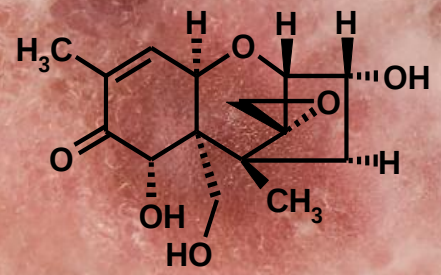
Modern Analytical Tools to Tackle an Old Problem: Mycotoxins in Food

Elisabeth Varga, Thomas Glauner, Emma Rennie, Bernhard Wuest, Michael Sulyok, Rainer Schuhmacher, Rudolf Krska, Franz Berthiller

Christian Doppler Laboratory for Mycotoxin Metabolism
& Center for Analytical Chemistry
Department of Agrobiotechnology (IFA-Tulln)
University of Natural Resources and Life Sciences, Vienna (BOKU), Austria

Mycotoxins are….



… toxic secondary fungal metabolites

***Aspergillus* spp.**

***Penicillium* spp.**

***Fusarium* spp.**

***Alternaria* spp.**

***Claviceps* spp.**

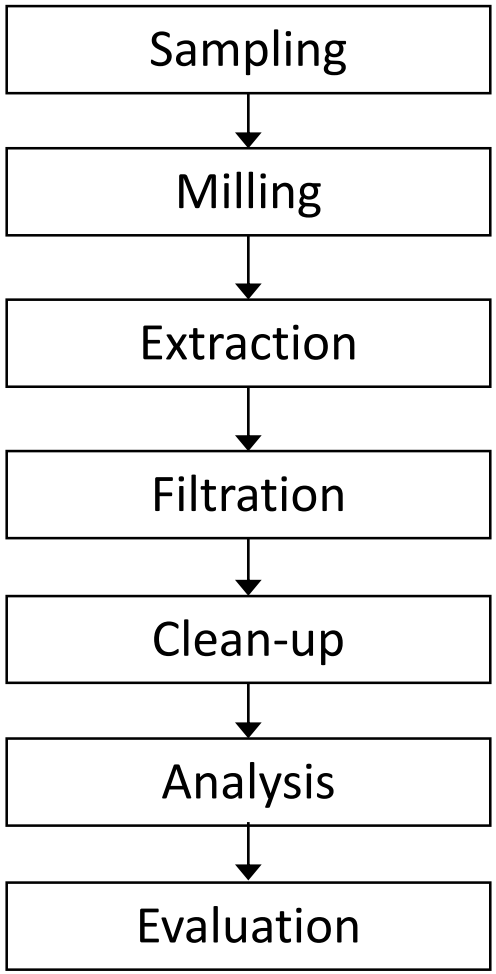
etc.

Mycotoxins
History

- Epidemics of ergotism have been reported as early as **857 A.D.** resulting from the ingestion of cereals infected with ergot
- **1960:** Turkey X Disease
  Peanuts infected with ***Aspergillus flavus***
- Isolation of the highly toxic fungal metabolite **aflatoxin** and **intensive research** on other fungi and toxins produced by them
- Effects: acute toxic, nephrotoxic, **carcinogenic**, immunosuppressive, estrogenic, hepatotoxic,…
- Annual losses of **several hundred million tons of food and feed** worldwide
- 100+ countries have **regulations** for the control of mycotoxins in food and feed

Determination of Mycotoxins

Analytical Scheme



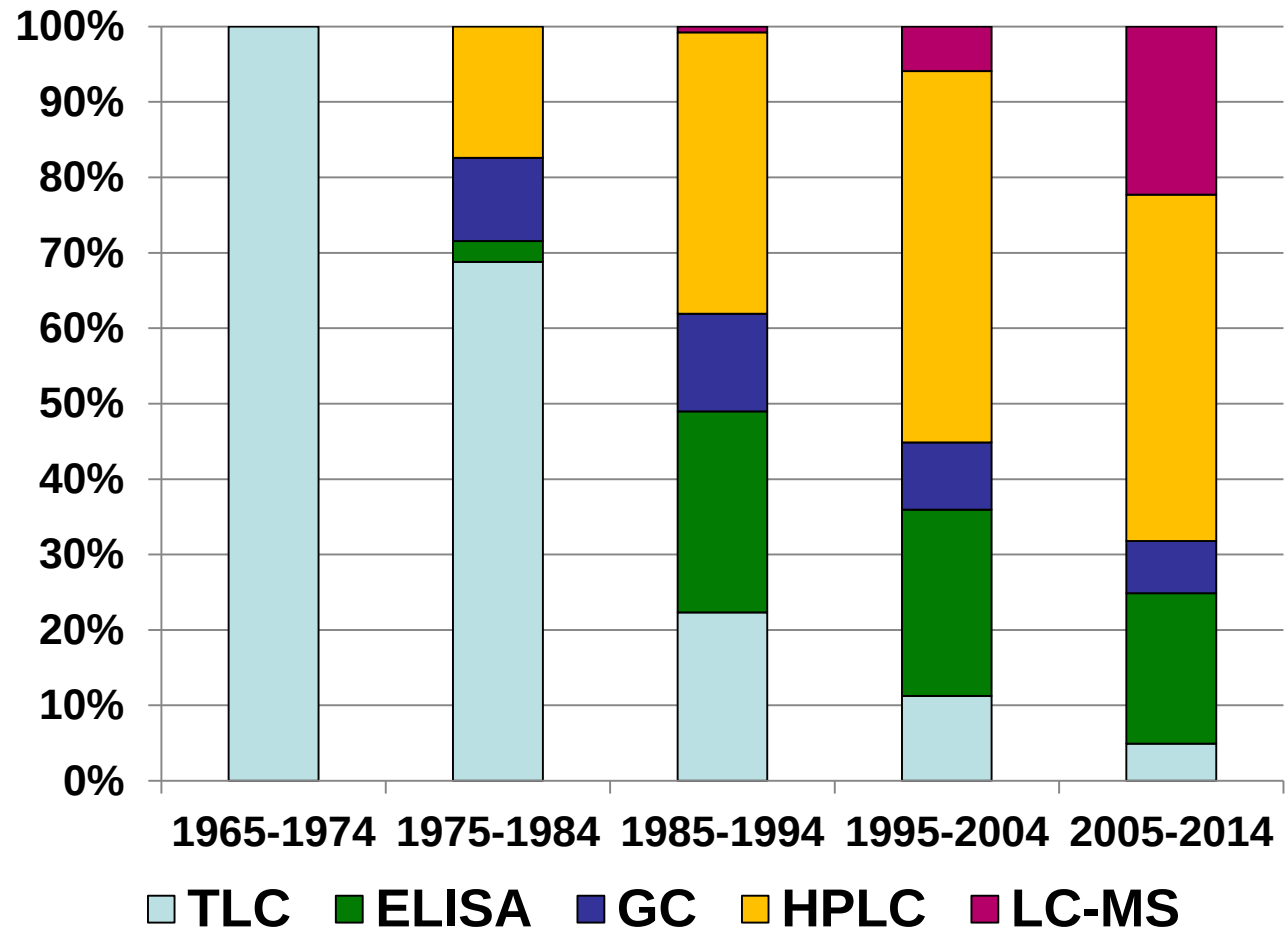
- Sampling is major source of error
  - regulation EC 401/2006: Methods of sampling and analysis for the official control of the levels of mycotoxins in foods
- Careful choice of extraction solvents
  - typically mixtures of water with organic solvents (MeOH, ACN, acetone)
- SPE, IAC, MycoSep®, QuEChERS, dilute & shoot
- Rapid methods (mostly antibody-based) or
- **Chromatographic methods**

50 years of Mycotoxin Analysis

A Transition of Methods

Transition of methods over the last 50 years used in mycotoxin analysis based on the Web of Science®

Keywords: mycotox*
& TLC
& ELISA
& GC
& HPLC
& LC-MS



LC-MS Multi-Mycotoxin Methods

Reasons

- Unified methods save time and costs

- For **accurate quantification** of regulated mycotoxins
  - high degree of accuracy
  - easy to handle, cost effective

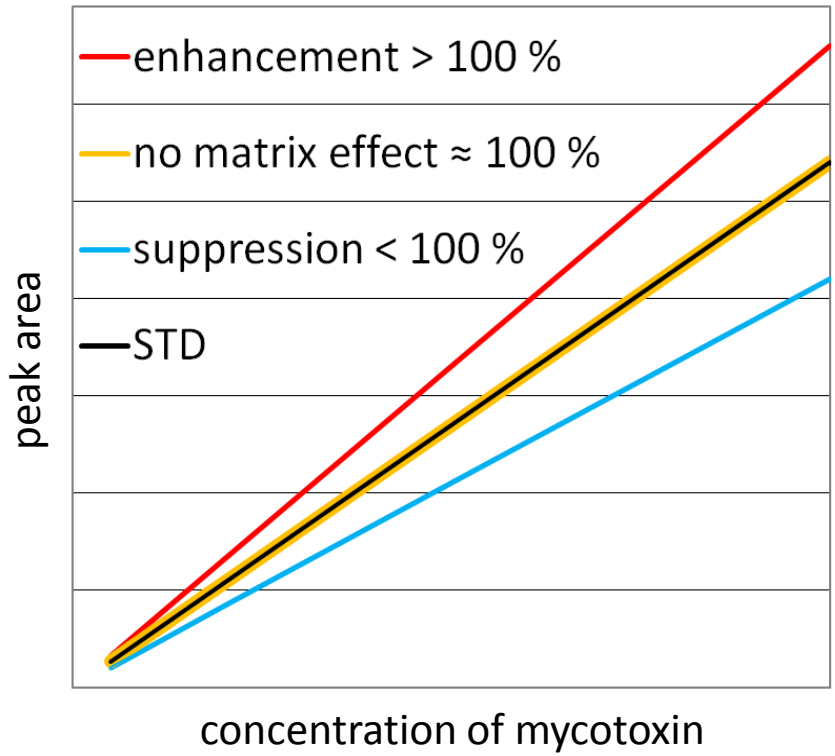
Accurate Mycotoxin Quantification

Reasons

- European Commission Regulation (EC) No 1881/2006 maximum levels in maize for
  - aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2)
  - fumonisins (FB_1, FB_2)
  - deoxynivalenol
  - ochratoxin A
  - zearalenone
  - (HT-2 toxin and T-2 toxin)

BUT:

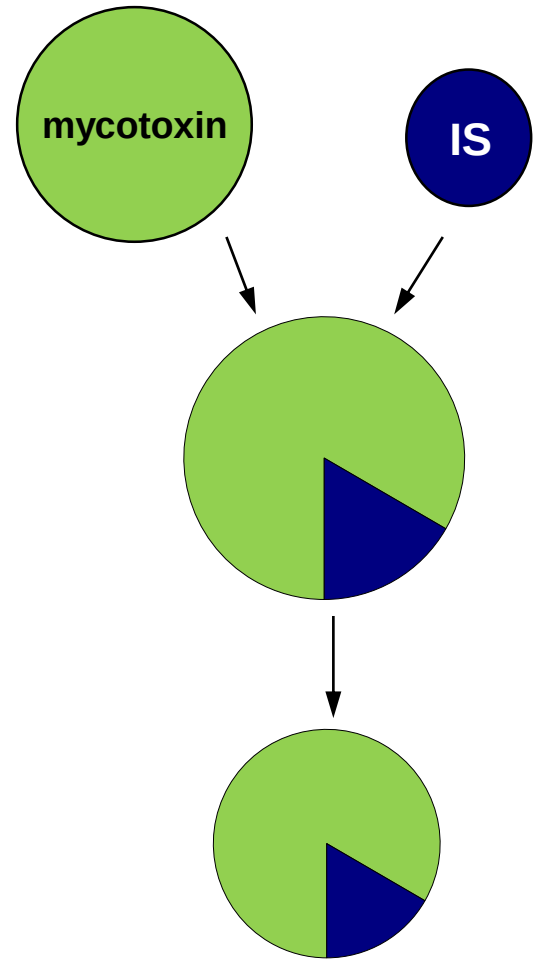
- Electrospray ionisation (ESI)
  - matrix effects hamper accurate mass spectrometric quantification



Accurate Mycotoxin Quantification

Approaches

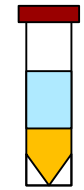
- Dilution of the sample
  - loss of sensitivity
- Matrix matched calibration
  - needed for each individual matrix
- Standard addition to each sample
  - multiple injections needed
- Internal calibration
  - ideally: internal standard (IS) behaves exactly the same as the analyte, but still distinctive
  - structurally related or similar compounds zearalanone (ZAN) for zearalenone (ZEN)
  - [^{2}H]- or [^{13}C]-labelled compounds

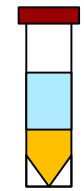


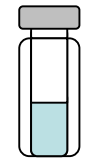
Stable Isotope Dilution Assay (SIDA)

Multiple Mycotoxins - Sample Preparation

Milling	grind and homogenise 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	
Transfer (+IS)	transfer 80 µL aliquot to HPLC vial with microinsert and add 20 µL ISTD-mix
Analysis	UHPLC-MS/MS



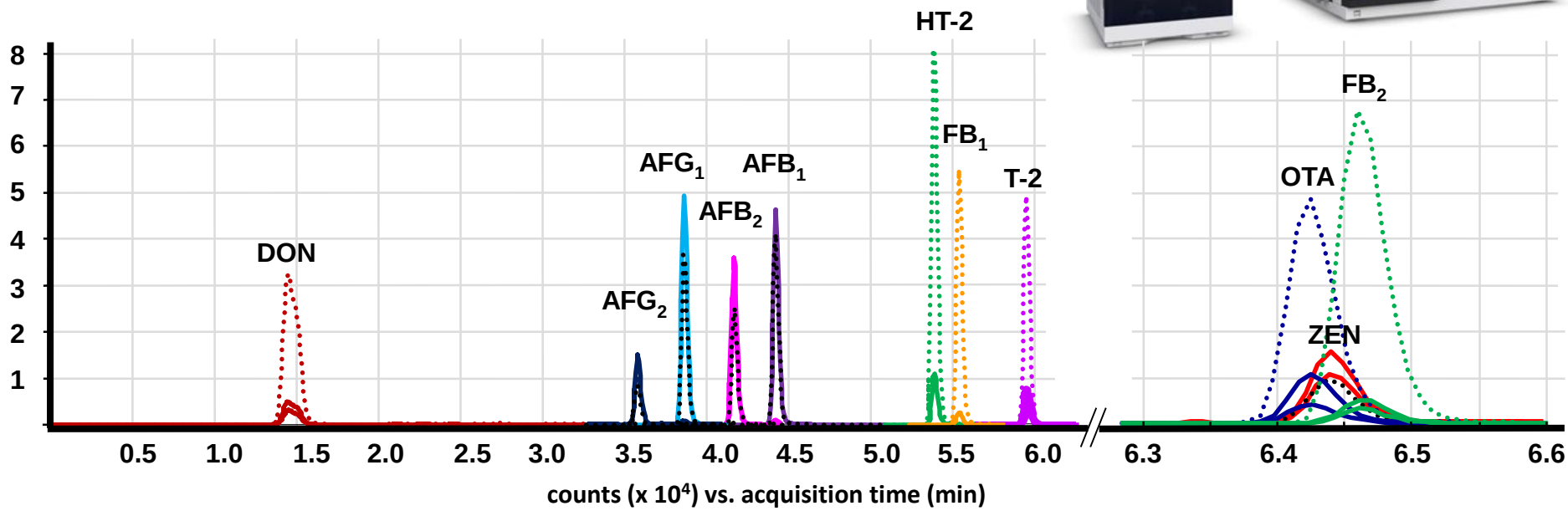




Stable Isotope Dilution Assay

UHPLC-MS/MS Setup

- 1290 Infinity UHPLC (Agilent Technologies)
  - C18 column
  - methanol-water gradient
- 6490 QQQ MS/MS (Agilent Technologies)
  - single run, fast polarity switching, dynamic MRM



SIDA Method

Validation Results – Maize

Analyte	External calibration R_A [%] ± RSD [%] [1)]
Aflatoxin B_1	35 ± 4
Aflatoxin B_2	42 ± 4
Aflatoxin G_1	46 ± 5
Aflatoxin G_2	40 ± 6
Deoxynivalenol	41 ± 10
Fumonisin B_1	330 ± 6
Fumonisin B_2	181 ± 7
HT-2 toxin	134 ± 6
Ochratoxin A	167 ± 14
T-2 toxin	118 ± 5
Zearalenone	91 ± 6

suppression

enhancement

[1)] Apparent recovery ± relative standard deviation

SIDA Method

Validation Results – Maize

Analyte	External calibration R_A [%] ± RSD [%] [1)]	Internal calibration R_A [%] ± RSD [%] [1)]
Aflatoxin B_1	35 ± 4	105 ± 6
Aflatoxin B_2	42 ± 4	100 ± 4
Aflatoxin G_1	46 ± 5	101 ± 5
Aflatoxin G_2	40 ± 6	101 ± 8
Deoxynivalenol	41 ± 10	99 ± 9
Fumonisin B_1	330 ± 6	101 ± 10
Fumonisin B_2	181 ± 7	88 ± 7
HT-2 toxin	134 ± 6	98 ± 7
Ochratoxin A	167 ± 14	93 ± 7
T-2 toxin	118 ± 5	99 ± 6
Zearalenone	91 ± 6	103 ± 11

[1)] Apparent recovery ± relative standard deviation

SIDA Method

Validation Results – Maize

Analyte	External calibration R_A [%] ± RSD [%] [1)]	Internal calibration R_A [%] ± RSD [%] [1)]	R_E [%] [2)]
Aflatoxin B_1	35 ± 4	105 ± 6	108
Aflatoxin B_2	42 ± 4	100 ± 4	107
Aflatoxin G_1	46 ± 5	101 ± 5	109
Aflatoxin G_2	40 ± 6	101 ± 8	111
Deoxynivalenol	41 ± 10	99 ± 9	107
Fumonisin B_1	330 ± 6	101 ± 10	103
Fumonisin B_2	181 ± 7	88 ± 7	97
HT-2 toxin	134 ± 6	98 ± 7	109
Ochratoxin A	167 ± 14	93 ± 7	102
T-2 toxin	118 ± 5	99 ± 6	110
Zearalenone	91 ± 6	103 ± 11	109

1) Apparent recovery ± relative standard deviation

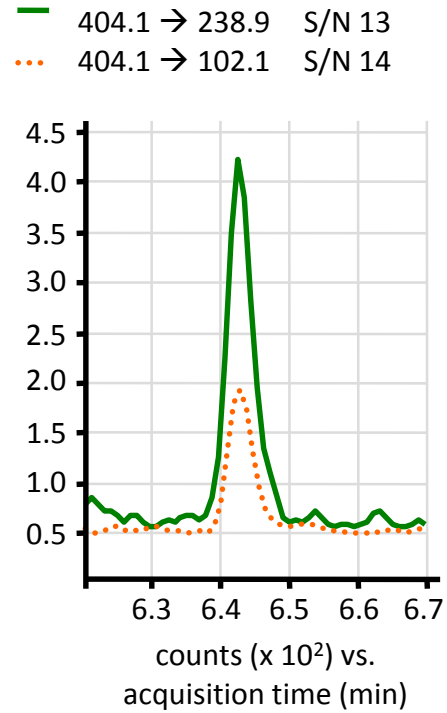
2) Extraction recovery

SIDA Method

Limits of Quantification

Analyte	LOQ (µg/kg) [1]	MLs (µg/kg) [2]
Aflatoxin B_1	0.1	2.0 – 5.0 (**0.1**)
Aflatoxin B_2	0.2	sum aflatoxins 4.0 – 10.0
Aflatoxin G_1	0.1	
Aflatoxin G_2	0.35	
Deoxynivalenol	12	500 – 1750 (200)
Fumonisin B_1	4.5	sum fumonisins 800 – 4000 (200)
Fumonisin B_2	4	
HT-2 toxin	3	indicative values [3] sum 15-1000
T-2 toxin	0.3	
Ochratoxin A	0.4	3.0 – 5.0 (**0.5**)
Zearalenone	3	50 (20)

OTA: 0.45 µg/kg



1) Limit of quantification (S/N = 10) for maize
2) Maximum levels for various commodities according to European Union Commission Regulation 1881/2006 and its amendments (numbers in brackets: baby food)
3) Indicative values in various cereals according to European Union Recommendation 165/2013

Stable Isotope Dilution Assay

Summary

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

Application Note 5991-2808

Anal Bioanal Chem (2012) 402:2675–2686
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 Krska · Franz Berthiller

Open access

LC-MS Multi-Mycotoxin Methods

Reasons

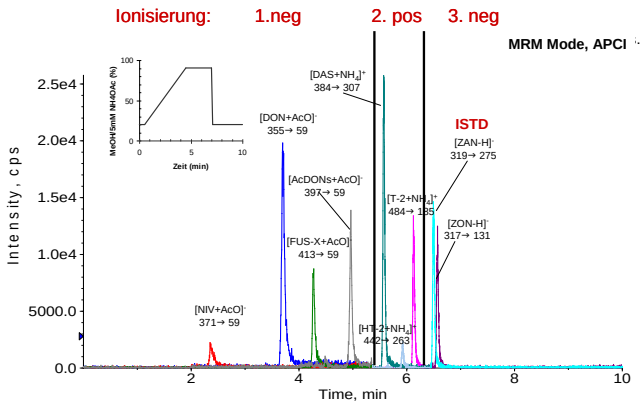
- Unified methods save time and costs

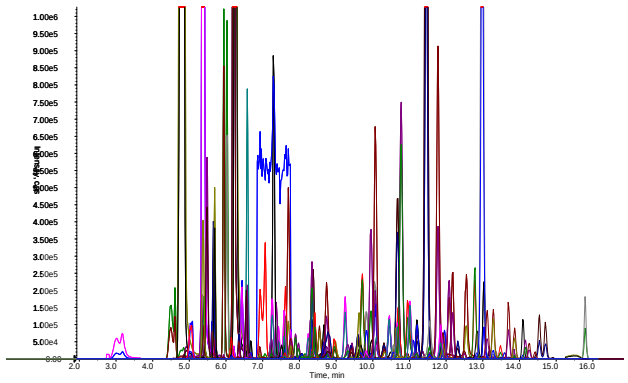
- For **accurate quantification** of regulated mycotoxins
  - high degree of accuracy
  - easy to handle, cost effective

- For **(semi-)quantification of 200+ mycotoxins**
  - analysis of food and feed mixtures of several commodities
  - monitoring of changes in regional fungal spread (climate change)

Multi-Mycotoxin Quantification

The Need to Determine More Toxins in Food





Berthiller et al. (2005) J. Chrom. A
- 9 mycotoxins, APCI, polarity switching
- Mycosep clean-up
- LOD deoxynivalenol: 65 pg on column

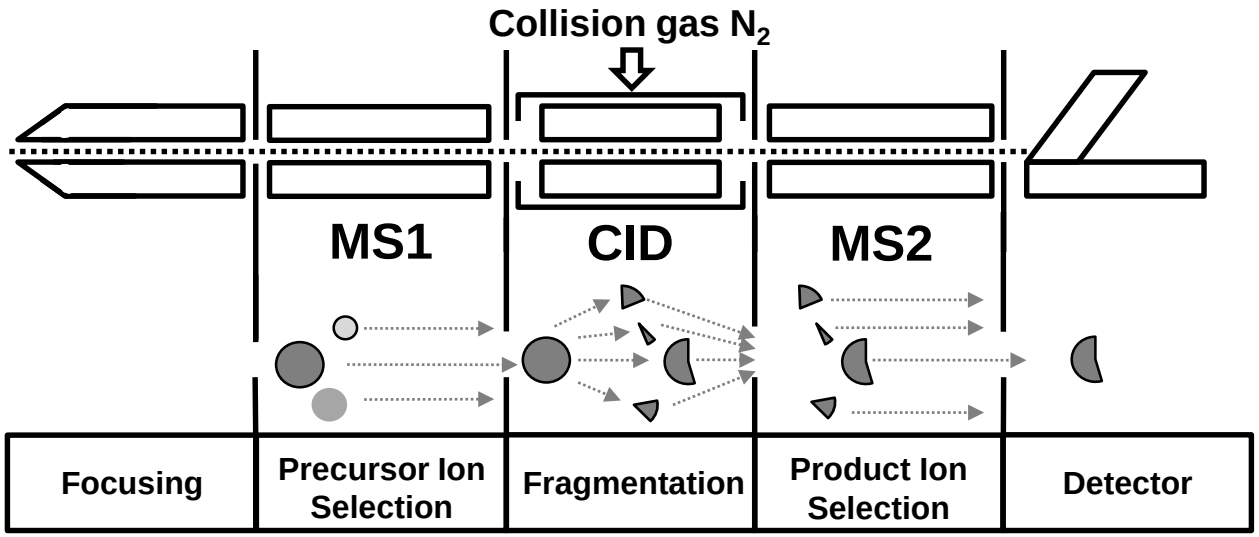
Sulyok et al. (2006) Rapid Commun. Mass Spectrom.
- 39 mycotoxins, ESI, 2 runs (pos. and neg.)
- no clean-up
- LOD deoxynivalenol: 10 pg on column

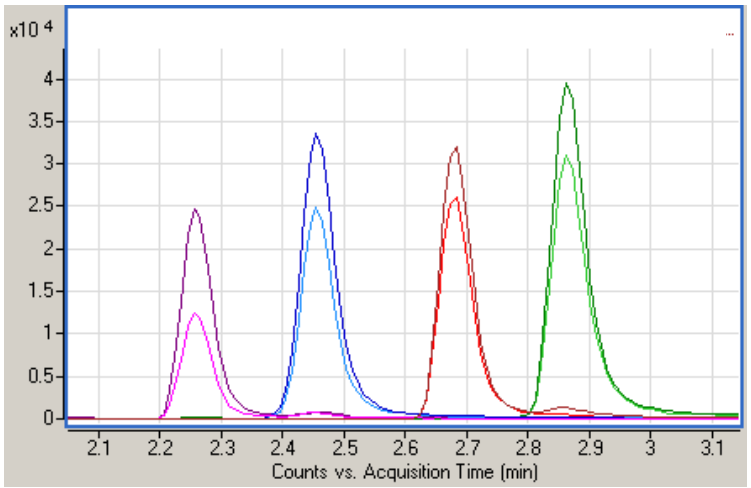
extended to:
- 87 toxins (Sulyok et al., 2007)
- 186 metabolites (Vishwanath et al., 2009)

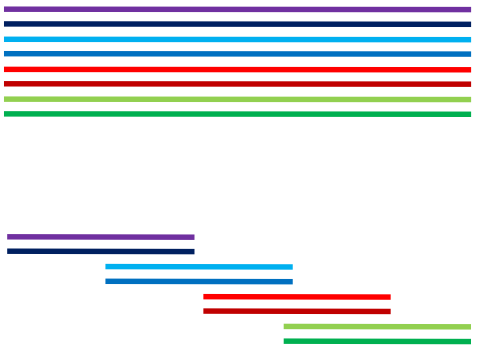
Malachová et al. (2014) J. Chrom. A
- 295 fungal and bacterial metabolites
- LOD deoxynivalenol: 0.3 pg on column

LC-Tandem-Mass Spectrometry

Multiple Reaction Monitoring (MRM)







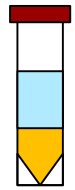
static (classical) MRM

dynamic (scheduled) MRM

Multi-Mycotoxin Quantification

Method Workflow

Sampling	representative sample
Milling	Osterizer Blender (household mill)
Extraction	acetonitrile:water:acetic acid (79:20:1, v:v:v) 90 min at RT on rotary shaker
Filtration	
Clean-up	
Analysis	
Evaluation	



based on: Sulyok *et al.* (2006) *Rapid Commun. Mass Spectrom.* 20(18): 2649-2659

Multi-Mycotoxin Quantification

Method Workflow

Sampling	representative sample
Milling	Osterizer Blender (household mill)
Extraction	acetonitrile:water:acetic acid (79:20:1, v:v:v) 90 min at RT on rotary shaker
~~Filtration~~	
~~Clean-up~~	
Analysis	“dilute and shoot” approach acetonitrile:water:acetic acid (20:79:1, v:v:v)
Evaluation	

based on: Sulyok *et al.* (2006) *Rapid Commun. Mass Spectrom.* 20(18): 2649-2659

Multi-Mycotoxin Quantification

Method Workflow

Sampling

↓

Milling

↓

Extraction

↓

~~Filtration~~

↓

~~Clean-up~~

↓

Analysis

↓

Evaluation

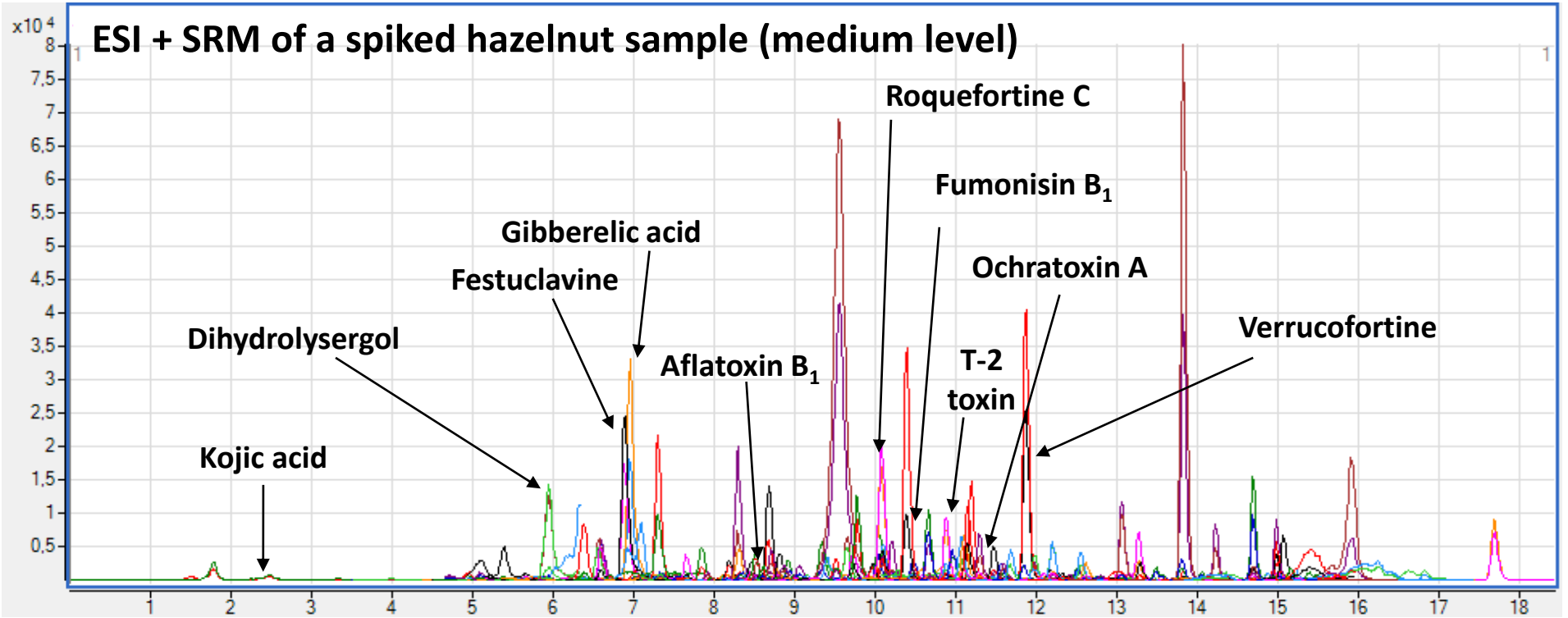
LC-MS/MS (1290 series UHPLC + QQQ 6460)
eluents (acidified methanol/water mixtures)
pos. and neg. ionisation mode

Mass Hunter (manual correction);
linear, 1/x weighted calibration curves

Multi-Mycotoxin Quantification

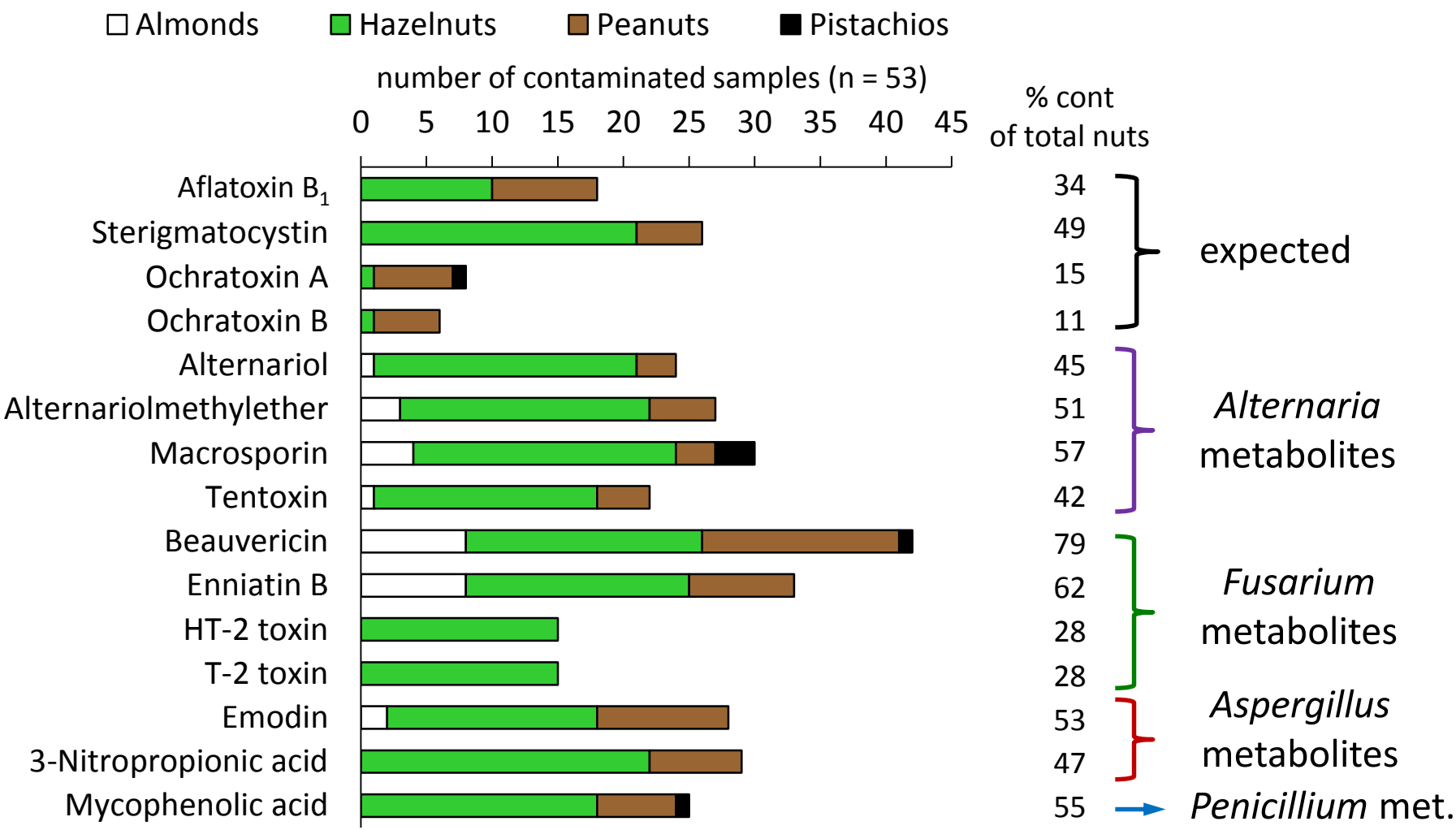
Chromatogram for a total of 191 compounds

- 65 analytes validated
  for almonds, hazelnuts, peanuts and pistachios
- Semi-quantitative method for 126 analytes



Multi-Mycotoxin Quantification

Market Survey - Nuts



Multi-Mycotoxin Quantification

Summary

Anal Bioanal Chem (2013) 405:5087–5104
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 Krska · Rainer Schuhmacher · Michael Sulyok

Screening and Quantitation of 191 Mycotoxins and Other Fungal Metabolites in Almonds, Hazelnuts, Peanuts, and Pistachios Using UHPLC/MS/MS

Application Note 5991-4991

LC-MS Multi-mycotoxin Methods

Reasons

- Unified methods save time and costs

- For **accurate quantification** of regulated mycotoxins
  - high degree of accuracy
  - easy to handle, cost effective

- For **(semi-)quantification of 200+ mycotoxins**
  - analysis of food and feed mixtures of several commodities
  - monitoring of changes in regional fungal spread (climate change)

- For **screening** of a huge variety of mycotoxins
  - identification of mycotoxins in unlikely matrices
  - screening of co-occurring analytes to evaluate synergistic effects

Contaminant Screening

Using HR-MS instruments

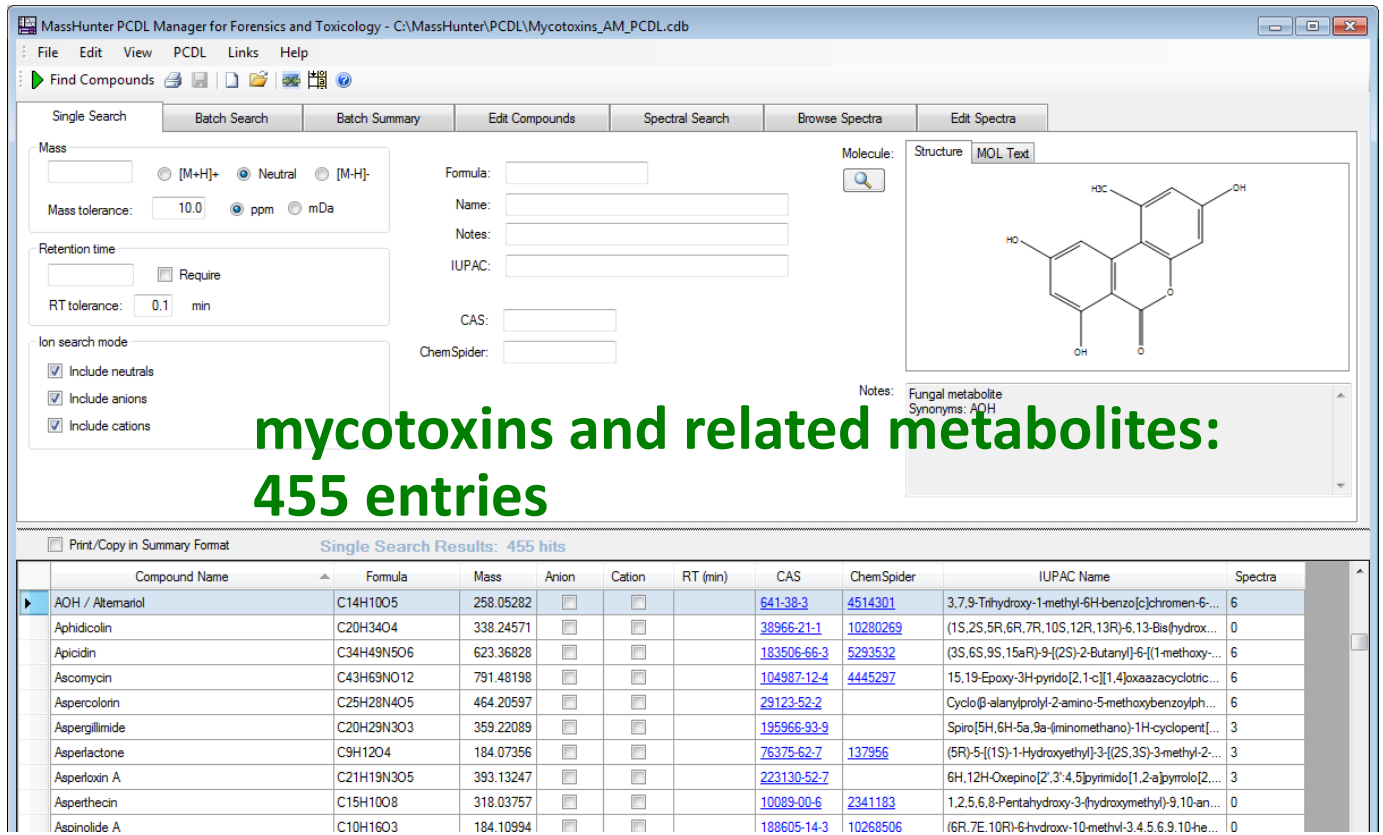
- Advantages
  - \+ in MS mode -> **fast data acquisition**
  - \+ **post-acquisition data analysis** (at least in MS mode)
  - \+ potential screening for **unknown contaminants**
    MS-mode: high mass accuracy -> sum formulas
    MS/MS-mode: sum formulas of fragments
  - \+ **unambiguous identification possible without standards**

- Disadvantages
  - \- higher limit of detections compared to QQQs

6550 iFunnel QTOF

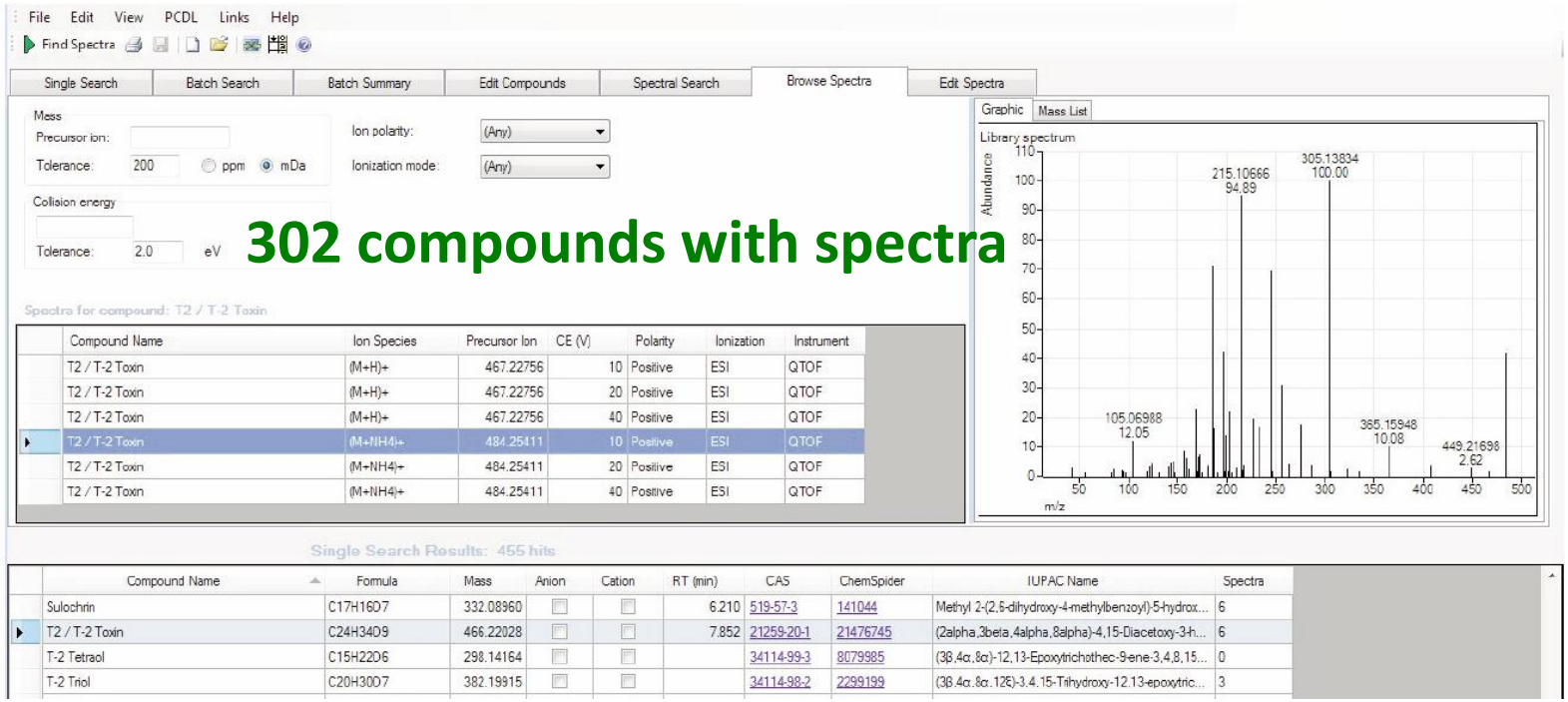
Mycotoxin Screening
Database

- Contains compound information:
  - name
  - formula
  - exact mass
  - retention time
  - external IDs (e.g. CAS)
  - IUPAC name
  - structural information



Screening
HR-MS/MS Library

- Acquiring target HR-MS/MS spectra from standards
  - import using mass correction based on (fragment) structures
  - only explainable fragments are imported
    → exact masses for precursors and fragments

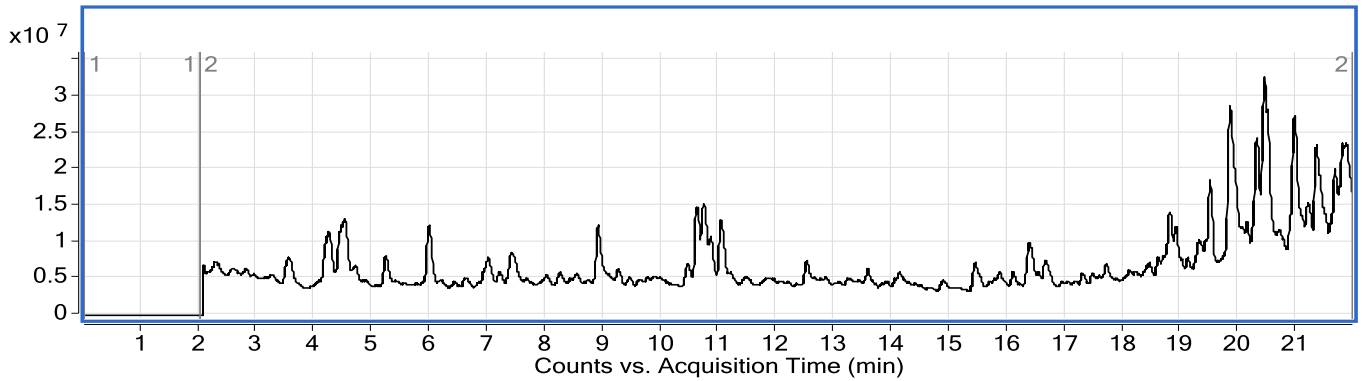


LC-HR-MS/MS Screening

Classical Workflow

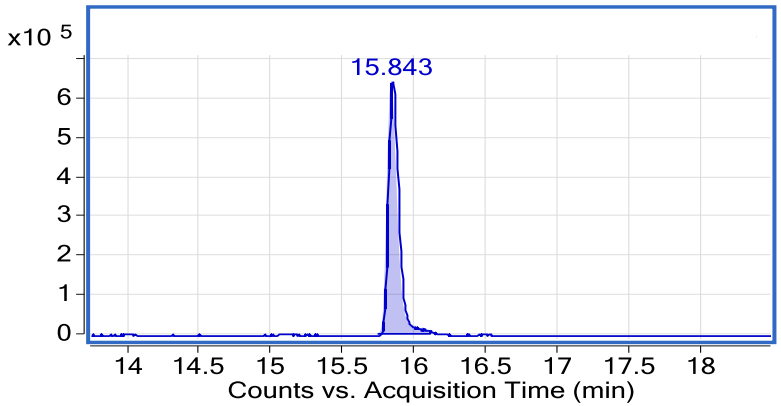
1) HR-MS full scan

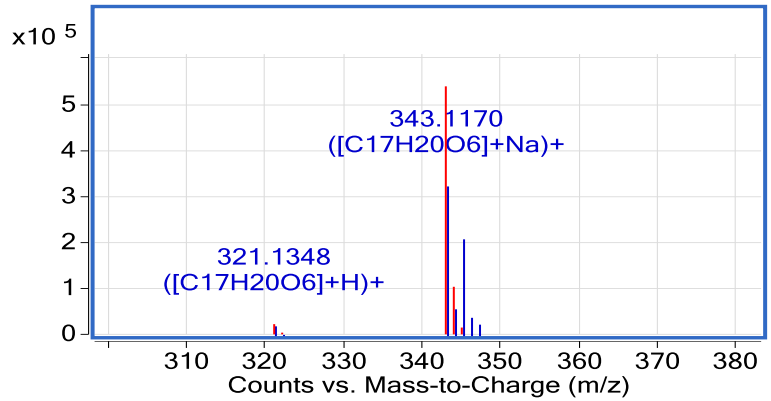
total ion chromatogram
of a naturally contaminated hazelnut sample



2) Find-by-Formula (FBF)

extracted ion chromatogram &
extracted MS spectrum of mycophenolic acid





LC-HR-MS/MS Screening

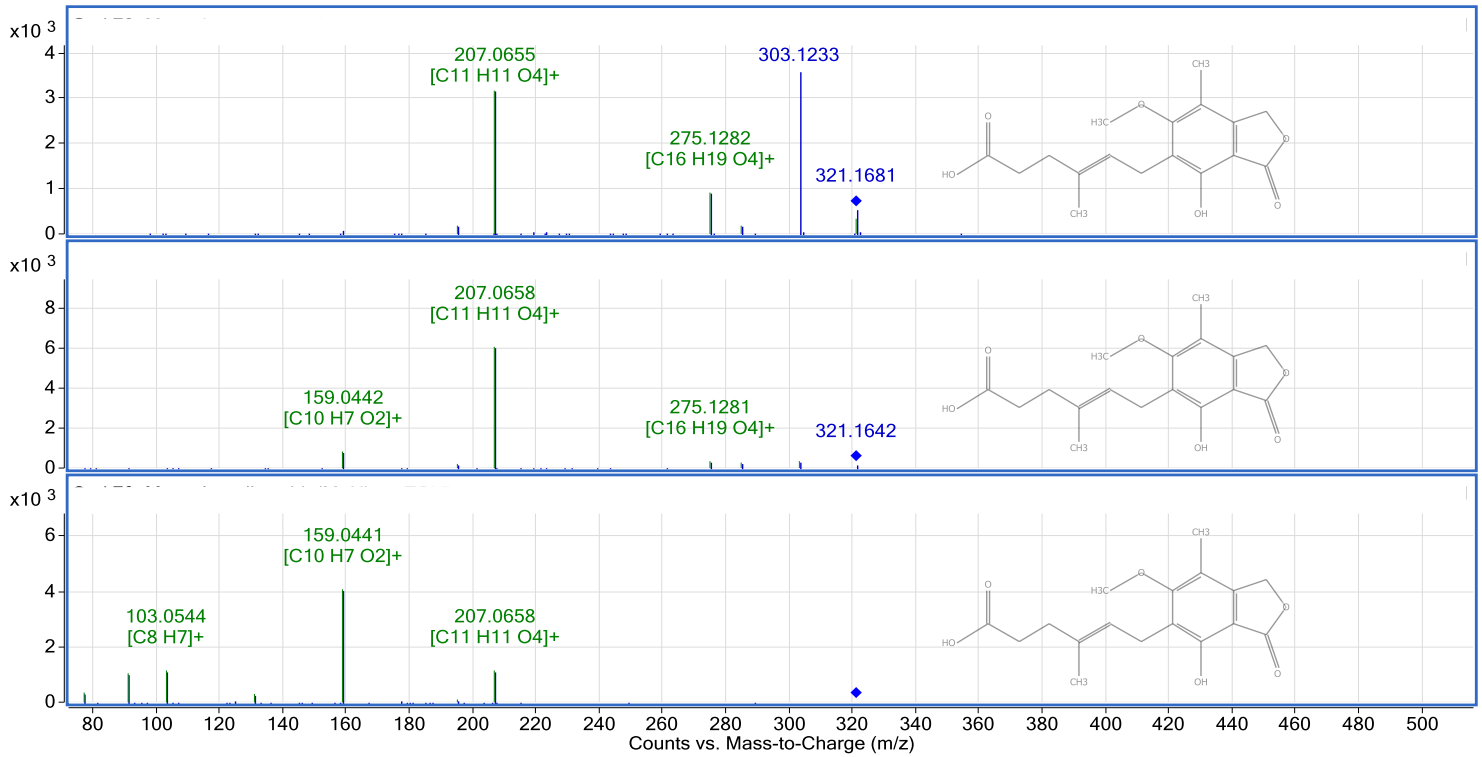
Classical Workflow

3) MS/MS scan

targeted MS/MS scan
specify precursor mass, retention time (RT), RT window and collision energy

4) Library confirmation

extracted MS/MS spectra at different collision energies



LC-HR-MS/MS Screening

Classical Workflow

- Library hit confirming the presence of the immunosuppressive mycotoxin mycophenolic acid in a hazelnut sample

Compound Identification Results: Cpd 70: Mycophenolic acid

Automatically Show Columns

ID Techniques Applied: LibSearch-FBF

Best	Name	Formula	Mass	Mass (Tgt)	Diff (ppm)	Diff (mD	RT	Score (Tg	m/z	Species	Score (Lib)	ID Source
	Mycophenolic acid	C17H20O6	320.1253	320.126	2.26	0.72	15.863	77.27	321.1349 343.11...	(M+H)+ (M+Na)+	95.13	ibSearch-FBF

CE	Name	Num Peaks	m/z (prec.)	Reverse Score	Score (Lib)
10	Mycophenolic acid	8	321.1333	97.41	97.41
20	Mycophenolic acid	6	321.1333	99.65	99.65
40	Mycophenolic acid	16	321.1333	92.29	92.29

Score (iso. abund)	Score (mass)	Score (MS)	Score (iso. spacing)	Abund	Species	m/z
89.64	85.77	77.27	45.42	41720.7	(M+H)+	321.1349
0.02	95.59	59.55	58.92	812708.5	(M+Na)+	343.1174

mycophenolic acid ($C_{17}H_{20}O_6$)
produced by several *Penicillium* spp.
low acute toxicity, immunosuppressive

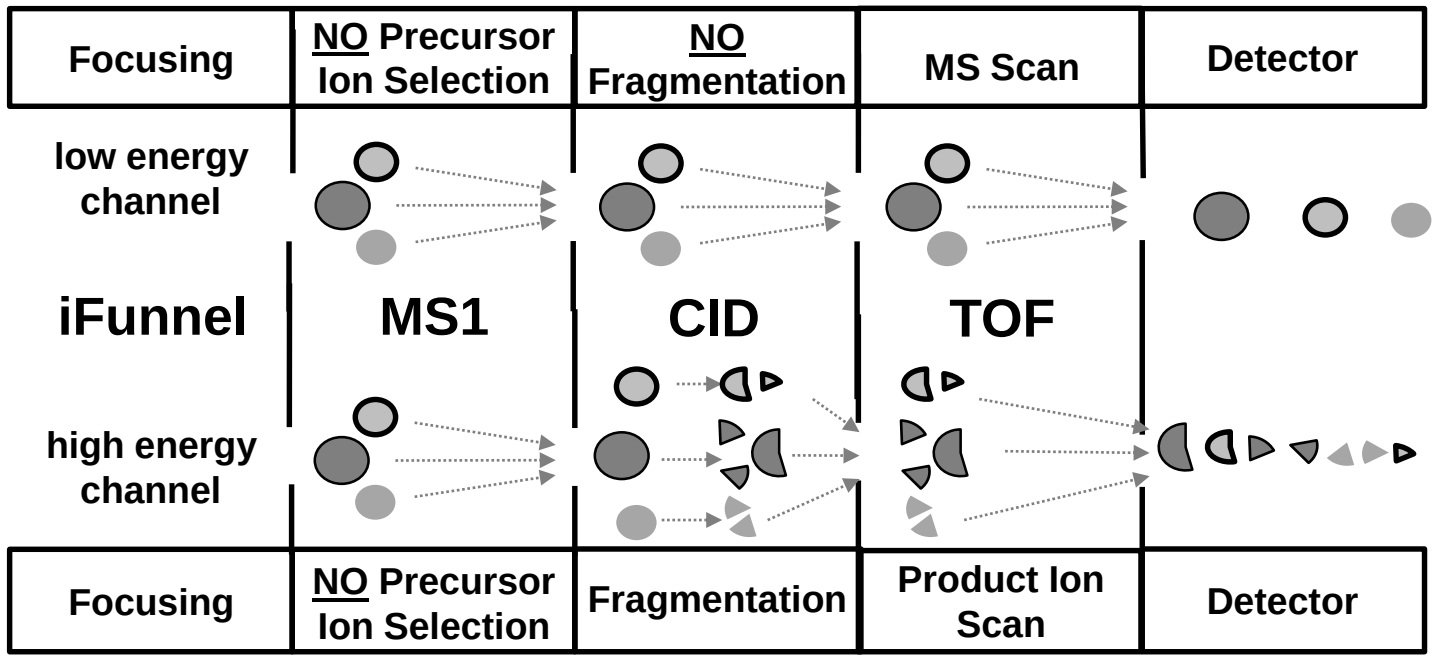
LC-HR-MS/MS Screening

All Ions Approach

1) HR-All Ions MS/MS acquisition

Fragmentation without precursor selection

Measurement with “low energy channel” (no collision energy, MS scan) and at least one “high energy channel” (collision energy e.g. 20 eV)



LC-HR-MS/MS Screening

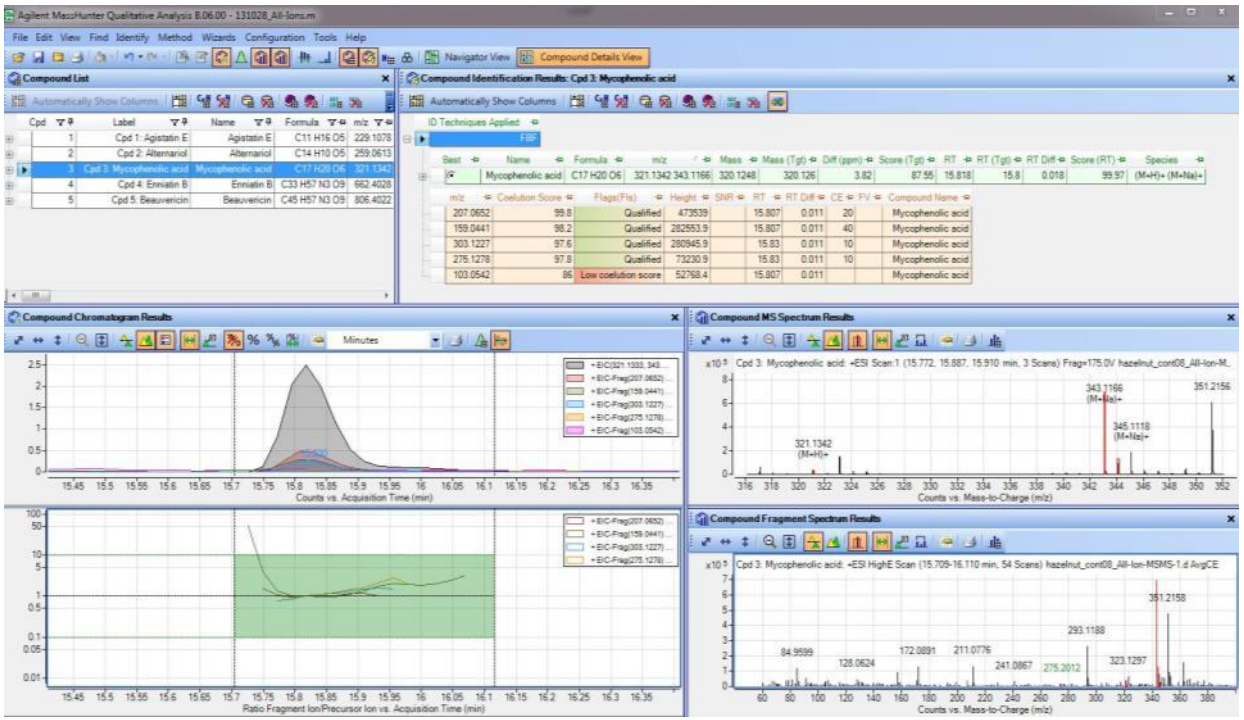
All Ions Approach

2) Database search and library confirmation

I) Database search: applying “Find by Formula” algorithm on low channel

II) Automatic extraction of fragment ion chromatograms in high energy channel

III) Co-elution scoring of fragment and parent EICs for qualification and library confirmation



LC-HR-MS/MS Screening

All Ions Approach

Co-elution score

Major function to qualify compounds based on similarity of peak shapes

Compound Identification Results: Cpd 3: Mycophenolic acid

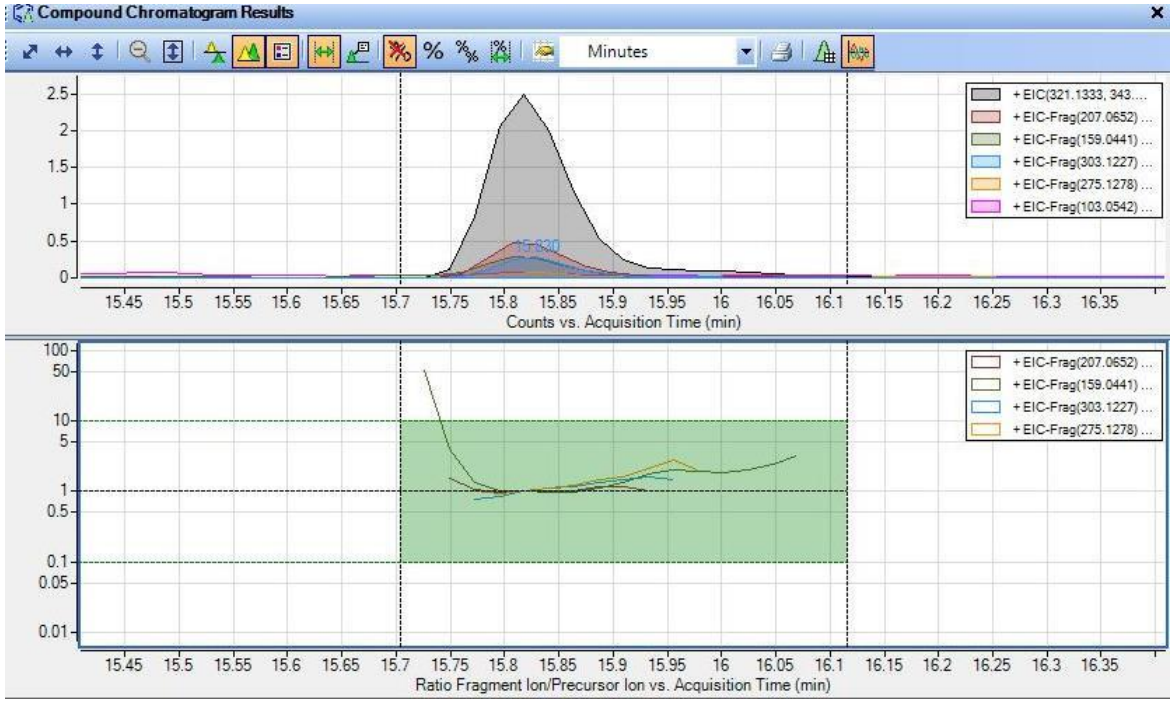
Automatically Show Columns

ID Techniques Applied

FBF

Best	Name	Formula	m/z	Mass	Mass (Tgt)	Diff (ppm)	Score (Tgt)	RT
	Mycophenolic acid	C17 H20 O6	321.1342 343.1166	320.1248	320.126	3.82	87.55	15.818

m/z	Coelution Score	Flags(FIs)	Height	SNR	RT	RT Diff	CE	FV	Compound Name
207.0652	99.8	Qualified	473539		15.807	0.011	20		Mycophenolic acid
159.0441	98.2	Qualified	282553.9		15.807	0.011	40		Mycophenolic acid
303.1227	97.6	Qualified	280945.9		15.83	0.011	10		Mycophenolic acid
275.1278	97.8	Qualified	73230.9		15.83	0.011	10		Mycophenolic acid
103.0542	86	Low coelution score	52768.4		15.807	0.011			Mycophenolic acid



All Ions approach

- worse limits of detection (more noise)
- limited with collision energies

\+ only one injection for each polarity

\+ post-acquisition MS/MS information

LC-HR-MS/MS Screening

Summary

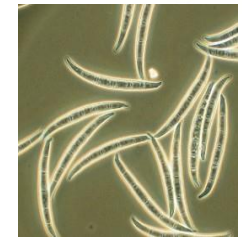
- **General unknown screening** using HR-MS instrumentation
- Identification by **HR-MS/MS library spectra**
- Standards not mandatory for unambiguous identification
- **Retrospective data-analysis** also in MS/MS mode possible

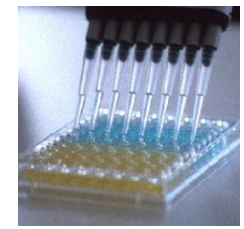
Screening and Verifying Mycotoxins in Food with Q-TOF LC/MS and an Accurate Mass Library

Application Note 5991-5667

Conclusions

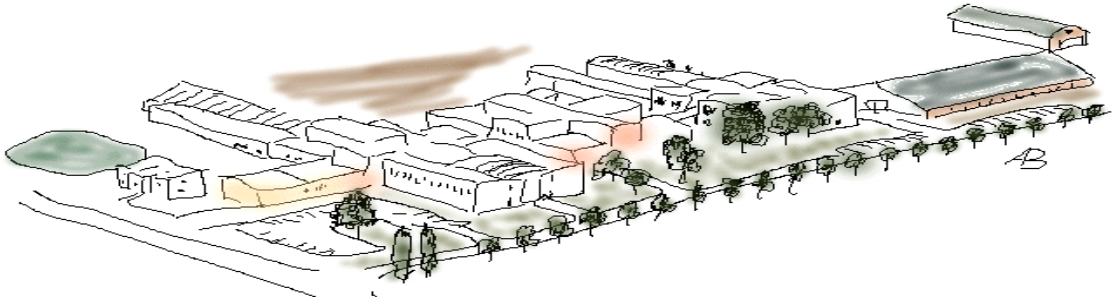
Summary





- **LC-MS/MS** is increasingly used for the **simultaneous quantification and identification** of mycotoxins and other food contaminants
- **Stable Isotope Dilution Analysis** is used for quantification of mycotoxins in food and feed offering **highest possible accuracy** at affordable additional costs for internal standards
- (Semi-)quantitative **LC-MS/MS methods allow** for screening and **quantification of 200+ mycotoxins**
- **LC-HR-MS screening** with exact mass MS/MS libraries allow **unambiguous identification for a practically unlimited number of compounds** even without standards and **in retrospective data analysis**

Thanks for the attention!



Christian Doppler Laboratory for Mycotoxin Metabolism
& Center for Analytical Chemistry
Department of Agrobiotechnology (IFA-Tulln)
University of Natural Resources and Life Sciences, Vienna (BOKU), Austria

franz.berthiller@boku.ac.at