Accurate Quantitation of Regulated Mycotoxins by UHPLC/MS/MS

Dr. Thomas Glauner

Senior LC/MS
Applications Scientist

EMEA Market
Development Team

Agenda

- Mycotoxins
- EU Regulations
- Stable isotope dilution assay (SIDA) for the mycotoxins regulated in the European Union
  - Reasons
  - Approaches
  - Sample Preparation and Method
  - Results
- Summary

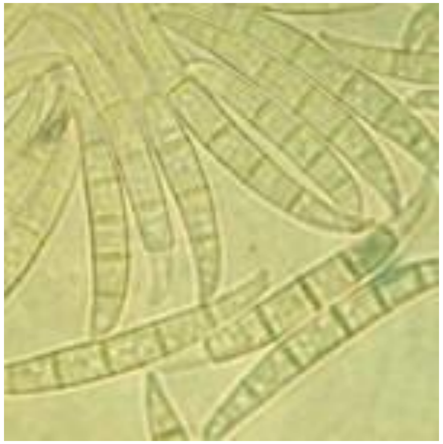
Mycotoxins
Background

myces (Greek) = fungus

toxicum (Latin) = toxic

= Mycotoxin

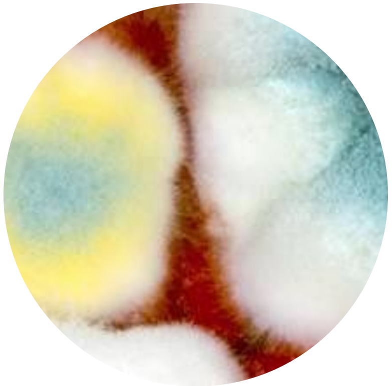
- low molecular weight, toxic, secondary metabolites of fungi
- produced by e.g.:
  - *Fusarium sp., Aspergillus sp., Penicillium sp.*
- toxicity:
  - acute toxic, carcinogenic, mutagenic, teratogenic, estrogenic and immunotoxic effects



provided by Prof. Bürstmayr

Mycotoxins

How many mycotoxins are there ?



- Hundreds of compounds
- 2 Main classes:

Major Mycotoxins

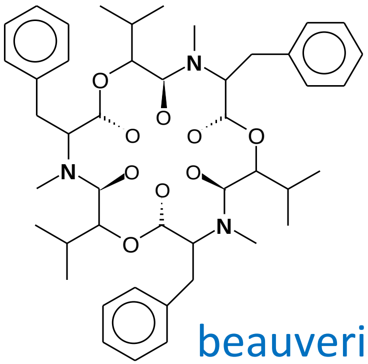
- Aflatoxins
- Ochratoxins (OTA)
- Trichothecenes
- Zearalenone
- Fumonisins
- Patulin

Minor Mycotoxins

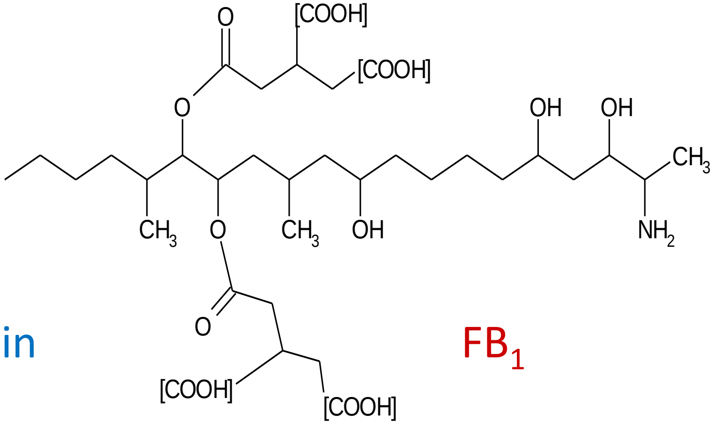
- Ergot alkaloids
- Citrinin
- Cyclopiazonic acid
- Sterigmatocystin
- Monoliformin
- Gliotoxin
- Citreoviridin
- Tremorgenic mycotoxins
- Penicillic acid
- Roquefortine
- 3-Nitropropionic acid
- Fusaproliferin

Mycotoxins

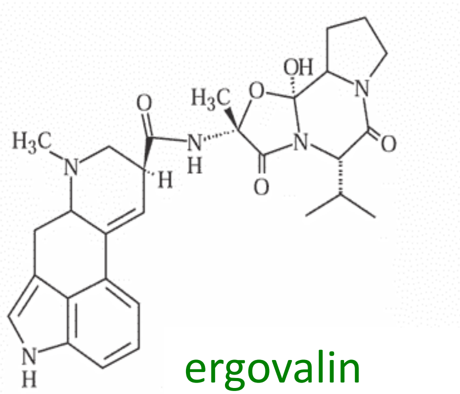
Chemical diversity – a challenge for the sample prep



beauvericin



FB$_1$



ergovalin

Enniatins	Fumonisins	Ergot alkaloids
beauvericin enniatin A, A$_1$, B, B$_1$	fumonisin B$_1$, FB$_2$, FB$_3$, hydrolyzed FB$_1$	ergotamin, ergocornin, ergovalin, dihydroergosin
APOLAR	POLAR, ACIDIC	POLAR, BASIC

Mycotoxins

Why are they an issue?

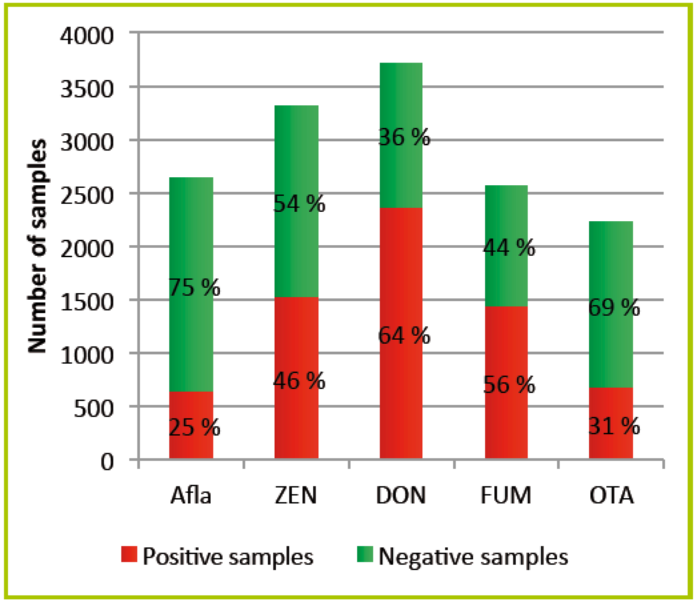
- 100+ countries have regulations for the control of mycotoxins in food and feed

- >25% of all agricultural commodities are contaminated with mycotoxins
- annual losses of several hundred million tons of food worldwide
- annual economical losses: 1 billion USD (US only)

Mycotoxins

Infected food products

- Found in cereals, dried fruits, spices, grape, coffee, cocoa, fruit juices
- Secondary contamination in milk, eggs, meat

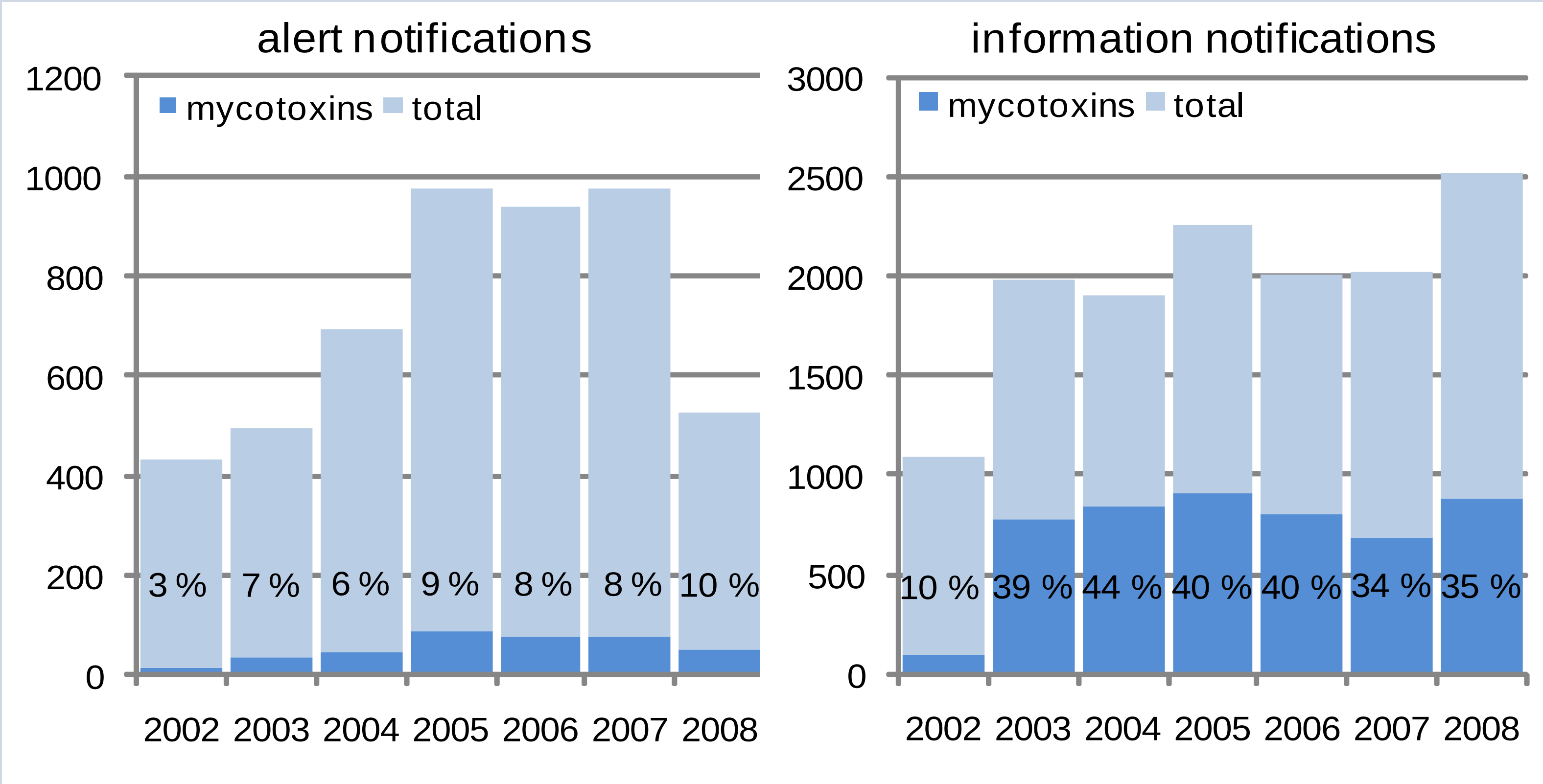


BIOMIN‘s Mycotoxin Survey Program 2012
Percentage of positive and negative samples worldwide
BIOMIN Newsletter, Vol. 11, No. 130

- Resistant to home cooking

Mycotoxins
Relevance for food control

- Notifications concerning mycotoxins
  - (RASFF-Annual reports 2002-2008)



Regulations for Mycotoxins

European Commission Regulation 1881/2006 (EC)

Analytes	**MLs µg/kg (EC Reg. No 1881/2006)**	**Commodities**
Aflatoxin B_1	0.1	processed cereal-based baby food
	2.0-12 sum of aflatoxins: 4.0-15.0	nuts and cereals
Deoxynivalenol	200 500 - 1750	processed cereal-based baby food processed / unprocessed cereals, bread, pasta, breakfast cereals
Fumonisin B_1	200 800 1000 / 4000	processed maize-based baby food maize-based breakfast cereals maize / unprocessed maize
Fumonisin B_2		
Patulin	10-50	fruit juices, apple products, baby food other than processed cereal-based foods
Ochratoxin A	0.5 3.0 / 5.0 10.0 15 / 20 / 80	processed cereal-based baby food processed / unprocessed cereals dried vine fruit spices / liquorice root / extract
Zearalenone	20 50 75-350	processed cereal-based baby food bread, biscuits, breakfast cereals processed / unprocessed cereals

Regulations for Mycotoxins
FDA Regulatory guidelines

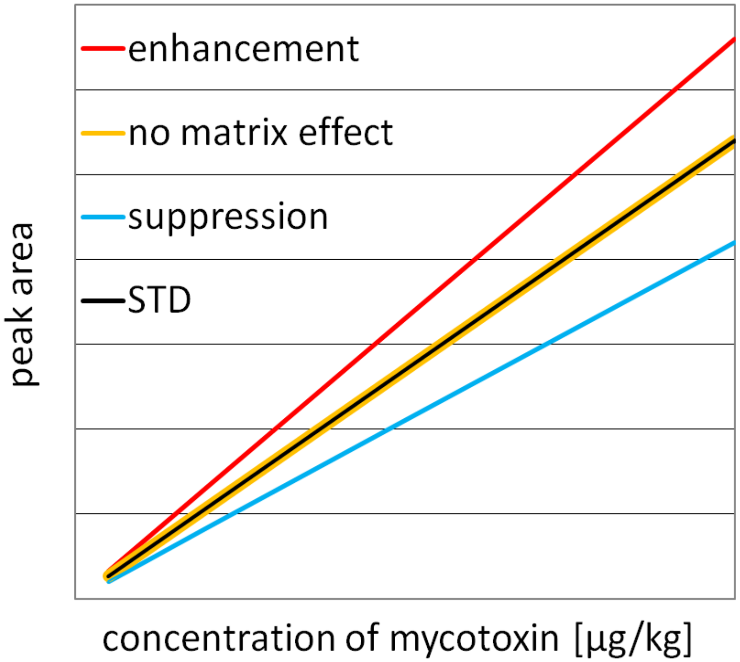
Analytes	Limit µg/kg	Commodities
Aflatoxins, sum	20	All foods except milk
Patulin	50	Apple juice, apple juice concentrate, apple components in processed food
Deoxynivalenol	1000	Finished wheat products
Fumonisins sum of B_1, B_2, B_3	2000	degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour)
	3000	cleaned corn intended for popcorn
	4000	whole of partially degermed dry milled corn products (e.g. flaking grits, corn grits, corn meal, corn flour
Aflatoxin M_1	0.5	milk

Accurate quantitation of mycotoxins
Reasons

- European Commission Regulation (EC) No 1881/2006
  - set maximum limits (ML) for mycotoxins
- Single target versus multi-target methods

BUT:

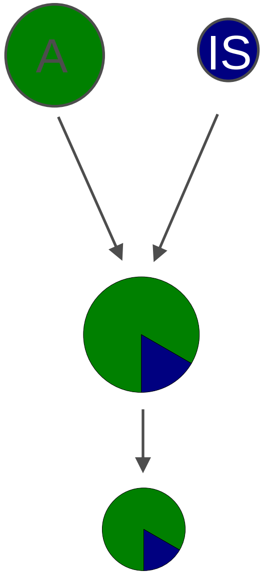
- Electrospray ionisation (ESI)
  - matrix effects hamper accurate mass spectrometric quantification
- Quantification of regulated mycotoxins at a very high degree of accuracy is required



Matrix effects in ESI-MS and quantitation

Approaches

- Dilution of the sample
  - method less sensitive
- Matrix matched calibration
  - tedious
  - differences within one commodity not compensated
- Standard addition to each sample
  - more runs
  - more costs (time and standards)
- Internal calibration
  - similar compounds (ZAN for ZEN)
  - deuterium or ^{13}C-labelled compounds
  - In the past: only single analyte or group analyte IS-addition
  - usually associated with rather high costs

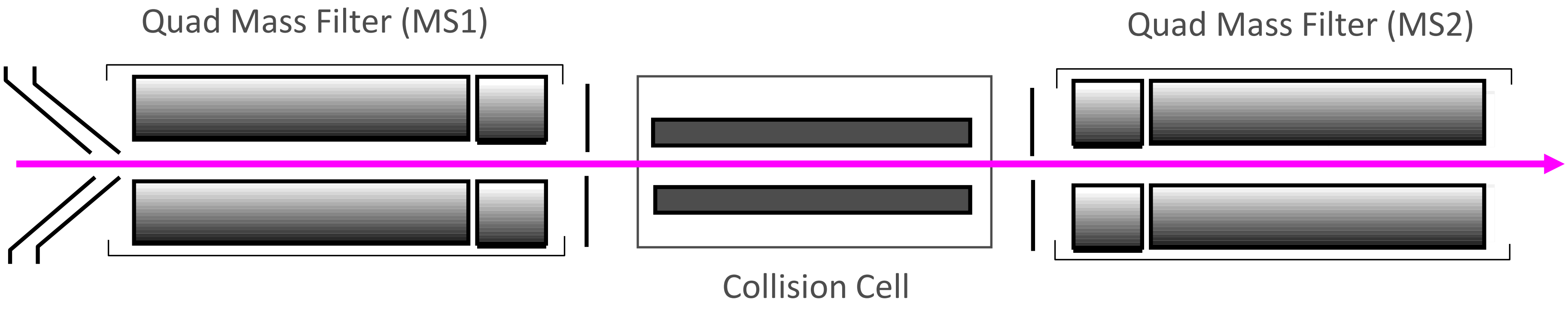


Stable Isotope Dilution Assay (SIDA) Aims

- Development of a method fulfilling:
  - covering all regulated mycotoxins in solid food matrices
  - providing best possible accuracy
  - easy to handle
  - cost effective
- Stable isotope dilution assay (SIDA) for LC-MS/MS
  - 11 mycotoxins
  - ^{13}C-labelled compounds as internal standards
  - validation of the method for maize

Multiple Reaction Monitoring

Principles

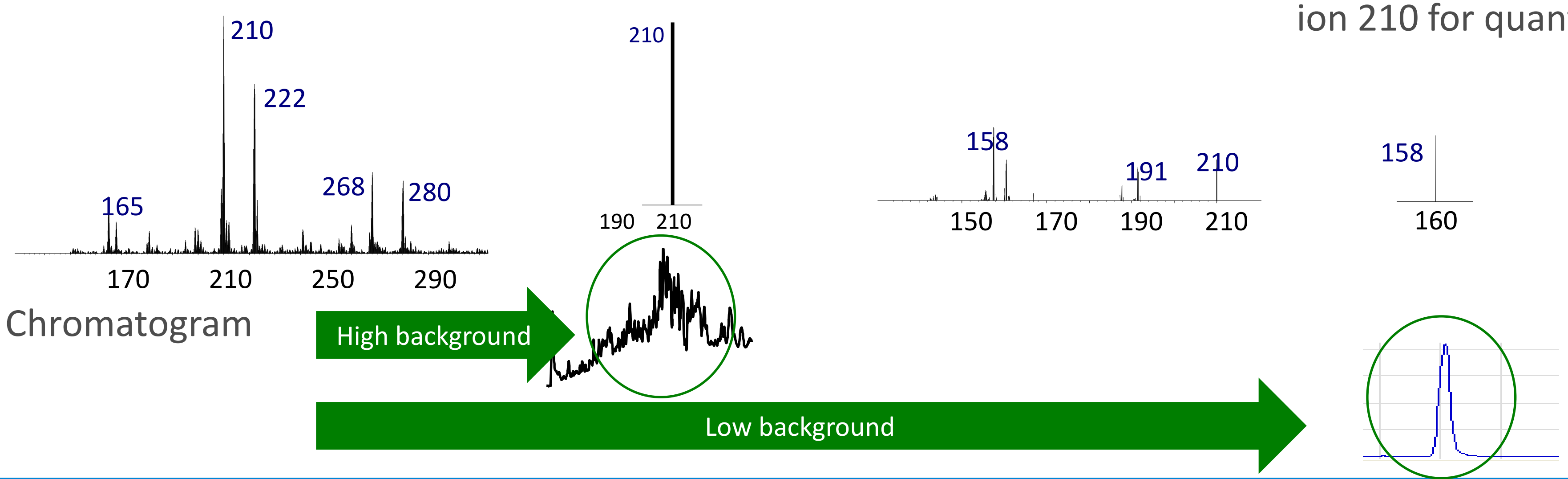


Spectrum with <u>background ions</u> (from ESI)

Q1 lets only target ion 210 pass through

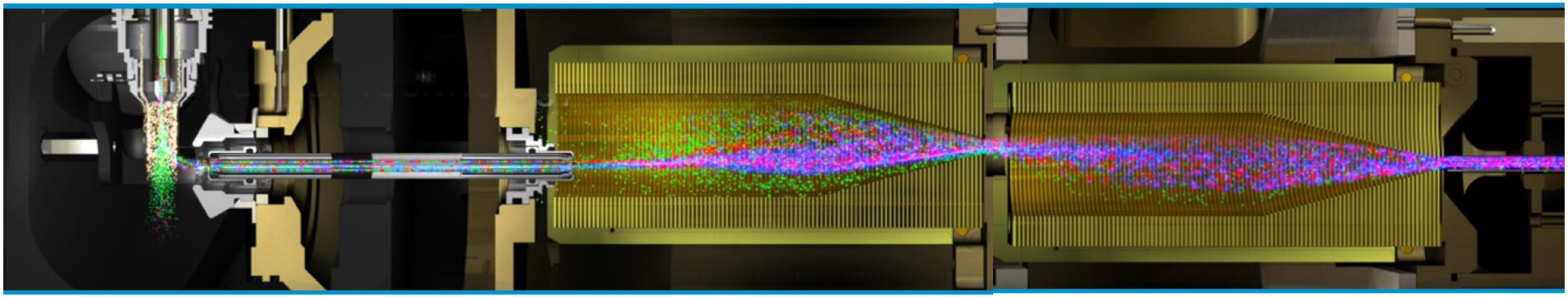
Collision cell breaks ion 210 apart

Q3 monitors only characteristic fragments 158 from ion 210 for quant



iFunnel Technology Revolutionizes Ion Sampling

Proven Sensitivity Breakthrough



Agilent Jet Stream

Hexabore Capillary

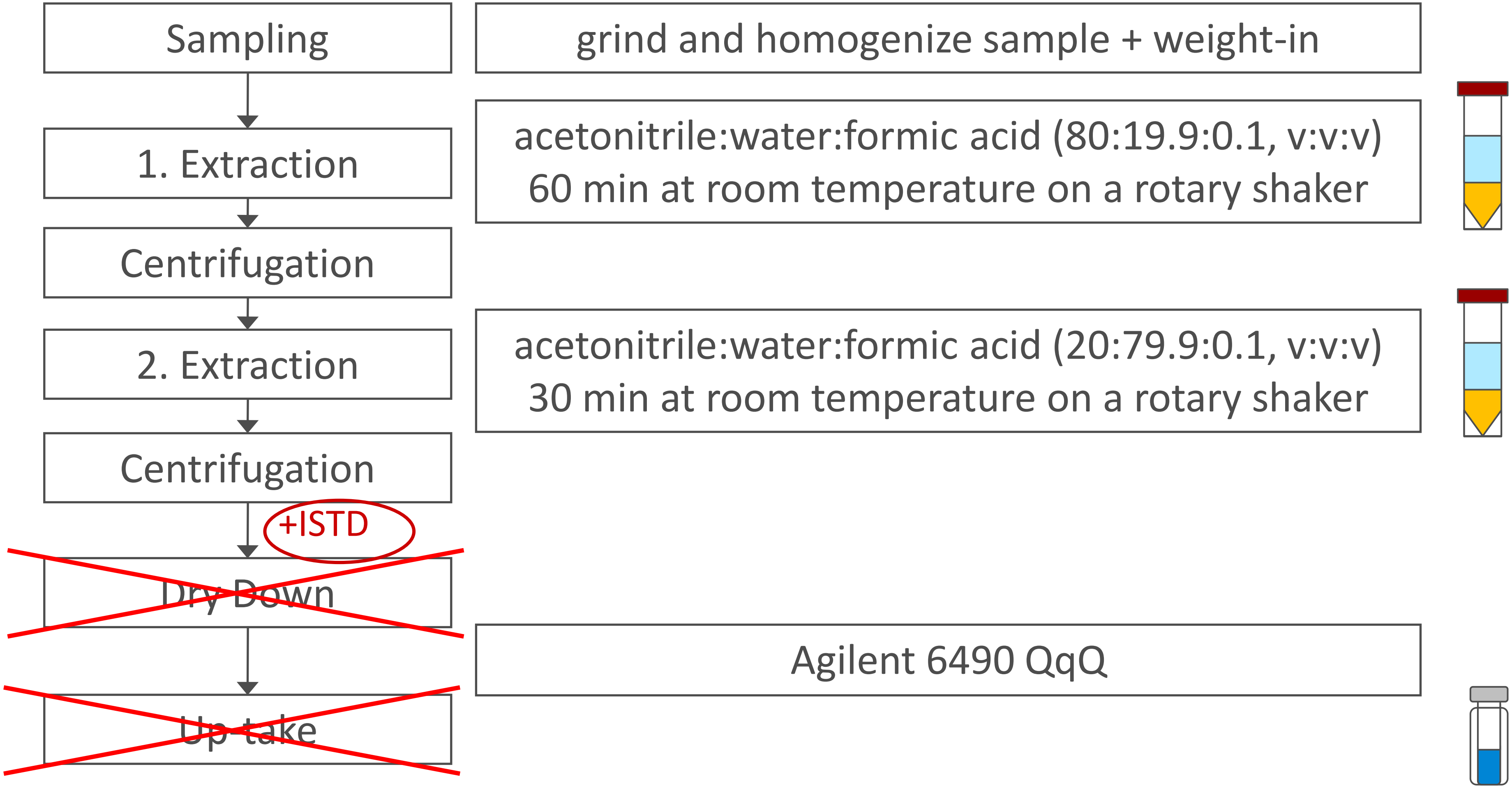
Dual Stage Ion Funnel

10X Higher Gas Sampling

iFUNNEL TECHNOLOGY
REVOLUTIONIZES
ATMOSPHERIC SAMPLING

Sample preparation

Universal extraction procedure



Sampling → grind and homogenize sample + weight-in

1. Extraction → acetonitrile:water:formic acid (80:19.9:0.1, v:v:v)
60 min at room temperature on a rotary shaker

Centrifugation

2. Extraction → acetonitrile:water:formic acid (20:79.9:0.1, v:v:v)
30 min at room temperature on a rotary shaker

Centrifugation

+ISTD

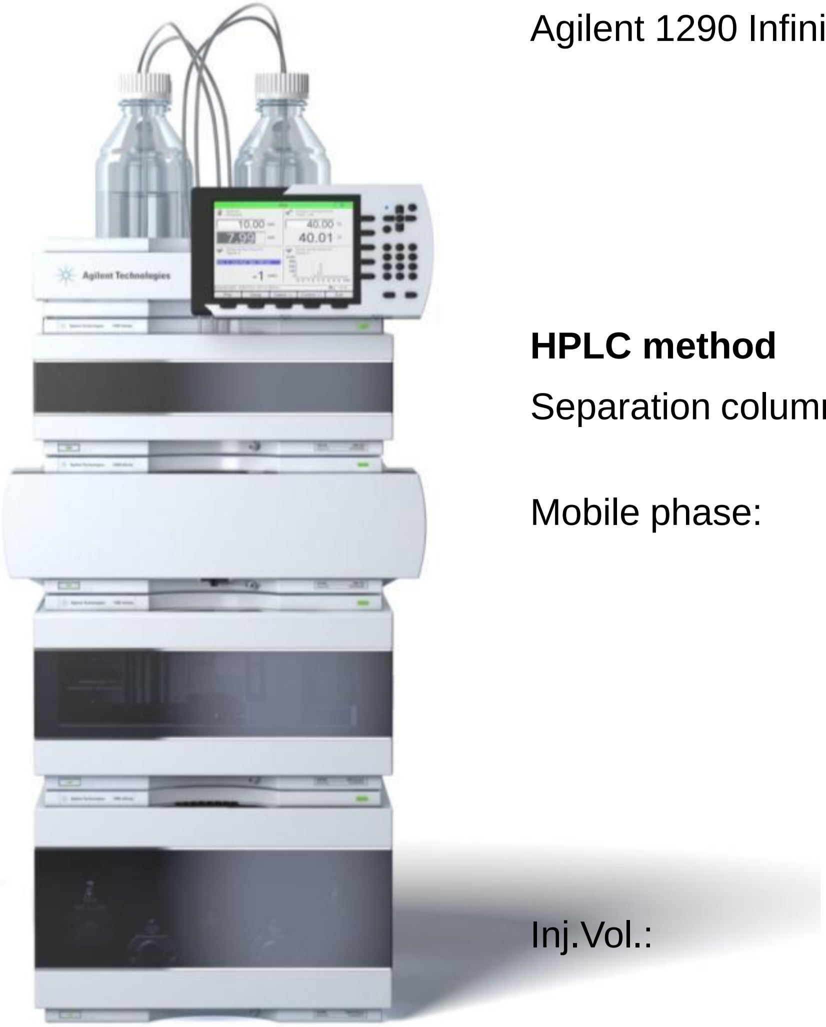
~~Dry Down~~

~~Up-take~~

Agilent 6490 QqQ

Stable Isotope Dilution Assay (SIDA)

HPLC method



Agilent 1290 Infinity LC system consisting of:

- binary pump
- wellplate sampler
- column compartment

HPLC method

Separation column: ZORBAX Eclipse Plus C-18 RRHD column, 100 x 2.1 mm, 1.8 µm @ 30°C

Mobile phase:
A: 5 mM $HCOONH_4$+ 0.1% formic acid
B: methanol + 5 mM $HCOONH_4$ + 0.1% formic acid

Flow: 0.35 ml/min

Gradient:		
	0.00 min	30 % B
	0.50 min	30 % B
	8.00 min	100 % B
	9.50 min	100 % B
	9.60 min	30 % B

Inj.Vol.: 3 µl

Stable Isotope Dilution Assay (SIDA)

MS method

Spray chamber conditions:

Gas temp.:	140°C
Dry gas:	16 l/min
Nebulizer:	25 psi
Sheath gas temp:	350°C
Sheath gas flow:	11 l/min

	Positive	**Negative**
CapVoltage:	4000 V	3000 V
Nozzle voltage	0 V	0 V

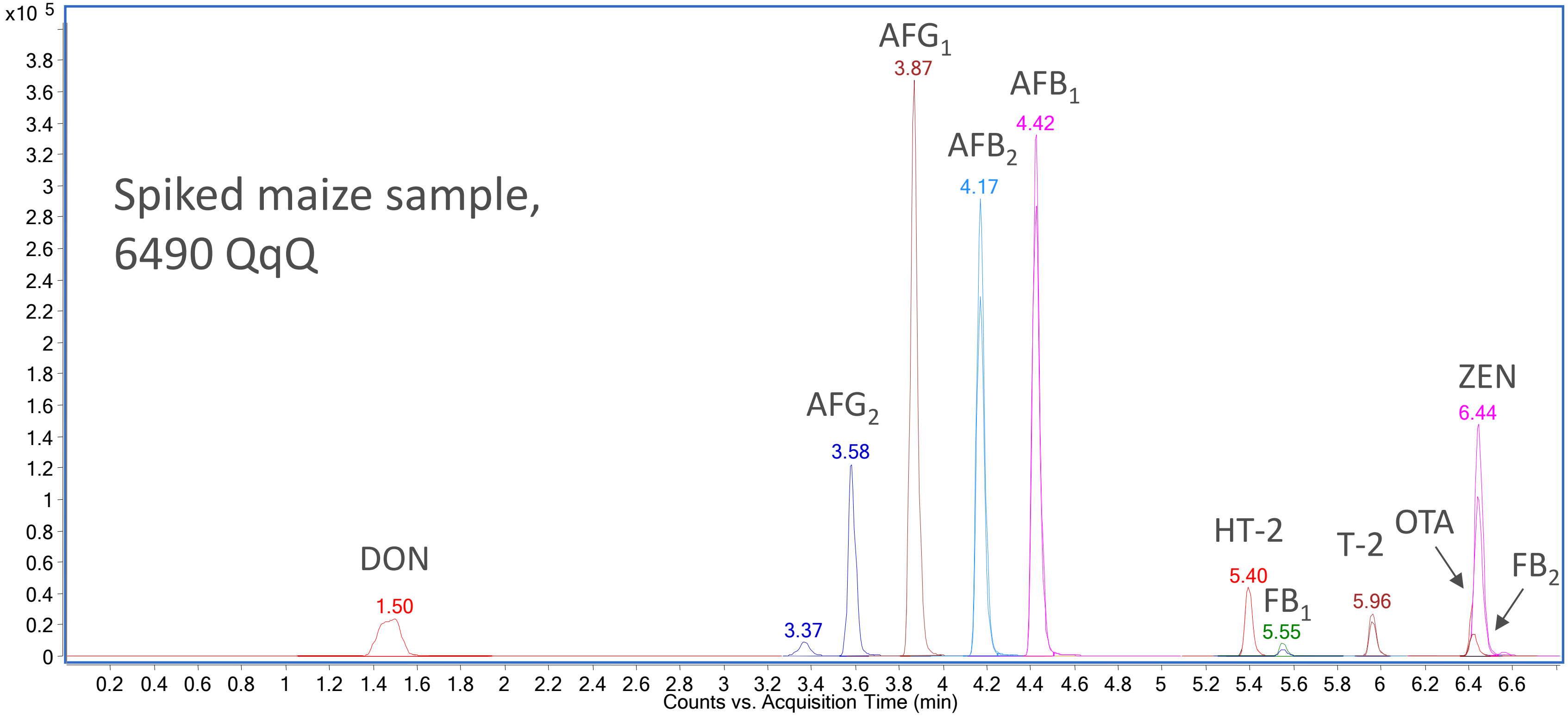
Automatic setup of MRM tables based on selected cycle time, retention times and retention time windows for the individual compounds

• Cycle time	400 ms
• Interscan delay	3.5 ms
• Total No. of MRMs	33
• Maximum No. Of concurrent MRMs	12
• Minimum Dwell time	39.8 ms
• Maximum Dwell time	196.5 ms

Stable Isotope Dilution Assay (SIDA)

Chromatogram

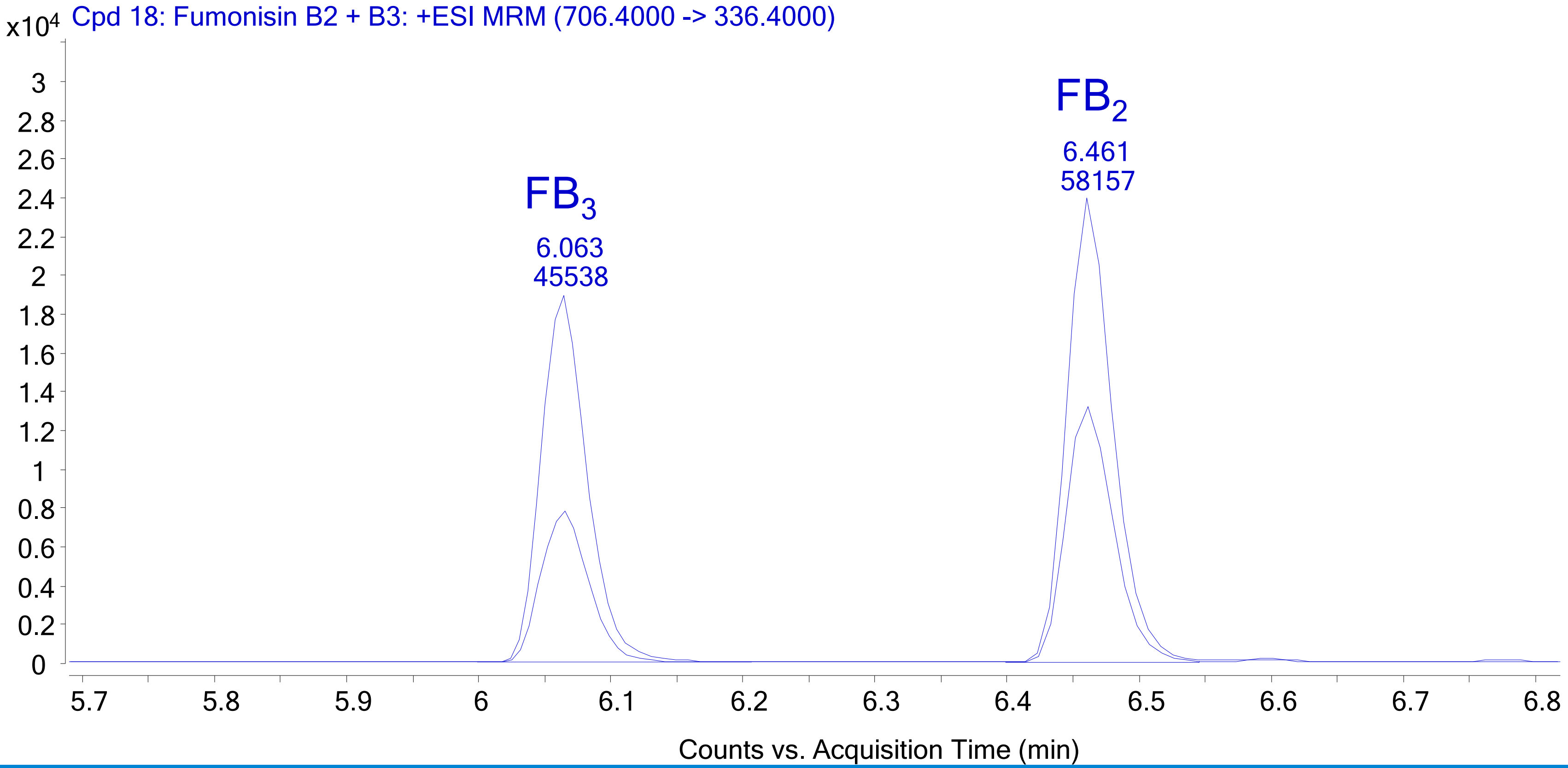
- due to same MRM transitions baseline separation required for:
  - aflatoxin G1 and ^{13}C-aflatoxin B1
  - aflatoxin G2 and ^{13}C-aflatoxin B2



Stable Isotope Dilution Assay (SIDA)

Chromatogram

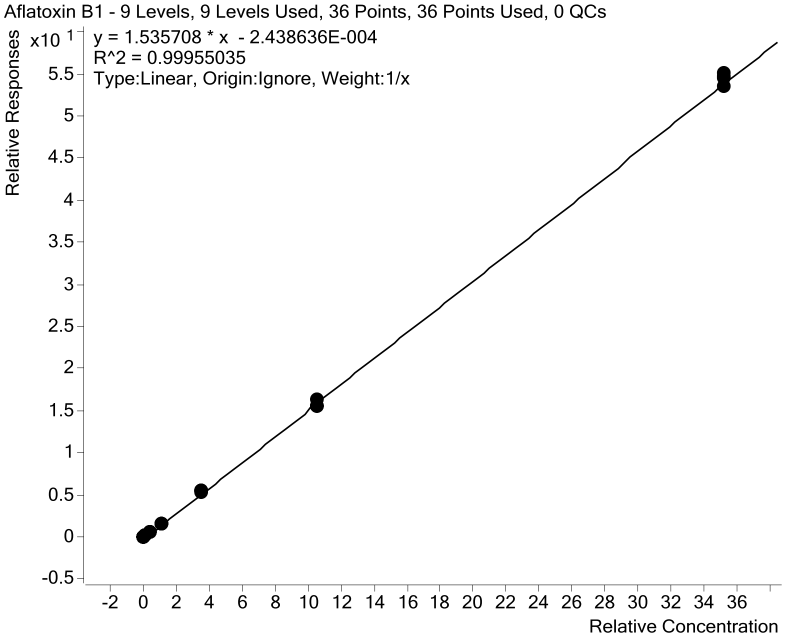
- due to same MRM transitions baseline separation required for:
  - fumonisin B2 and B3



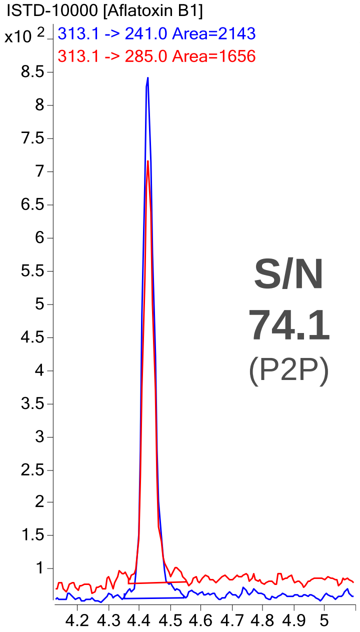
Internal calibration in solvent

Aflatoxin B1

- Challenging compound due to low MLs
  - 0.1 µg/kg in processed cereal based baby food
  - 2 to 12 µg/kg in nuts and cereals

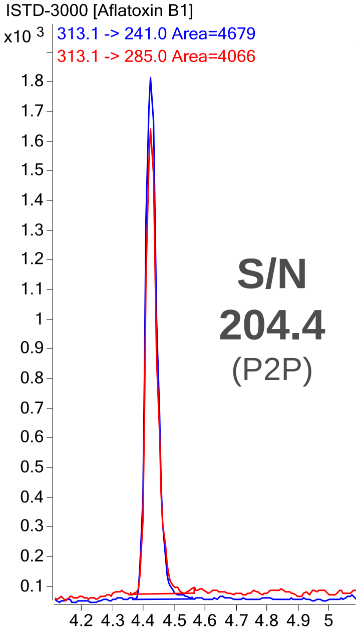


Overlay of 4 individual calibrations acquired within 45 hour worklist.



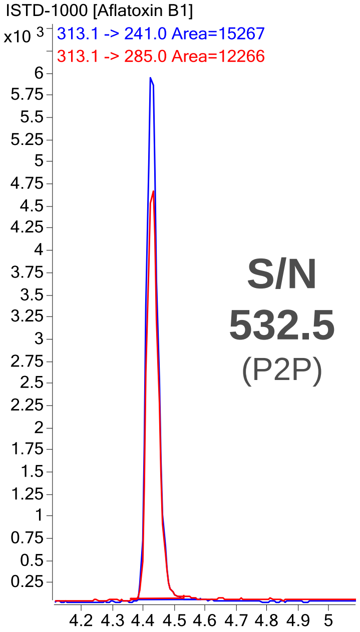
S/N 74.1 (P2P)

0.0075 ng/ml



S/N 204.4 (P2P)

0.0225 ng/ml



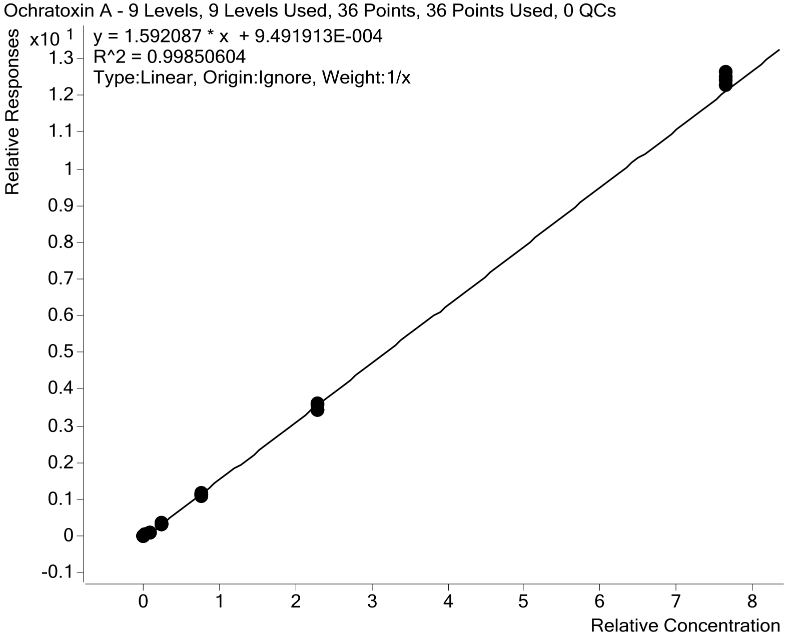
S/N 532.5 (P2P)

0.075 ng/ml

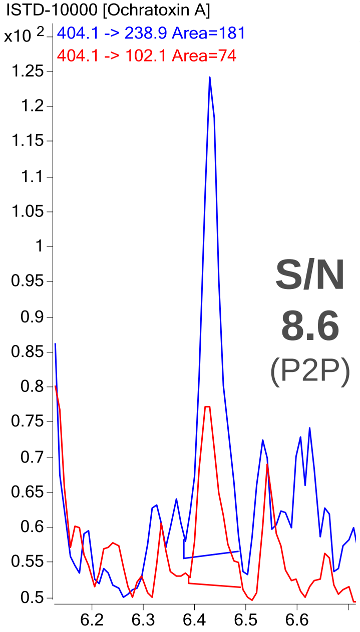
Internal calibration in solvent

Ochratoxin A

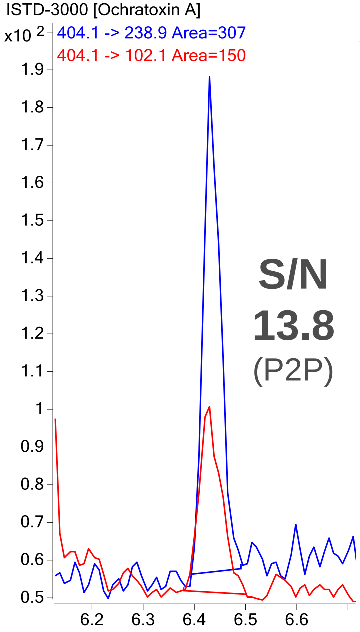
- Challenging compound due to low MRLs
  - 0.5 µg/kg in processed cereal based baby food
  - 3.0 / 5.0 µg/kg in processed / unprocessed cereals
  - 10.0 µg/kg in dried vine fruit



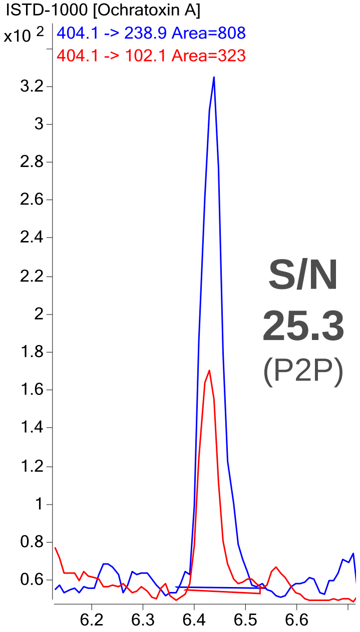
Overlay of 4 individual calibrations acquired within 45 hour worklist.



0.0077 ng/ml



0.0230 ng/ml



0.0765 ng/ml

Validation of SIDA method

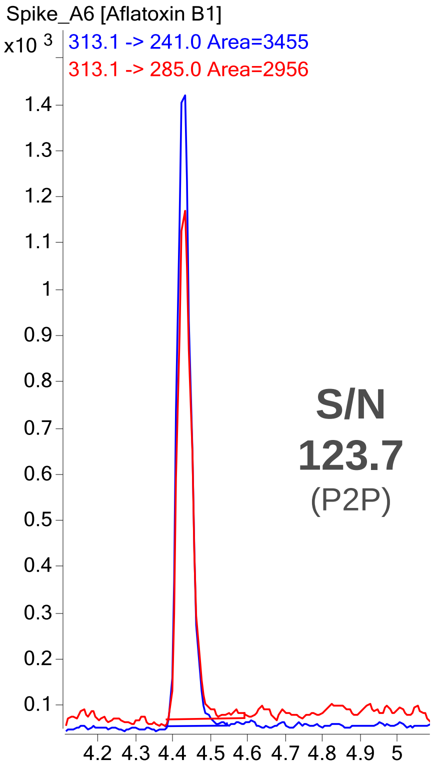
Experimental setup and results

- Linear range (external calibration in solvent)
  - 4 orders of magnitude for all toxins, 5 orders for Aflatoxins, T-2, and ZEN
- Costs
  - Additional price per IS per sample is between US$0.03 to 0.66
  - Price for all 11 IS per sample < US$5.23
- Full validation for maize
  - Maize: - matrix for which most mycotoxins are regulated
- known for matrix effects and matrix interferences
  - Spiking with native mycotoxins before extraction
  - Six concentration levels with 3 replicates
  - Spiking with ^{13}C-labelled mycotoxins before analysis to compensate matrix effects in ESI
  - No sample clean-up

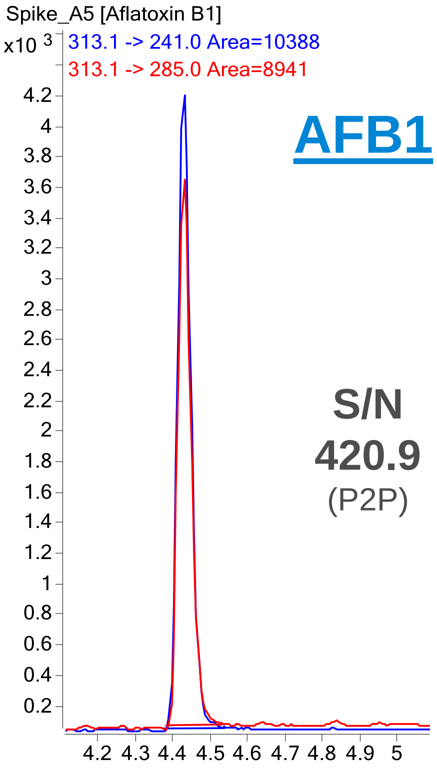
Validation of SIDA method in maize

Extraction of spiked blank maize and reference materials

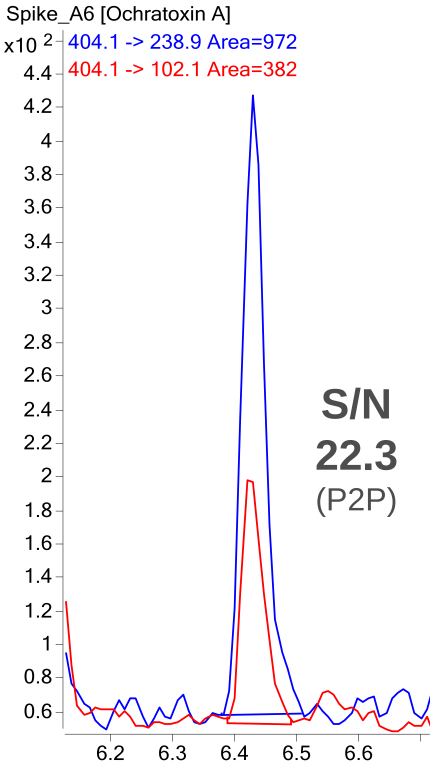
- Blank maize sample spiked with native mycotoxins before extraction
  - includes 10-fold dilution of matrix in the final extract due to extraction procedure


Spike_A6 [Aflatoxin B1]

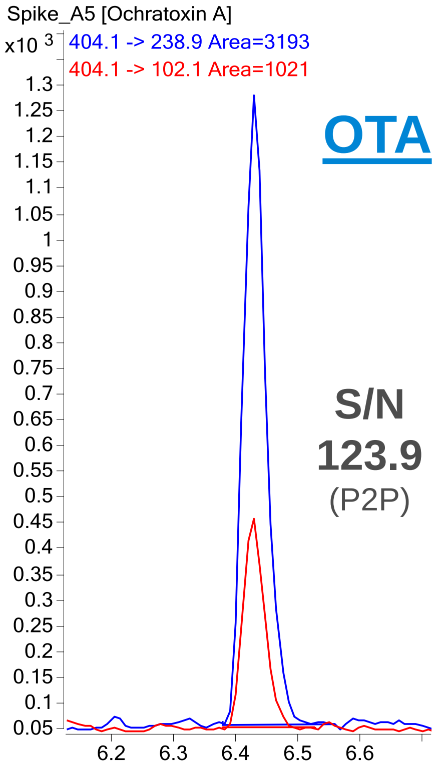
0.5 µg/kg


Spike_A5 [Aflatoxin B1]

1.5 µg/kg


Spike_A6 [Ochratoxin A]

0.5 µg/kg


Spike_A5 [Ochratoxin A]

1.5 µg/kg

Validation of SIDA method

Results – Sample preparation

- Extraction efficiency
  - Determined by spiking of blank samples before extraction
  - First extraction: 80% acetonitrile content (60 min)
    - recovery between 80 and 110% except for FB1 and FB2
  - Second extraction: 20% acetonitrile content (30 min)
    - improved extraction recovery for FB1 and FB2 to approx. 90%
- Matrix effects
  - Signal suppression
    - 50 to 60% aflatoxins
    - 50% DON
  - Signal enhancement
    - Fumonisins, HT-2, T2, OTA
  - Effectively compensated by ISTD

Validation of SIDA method

Results for maize

Analyte	LOQ in µg/kg	R_A** in % ± RSD in %	
Aflatoxin B_1	0.04	105	6
Aflatoxin B_2	0.04	100	4
Aflatoxin G_1	0.05	101	5
Aflatoxin G_2	0.24	101	8
Deoxynivalenol	2.5	99	9
HT-2 toxin	2.0	98	7
T-2 toxin	0.17	99	6
Ochratoxin A	0.23	93	7
Zearalenone	0.97	103	11
Fumonisin B_1	2.5	101	10
Fumonisin B_2	0.64	88	7

** average for triplicate samples and 6 spiking levels

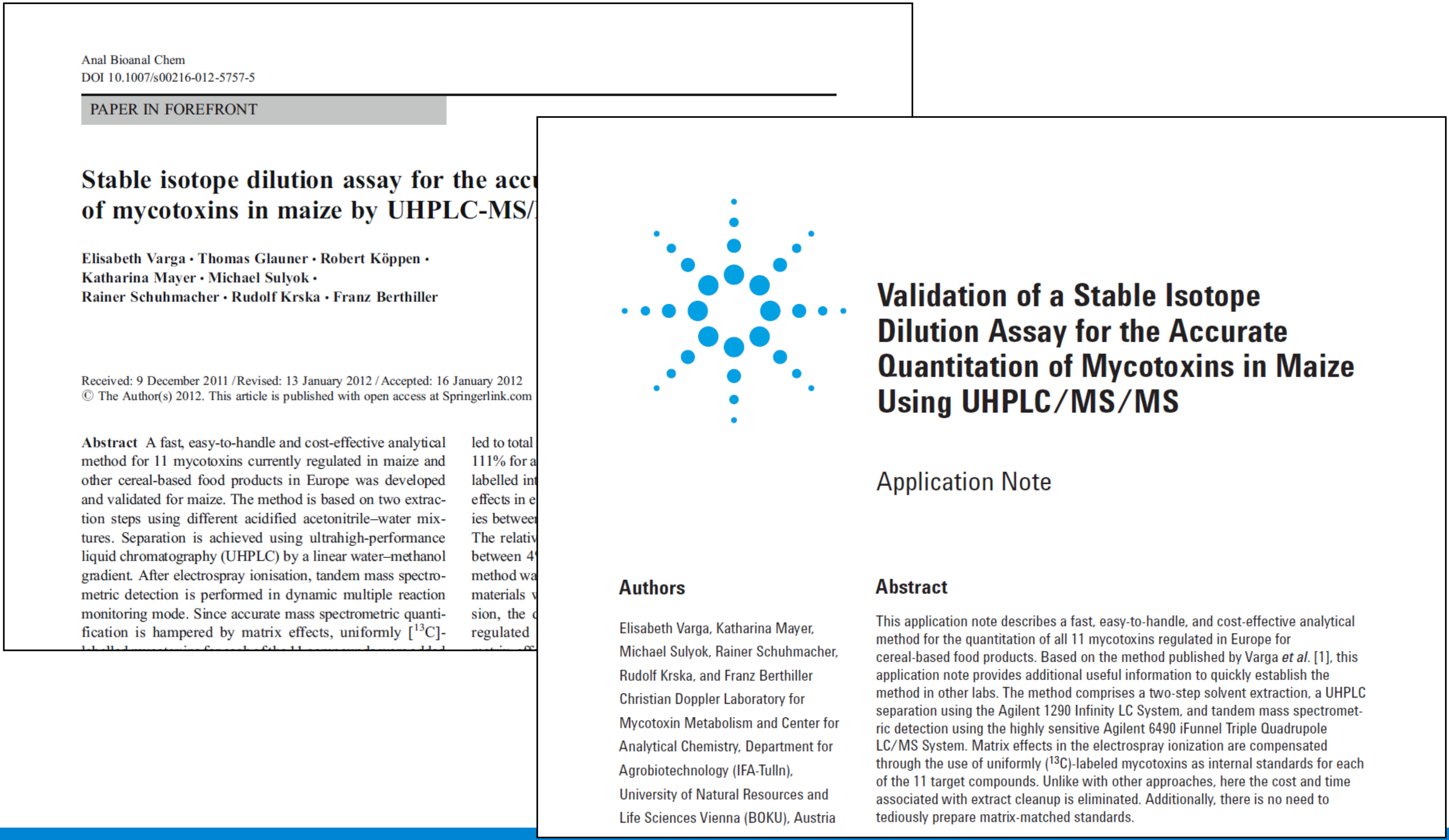
Validation of SIDA method

Results for official test materials

No	Analyte	assigned value [a] ± STDEV [μg kg^{-1}]	measured value [b] ± STDEV [μg kg^{-1}]	Status [c]
TM_01	ZEN	83 ± 4.5	86 ± 10	ok
TM_02	Sum AFs	3.79 ± 1.67	4.6 ± 0.2	ok
	AFB$_1$	1.87 ± 0.83	2.3 ± 0.1	ok
	AFB$_2$	0.51 ± 0.23	0.6 ± 0 03	ok
	AFG$_1$	0.96 ± 0.43	1.0 ± 0 1	ok
	AFG$_2$	0.52 ± 0.23	0.7 ± 0.1	ok
TM_03	FB$_1$	1650 ± 53	1960 ± 198	+
	FB$_2$	461 ± 16	496 ± 32	ok
TM_04	DON	1714 ± 64	1660 ± 145	ok
TM_05	DON	901 ± 55	908 ± 79	ok
	ZEN	79 ± 13	84 ± 10	ok
TM_06	FB$_1$	2630 ± 370	2300 ± 233	ok
	FB$_2$	690 ± 170	578 ± 38	ok
TM_07	FB$_1$	270 ± 55	223 ± 23	ok
	FB$_2$	< 80	55 ± 4	ok

Validation of SIDA method

Published in Anal. Bioanal. Chem, 402 (9) 2675-2686



Anal Bioanal Chem
DOI 10.1007/s00216-012-5757-5

PAPER IN FOREFRONT

Stable isotope dilution assay for the accu of mycotoxins in maize by UHPLC-MS/

**Elisabeth Varga • Thomas Glauner • Robert Köppen •
Katharina Mayer • Michael Sulyok •
Rainer Schuhmacher • Rudolf Krska • Franz Berthiller**

Received: 9 December 2011 /Revised: 13 January 2012 / Accepted: 16 January 2012

Abstract A fast, easy-to-handle and cost-effective analytical method for 11 mycotoxins currently regulated in maize and other cereal-based food products in Europe was developed and validated for maize. The method is based on two extraction steps using different acidified acetonitrile–water mixtures. Separation is achieved using ultrahigh-performance liquid chromatography (UHPLC) by a linear water–methanol gradient. After electrospray ionisation, tandem mass spectrometric detection is performed in dynamic multiple reaction monitoring mode. Since accurate mass spectrometric quantification is hampered by matrix effects, uniformly [^{13}C]-

led to total
111% for a
labelled in
effects in e
ies between
The relativ
between 4
method wa
materials v
sion, the c
regulated

Validation of a Stable Isotope Dilution Assay for the Accurate Quantitation of Mycotoxins in Maize Using UHPLC/MS/MS

Application Note

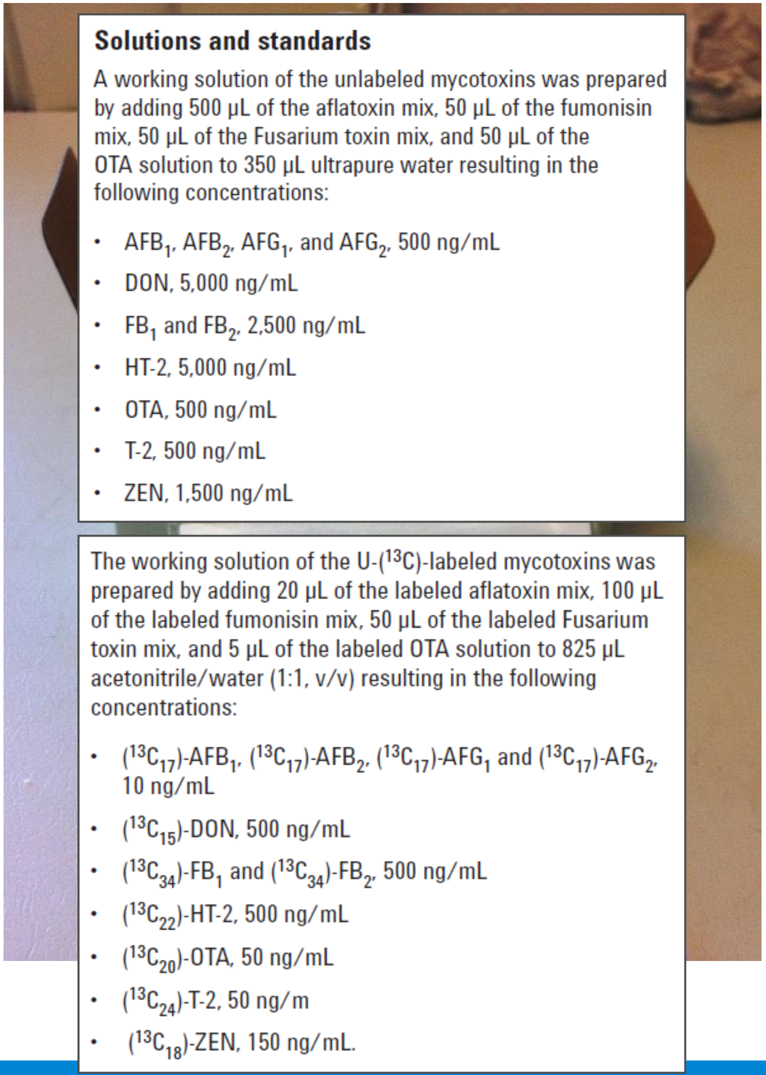
Authors

Elisabeth Varga, Katharina Mayer,
Michael Sulyok, Rainer Schuhmacher,
Rudolf Krska, and Franz Berthiller
Christian Doppler Laboratory for
Mycotoxin Metabolism and Center for
Analytical Chemistry, Department for
Agrobiotechnology (IFA-Tulln),
University of Natural Resources and
Life Sciences Vienna (BOKU), Austria

Abstract

This application note describes a fast, easy-to-handle, and cost-effective analytical method for the quantitation of all 11 mycotoxins regulated in Europe for cereal-based food products. Based on the method published by Varga *et al.* [1], this application note provides additional useful information to quickly establish the method in other labs. The method comprises a two-step solvent extraction, a UHPLC separation using the Agilent 1290 Infinity LC System, and tandem mass spectrometric detection using the highly sensitive Agilent 6490 iFunnel Triple Quadrupole LC/MS System. Matrix effects in the electrospray ionization are compensated through the use of uniformly (^{13}C)-labeled mycotoxins as internal standards for each of the 11 target compounds. Unlike with other approaches, here the cost and time associated with extract cleanup is eliminated. Additionally, there is no need to tediously prepare matrix-matched standards.

Implementation of SIDA method
Romer LC/MS Mycotoxin kit – developed to fit the application



Solutions and standards

A working solution of the unlabeled mycotoxins was prepared by adding 500 µL of the aflatoxin mix, 50 µL of the fumonisin mix, 50 µL of the Fusarium toxin mix, and 50 µL of the OTA solution to 350 µL ultrapure water resulting in the following concentrations:

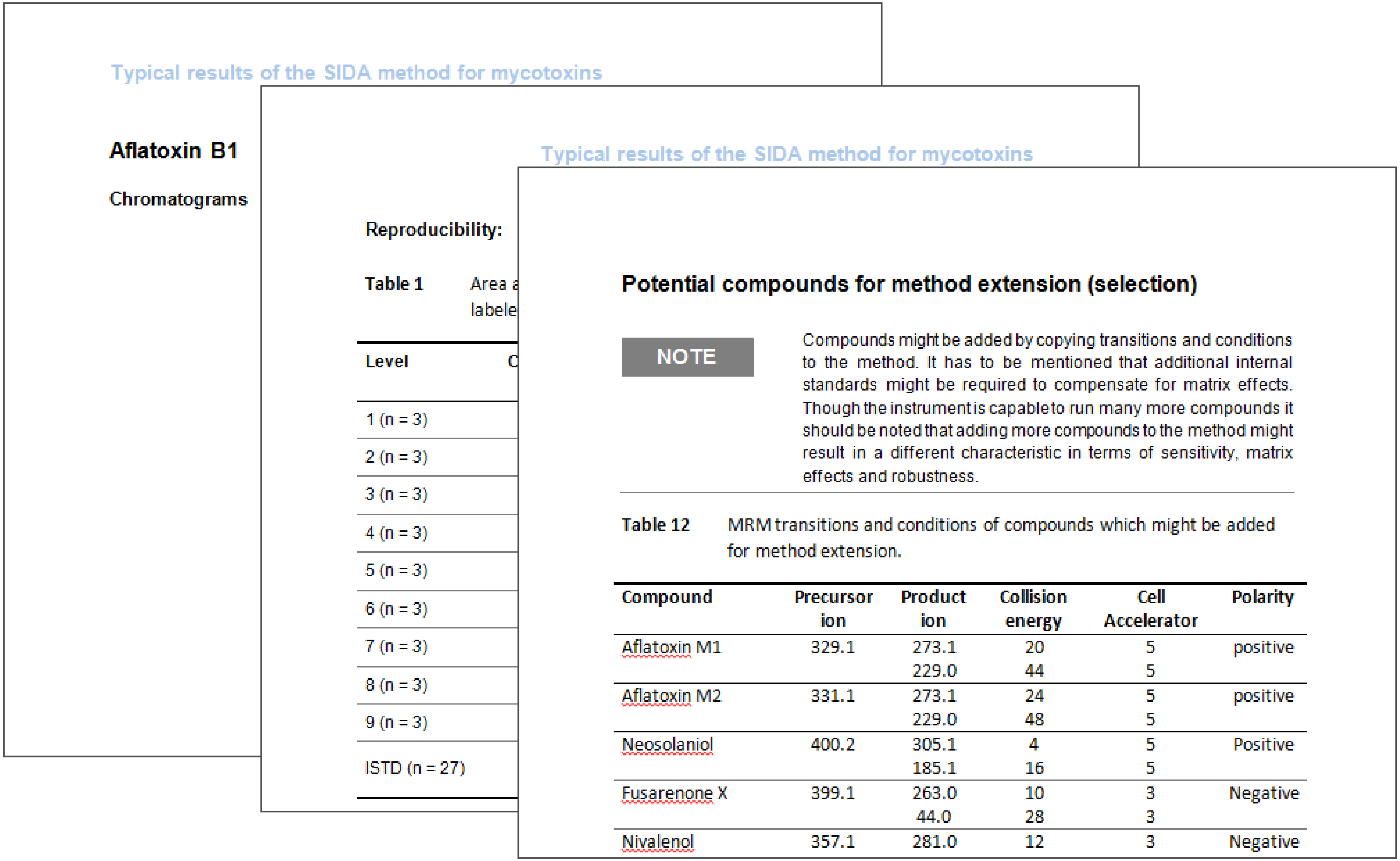
- AFB_1, AFB_2, AFG_1, and AFG_2, 500 ng/mL
- DON, 5,000 ng/mL
- FB_1 and FB_2, 2,500 ng/mL
- HT-2, 5,000 ng/mL
- OTA, 500 ng/mL
- T-2, 500 ng/mL
- ZEN, 1,500 ng/mL

The working solution of the U-(^{13}C)-labeled mycotoxins was prepared by adding 20 µL of the labeled aflatoxin mix, 100 µL of the labeled fumonisin mix, 50 µL of the labeled Fusarium toxin mix, and 5 µL of the labeled OTA solution to 825 µL acetonitrile/water (1:1, v/v) resulting in the following concentrations:

- $(^{13}C_{17})$-AFB_1, $(^{13}C_{17})$-AFB_2, $(^{13}C_{17})$-AFG_1 and $(^{13}C_{17})$-AFG_2, 10 ng/mL
- $(^{13}C_{15})$-DON, 500 ng/mL
- $(^{13}C_{34})$-FB_1 and $(^{13}C_{34})$-FB_2, 500 ng/mL
- $(^{13}C_{22})$-HT-2, 500 ng/mL
- $(^{13}C_{20})$-OTA, 50 ng/mL
- $(^{13}C_{24})$-T-2, 50 ng/m
- $(^{13}C_{18})$-ZEN, 150 ng/mL.

- Native mycotoxins:
  - Mix 3 (fumonisins)
  - Mix 9 (aflatoxins)
  - Mix 8 (fusarium toxins)
  - Ochratoxin
- ^{13}C labelled mycotoxins:
  - Mix 12 (^{13}C fumonisins)
  - Mix 11 (^{13}C aflatoxins)
  - Mix 10 (^{13}C fusarium toxins)
  - $[^{13}C_{20}]$-Ochratoxin

Typical performance during method implementation



Typical results of the SIDA method for mycotoxins

Aflatoxin B1

Chromatograms

Typical results of the SIDA method for mycotoxins

Reproducibility:

Table 1 Area a
labele

Level	C
1 (n = 3)	
2 (n = 3)	
3 (n = 3)	
4 (n = 3)	
5 (n = 3)	
6 (n = 3)	
7 (n = 3)	
8 (n = 3)	
9 (n = 3)	
ISTD (n = 27)	

Potential compounds for method extension (selection)

NOTE

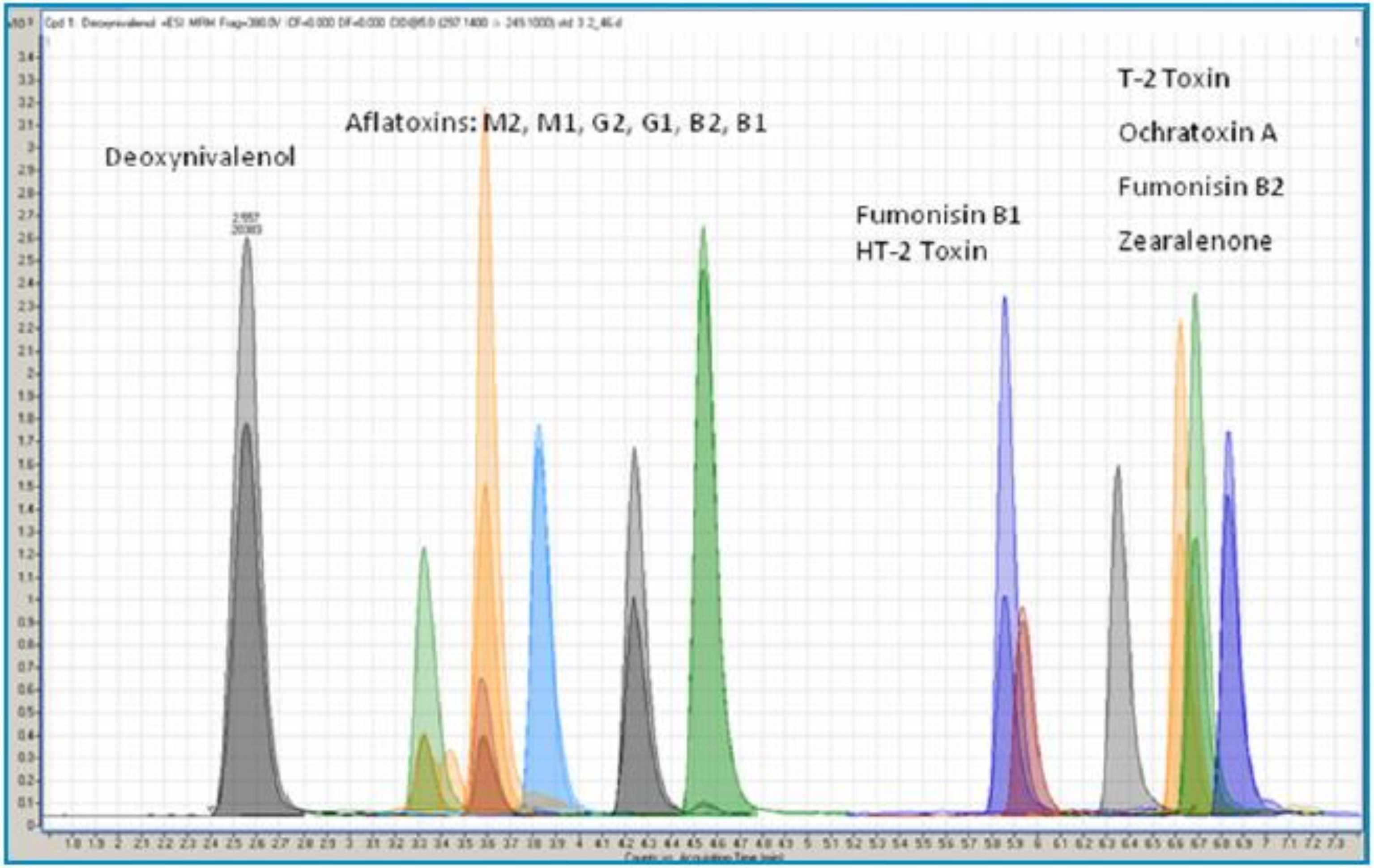
Compounds might be added by copying transitions and conditions to the method. It has to be mentioned that additional internal standards might be required to compensate for matrix effects. Though the instrument is capable to run many more compounds it should be noted that adding more compounds to the method might result in a different characteristic in terms of sensitivity, matrix effects and robustness.

Table 12 MRM transitions and conditions of compounds which might be added for method extension.

Compound	Precursor ion	Product ion	Collision energy	Cell Accelerator	Polarity
Aflatoxin M1	329.1	273.1	20	5	positive
		229.0	44	5	
Aflatoxin M2	331.1	273.1	24	5	positive
		229.0	48	5	
Neosolaniol	400.2	305.1	4	5	Positive
		185.1	16	5	
Fusarenone X	399.1	263.0	10	3	Negative
		44.0	28	3	
Nivalenol	357.1	281.0	12	3	Negative

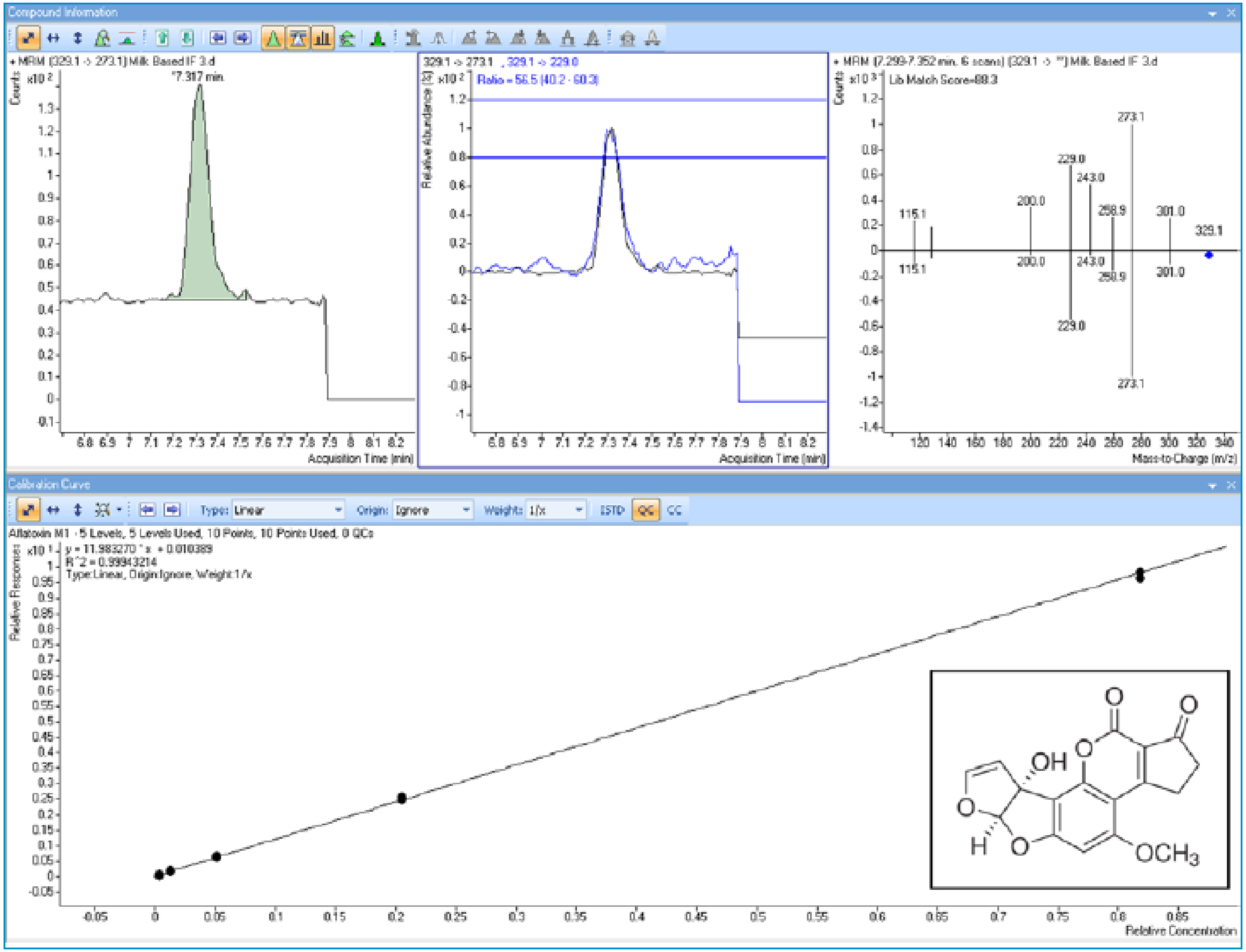
Analysis of regulated mycotoxins in infant formula

Poster presented at the NACRW 2013



Increased confidence by using triggered MRM

Aflatoxin M1 in Infant Formula, fortified at 0.02 µg/kg



+ MRM (329.1 -> 273.1) Milk Based IF 3.d

329.1 -> 273.1 , 329.1 -> 229.0

Ratio = 56.5 (40.2 - 60.3)

+ MRM (7.299-7.352 min, 6 scans) (329.1 -> **) Milk Based IF 3.d

Lib Match Score=88.3

Type: Linear

Origin: Ignore

Weight: 1/x

Aflatoxin M1 - 5 Levels, 5 Levels Used, 10 Points, 10 Points Used, 0 QCs

y = 11.983270 * x + 0.010389

R^2 = 0.99943214

Type:Linear, Origin:Ignore, Weight:1/x

Summary

- UHPLC-MS/MS method
  - Improved chromatographic resolution
- Multiple extraction steps
  - Enhancement of extraction efficiency especially for fumonisins
- Dynamic MRM with fast polarity switching
  - Most abundant ionization mode and maximized dwell times within a single run
- Addition of internal standards after extraction
  - Compensation for matrix effects
  - Minimized costs
- Apparent recoveries of 88 to 105% for all mycotoxins
  - Evaluated by extraction of spiked maize samples
  - Validated by correct quantitation of 12 official test materials covering most toxins
- Sensitivity suitable for MRLs
  - Improved sensitivity of G6490 allows to omit sample concentration resulting in easier handling and improved robustness

Thanks!

thomas_glauner@agilent.com

Acknowledgements

- Elisabeth Varga
- Katharina Mayer
- Franz Berthiller
- Rainer Schuhmacher, Michael Sulyok, Rudolf Krska
- Biopure Reference Materials, Romer Labs Diagnostic GmbH, Tulln