Development and application of an exact mass LC-MS/MS library for the screening of mycotoxins and fungal metabolites in food and feed

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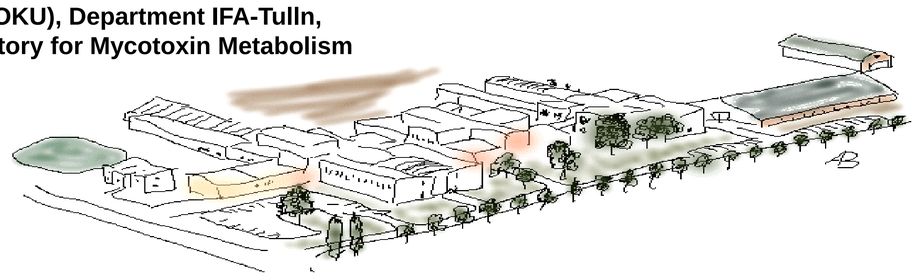

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Overview and Introduction

Purpose:

- creation of a exact mass HR-MS/MS library for mycotoxins and other fungal metabolites
- application for the screening and confirmation of mycotoxins in food and feed commodities

Methods:

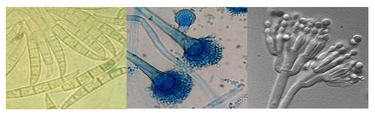
- HR-MS/MS spectral library → flow injection of single analyte solutions
- classical screening approach and All-Ions MS/MS approach

Results:

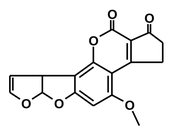
- HR-MS/MS database (> 400) and library (~ 150) for mycotoxins and fungal metabolites
- All-Ions MS/MS yields fragments without precursor selection and is a suitable tool for screening

Mycotoxins are secondary **fungal metabolites** capable of causing **various toxic effects** including hepatotoxicity, mutagenicity, carcinogenicity or estrogenicity [1].

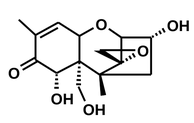
- produced by
  e.g. *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp.



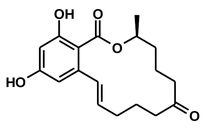
- detected in a **broad range of food and feed** e.g. cereals, beer, milk, spices, coffee, nuts, dried fruits [2]
- **400+ known** – many more unknown
- several are **regulated** in 100+ countries and standards are available for a limited number of substances [3]



aflatoxin B_1



deoxynivalenol



zearalenone

Accurate mass screening for food contaminants is of growing interest due to the complexity of the samples and the increasing number of relevant analytes (masked and emerging mycotoxins).

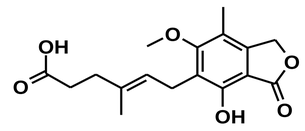
Creation of Database & MS/MS-Library

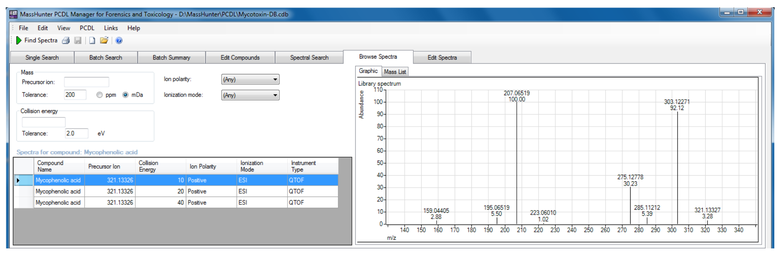
- acquired **HR-MS/MS library spectra** for about 150 mycotoxins and other fungal metabolites
→injection of individual standards

collision energies:	typically 10, 20 and 40 eV
acquisition rate:	MS: 5 spectra/s; MS/MS: 5 spectra/s
isolation width:	narrow (1.3 amu)
ion species:	pos. mode: $[M+H]^+$, $[M+Na]^+$; neg. mode: $[M-H]^-$, $[M+HCOO]^-$

→ import **spectra and structure formulae** into user defined database and library
→ **curation of fragment masses** based on fragment formulas and structures **using *in silico* fragmentation** (MassHunter Molecular Structure Correlator)
→ only **explainable fragments** are included in the database with their **exact *m/z* values**

Figure 1: Library spectrum and structure of mycophenolic acid

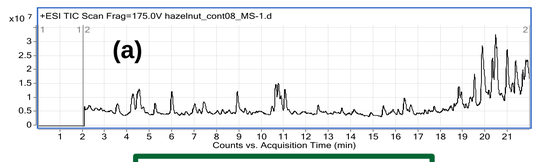




Classic Screening Approach

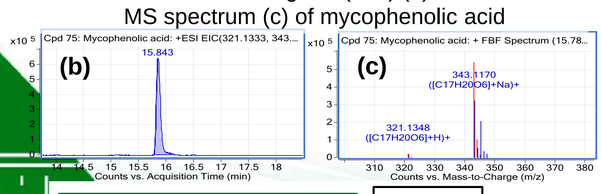
1) HR-MS full scan

total ion chromatogram (TIC) of a naturally contaminated hazelnut sample (a)



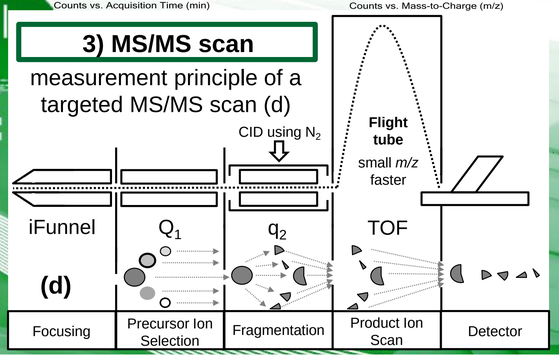
2) Database search

extracted ion chromatogram (EIC) (b) and extracted MS spectrum (c) of mycophenolic acid



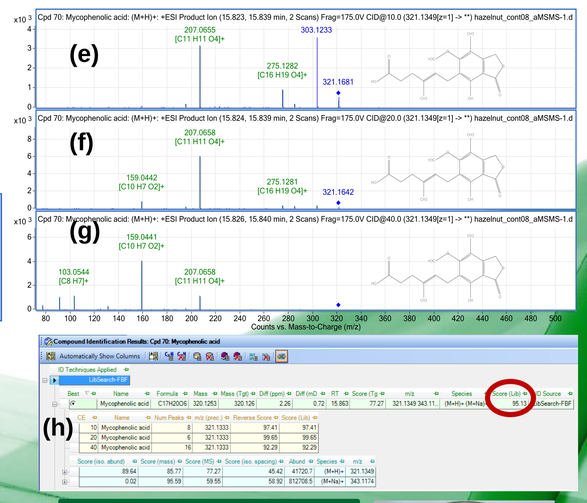
3) MS/MS scan

measurement principle of a targeted MS/MS scan (d)



4) Library confirmation

extracted MS/MS spectra at collision energies of 10 eV (e), 20 eV (f) and 40 eV (g) and library hit confirming the presence of the immunosuppressive mycotoxin mycophenolic acid (h)



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

Scheme 2: Example of an identification pathway using a classic screening approach

Sample Preparation and LC-MS-Method

Due to the huge **differences in chemical and physicochemical properties** of mycotoxins, a **general sample preparation procedure** is required. Extraction was performed according to [4] and no clean-up was performed to avoid discrimination of certain mycotoxins.

Method Details:

LC-MS system: 1290 Infinity UHPLC
6550 iFunnel QTOF (Agilent Technologies), correction of mass axis with reference masses during the whole run
column: Zorbax SB-C18 RRHD
(150 x 2.1 mm; 1.8 µm particle size)
eluents: water and methanol containing 0.1% formic acid
gradient: from 10% to 100% B in 19.5 min

Sampling	representative sample
Milling	grind, homogenise and weigh-in
Extraction	ACN:H_2O:HAc (79:20:1, v:v:v) 90 min @ RT on a rotary shaker
Clean-up	"(dilute and) shoot" approach
Analysis	LC-MS (pos./neg. ionisation mode)
Evaluation	qual. and quant. analysis

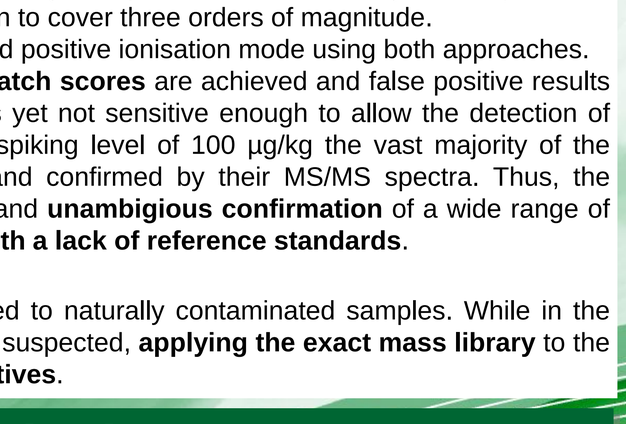
Scheme 1: Applied analytical scheme

All-Ions MS/MS Approach

Alternatively the "**All-Ions MS/MS**" acquisition was applied for elimination of potential false positives, which used **fragmentation without precursor selection**. The presence of mycotoxins was confirmed by the **co-elution of characteristic fragment ions**

1) HR-MS/MS All-Ions scan

Measurement with "low energy channel" (no collision energy) and at least one "high energy channel" (collision energy e.g. 20 eV) (a)



2) Database search and library confirmation

I) Database search: applying "Find by Formula" algorithm on low channel
II) Extraction of ion chromatograms (EICs) in high channel mode
III) Alignment EICs of fragments with parent EICs and correlate qualified by library confirmation

The coelution score is the major function to qualify compounds based on similarity of peak shapes.

(b) summary list; (c) fragment list; (d) overlaid EIC chromatograms; (e) co-elution plot;
(f) MS-scan results, (g) All-Ions MS/MS results

Scheme 3: Example of an identification pathway using the All-Ions approach

Validation and Application

Validation:

- **45 analytes were selected** to cover a **wide range of different properties**: ionisation mode (neg./pos.), molecular mass (100 to 1300 amu), polarity, regulated mycotoxins and representatives of the most important groups.
- Neat standard solution and three matrices (**maize, hazelnut and wine**) were spiked on several concentration levels after extraction to cover three orders of magnitude.
- All samples were measured in negative and positive ionisation mode using both approaches.

Preliminary results show, that **high library match scores** are achieved and false positive results are completely avoided. While the method is yet not sensitive enough to allow the detection of e.g. aflatoxins at the regulated levels, at a spiking level of 100 µg/kg the vast majority of the analytes were identified in the MS scans and confirmed by their MS/MS spectra. Thus, the method offers a possibility for the screening and **unambigious confirmation** of a wide range of different mycotoxins in food and feed **even with a lack of reference standards**.

Application:

Additionally, the two approaches were applied to naturally contaminated samples. While in the initial TOF screen several contaminants were suspected, **applying the exact mass library** to the MS/MS data efficiently **eliminated false positives**.

Conclusion

Accurate mass screening using a UHPLC-QTOF in combination with an **exact mass library** is a **suitable technique for routine screening and confirmation** of a wide range of mycotoxins in food and feed. The application of the All-Ions approach reduces the measurement time (only one injection instead of two) and allows also **post-acquisition evaluation including MS/MS** information. The database currently comprises **more than 400 mycotoxins** and fungal metabolites. One third of the toxins are already included with their MS/MS spectra.

References

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Pictures: *Fusarium sp.*: Marc Lemmens (BOKU); *Aspergillus sp.*: http://www.eapcri.eu/; *Penicillium sp.*: http://website.nbm-mnb.ca/mycologywebpages/Moulds/Penicillium.html; Maize: Marc Lemmens (BOKU); Cheese: http://www.yumsugar.com/Burning-Question-When-OK-Eat-Moldy-Food-4391316; Pistachio: http://www.buhlergroup.com/northamerica/en/process-technologies/optical-sorting/nut-sorting/pistachio-sorting.htm; Chromatograms and spectra: Mass Hunter qualitative and quantitative analysis (Agilent Technologies)