Mycotoxins in Danish cereals

- do we have a problem ?

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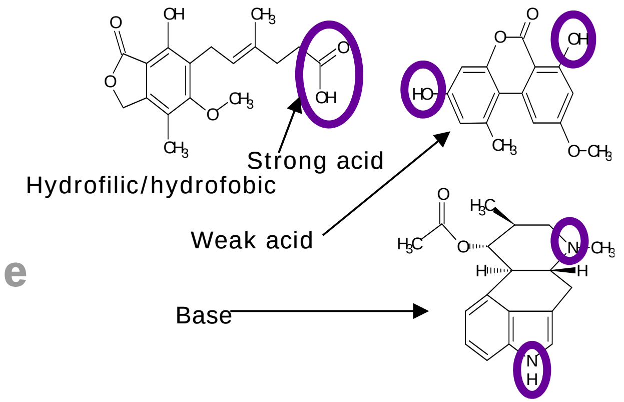
Content

- Mycotoxin problems – what do we know – and what do we need to know
- Results from the cocktail project, new fungal metabolites
- Future challenges – what can we expect in a climate changing world with more warm and rain

Development of generic methods – *sample extraction – often the critical step*

Multimethod based on the ''dilute and shoot'' pricinciple cannot fulfill the sensitivity requirements at the current time



Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) method

Aqueous organic solvents	**Extraction**
Specific clean-up on SPE-columns	**Purification**
Quantification by UHPLC-MS/MS	**Determination**

Sample preparation
Homogenisation with liquid nitrogen

1st extraction
Water | 1% Acetic acid in ACN | Na-Acetate

2nd extraction
Anhydrous $MgSO_4$

Centrifugation

Preparation for LC-MS/MS
Decant ACN phase | Filter in vials

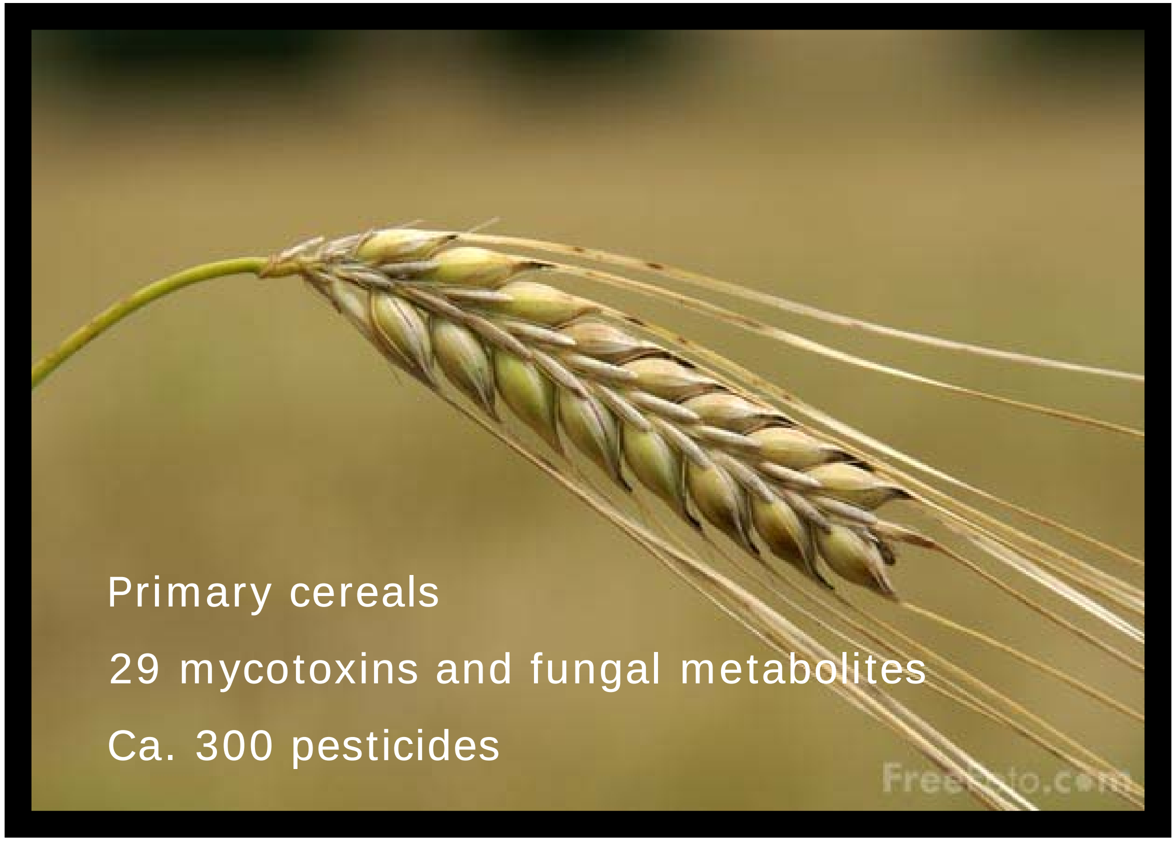
1) Anastassiades et al. (2003) *J. AOAC Int.* 86, 412-431
2) Rasmussen et al. Anal Bioanal Chem (2010)

Development of generic methods based on high resolution mass spectrometry

Multi methods

Screening methods

Characterization of new fungal metabolites



Validation levels: 10, 20 and 100 µg/kg

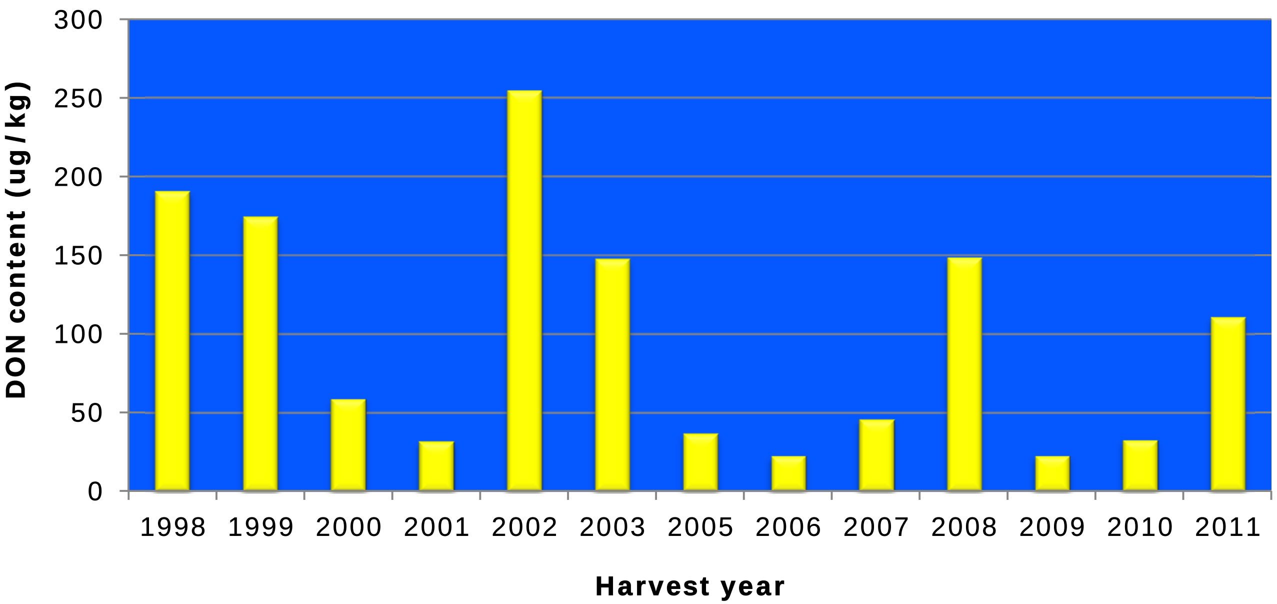
Other contaminats: plant toxins, veterinary drugs etc.

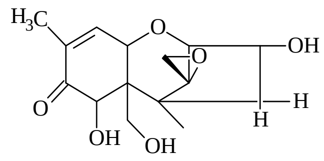
Mycotoxins (and other secondary fungal metabolites) are produced on grains in the field or during storage

EU regulated mycotoxins and the forchcoming ones

Mycotoxin	Tolerable daily intake (TDI)	Effects
Aflatoxins	”Low as possible”,	Strong carinogen, acute toxic to the liver
Ochratoxin A	EFSA: weekly intake of 120 ng/bw	Acute toxic to the kidney and liver and cancer
Patulin	0,4 µg/kg bw/dag	Reduced weight gain, carnigonen effects
Deoxynivalenol (DON)	1 µg/kg bw/dag	Inhibit protein synthesis, feed refusal, weigt loss, skin lesion , immuno supressive
Nivalenol	0-0,7 µg/kg bw/dag	As for DON but more toxic
HT-2 & T-2 toksin	60 ng/kg bw/dag	As for DON but more toxic carinogen (?)
Zearalenone	0,2 µg/kg bw/dag	Estrogen effects forplantningsforstyrrelser
Fumonisin B1 & B2	2 µg/kg bw/dag	Attach brain and lung, cancer ringogen
Ergot alkaloids	1-2 mg alkaloid/kg bw/dag	Acute toxic on nerves

Danish monitoring results of deoxynivalenol (DON). Annual variation in average concentration in flour wheat samples collected in Denmark.



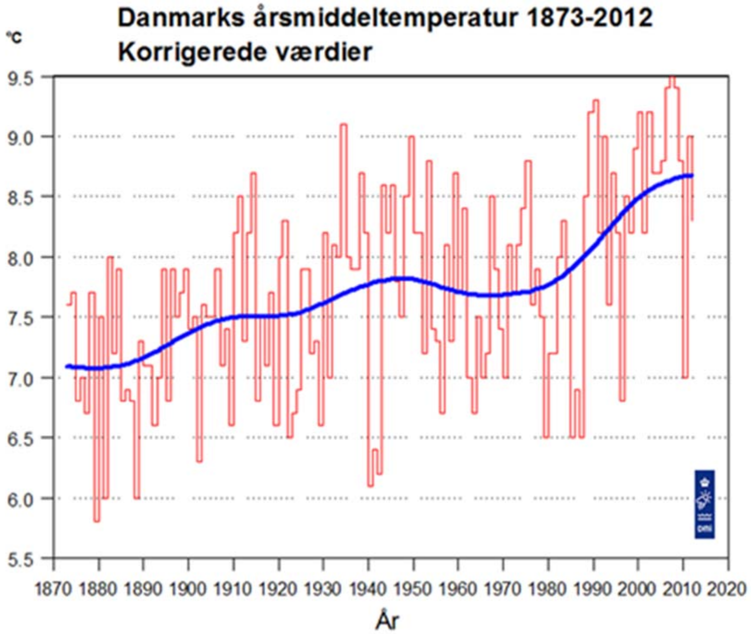


DON

Rasmussen et al., *Food Additives and Contaminants* 24(3): 315-325, 2007. H.L Madsen and P.H Rasmussen (Chemical contaminats, Report: Danish Veterinary and Food Administration (2013).

Types of effects related to climate changes

- Long time effects
  - Increased temperatures
  - Increased rainfall
  - Increased humidity



- Dramatic effects: extreme rainfalls, flooding, record warm temperatures

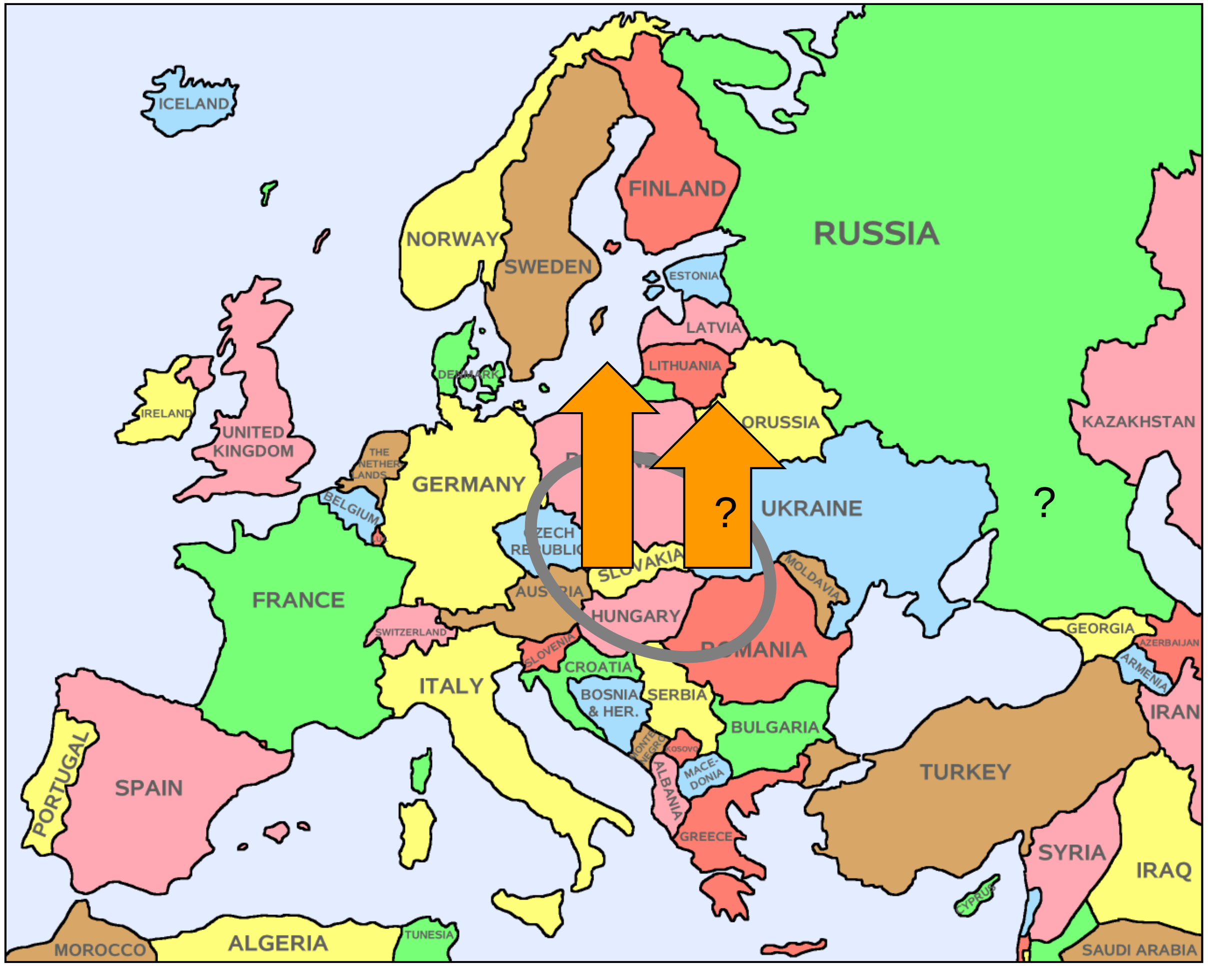
Occurrence and content of aflatoxin B1 in maize from the harvest 2013 (Hungary)

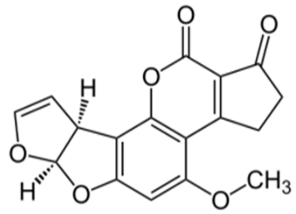
	Aflatoxin B1	Zearalenone	DON	FUM B1	OTA	T-2 toxin
Number of samples tested	**87**	74	85	49	49	56
Positive (%)	**52.9**	23.0	52.9	71.4	61.2	14.3
Maximum (µg/kg)	**39.3**	12.4	711.9	3100.0	237.6	52.6
Average (µg/kg)	**3.6**	1.2	88.8	300.6	10.6	5.5

Reference: Biomin/Romer Lab

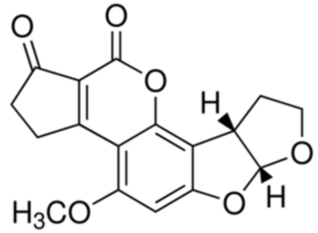
EU maximum limit for aflatoxin B1: 5 µg/kg

Aflatoxins in Maize Pose New Risk to Europe

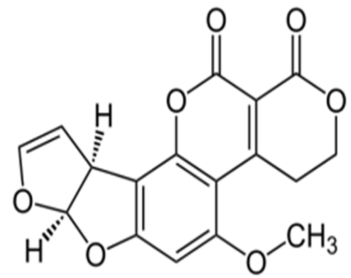




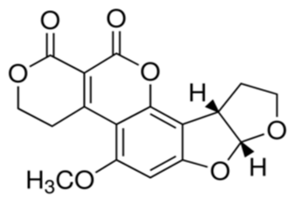
Aflatoxin B1



Aflatoxin B2



Aflatoxin G1

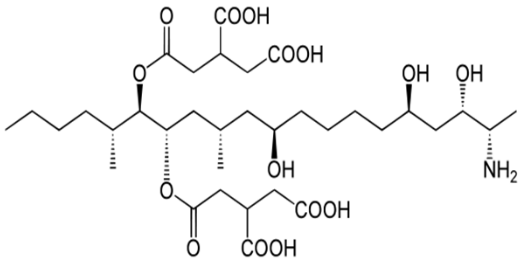


Aflatoxin G2

National Food Institute, Technical University of Denmark

What can we see out there in Denmark (and Scandinavia)

- More *F. graminearum* than *F. culmorum* – problem - more heavy producer of mycotoxins. But is it due to the climate or different cropping systems.
- More fumonisin producing *Fusarium* in maize in Denmark during recent years.
- *F. langsethiae* increased in the Scandinavian countries (more HT-2/T-2 toxin – problems in especially oat from Norway)
- Increased occurrence of *F. aveneacium* compared to earlier periods - heavy producer of enniatins and other secondary metabolites



Fumonisin B1

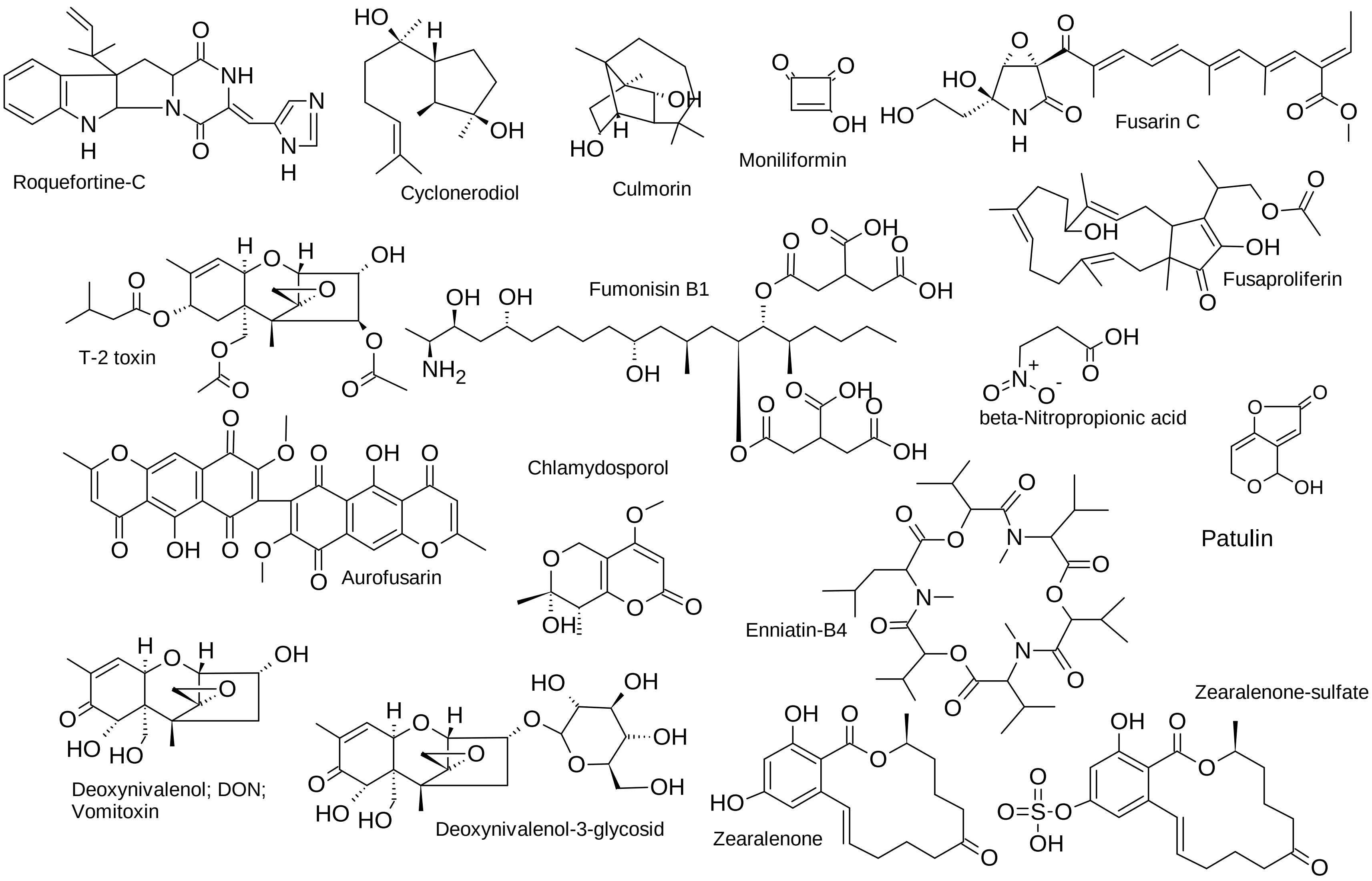
Risk assessment of mycotoxins in cereal grain in Norway (2013)

(Opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety)

''During the last ten years, *Fusarium* infections of cereal seed have increased by more than 100 % in oats, barley and spring wheat, compared with the three previous decades. Precipitation in the flowering period and during late summer before harvest promotes the occurrence of mycotoxins in cereals. In the last five growing seasons there has been more precipitation than normal in the flowering period for cereals in Norway. If such weather conditions are representative of the future climate, then we can expect significantly increased problems with mycotoxins in cereals in the years to come''.

VKM concludes that exceeding the TDI at mean or high exposures to DON in infants and children is of concern

Examples of mycotoxins and other secondary metabolites produced by fungi



Enniatins

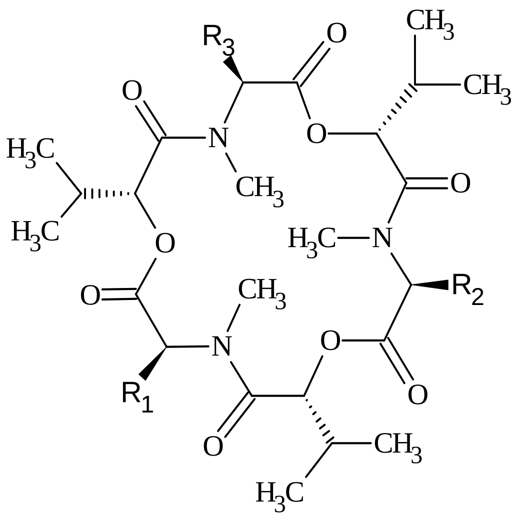
F.avenaceum

Enniatin A: R1=R2=R3= - CH(CH3)CH2CH3

Enniatin A1: R1=R2= -CH(CH3)CH2CH3, R3= -CH(CH3)2

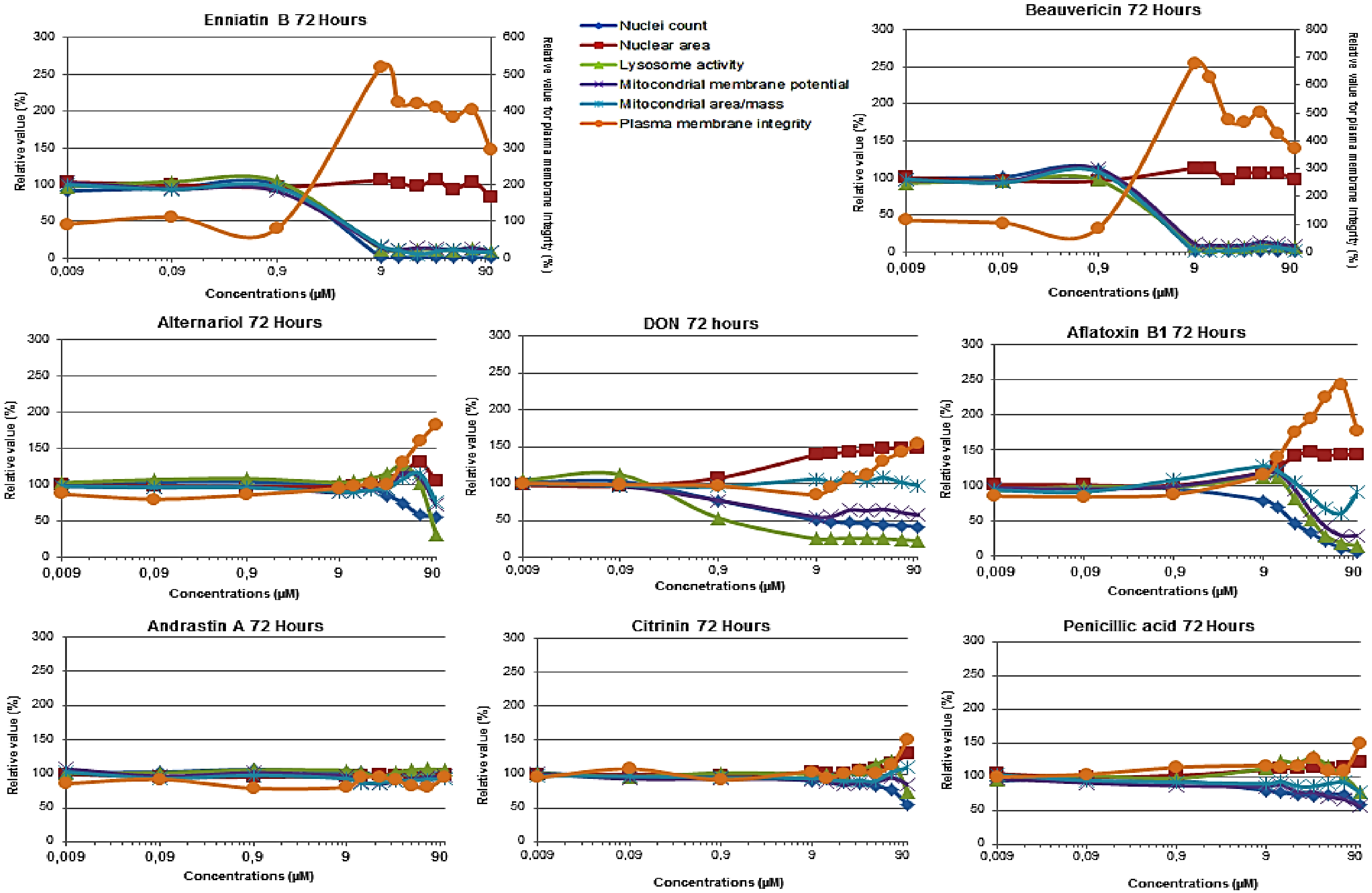
Enniatin B: R1=R2=R3= -CH(CH3)2

Enniatin B1: R1=R2= -CH(CH3)2, R3= -CH(CH3)CH2CH3



Cereal	Enniatin A (µg/kg)	Enniatin A1 (µg/kg)	Enniatin B (µg/kg)	Ennaitin B1 (µg/kg)
Wheat (n = 16)				
Positive	50 %	38 %	**88 %**	56 %
Mean (µg/kg)	46	27	**386**	88
Median (µg/kg)	10	n.d.	**316**	26
Range (µg/kg)	n.d. - 400	n.d. – 188	**3 - 1092**	3 – 300
Barley (n = 15)				
Positive (%)	80 %	100 %	**100 %**	100 %
Mean (µg/kg)	88	150	**755**	276
Median (µg/kg)	140	180	**877**	287
Range (µg/kg)	8 - 142	72 -202	**206 - 932**	156 – 340
Oat (n = 4)				
Positive	100 %	75 %	**100 %**	75 %
Mean (µg/kg)	116	164	**382**	218
Median (µg/kg)	142	205	**446**	273
Range (µg/kg)	36 - 144	n.d. – 226	**100 - 536**	n.d. – 324
Rye (n=2)				
Positive	50 %	50 %	**100 %**	50 %
Mean (µg/kg)	70	98	**747**	195
Median (µg/kg)	70	98	**747**	195
Range (µg/kg)	n.d. - 140	n.d. – 196	**68 - 1426**	n.d. – 390
Triticale (n = 12)				
Positive	92 %	92 %	**100 %**	100 %
Mean (µg/kg)	59	108	**1080**	327
Median (µg/kg)	16	127	**1220**	304
Range (µg/kg)	n.d. - 156	n.d. - 320	**298 - 2569**	158 – 746

8 mycotoxins tested *in vitro* assays based on human HepG2 and high content imaging (HCI)



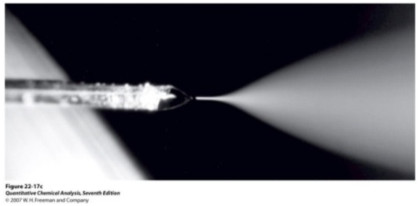
You are only finding what you are searcing for……….

but what about all the other things ?

Looking for new fungal metabolites in cereal grain

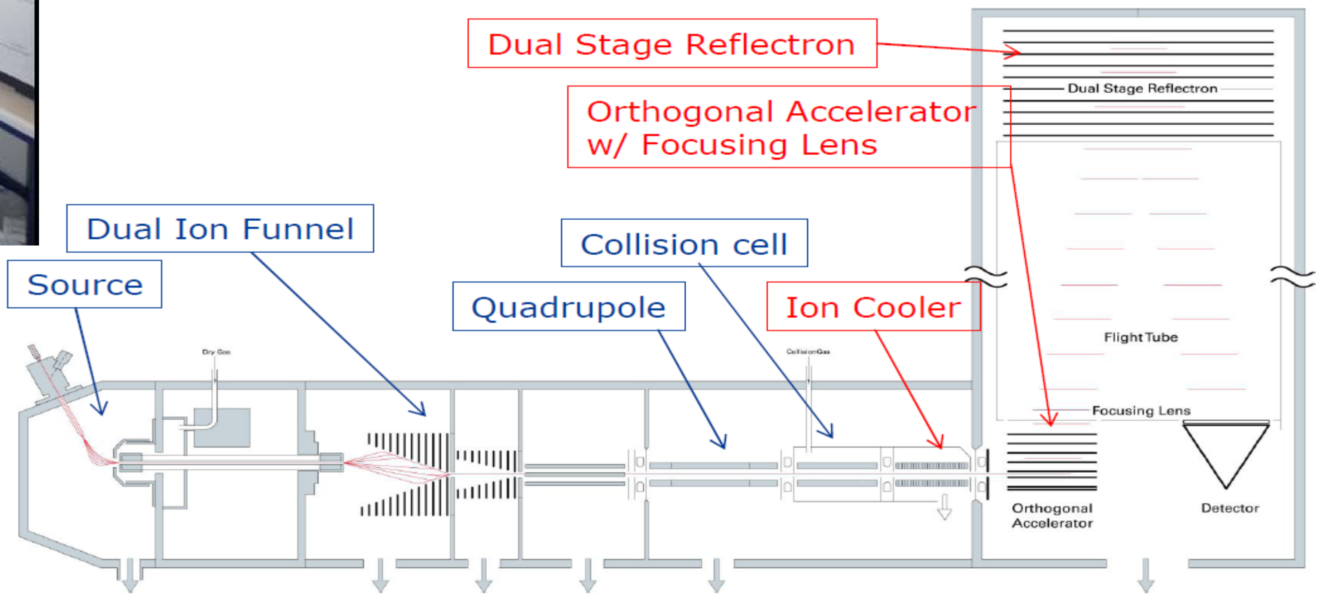
maXis QTOF

- Most probably the best instrument on the market with the highest mass resolution -

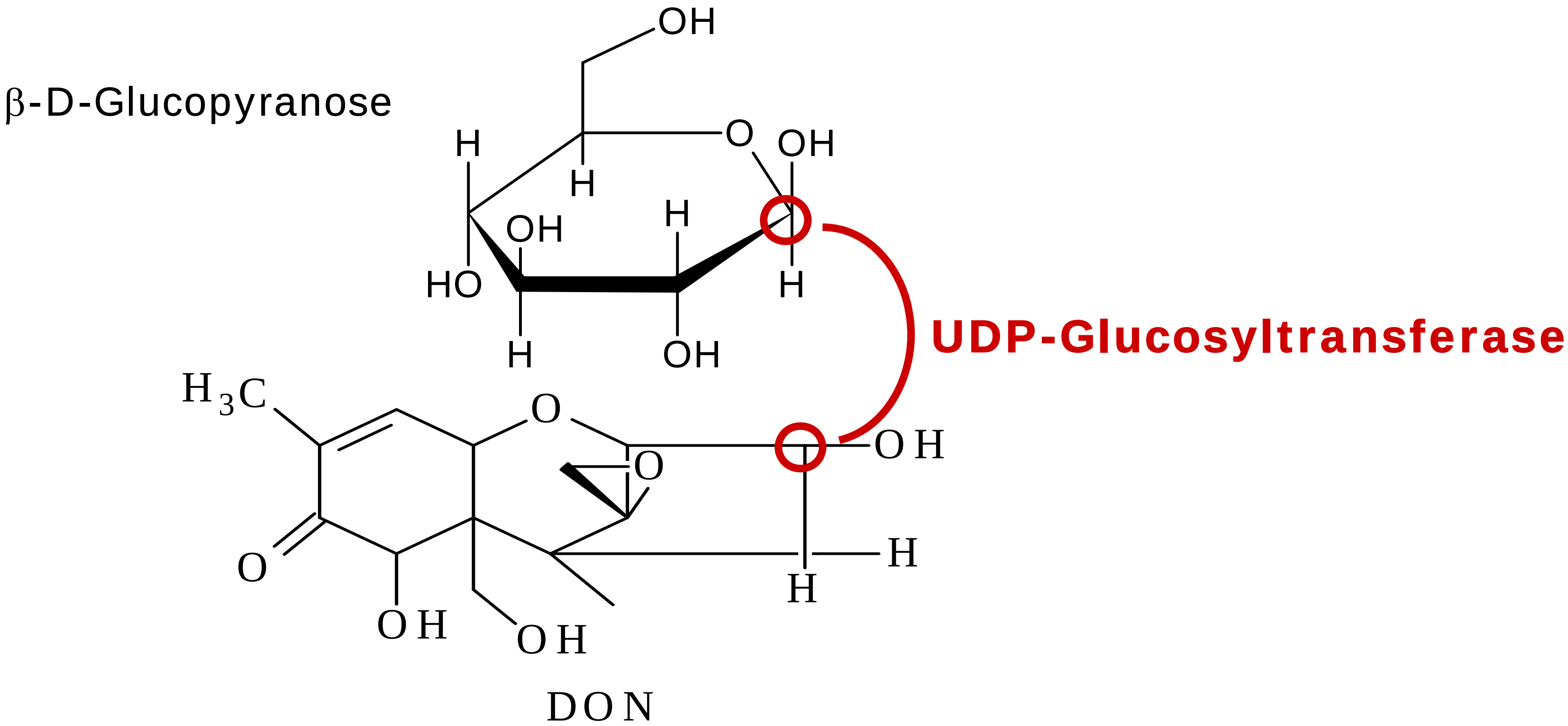


Electrospray

(ionization, positive mode)



Transformation of deoxynivalenol into 3-ß-D-glucoside by plants (masked mycotoxin)



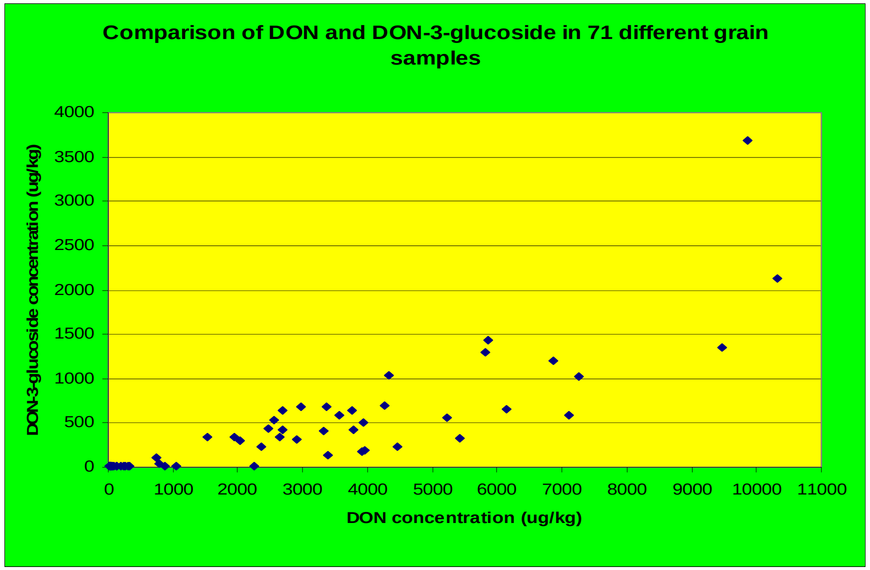
Poppenberger et al. J Biol Chem (2003)

Background

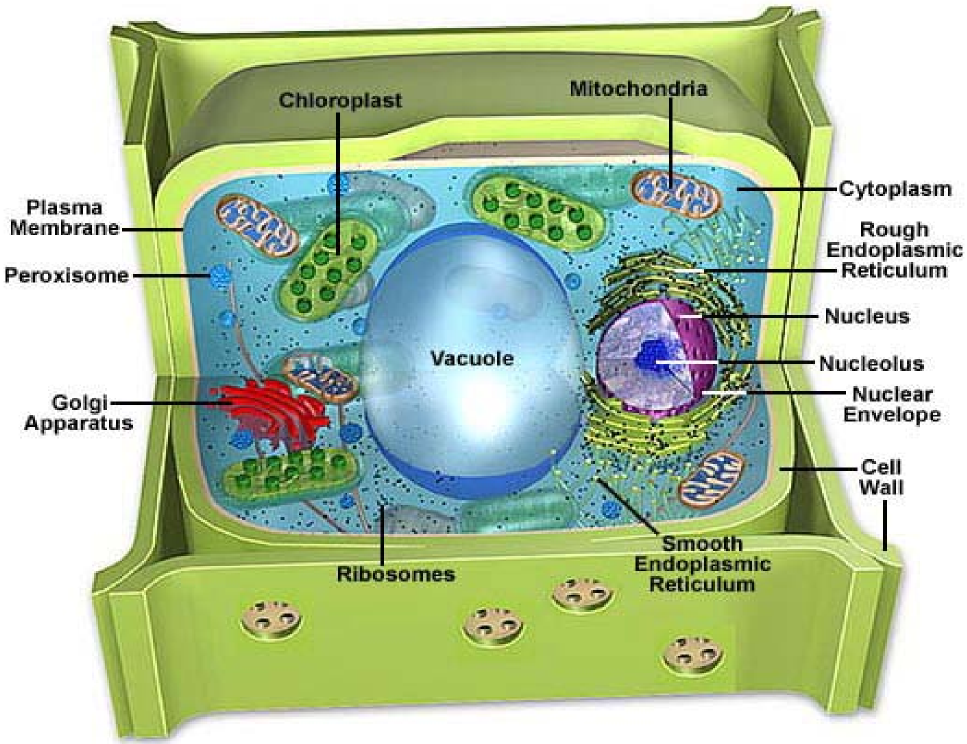
DON-3-glucoside (masked DON) has been found in naturally and artificially contaminated Danish cereal grains of barley, wheat, oat, rye and triticale (2006 – 2010).

Results

DON-glucoside and DON co-occured, especially in highly contaminated samples, the concentration og the glucoside can be relatively high, corresponding to ca. 37% of the DON concentration. DON-3-glucoside is positively correlated to the DON content.



Rasmussen et al, Mycotoxin Research (2012)



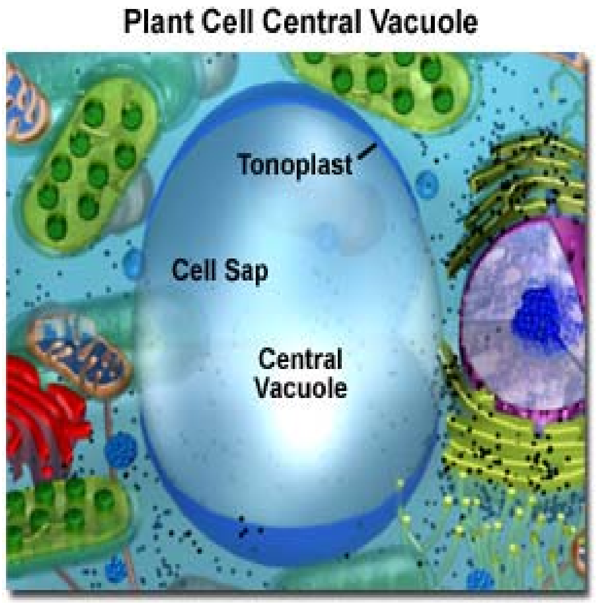


Figure 1

Modified after Novikoff A.B., Holtzman E., and others. Cells and Organelles

(1976, 1982, www.
(http://www.moleculareexpressions.com)

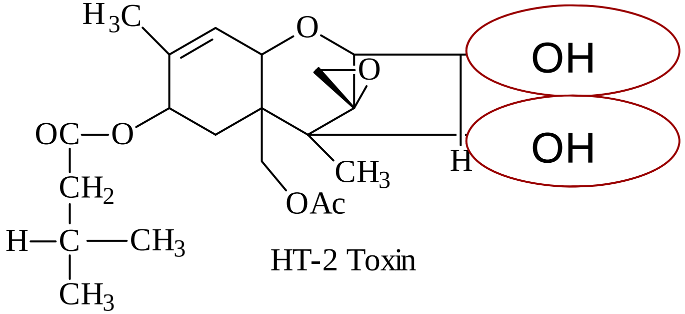
Masked mycotoxins

by UDP- glucosyltransferease activity

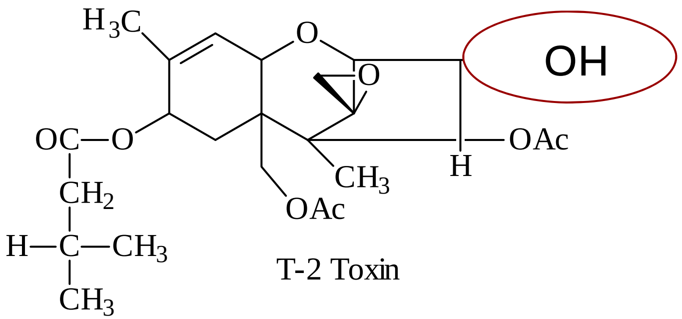
in the plant.

***F. Langsethiae* grown in culture can also**

produce HT-2 glucoside



Identified (2012/13)



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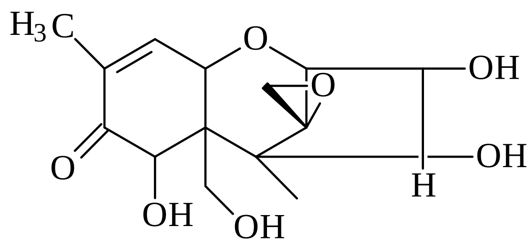
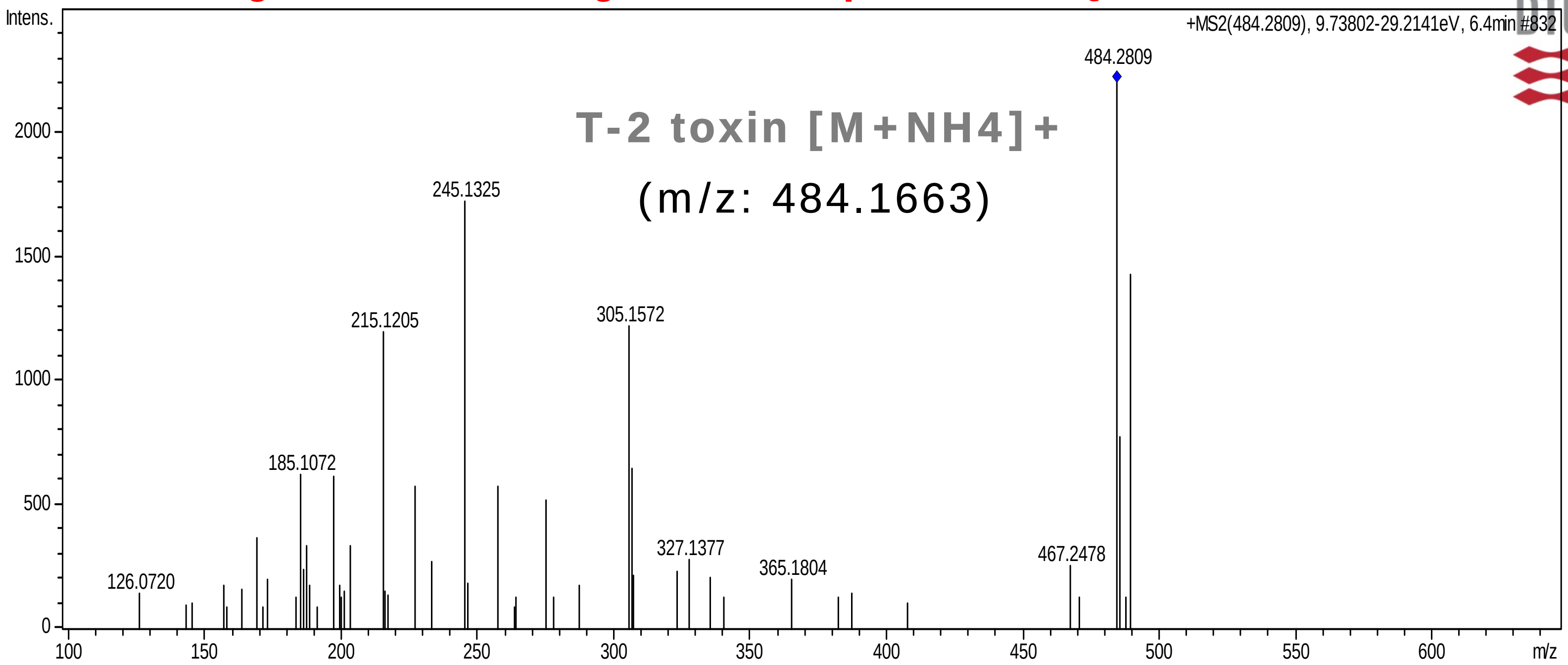
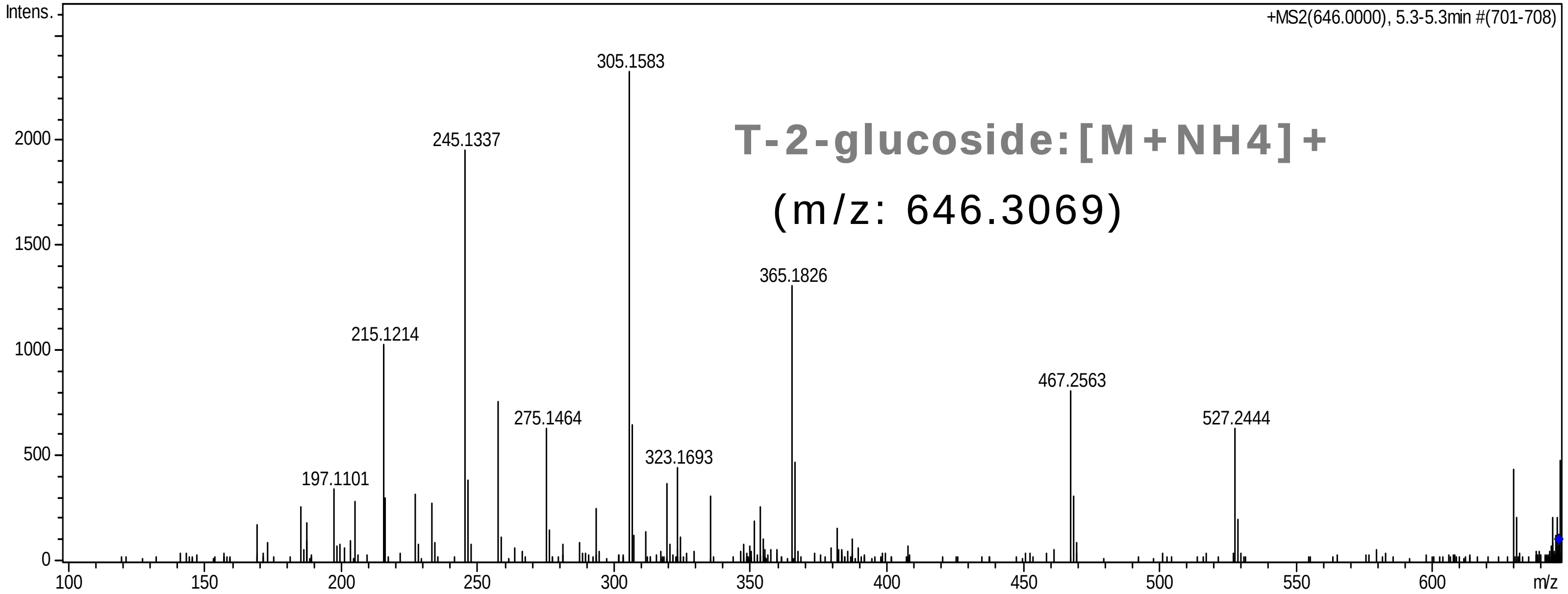


NIV

Identified (2012/13)

High resolution fragmentation pattern on QTOF





Occurrence of HT-2 and T-2 glucosides in naturally contaminated barley from Denmark: in total 12 samples

Sample identity number	HT-2 (µg/kg)	T-2 (µg/kg)	HT-2-glucoside/HT-2 (peak area ratio x 100)	T-2-glucoside/T-2 (peak area ratio x 100)
201	222	128	28	0
206	77	42	0	0
208	164	49	76	0
209	45	12	24	0
210	312	118	147	82
212	66	7	39	0
213	72	21	40	0
214	46	7	76	0
217	41	15	84	0
220	43	8	100	0
222	62	15	118	0
PD0	446	221	34	12

0: glucoside not found

Final remarks

- General very sparse information to the toxicity of the not regulated mycotoxins
- There is a total lack of knowledge related to the effects of combinations of mycotoxins
- The role of ''masked mycotoxins'' needs to be clarified as the content in cereals can be high
- Several new secondary fungal metobolites have been found in cereal grain
- Climate changes will most probably favour formation of more mycotoxins and thereby also increase the use of pesticides against fungal disease
- Further development of multimethods for simultaneous determination of pecticides and mycotoxins in grains and other food and feed matrices

Ackknowledgement

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