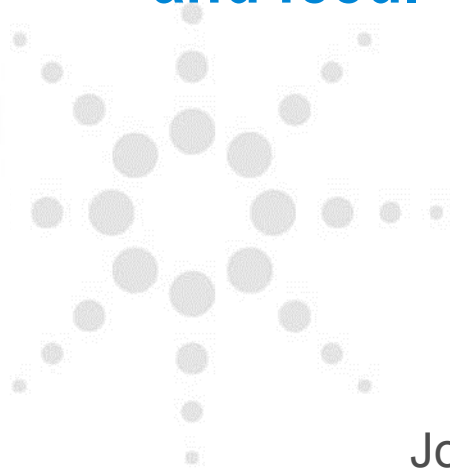




**Accurate quantification of
regulated mycotoxins by
UHPLC-MS/MS and screening
for 200+ mycotoxins in food
and feed.**



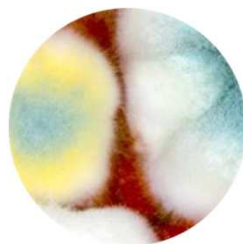
John Lee
Global Food Market Manager



Agilent Technologies

Mycotoxins

Background



myces (Greek) = fungus

toxicum (Latin) = toxin

} **Mycotoxin**

- secondary metabolites of fungi
 - Fusarium, Aspergillus, Penicillium.
- toxicity:
 - acute toxic, carcinogenic, mutagenic, teratogenic, estrogenic and immunotoxic effects
- resistant to home cooking
- Found in cereals, nuts, dried fruits, spices, grape, coffee, cocoa, fruit juice

Major Mycotoxins

- Aflatoxins
- Ochratoxins (OTA)
- Tricothecenes
- Zearalenone
- Fumonisin
- Patulin

Minor Mycotoxins (100's)

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



EU Regulations

Analytes	MRLs µg/kg (EC Reg. No 1831/2003)	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	processed maize-based baby food maize-based breakfast cereals maize / unprocessed maize
Fumonisin B ₂	800 1000 / 4000	
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

US (FDA) Regulations

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
Fumonisin 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



Project in collaboration with IFA Tulln:

Elisabeth Varga, Michael Sulyok, Rainer Schuhmacher, Rudolf Krska, Franz Berthiller

Thomas Glauner: Agilent



Project Aim:

To create a fast LCMS method covering all mycotoxins regulated in cereals.



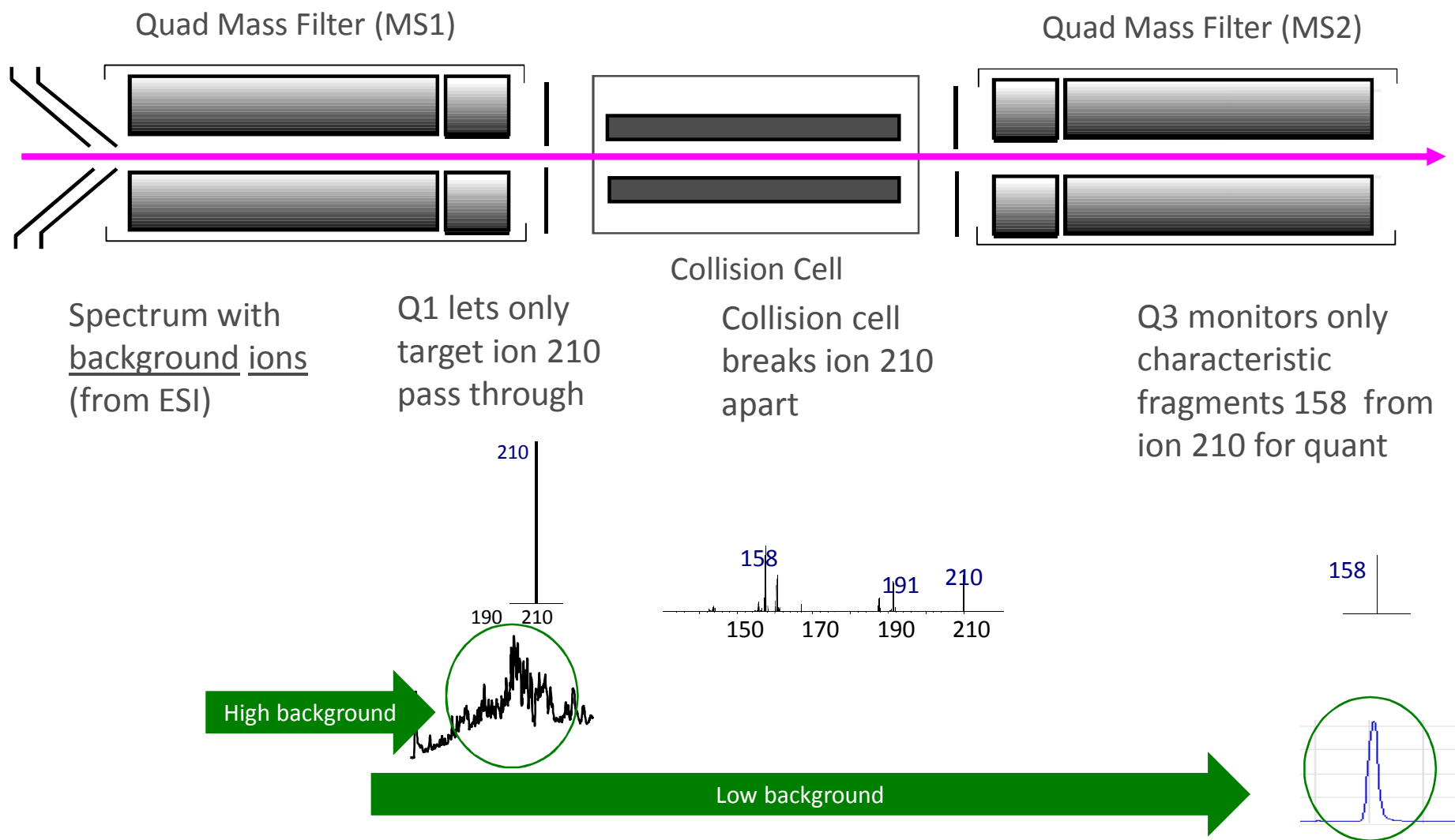
1290 UHPLC
6490 QQQ Mass Spec



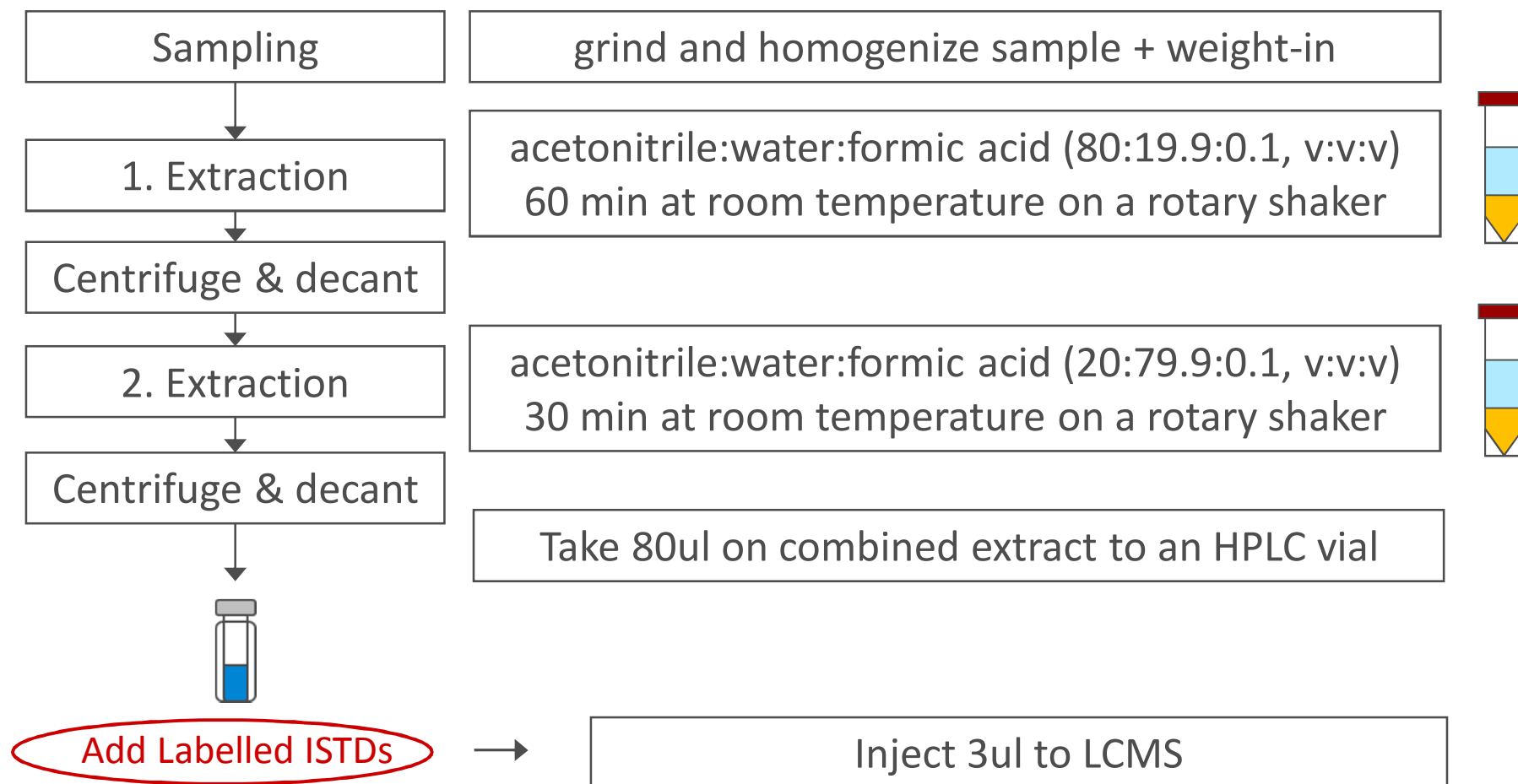
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Multiple Reaction Monitoring

Principles



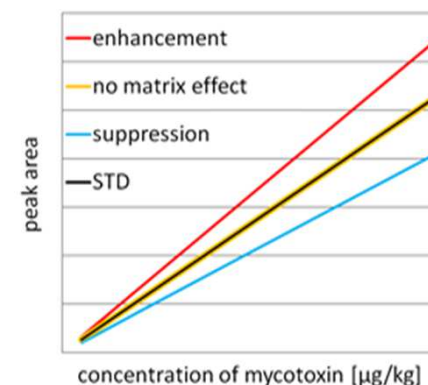
Extraction Protocol



Stable Isotope Dilution. Why?

■ The challenge

- Without clean up of extracts, direct comparison to a solvent standard won't be accurate.
- Clean-up based on immuno-affinity or other affinity are never selective for all.
- Matrix standards do not account for sample to sample variation.

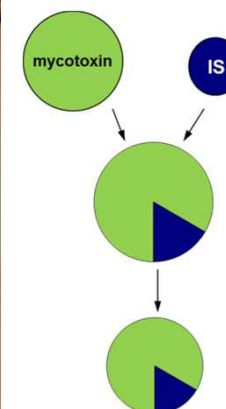


■ Stable isotope dilution assay (SIDA) for LC-MS/MS

- 11 native mycotoxin standards
- 11 mycotoxins as ^{13}C -labelled compounds : internal standards

U- $^{13}\text{C}_{17}$ -aflatoxin B₁
U- $^{13}\text{C}_{17}$ -aflatoxin G₁
U- $^{13}\text{C}_{34}$ -fumonisin B₁

U- $^{13}\text{C}_{17}$ -aflatoxin B₂
U- $^{13}\text{C}_{17}$ -aflatoxin G₂
U- $^{13}\text{C}_{34}$ -fumonisin B₂
U- $^{13}\text{C}_{20}$ -ochratoxin A
U- $^{13}\text{C}_{24}$ -T-2 toxin
U- $^{13}\text{C}_{22}$ -HT-2 toxin
U- $^{13}\text{C}_{15}$ -deoxynivalenol
U- $^{13}\text{C}_{18}$ -zearalenone



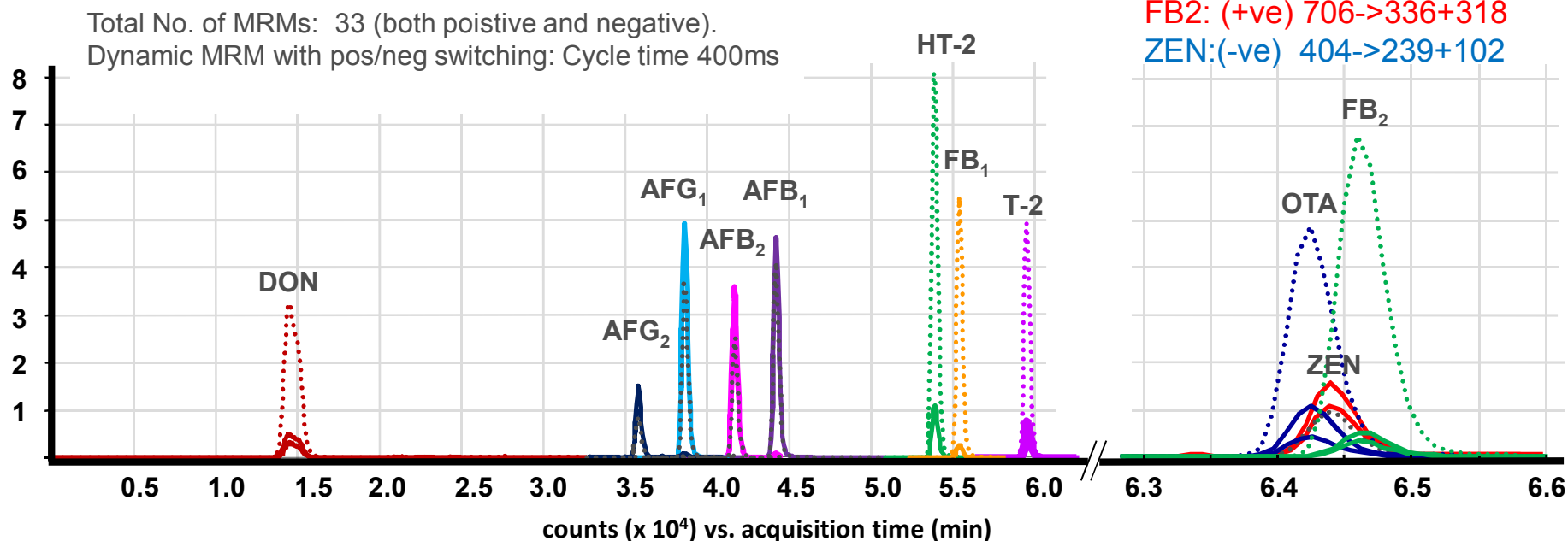
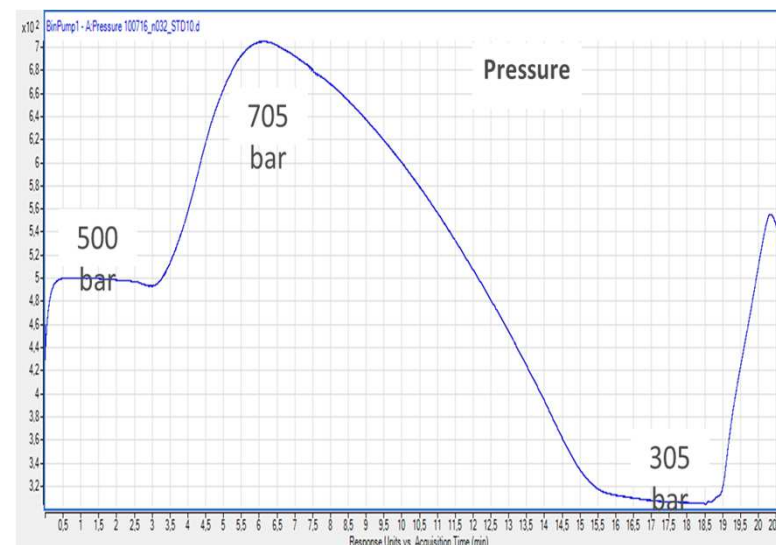
UHPLC-MS/MS Setup

1290 Infinity UHPLC

- ZORBAX Eclipse Plus C-18 : 100 x 2.1 mm, 1.8 μm
- Methanol-water gradient: 3ul injection

6490 QQQ Infinity UHPLC

- Single run possible with fast polarity switching



OTA (+ve): 317->131+272

FB2: (+ve) 706->336+318

ZEN:(-ve) 404->239+102



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Validation of SIDA method based on Maize



- Does the method perform over the whole range of interest in maize?
- Is the method accurate and precise?
- ✓ Range and linearity of Calibration.
- ✓ Spiking blank maize (six levels in triplicate).



Results: LOQ's versus requirements

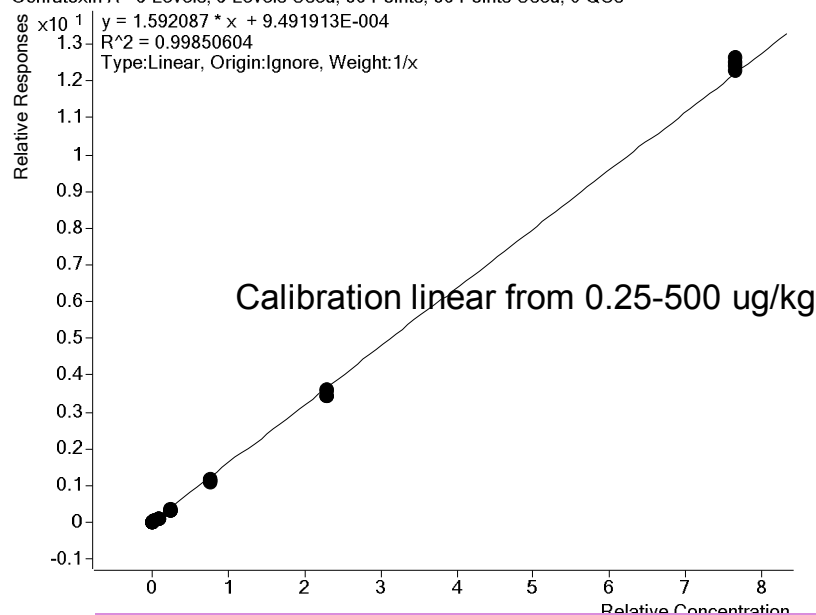
Analytes	Linear range ng/mL	LOQs (maize) µg/kg	MRLs (EC Reg. No 1881/2006)	Commodities
Aflatoxin B ₁	0.0075 - 74.6	0.05	0.1	processed cereal-based baby food
			2.0-12 sum of aflatoxins: 4.0-15.0	nuts and cereals
Deoxynivalenol	0.23 - 225	2.5	200 500 - 1750	processed cereal-based baby food processed / unprocessed cereals, bread, pasta, breakfast cereals
Fumonisin B ₁	0.075 - 249	2.5	200 800 1000 / 4000	processed maize-based baby food maize-based breakfast cereals maize / unprocessed maize
Fumonisin B ₂	0.075 - 251	0.64		
HT-2 toxin	0.2 - 202	2.0	implementation of MRLs is expected in the near future	unprocessed cereals and cereal products
T-2 toxin	0.023 - 75.4	0.17		
Ochratoxin A	0.023 – 23.0	0.25	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	0.076 - 252	1.0	20 50 75-350	processed cereal-based baby food bread, biscuits, breakfast cereals processed / unprocessed cereals



Results: Ochratoxin A

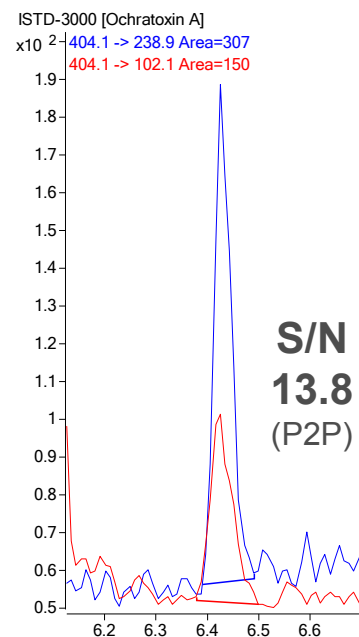
- Challenging compound due to low MRLs
- 0.5 µg/kg in processed cereal based baby food
- 3.0 / 5.0 µg/kg in processed / unprocessed cereals
- 10.0 µg/kg in dried vine fruit

Ochratoxin A - 9 Levels Used, 36 Points Used, 0 QCs



Overlay of 4 individual calibrations
acquired within 45 hour worklist.

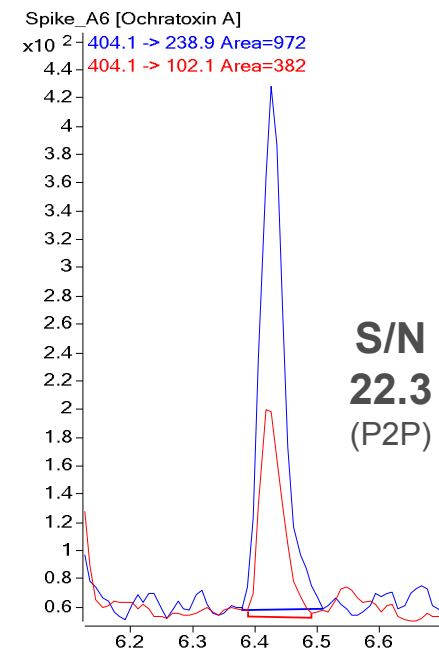
In solvent



0.0230 ng/ml

0.230 µg/kg

Spiked Maize



0.5 µg/kg

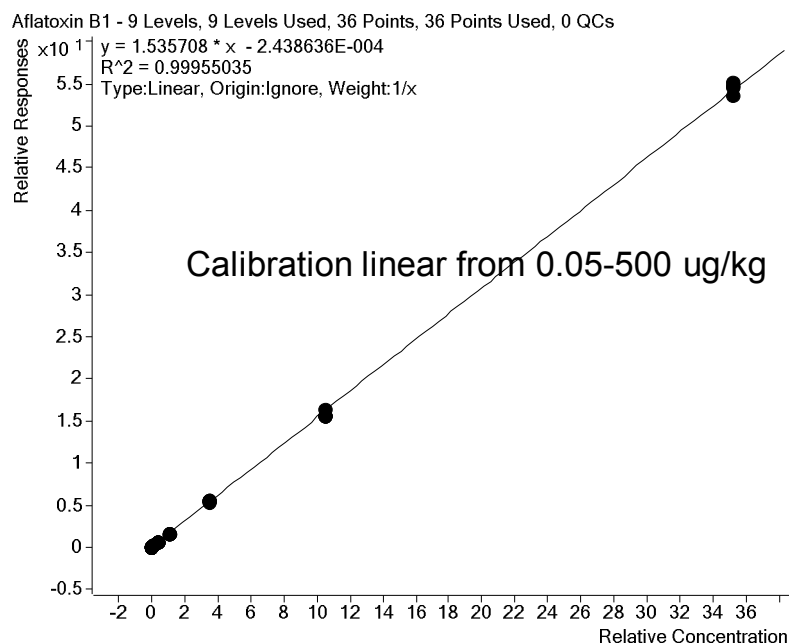


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Results: Aflatoxin B1

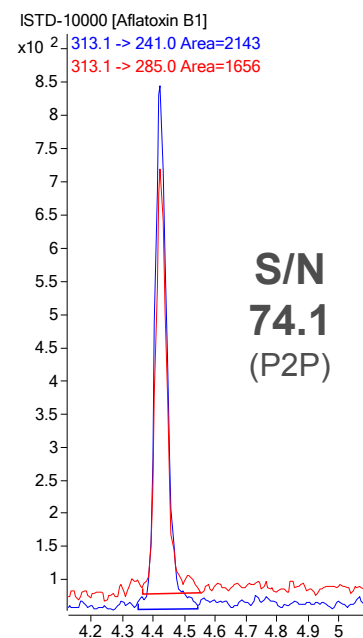
- Challenging compound due to low MRLs

- 0.1 µg/kg in processed cereal based baby food
- Typically 2 to 12 µg/kg in nuts and cereals

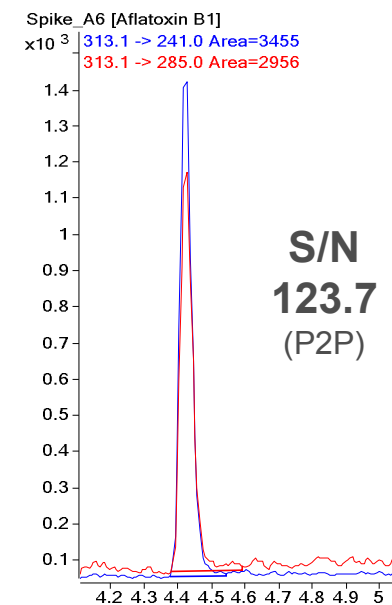


Overlay of 4 individual calibrations
acquired within 45 hour worklist.

In solvent



Spiked Maize



Results – Validation

Analyte	External calibration R_A [%] \pm RSD [%] ¹⁾	Internal calibration R_A [%] \pm RSD [%] ¹⁾
Aflatoxin B ₁	35 \pm 4	105 \pm 6
Aflatoxin B ₂	42 \pm 4	100 \pm 4
Aflatoxin G ₁	46 \pm 5	101 \pm 5
Aflatoxin G ₂	40 \pm 6	101 \pm 8
Deoxynivalenol	41 \pm 10	99 \pm 9
Fumonisin B ₁	330 \pm 6	101 \pm 10
Fumonisin B ₂	181 \pm 7	88 \pm 7
HT-2 toxin	134 \pm 6	98 \pm 7
Ochratoxin A	167 \pm 14	93 \pm 7
T-2 toxin	118 \pm 5	99 \pm 6
Zearalenone	91 \pm 6	103 \pm 11

¹⁾ Apparent recovery \pm Relative Standard Deviation for triplicate samples and 6 spiking levels

Summary

- **Two step extraction (90 minute total)**
 - Enhancement of extraction efficiency especially for fumonisins
- **UHPLC-MS/MS method with Dynamic MRM**
 - Good resolution between aflatoxins
 - Positive & negative ions analysed together even with co-elution.
 - Segmentation is eliminated through simple Dynamic MRM
- **Sensitivity suitable for MRLs even for Baby food.**
 - 6490 sensitivity allows for this even though sample is diluted 10 fold in sample preparation.
- **Robust**
 - 4 orders of linearity for all toxins.
 - Recoveries 88-105% for all mycotoxins. RSD's 5-10%.
- **Low running costs**
 - Additional price per IS per sample is between 0.01 to 1.40 €
 - Price for all 11 IS per sample 3-4 €

Results for official test materials

No	Analyte	assigned value ± STDEV µg kg ⁻¹	measured value ± STDEV µg kg ⁻¹
TM_01	ZEN	83 ± 4.5	86 ± 10
TM_02	Sum AFs	3.79 ± 1.67	4.6 ± 0.2
	AFB ₁	1.87 ± 0.83	2.3 ± 0.1
	AFB ₂	0.51 ± 0.23	0.6 ± 0.03
	AFG ₁	0.96 ± 0.43	1.0 ± 0.1
	AFG ₂	0.52 ± 0.23	0.7 ± 0.1
TM_03	FB ₁	1650 ± 53	1960 ± 198
	FB ₂	461 ± 16	496 ± 32
TM_04	DON	1714 ± 64	1660 ± 145
TM_05	DON	901 ± 55	908 ± 79
	ZEN	79 ± 13	84 ± 10
TM_06	FB ₁	2630 ± 370	2300 ± 233
	FB ₂	690 ± 170	578 ± 38
TM_07	FB ₁	270 ± 55	223 ± 23
	FB ₂	< 80	55 ± 4



Customize your mycotoxin method

- Other sample types.
- Inclusion of Aflatoxin M1 and M2.
- Inclusion of MSMS library confirmation.

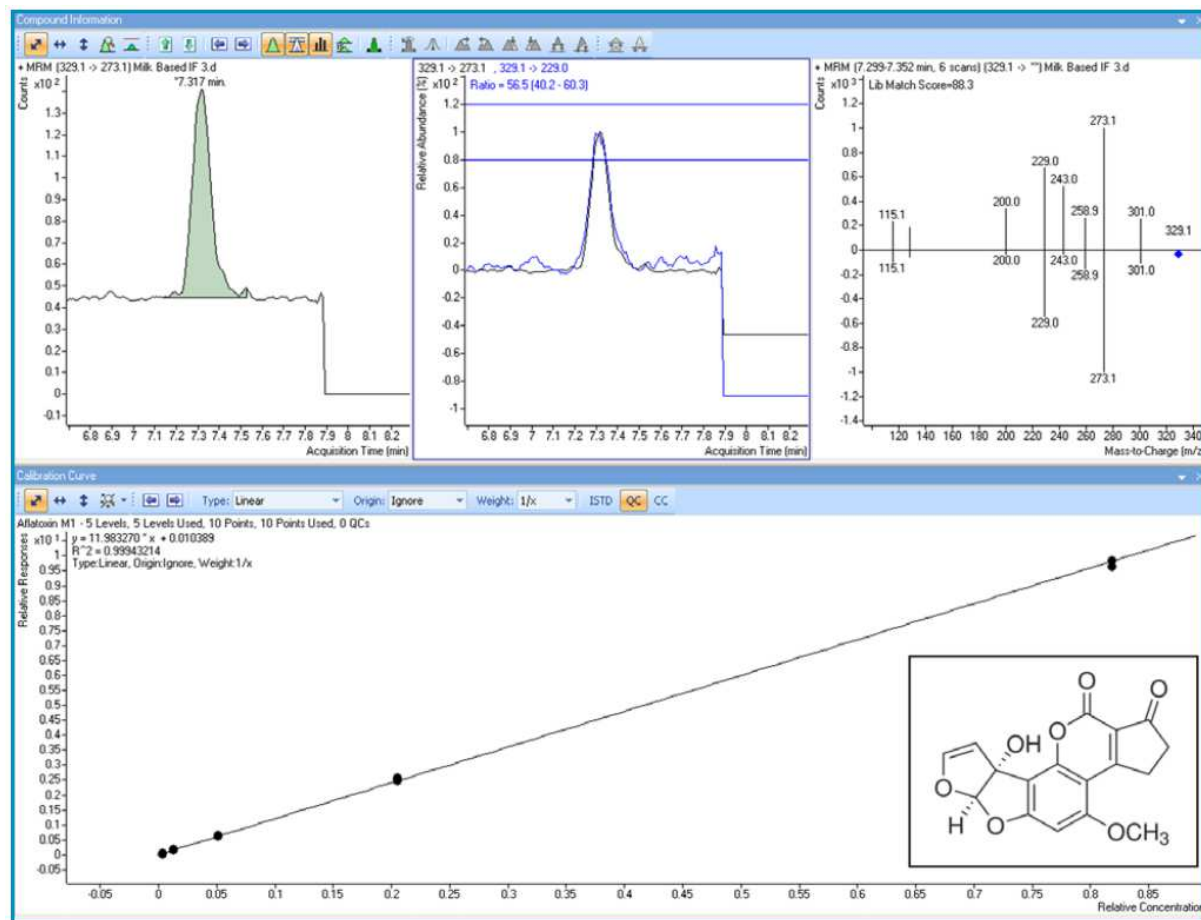


Figure 2. Aflatoxin M1 in Infant Formula, fortified at 0.02 µg/kg.

This LC-MS/MS method has been proven to be effective for the analysis of regulated mycotoxins in **infant formula samples**.

Acceptable Results have been achieved for calibration curves, precision and accuracy.

The use of dMRM with two MS/MS transitions satisfies requirements for positive compound identification, while the use of **tMRM gives greater confidence in analyte identification** without sacrificing sensitivity.

COVANCE



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Modified QuEChERS Method Coupling with UHPLC-ESI/QQQ Mass Spectrometry for Simultaneous Determination of Multiple Mycotoxins in Sesame Butter

Conclusion

A methods for simultaneous determination of twenty-six mycotoxins in sesame butter were developed. These compounds cover most of common mycotoxins in cereal products

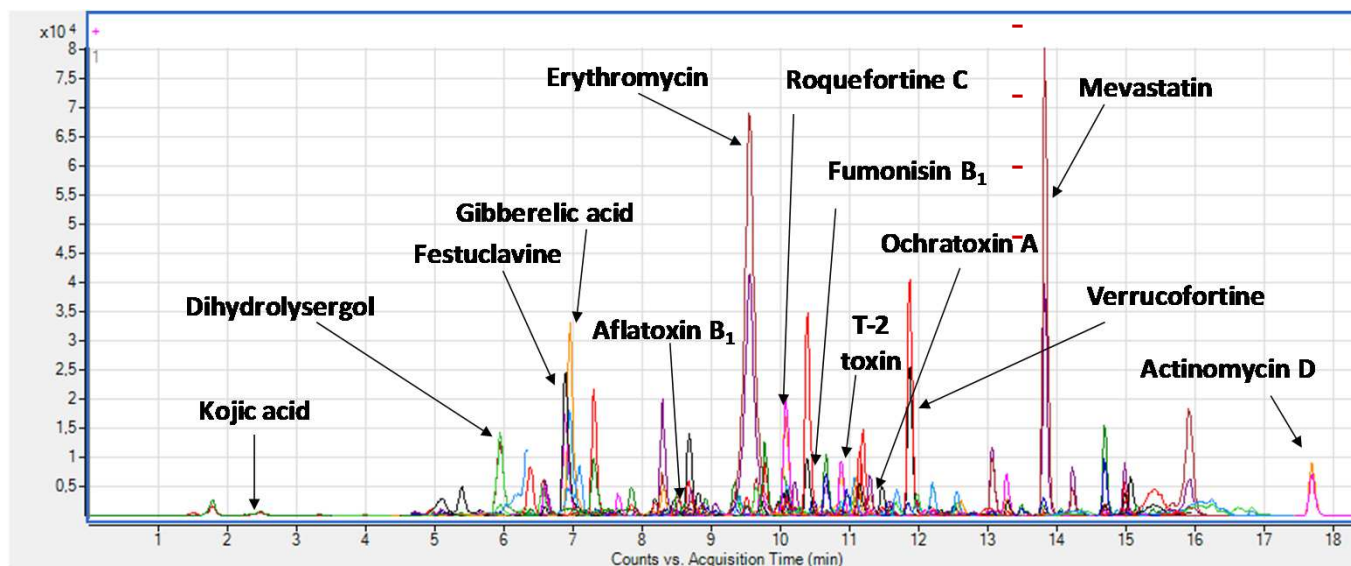


By combining modified QuEChERS clean-up with UHPLC-
QQQ MRM monitoring, the method is more sensitive and
more rapid. Compounds can be accurately quantitated within
the range of **0.21-21.74 ng/mL(0.7-71.74 ug/kg)**

Customize your mycotoxin method

DMRM database for mycotoxins

- Multi-mycotoxin method for 191 mycotoxins and other fungal metabolites has been developed
 - Validated for different nuts: almond, hazelnut, pistachio, and peanut
 - Transitions are shortly available as DMRM database.
- 50 nut samples were screened.
- A naturally contaminated hazelnut sample contained aflatoxins, Alternaria toxins, mycophenolic acid & T2 toxin (found for the first time in hazelnuts).
- >40 different mycotoxins found in all samples
 - hazelnut (36) peanuts (30) almonds (13), pistachios (5).
 - one hazelnut sample with 26 mycotoxins, inc



Beauvericin
enniatin B
macrosporin
3-nitropropionic acid



Christian Doppler
Forschungsgesellschaft



Agilent Technologies

Thank you for your attention

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

PAPER IN FOREFRONT

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

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

Please also see
Agilent application note
5991-2808EN

Anal Bioanal Chem
DOI 10.1007/s00216-013-6831-3

RESEARCH PAPER

Development and validation of a (semi-)quantitative UHPLC-MS/MS method for the determination of 191 mycotoxins and other fungal metabolites in almonds, hazelnuts, peanuts and pistachios

Elisabeth Varga • Thomas Glauner • Franz Berthiller •
Rudolf Krška • Rainer Schuhmacher • Michael Sulyok

Please also see
Agilent application note
5991-4991EN

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Modified QuEChERS method combined with ultra-high performance liquid chromatography tandem mass spectrometry for the simultaneous determination of 26 mycotoxins in sesame butter

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