg Myco7



(1). Reference Spot, (2). Correction Spot, (10). Correction Spot (8). Aflatoxin B_1/B_2 (9). Zearalenone
(3). Fumonisins (4). Ochratoxin A (5). Aflatoxin G_1/G_2 (6). Deoxynivalenol (7). T-2/HT-2 Toxin

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All on one platform

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Sample Preparation

1. 5g of milled homogenised sample
2. Add 25ml of solvent (Acetonitrile:Methanol:Water; 50:40:10, v/v/v)
3. Vortexed and shaken for 10 minutes
4. Centrifuged for 2 minutes at 1500 rcf
5. Diluted 50µl sample in 150µl diluted wash buffer

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Assay Procedure

1. Pipette 150 µl of Assay Buffer into each well
2. 50 µl of Standards and samples were added to the Biochips
3. Incubation at 25°C for 30mins in a Thermoshaker
4. HRP-Conjugate was added & incubated for 1 hr at 25°C
5. Biochips were washed with Wash Buffer
6. Luminol/Peroxide (substrate) was added & incubated for 2 min in the dark

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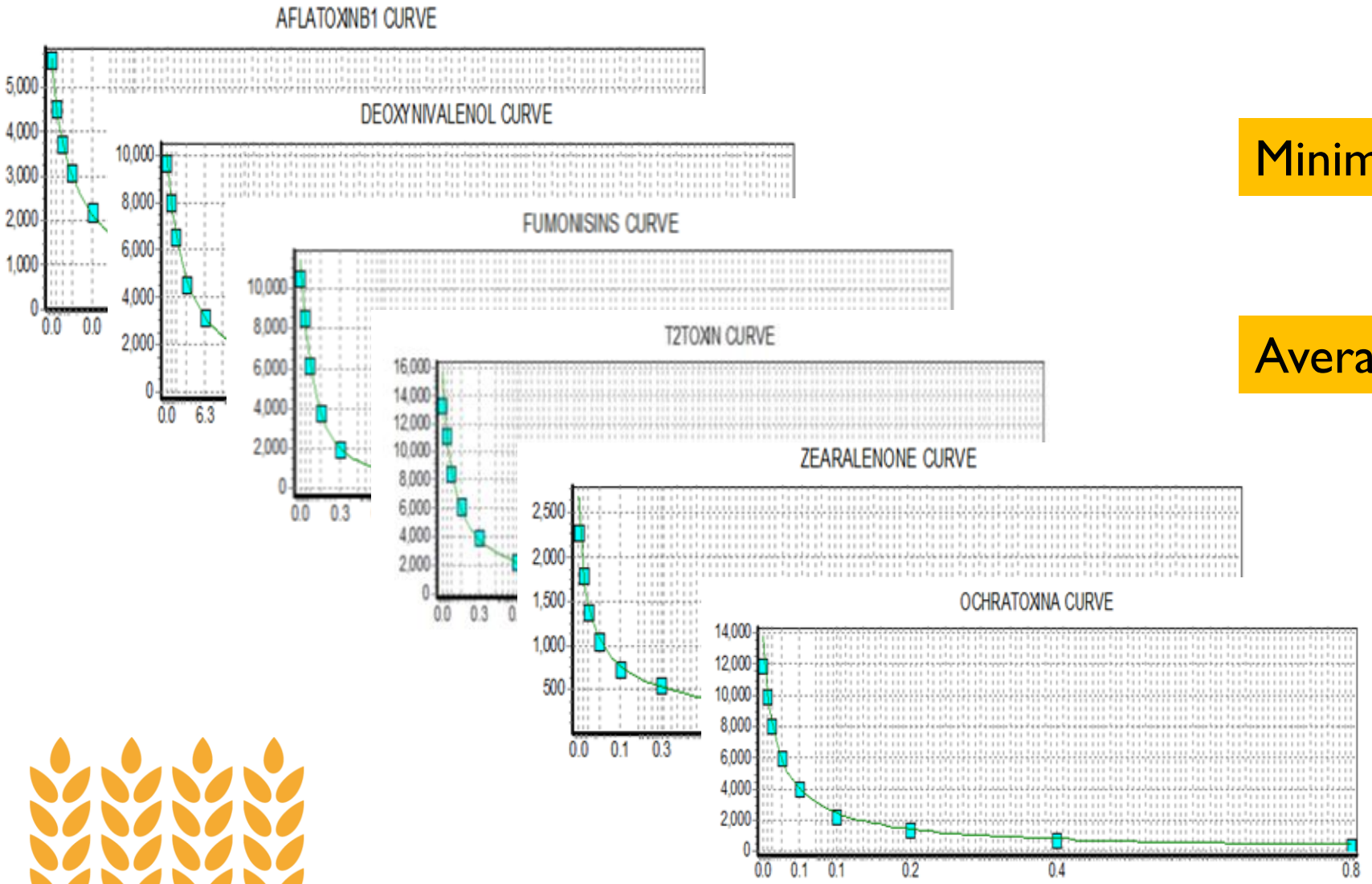
Calibration Curve



Minimum Curve Fit = 95%

Average Regression = 99%

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Current User : gillian

Time : 10:51:32

Sample Entry | Result History | Calibration | QC | Archiving | Error Log | Store Work | Retrieve Work | LIMS | Service | Log Off | Close

Result History

General search by Array, Date, Operator and Batch

Array	Date	Operator	Batch Id
Myco 7	02/19/2019	gillian	1557

Specific Search

Sample

Search

Selected Samples

	Sample	Time	Cal Id	LIMS
✓	1	11:54:47	75	
✓	2	11:54:47	75	
✓	3	11:54:47	75	
✓	4	11:54:47	75	
✓	5	11:54:47	75	
✓	6	11:54:47	75	
✓	7	11:54:47	75	
✓	8	11:54:48	75	
✓	9	11:54:48	75	
✓	1	11:57:35	75	
✓	2	11:57:35	75	
✓	3	11:57:35	75	
✓	4	11:57:35	75	
✓	5	11:57:35	75	
✓	6	11:57:35	75	
✓	7	11:57:35	75	
✓	8	11:57:36	75	
✓	9	11:57:36	75	
✓	1	12:05:03	76	
✓	2	12:05:03	76	
✓	3	12:05:03	76	
✓	4	12:05:03	76	
✓	5	12:05:03	76	
✓	6	12:05:03	76	
✓	7	12:05:03	76	
✓	8	12:05:04	76	
✓	9	12:05:04	76	
✓	1	12:07:49	76	
✓	2	12:07:49	76	
✓	3	12:07:49	76	
✓	4	12:07:49	76	
✓	5	12:07:49	76	
✓	6	12:07:49	76	
✓	7	12:07:49	76	
✓	8	12:07:49	76	
✓	9	12:07:50	76	

Selected Samples Results View

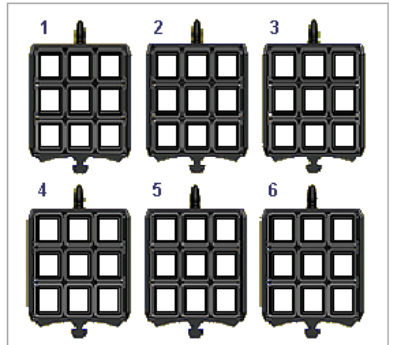
Sample Code : 6

Date : 02/19/2019 11:54:47

Analyte	Units	Result	Error	DFactor
FUMONISINS	ppb	0.289	0	1
OCHRATOXINA	ppb	0.0523	0	1
AFLATOXING1	ppb	0.0644	0	1
DEOXYNIVALENOL	ppb	6.535	0	1
T2TOXIN	ppb	0.226	0	1
AFLATOXINB1	ppb	0.0081	0	1
ZEARALENONE	ppb	0.133	0	1

Worklist Array

Awaiting Loading



Worklist Summary

Carrier	Array	No.Samples

Camera Status

No Work (Waiting)

-60 -50 -40 -30 -20 -10 0 10 20 30

Investigator Cycle Status

Awaiting Work

Accept Samples | Open Door

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Final Results Print-Off

Myco 9 Report

Array Details:	Myco 9
Sample(s) Run By:	gillian craig
Batch Details:	2101
Date of Sample(s):	09/25/2019 16:44:28
Date of Calibration:	09/24/2019 01:01 PM
Calibrator Lot No:	2101

		ppb	ppb	ppb	ppb	ppb	ppb
SampleCode	(DF)	PAX	FUM	OTA	AFG1	DAS	DON
8	20	1.678	221.583	2.7541	0.0234	1.530	754.082

		ppb	ppb	ppb
SampleCode	(DF)	T2	AFB1	ZEA
8	20	15.719	0.0089	1.572

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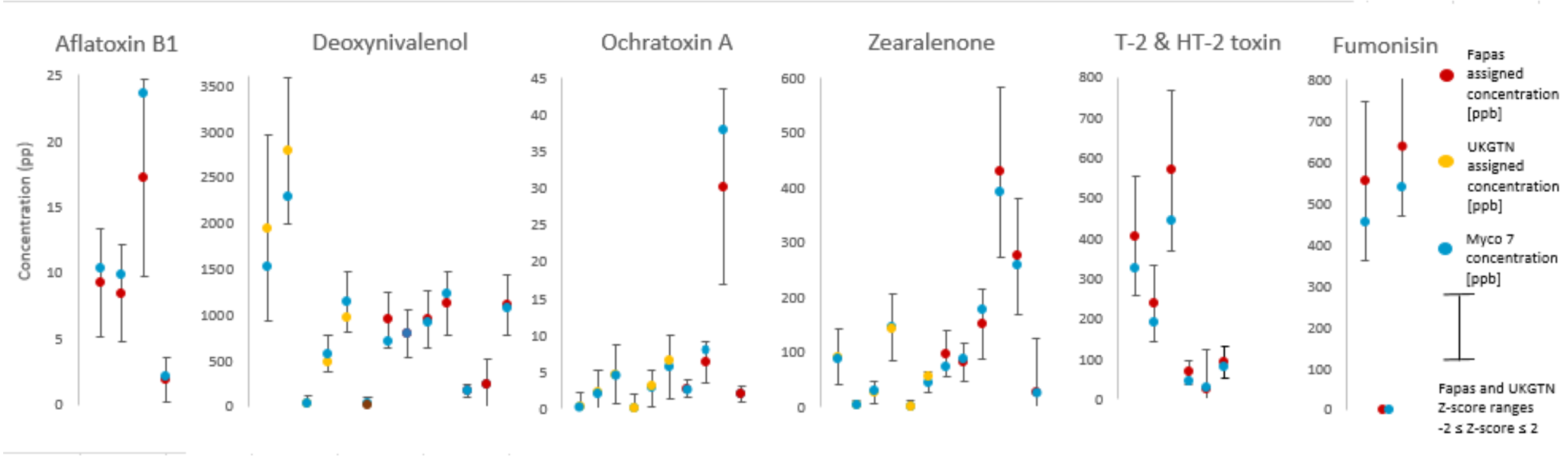
Myco 7 Validation – Samples assessment

Proficiency testing scheme:

- **All the samples were within the accepted -2≤ Z-score ≤2.**
- **Fapas® samples tested: maize, maize flour, cereal based animal feed, wheat flour, oat flour.**
- **UKGTN samples tested: wheat and barley.**



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Myco biochip products. Benefits:

- A simple, single extraction step.
- **Reliable** quantitative detection of prominent, masked and emerging mycotoxins
- Detect co-occurrence of **multiple mycotoxins** from a single sample
- Increased **detection capacity** (multi-mycotoxins detection of 45 samples per 1 kit)
- Detect multiple mycotoxins at very **low concentrations**
- Reduced number of samples requiring confirmatory analysis
- **Flexibility** of this technology allows the extension of the analytical profile for new, more restrictive jurisdictions to meet future challenges.

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Chromatography

Sample Preparation

Weigh sample

Add extraction solution and shake

Centrifuge and use supernatant

Sample Preparation for LC-MS/MS

Sample (25 g) is ground to a fine powder.

It is extracted with 50 ml of 80:20 methanol water.

The mixture is shaken for 30 – 60mins at high speed.

The mixture is centrifuged or filtered through Whatman No. 4 filter paper.

The supernatant of filtrate diluted to <30% MeOH with $NaCHO_3$.

Diluted extract is extracted over IACs, or QuEChERS or some form of Bonded Silica or MIP.

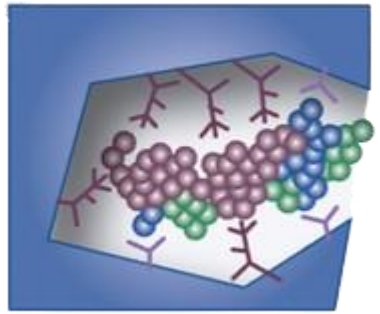
Sample Clean-Up

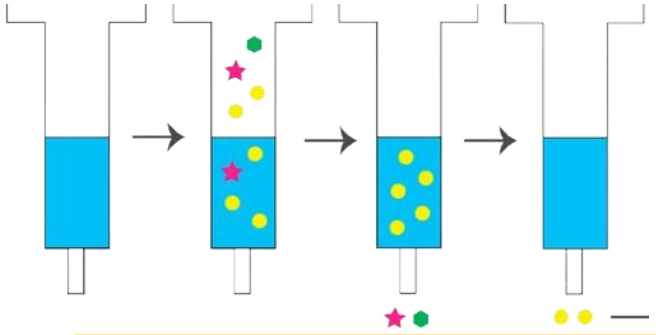
Solvent Extraction:

- Most mycotoxins are polar (conferred either by reactive groups or by polar charge).
- Extraction solvents vary from polar organic solvents to 100% water.
- Extraction solvents are not mycotoxin specific and do not discriminate against matrices.

Clean-Up:

- Solid Phase Clean-Up
  - (i) Immunoaffinity Columns (IAC) (AFLAs, OTA, DON, ZEA, FUM, T2/HT-2 e.t.c).
  - (ii) QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)
  - (iii) Solid Phase Imprinted Polymers





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HPLC

Zearalenone – FLD signal (Excitation 274 nm, Emission 446 nm)

Ochratoxin A - FLD signal (Excitation 333 nm, Emission 460 nm)

Fumonisins – FLD signal (Excitation 335 nm , Emission 440 nm)

Aflatoxins - FLD signal (Excitation 360nm, Emission 455nm)

Deoxynivalenol – FLD signal (Excitation 360 nm, Emission 470 nm)

T2/HT-2 Toxin – FLD signal (excitation 381 nm, Emission 470 nm)

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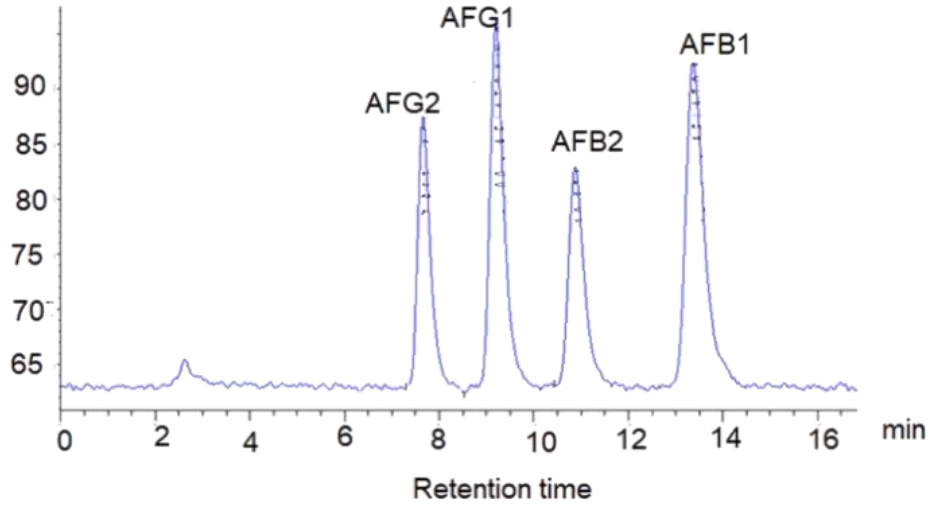
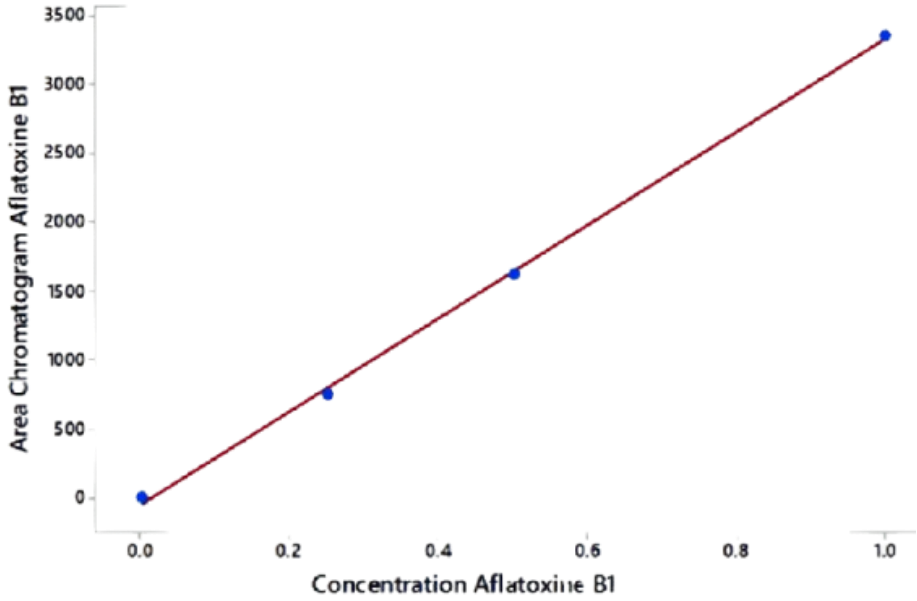
Disadvantages of Current Strategies

HPLC





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Performance Data of HPLC

	AFB_1	AFB_2	AFG_1	AFG_2	OTA	ZEA	T2/HT-2	FB_1	FB_2	FB_3	DAS	DON	15-A-DON	3-A-DON	NIV
LOD	0.14	0.09	0.12	2.50	0.13	4.00	3.00	0.60	1.20	1.50	15.00	14.00	12.00	12.00	20.00

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Tsquar System
Tsquar System

Sample Preparation for LC-MS/MS

Sample (25 g) is ground to a fine powder.

It is extracted with 50 ml of 80:20 methanol water and 2.5 g of NaCl.

The mixture is stirred for 2 hrs at high speed on a magnetic stirrer.

The extract is filtered through Whatman No. 1 filter paper.

The filtered extract is diluted 5 times with mobile phase buffer A (0.1% acetic acid in 10% methanol/H_2O).

Matrix-Matched calibration of 0.1- 450 ppb of Multi-Mycotoxin mix is done in the sample extract.

One of the mycotoxins is used as an internal standard at e.g., 75 ppb.

Sample Preparation

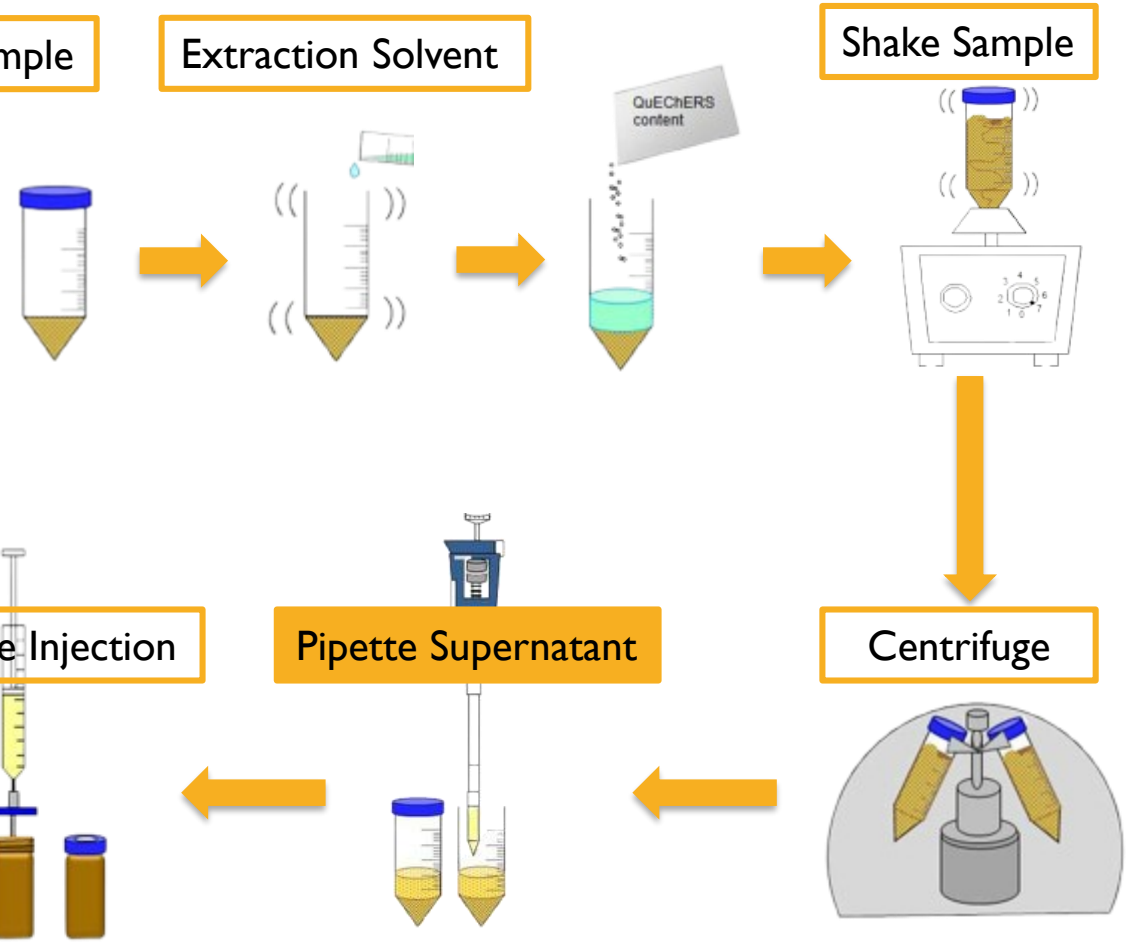
Targeted

Aqueous/Organic Solvent Extr. Solution

Only 6 Mycotoxins

Elute, Evaporate, Resuspend & Analyse

Un-Targeted



LC Conditions

Column	C-18 Bonded (5µm, 150 mm x3.0 mm)
Buffer A	0.1% CH_3 COOH in 10% MeOH/H_2O
Buffer B	0.1% CH3 COOH in 10% MeOH/H_2O
Injection Solvent	0.1% CH_3 COOH in 10% MeOH/H_2O
Injection Volume	10µl

API Conditions

Ionization Mode	ESI (positive and negative)
Collision Gas	1.8 mTorr Argon
API Drying Gas	30 psi at 250°C
API Nebulizing Gas	50 psi
SIM Width	0.7 amu
Needle	4500 V
Capillary	Scanning
Shield	600 V
Detector	1900 V

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LC-MS/MS Condition

Mobile Phase

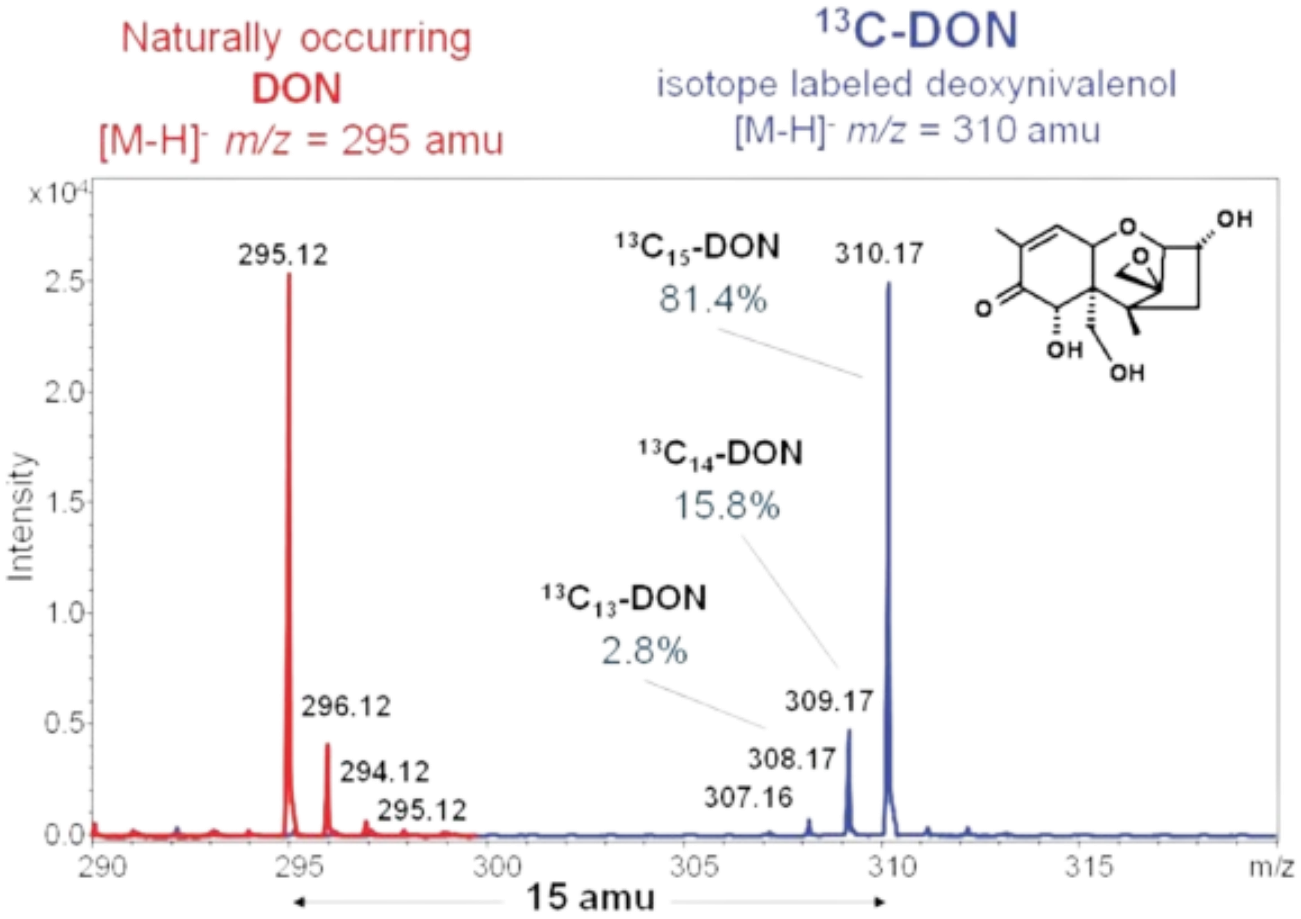
ACN/MeOH with Ammonium Acetate

MeOH helps baseline separate ZEA metabolites

$NH_4CH_3CO_2$ intensifies the signal (promotes ionization)

Stationary Phase

C-18 Bonded phase for polar retention & a-polar molecules
Typically – 2 x150mm, 3µm



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LC-MS/MS Conditions

ESI Source (Ionization Parameters)

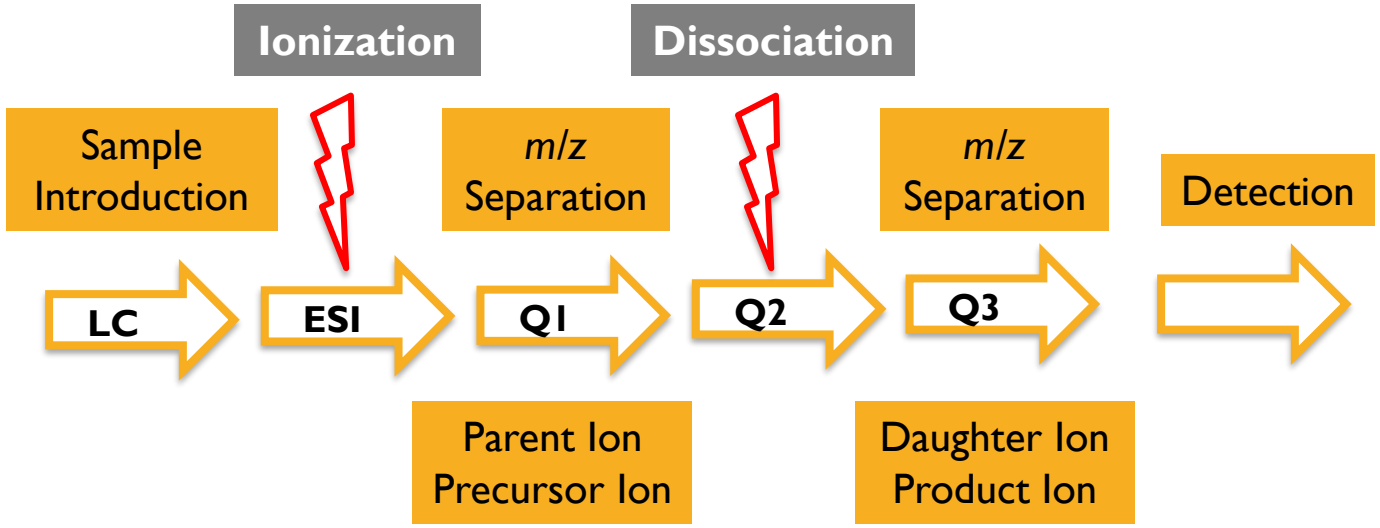
Source temperature 350°C

Curtain gas 35 psi, Gas 1 60psi, Gas 2 35psi

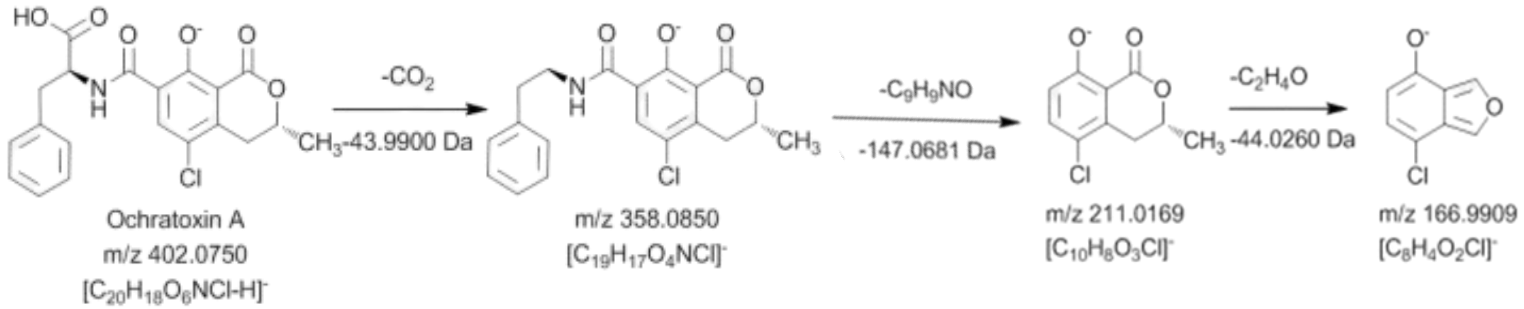
Dissociation

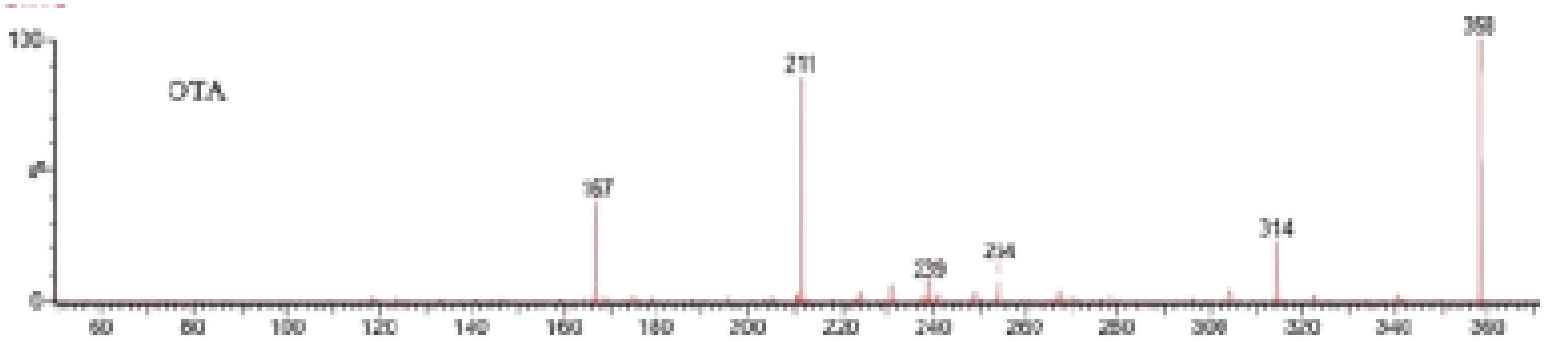
Ion spray voltage - ±4,000 V (Nitrogen)

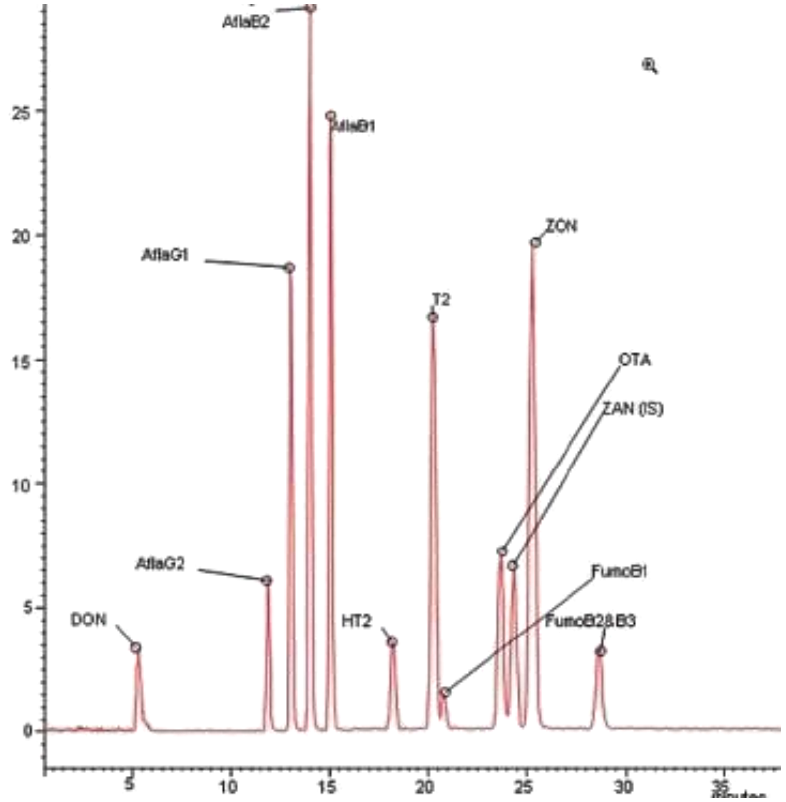
Ion spray voltage - ±4,000 V



MS/MS Fragmentation & Detection







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Outstanding in its field

Name	Capillary (V)	Q1 (m/z)	Ion Polarity	Q3 (1) (m/z)	CE1(V)	Q3(1) (m/z)	CE2 (V)	Dwell (sec)
Nivalenol	59	211.1	[M-H]-	291.0	8.00	191.0	19.0	0.3
Patulin	40	153.1	[M-H]-	108.9	7.00	81.00	10.0	0.3
DON	40	295.0	[M-H]-	265.0	11.5	138.0	11.5	0.3
Fus X	40	353.0	[M-H]-	187.0	29.0	205.0	9.50	0.3
AFG_2	90	331.0	[M+H]+	245.0	16.5	275.0	11.5	0.3
AFG_1	90	329.0	[M+H]+	283.0	25.5	243.0	25.5	0.3
AFB_2	63	315.0	[M+H]+	287.0	21.5	259.0	10.0	0.3
AFB_1	63	313.0	[M+H]+	245.0	16.5	241.0	22.0	0.3
FB_1	54	720.9	[M-H]-	157.0	33.0	563.0	24.0	0.3
FB_2	95	704.8	[M-H]-	157.0	33.0	548.0	22.0	0.3
FB_3	95	704.6	[M-H]-	546.0	33.0	159.0	33.0	0.3
T2 toxin	85	484.0	$[M+NH_4]+$	305.0	18.5	117.0	13.5	0.3
OTA	70	402.7	[M-H]-	358.0	18.5	167.0	34.0	0.3
ZEA	85	319.0	[M-H]-	136.0	26.5	187.0	28.5	0.3
α-Zearalenol	90	319.0	[M-H]-	174.0	25.5	159.0	27.5	0.3
β-Zearalenol	100	319.0	[M-H]-	275.0	16.0	187.0	23.0	0.3
ZEA (IS)	70	317.0	[M-H]-	185.0	24.5	187.0	18.5	0.3

Performance Data of LC-MS/MS

	AFB_1	AFB_2	AFG_1	AFG_2	OTA	ZEA	T2/HT-2	FB_1	FB_2	FB_3	DAS	DON	15-A-DON	3-A-DON	NIV	BEA
LOQ	0.20	0.12	0.20	0.12	0.8	1.0	0.50	1.75	1.00	1.00	0.5	3.50	8	2	6.25	0.2

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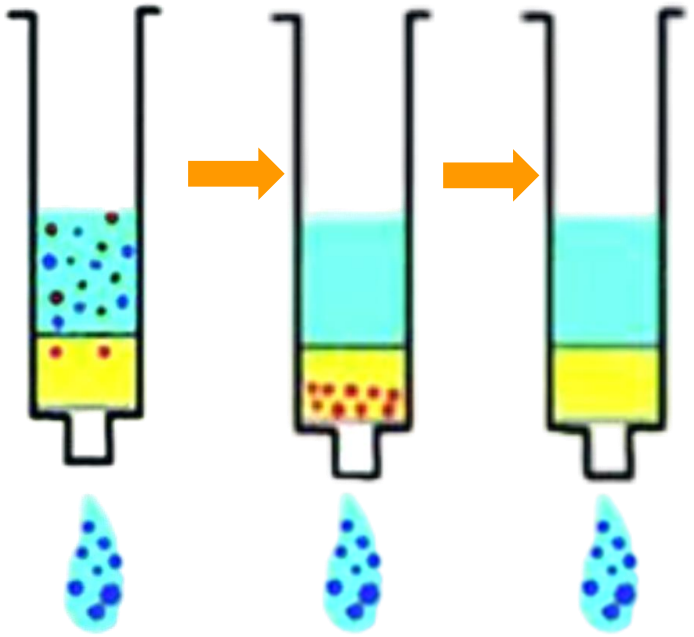
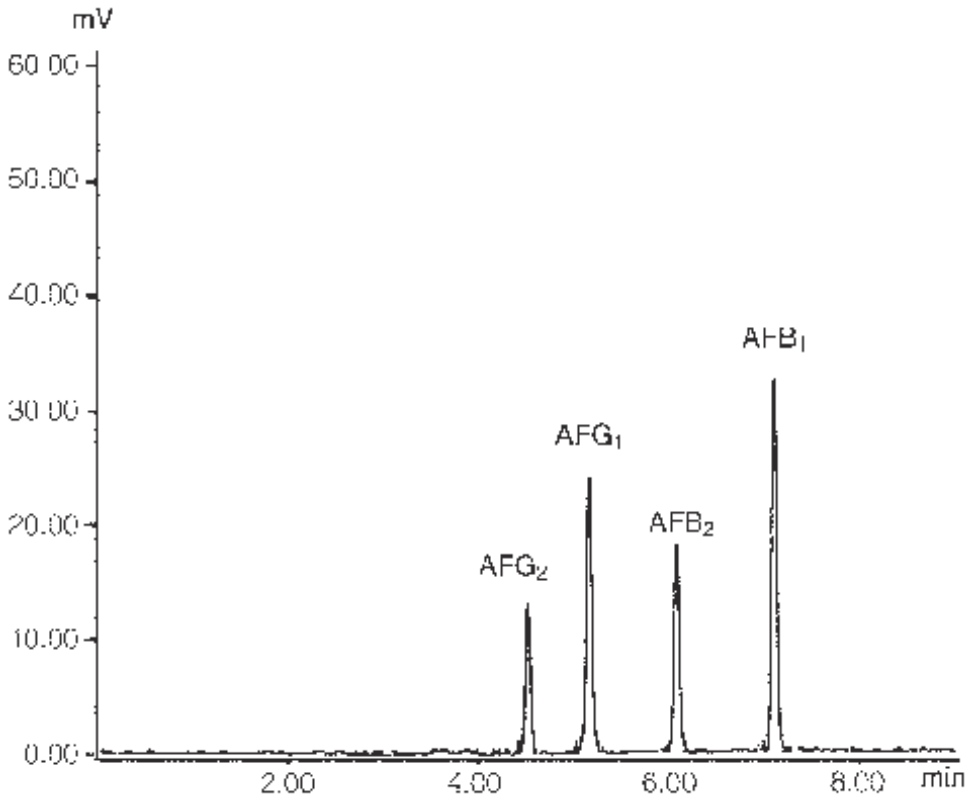
HPLC Cost of Analysis



Sample preparation cost



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Thank you for listening

Do you have any questions

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