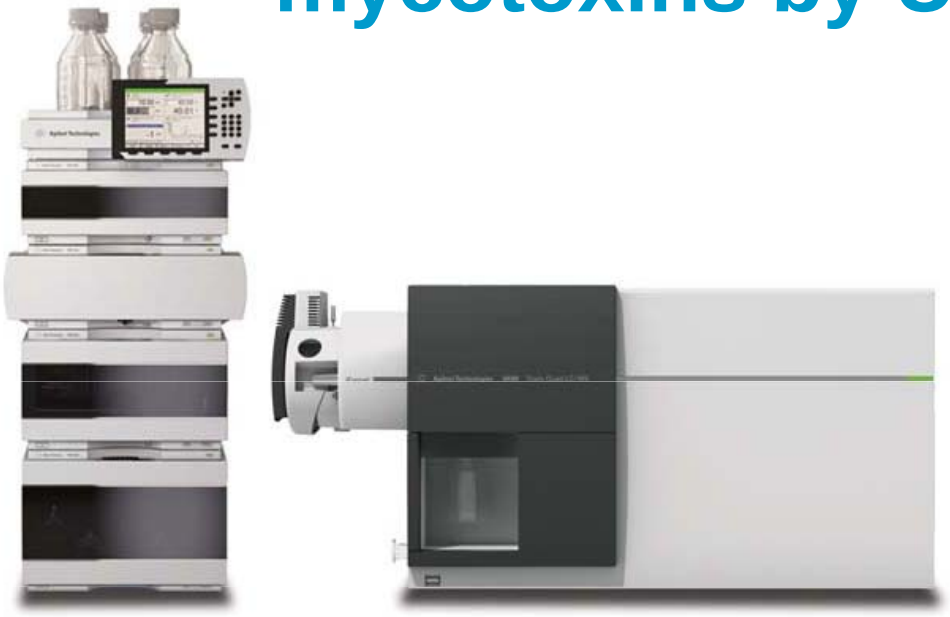
Accurate quantitation of regulated mycotoxins by UHPLC-MS/MS



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Outline

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

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

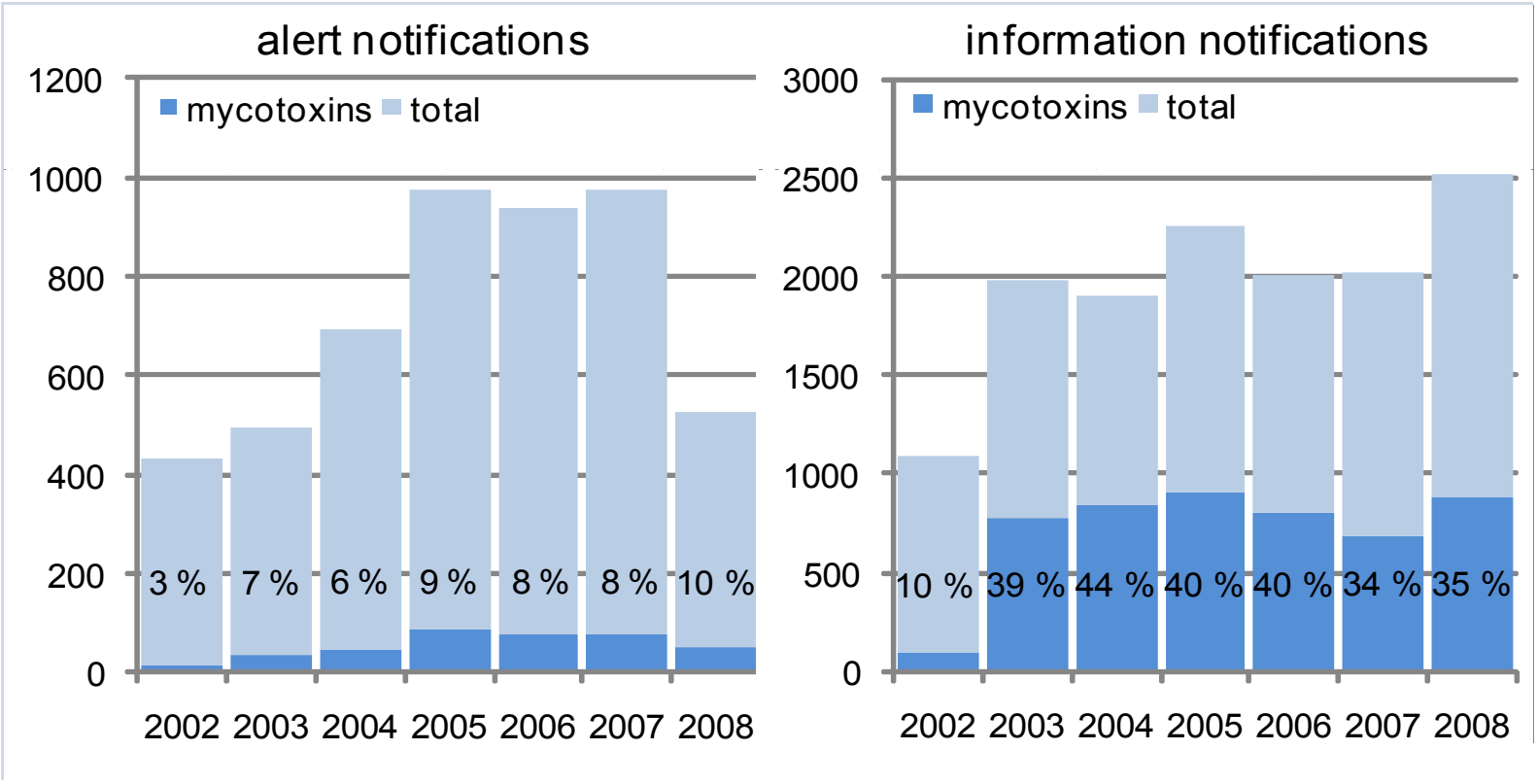
Why are they an issue?

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

Mycotoxins

Relevance for food control

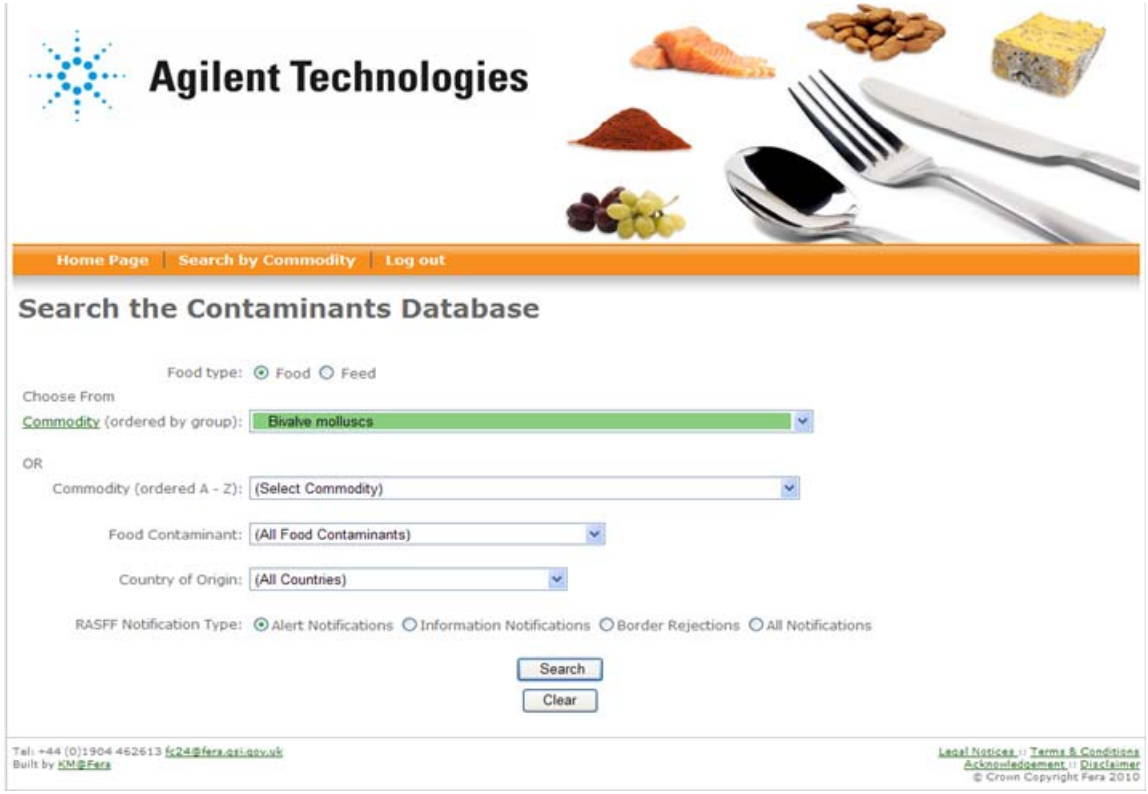
- Notifications concerning mycotoxins
  - (RASFF-Annual reports 2002-2008)



Food contaminants online

FERA FC24 database access

- Free access to the RASFF alerts and notifications via FC24 database
- Registration through Agilent website (http://www.chem.agilent.com/en-US/solutions/foodtesting-agriculture/Pages/default.aspx)

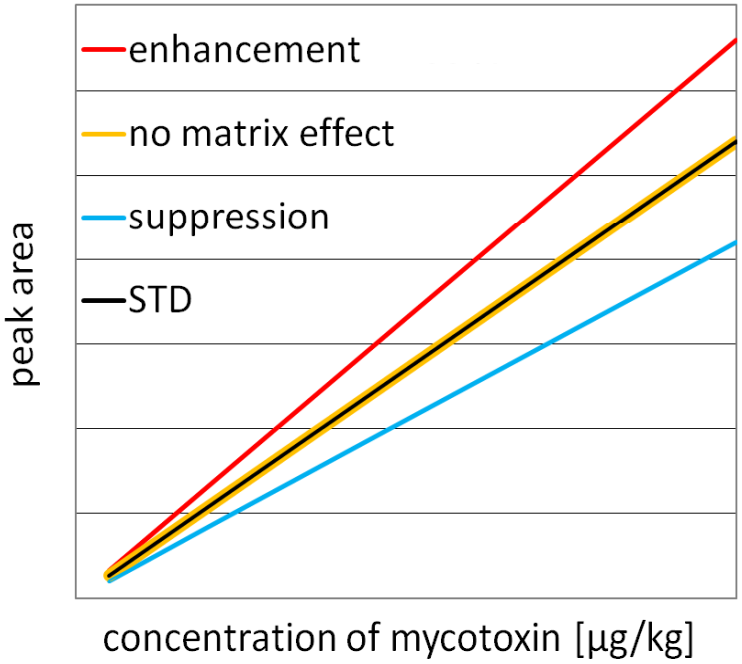


Accurate quantitation of mycotoxins
Reasons

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

BUT:

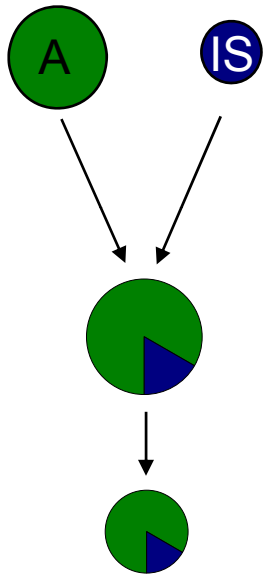
- Electrospray ionisation (ESI)
  - matrix effects hamper accurate mass spectrometric quantification
- Quantification of regulated mycotoxins at a very high degree of accuracy is required



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
  - until this year: only single analyte or group analyte IS-addition
  - usually associated with rather high costs



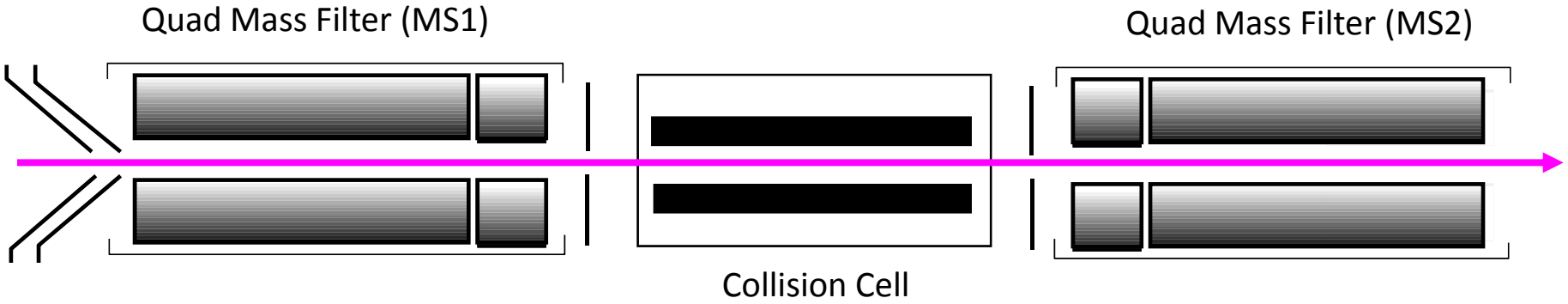
Stable Isotope Dilution Assay (SIDA)

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

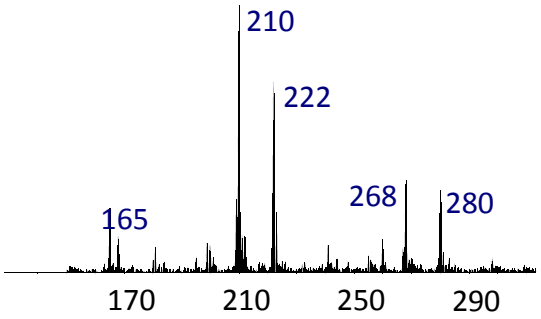


Spectrum with background ions (from ESI)

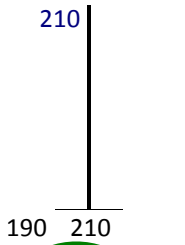
Q1 lets only target ion 210 pass through

Collision cell breaks ion 210 apart

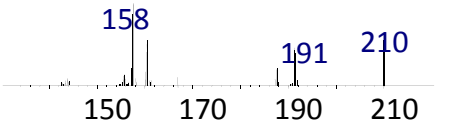
Q3 monitors only characteristic fragments 158 from ion 210 for quant

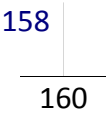


Chromatogram

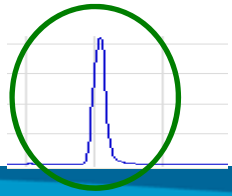


High background





Low background



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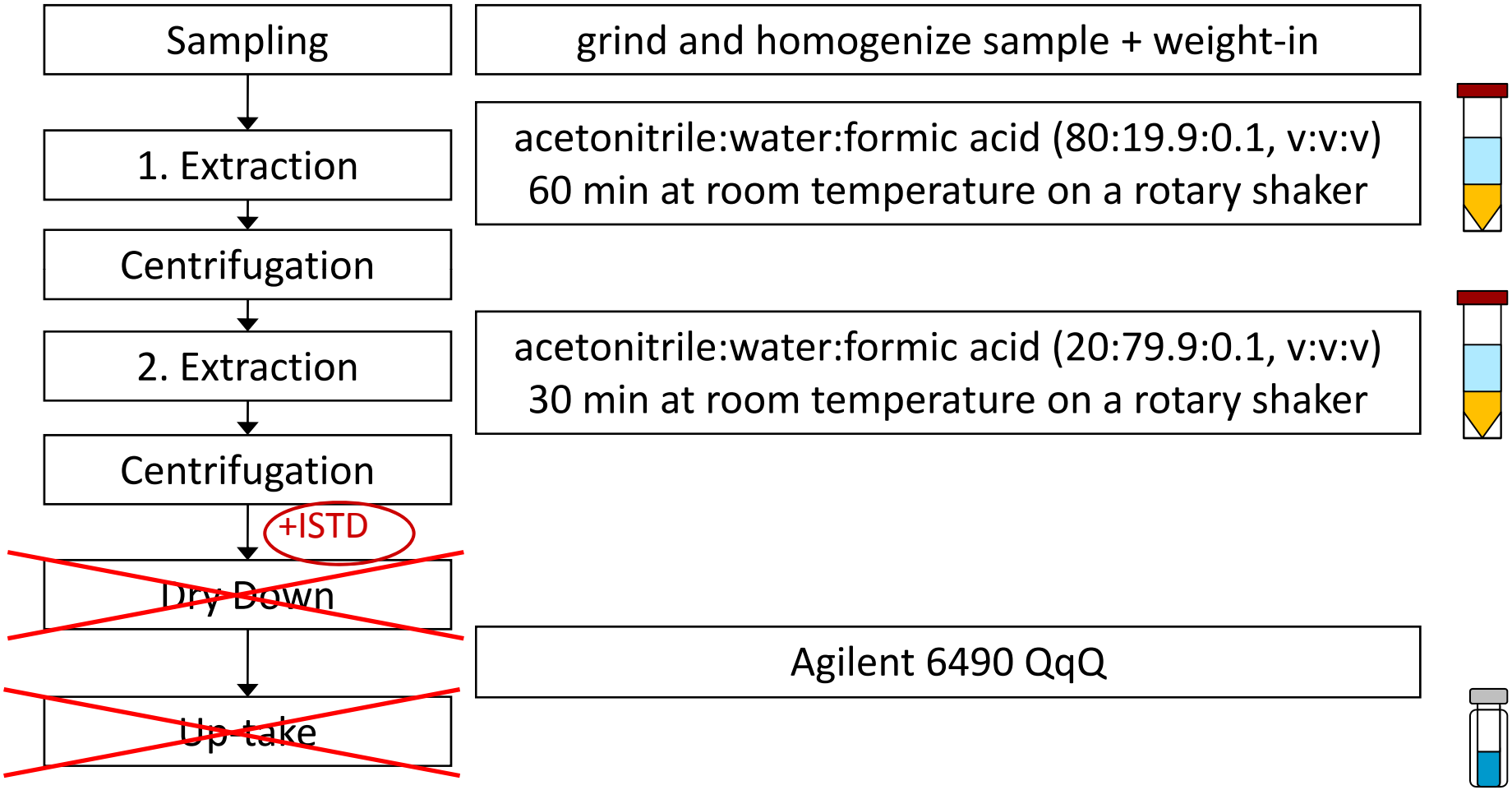
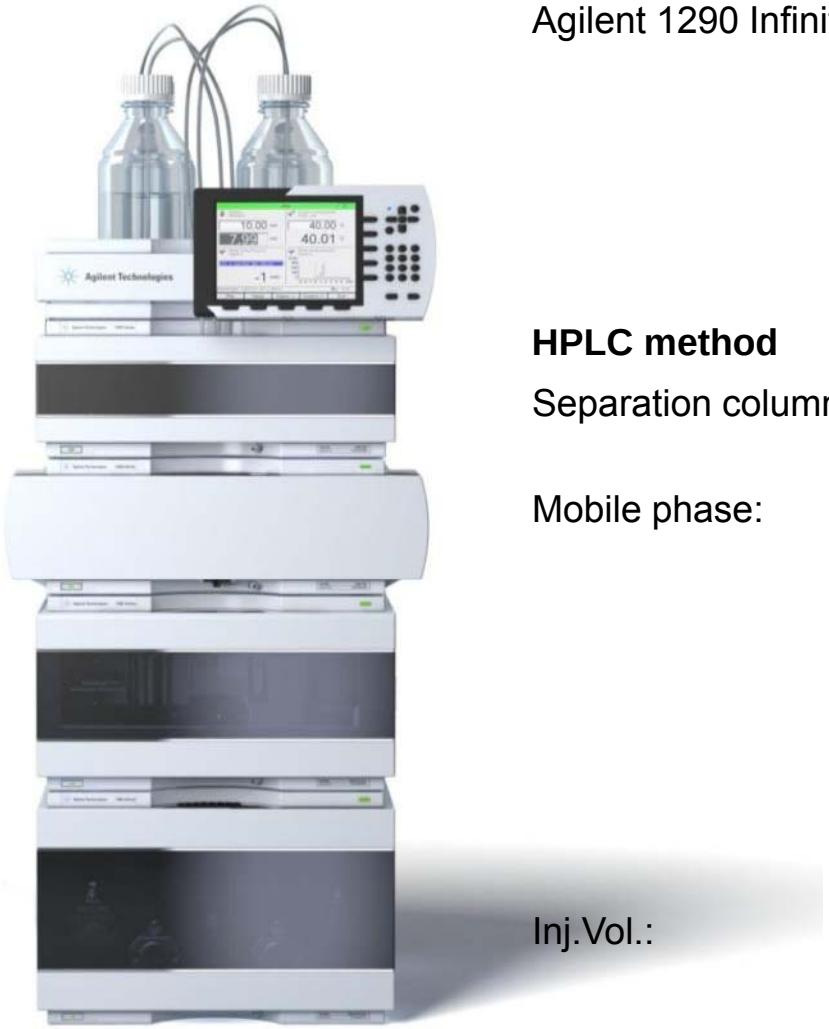
Sample preparation

Universal extraction procedure



Stable Isotope Dilution Assay (SIDA)

HPLC method



Agilent 1290 Infinity LC system consisting of:

- binary pump
- wellplate sampler
- column compartment
- diode array detector (not used)

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

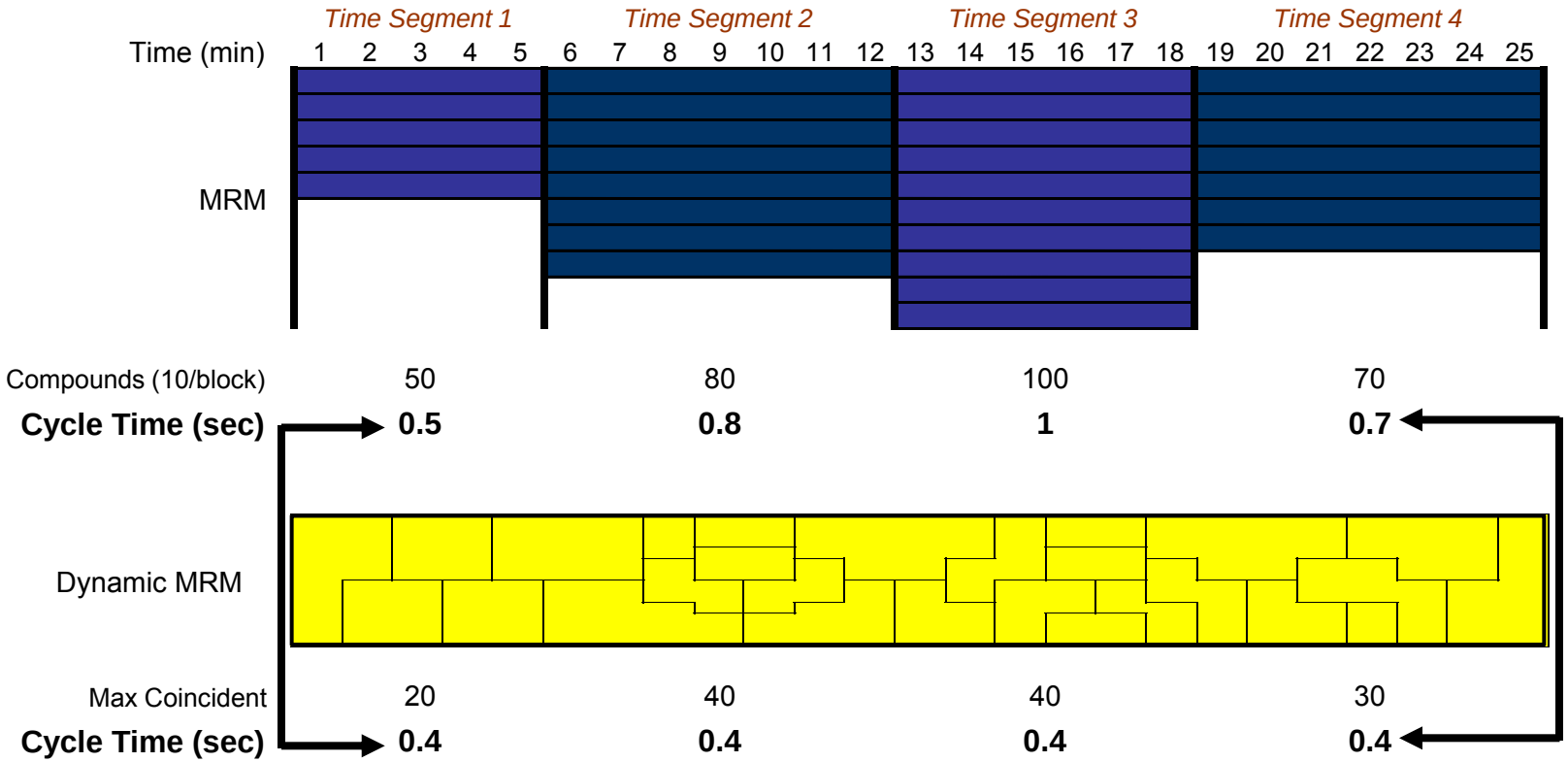
	Positive	**Negative**
CapVoltage:	4000 V	3000 V
Nozzle voltage	0 V	0 V

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

Dynamic MRM functionality

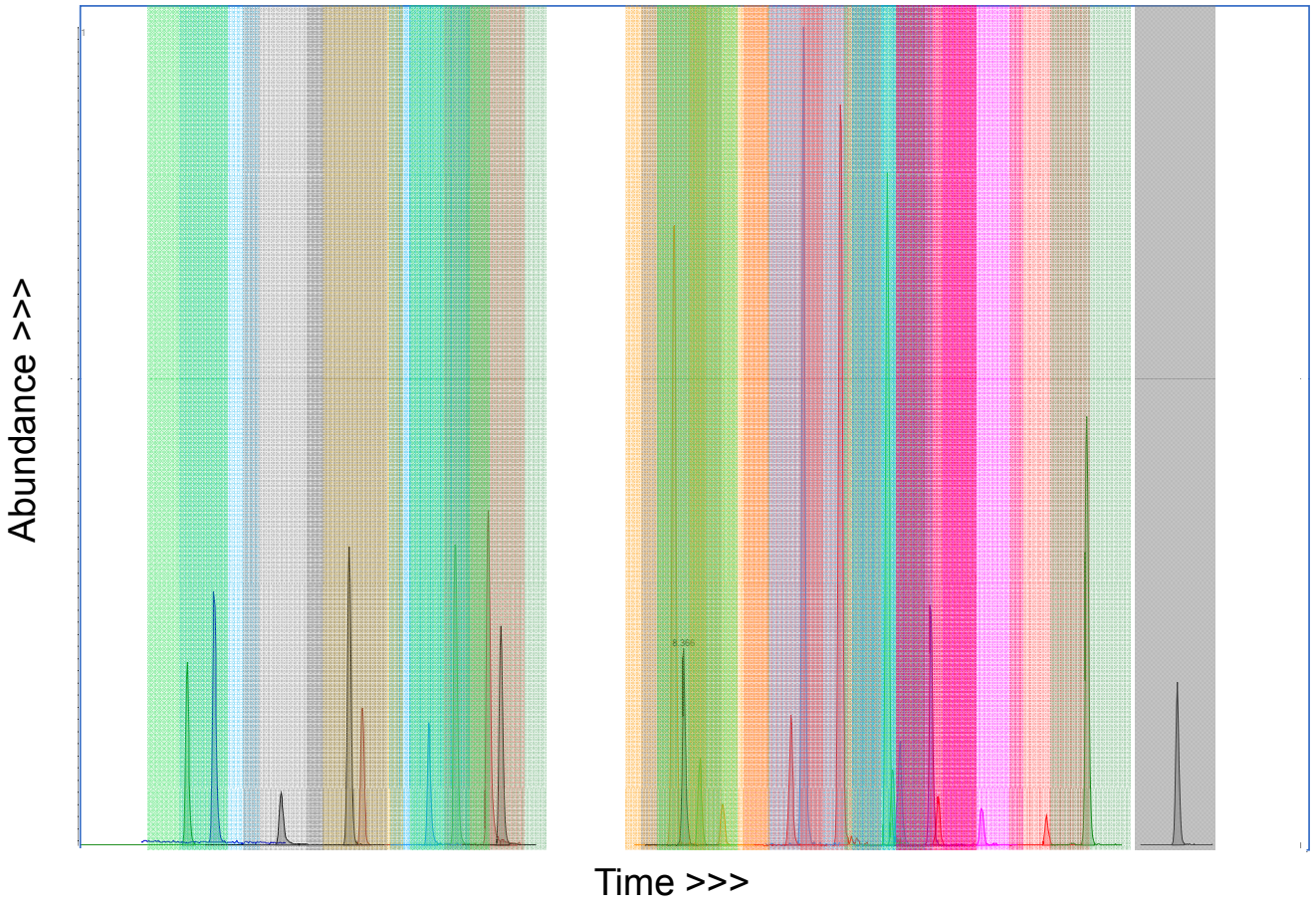
Comparison of MRM and DMRM



- 2 x shorter cycle times supports narrow chromatographic peaks, more analytes or longer dwell per analyte.

Dynamic MRM functionality

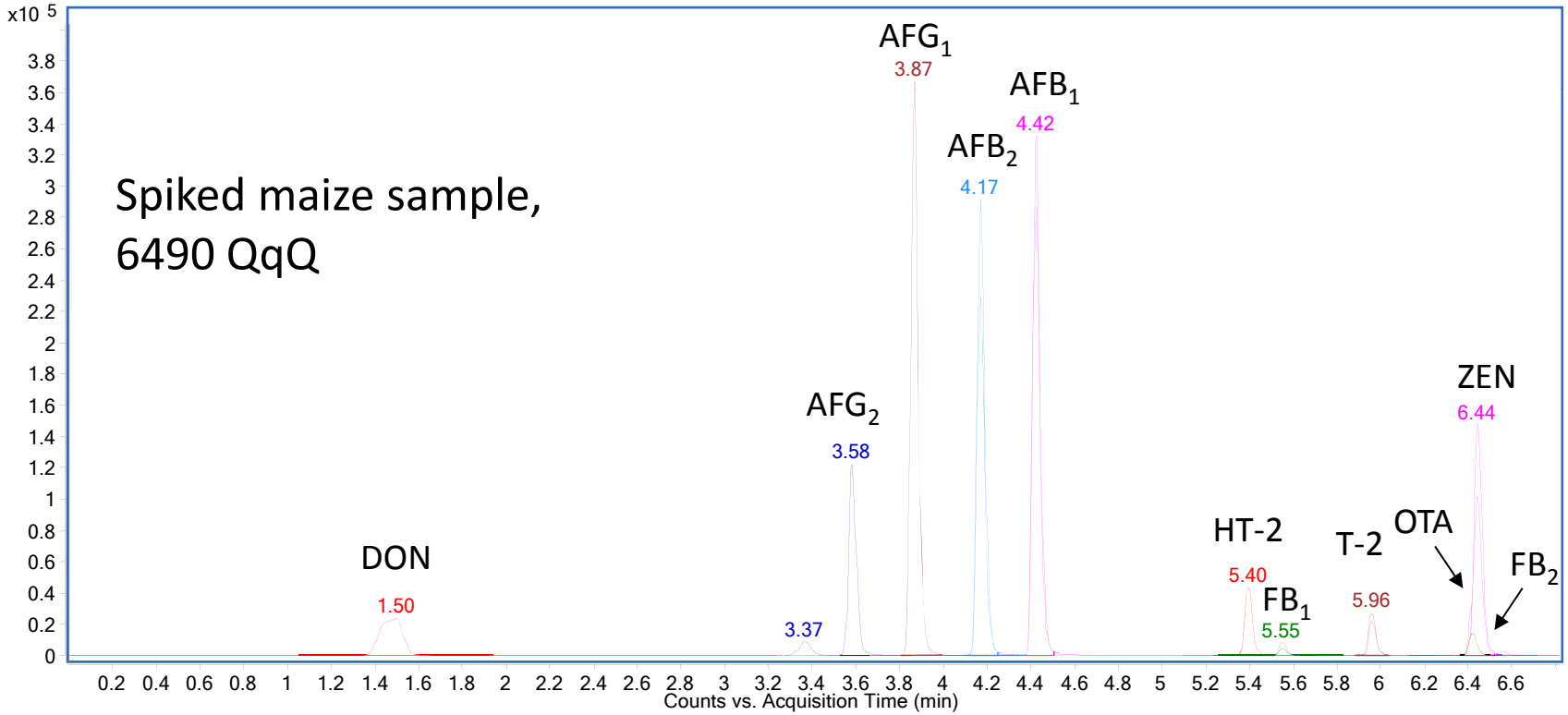
DMRM simulation



Stable Isotope Dilution Assay (SIDA)

Chromatogram

- due to same MRM transitions baseline separation required for:
  - fumonisin B2 and B3
  - aflatoxin G1 and ^{13}C-aflatoxin B1
  - aflatoxin G2 and ^{13}C-aflatoxin B2



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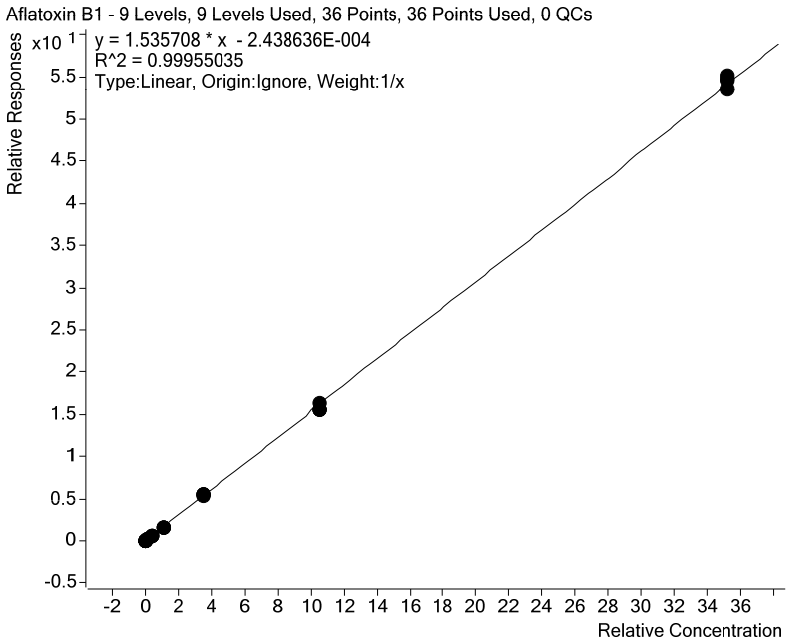
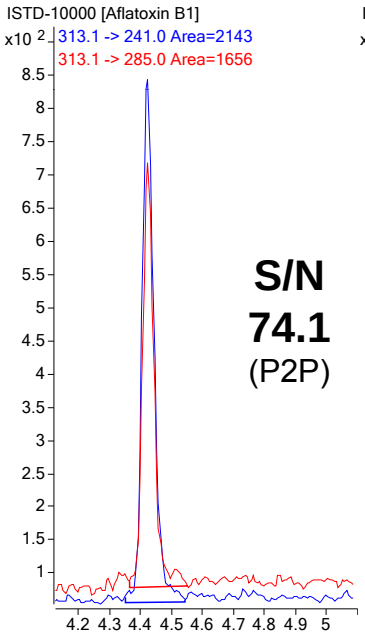
Internal calibration in solvent

Aflatoxin B1

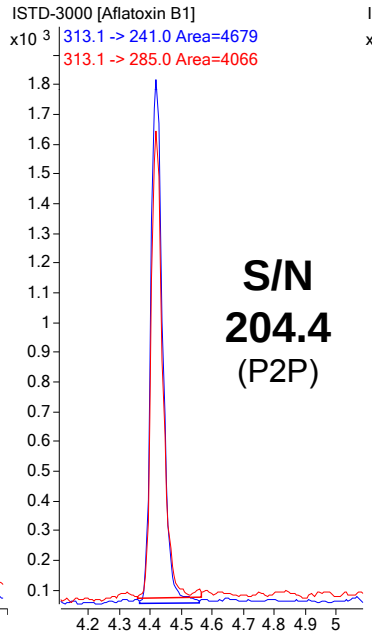
- Challenging compound due to low MRLs
  - 0.1 µg/kg in processed cereal based baby food
  - 2 to 12 µg/kg in nuts and cereals





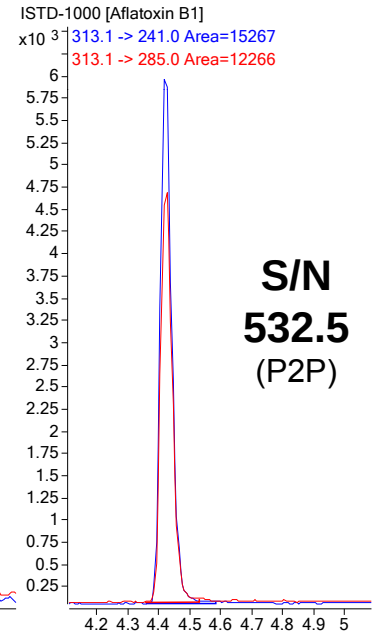
S/N 74.1 (P2P)

0.0075 ng/ml



S/N 204.4 (P2P)

0.0225 ng/ml



S/N 532.5 (P2P)

0.075 ng/ml

Overlay of 4 individual calibrations acquired within 45 hour worklist.

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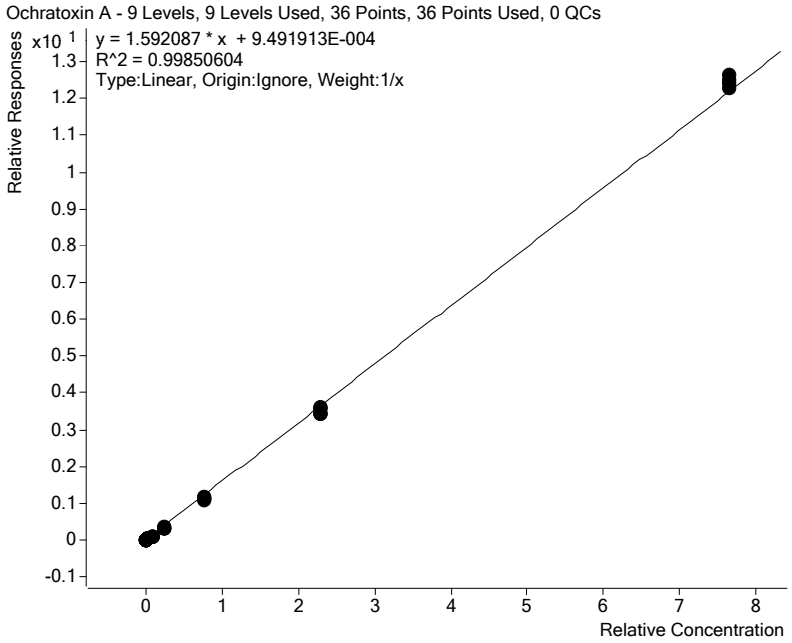
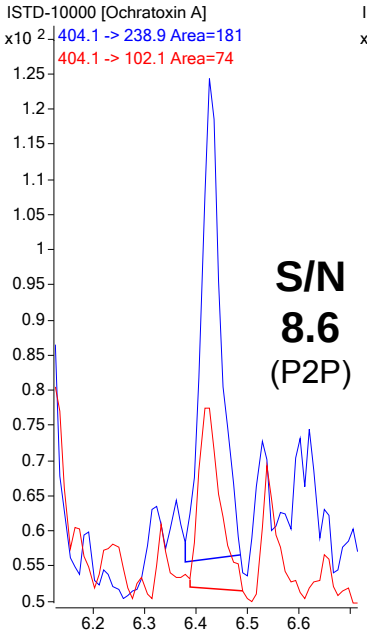
Internal calibration in solvent

Ochratoxin A

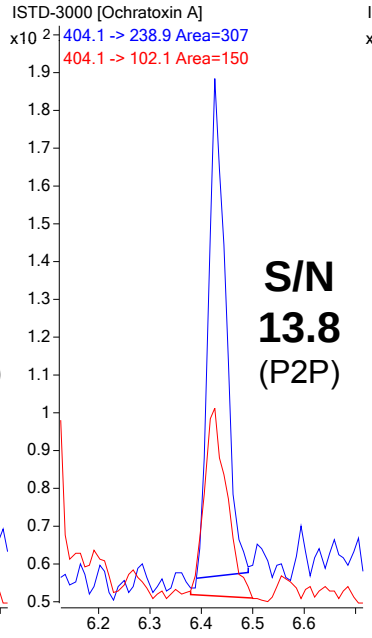
- Challenging compound due to low MRLs
  - 0.5 µg/kg in processed cereal based baby food
  - 3.0 / 5.0 µg/kg in processed / unprocessed cereals
  - 10.0 µg/kg in dried vine fruit





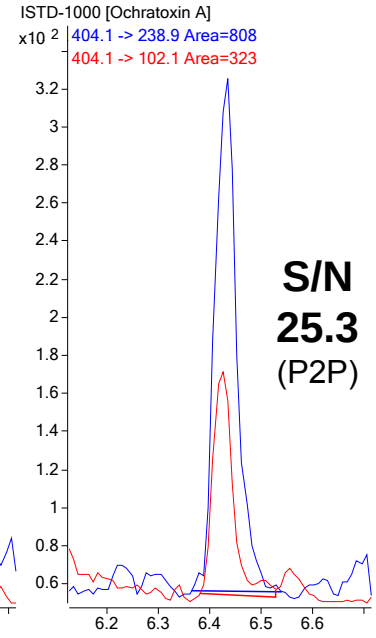
S/N 8.6 (P2P)

0.0077 ng/ml



S/N 13.8 (P2P)

0.0230 ng/ml



S/N 25.3 (P2P)

0.0765 ng/ml

Overlay of 4 individual calibrations acquired within 45 hour worklist.

Validation of SIDA method

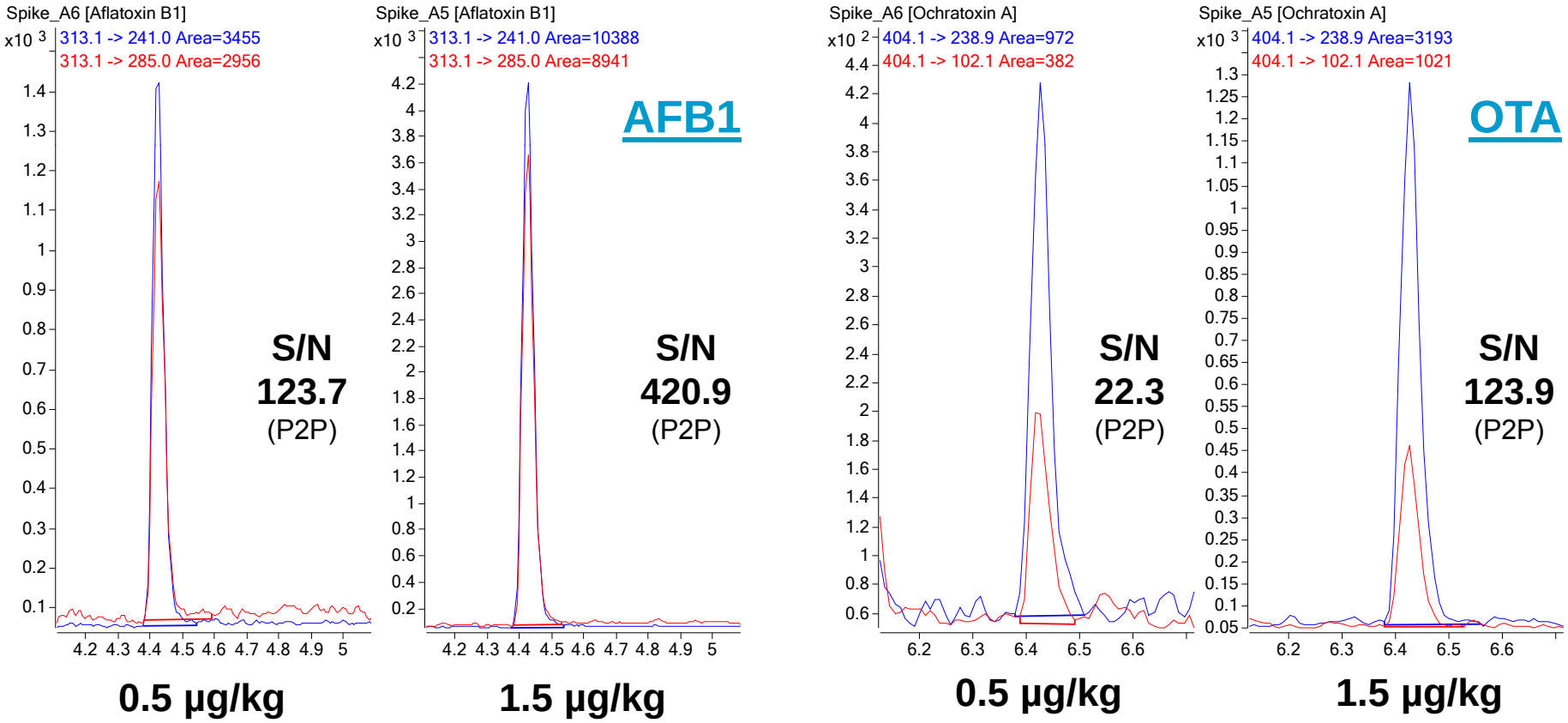
Experimental setup and results

- 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 0.01 to 1.40 €
  - Price for all 11 IS per sample < 2.00 €
- Full validation for maize
  - Maize: - matrix for which most mycotoxins are regulated
    - known for matrix effects and matrix interferences
  - more costs (time and standards)
  - 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



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

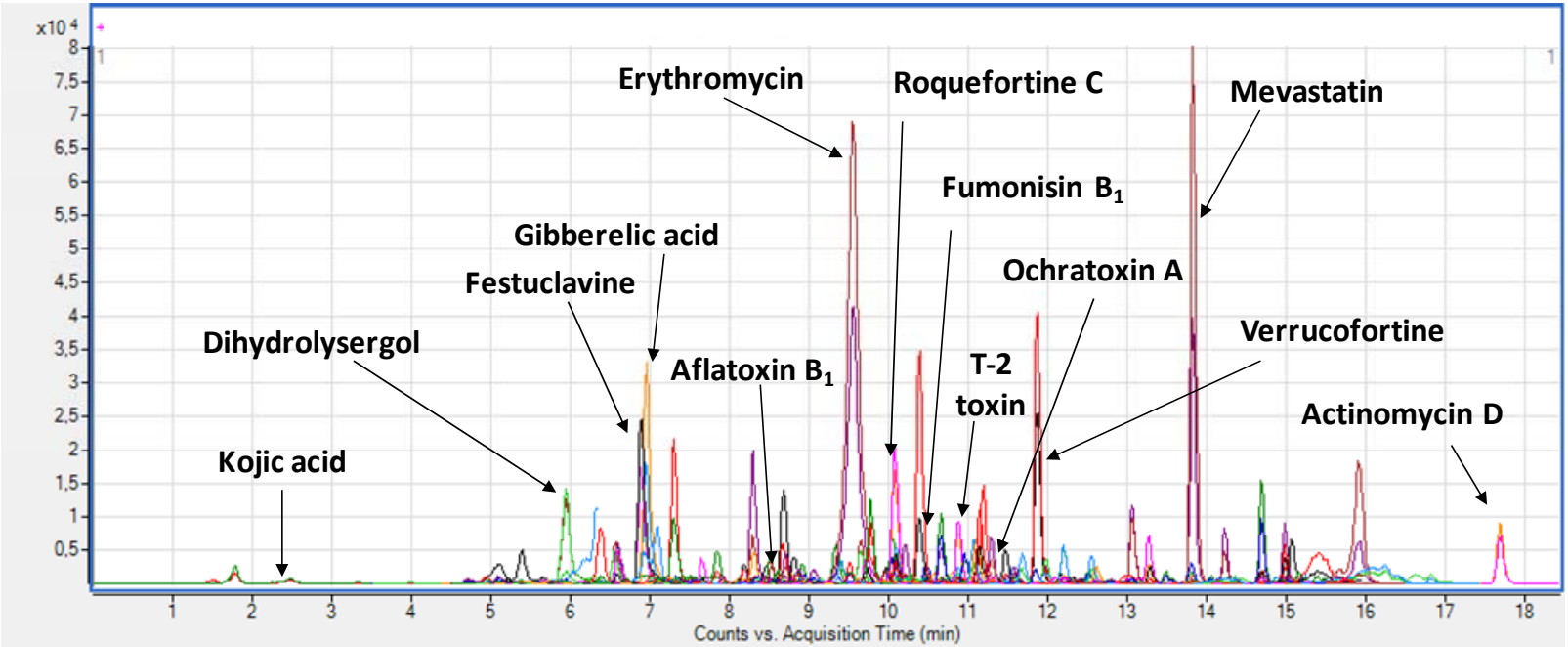
Analyte	LOQ in µg/kg	R_A** in % ± RSD in %	
Aflatoxin B_1	0.04	105	6
Aflatoxin B_2	0.04	100	4
Aflatoxin G_1	0.05	101	5
Aflatoxin G_2	0.24	101	8
Deoxynivalenol	2.5	99	9
HT-2 toxin	2.0	98	7
T-2 toxin	0.17	99	6
Ochratoxin A	0.23	93	7
Zearalenone	0.97	103	11
Fumonisin B_1	2.5	101	10
Fumonisin B_2	0.64	88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

** average for triplicate samples and 6 spiking levels

DMRM database for mycotoxins

Customize your mycotoxin method

- Multi-mycotoxin method for 242 mycotoxins and other fungal metabolites has been developed
  - Validated for different nuts
  - Transitions are shortly available as DMRM database



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 18 reference materials covering all toxin groups
- Sensitivity suitable for MRLs
  - Improved sensitivity of G6490 allows to omit sample concentration resulting in easier handling and improved robustness

Questions

Validation of SIDA method

Method characteristics and requirements

Analytes	Linear range ng/mL	LOQs (maize) µg/kg	MRLs (EC Reg. No 1881/2006)	Commodities
Aflatoxin B_1	0.0075 - 74.6	0.04	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	0.23	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