Mycotoxins in maize silage
Detection of toxins and toxicological aspects

PhD defense by
Rie Romme Rasmussen
September 20th 2010
Outline

• Introduction
• Aims of the study
• Experiments and Results:
  ➢ Cytotoxicity of fungal metabolites
  ➢ Detection of mycotoxins
  ➢ Occurrence in maize silage
• Conclusions
• Perspectives
Maize silage a feed product

Natural fermentation of maize plants by lactic acid bacteria

- low pH = 3-4
- low $\text{O}_2 < 2\%$
- high $\text{CO}_2 \approx 20-90\%$

Stored for up to 14 months

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Maize silage a feed product

Dairy cow
~ 40 kg silage pr. day
50-75% of the daily diet

Unexplained health problems
Decrease in milk yield
Illness, death

Mycotoxins from fungi
Maize silage

Når råmælk bliver farlig

Toksiner fra ensilage er akut forgiftede for køer, og de dør i vælden, skyldes toksiner fra fodernet.

Dairy cow

6,000,000 tonnes DK production

Dairy cow

~ 40 kg silage pr. dag

50 - 75% of the daily diet

Milk production is strongly reduced

Unexplained health problems

Decrease in milk yield

Illness, death

Mycotoxins from fungi

4,000,000

3,000,000

End of...
Mycotoxins

- **Toxic** secondary metabolites from filamentous fungi
- Cancer, hormonal imbalance, liver and kidney damage or reduced immune defence
- Production
  - type of fungi (species)
  - growth conditions
Maize silage pre-harvest fungi

Fusarium infection

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Maize silage pre-harvest fungi

- **Fusarium**
- **Alternaria**
- **Epicoccum**
- **Phoma**
- **Aspergillus flavus**
- **Aspergillus parasiticus**

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Maize silage post-harvest fungi

Penicillium infection

Photo: Kalkrup H

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Maize silage post-harvest fungi

Penicillium roqueforti
Penicillium paneum
Zygomycetes (Mucor and Rhizopus)
Aspergillus fumigatus
Byssochlamys nivea
Monascus ruber
Maize silage post-harvest fungi

- *Penicillium roqueforti* / *Penicillium paneum*
  - Very common fungal contaminant
  - Optimal pH: 4-5
  - Growth at 0.3% O$_2$, 25% CO$_2$
  - Growth at 5°C

- Secondary metabolites
  - PR-toxin (acute toxic)
  - Roquefortine C (neurotoxic)
  - Mycophenolic acid (immunosuppressive)
  - Patulin (genotoxic, immunosuppressive)
  - Marcfortines (no adverse effects described)
  - Andrastines
  - and more...

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Joint project (2005-2010)

“Mycotoxin carry-over from maize silage via cattle into dairy products”

**Preharvest** fungi and their mycotoxins in maize, Sørensen, JL (2009)

**Post-harvest** fungal spoilage of maize silage, Storm IMLD (2009)

National Food Institute, Technical University of Denmark
Joint project (2005-2010)

“Mycotoxin carry-over from maize silage via cattle into dairy products”

Preharvest fungi and their mycotoxins in maize, Sørensen, JL (2009)

Post-harvest fungal spoilage of maize silage, Storm IMLD (2009)

Aim

Mycotoxin hazard for cattle feeding on maize silage evaluated by chemical and cyto-toxicity test methods

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Experiments – mycotoxins and other fungal metabolites

Cytotoxicity
Comparison of
- Mycotoxins
- Fungal agar
- Maize silage

Detection
Method development & validation
- Silage extraction
- LC-MS/MS

Occurrence
27 fungal metabolites
- Hot-spots
- Fresh & ensiled maize silage

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Cytotoxicity assay

- Cell viability assay => model system for acute toxicity
- Limit: Absorption, distribution, metabolism, excretion
- Dairy cows
  - Rumen fermentation by bacteria, protozoa and fungi

Drawings: The Department of Animal Science at Texas A&M University
Cytotoxicity assay

**Caco-2** = human colon cancer cell line

Alamar Blue/resazurin assay

- 10,000 cells/well
- Growth medium
- 5% CO₂, 37°C
- 48 h toxin exposure

Viable cell

Resazurin → Reduction by mitochondria cell enzyme → Resorufin, fluorescent
Cytotoxicity of mycotoxin standards

Concentration-response graph

48 h exposure
Two distinct test days

IC$_{50}$ = 0.6 µg/mL

Patulin (µg/mL)
Cytotoxicity of mycotoxin standards

**Andrastine A**
- Viability (%) vs. Concentration (μg/mL)
- IC$_{50}$ > 50 μg/mL

**Roquefortine C**
- Viability (%) vs. Concentration (μg/mL)
- IC$_{50}$ = 48 μg/mL

**Patulin**
- Viability (%) vs. Concentration (μg/mL)
- IC$_{50}$ = 0.6 μg/mL
- 50% viability at 1 μg/mL

**Mycophenolic acid**
- Viability (%) vs. Concentration (μg/mL)
- IC$_{50}$ > 100 μg/mL

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# Cytotoxicity of mycotoxin standards

<table>
<thead>
<tr>
<th>Toxin, 48 h</th>
<th>Caco-2 cells, IC$_{50}$ (µg/ml)</th>
<th>SD</th>
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</thead>
<tbody>
<tr>
<td>T-2 toxin</td>
<td>0.0037 +/- 0.0008</td>
<td></td>
</tr>
<tr>
<td>Gliotoxin</td>
<td>0.035 +/- 0.003</td>
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</tr>
<tr>
<td>Deoxynivalenol</td>
<td>0.29 +/- 0.17</td>
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</tr>
<tr>
<td>Patulin</td>
<td>0.62 +/- 0.07</td>
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</tr>
<tr>
<td>Roquefortine C</td>
<td>48 +/- 2</td>
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</tr>
<tr>
<td>Zearalenone</td>
<td>58 +/- 6</td>
<td></td>
</tr>
<tr>
<td>Citrinin</td>
<td>83 +/- 32</td>
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</tr>
<tr>
<td>N6-formyl-roquefortin-C</td>
<td>&gt;46</td>
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</tr>
<tr>
<td>Andrastin A</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>1-hydroxyremophil-7(11),9(10)-dien-8-one</td>
<td>&gt;280</td>
<td></td>
</tr>
</tbody>
</table>

**IC$_{50}$** = 50% inhibit concentration  
SD = sample standard deviation of 2 independent experiments
Cytotoxicity of fungal agar extracts

- **Crude extracts (8 fungal species)**
  
  _Alternaria tenuissima_, _Fusarium avenaceum_, _F. graminearum_, _Aspergillus fumigatus_, _Byssochlamys nivea_, _Monascus ruber_, _Penicillium paneum_, _P. roqueforti_

- 13-14 days old cultures, 25º C

  - **YES** Yeast extract sucrose agar
  - **PDA** Potato Dextrose agar
  - **CYA** Czapek yeast extract agar
  - **SA** Maize silage agar

- 15 cm² plugs with fungal growth extracted with

  EtAc:CH₃Cl₂:MeOH (3:2:1), 1% (v/v) formic acid

- Re-dissolved in 2 ml MeOH

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Cytotoxicity of *Penicillium paneum*

Viability %

<table>
<thead>
<tr>
<th>Sample</th>
<th>YES</th>
<th>CYA</th>
<th>PDA</th>
<th>SA</th>
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<tr>
<td>28545</td>
<td>120</td>
<td>100</td>
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<tr>
<td>28544</td>
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<tr>
<td>28543</td>
<td>20</td>
<td>40</td>
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</tbody>
</table>

Mean of 2 tests

Fungal extract of IBT number

Patulin >2.9 µg/mL

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Cytotoxicity of *Penicillium paneum*

Major metabolites: YES, CYA, PDA and SA agar

- patulin $> IC_{50}$
- marcfortine A
- citreoisoumarin $< IC_{50}$
- roquefortine C $< IC_{50}$
- andrastin A $< IC_{50}$

Cytotoxic Non-toxic extracts

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# Cytotoxicity

## Fungal agar and silage extracts

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Sec. metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillium paneum</td>
<td><em>patulin</em>, <em>roquefortine C</em>, <em>andrastin A</em>, citreoisocoumarin, macfortine A, ...</td>
</tr>
<tr>
<td>Penicillium roqueforti</td>
<td><em>(mycophenolic acid)</em>, <em>roquefortine C</em>, <em>andrastin A</em>, Interactions or Other; PR-toxin, roquefortine A, ...</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td><em>gliotoxin</em>, + Other; verrucologen, fumitremorgin A &amp; B, fumigaclavine C, ...</td>
</tr>
<tr>
<td>Byssochlamys nivea</td>
<td><em>patulin</em>, <em>mycophenolic acid</em>, byssochlamic acid, ...</td>
</tr>
<tr>
<td>Monascus ruber</td>
<td><em>Citrinin</em>, Other; monacolin K, ankaflavin, ...</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
<td><em>Zearalenone</em>, <em>deoxynivalenol</em>, Interactions or Other; Aurofusarin, fusarin C, rubrofusarin, ...</td>
</tr>
</tbody>
</table>

Red = present in cytotoxic concentrations  
Gray = present in non-toxic concentrations  
Black = present

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Cytotoxicity of *Penicillium roqueforti*

Major cytotoxic metabolite in fungal YES extracts?

1-Hydroxyeremophil-7(11),9(10)-dien-8-one

Fractions
- by RT time
- targeting peaks

PR-toxin analogues (?)

→ PR-toxin
**Cytotoxicity of maize silage**

Inoculated silage

*Byssochlamys nivea*

Photo: Birgitte ML Raun

QuEChERS extract of maize silage
Evaporated and re-dissolved in MeOH

**Secondary metabolites**
+ Byssochlamamic acid
+ Mycophenolic acid
+ Other undescribed metabolites
- Patulin

*No significant cytotoxicity*

Penicillium roqueforti (+ PR-toxin)
Penicillium paneum (- patulin)
Aspergillus fumigatus (- gliotoxin)
Monascus ruber (+ citrinin)
**Summery of cytotoxicity**

- Caco-2 resazurin assay ≈ sensitivity as other *in vitro* assays
- Applications:
  - ☺ fungal agar extracts, screening
  - ☵ fraction of fungal agar extracts →
  - PR-toxic an important toxic metabolite from *P. roqueforti*
  - ☹ silage extracts
- Cytotoxic pre- and post-harvest species
  - *Alternaria tenuissima, Fusarium avenaceum, F. graminearum, Aspergillus fumigatus, Byssochlamys nivea, Monascus ruber, Penicillium paneum, P. roqueforti*
- Interactions or other metabolites than tested here often contributed to the cytotoxicity
Experiments – mycotoxins and other fungal metabolites

Cytotoxicity

Comparison of
- Mycotoxins
- Fungal agar
- Maize silage

Detection

Method development & validation
- Silage extraction
- LC-MS/MS

Occurrence

- 27 fungal metabolites
- Hot-spots
- Fresh & ensiled maize silage

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Detection techniques

High performance liquid chromatograph
(separation of analytes)

Mass spectrometer
(detector)

Photo: agilent.com

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LC-MS/MS

Electrospray (ionization)

Quadrupole 1 (mass filter)

Quadrupole 2 (Collision cell)

Quadrupole 3 (mass filter)

Sample, HPLC eluent

Capillary, high voltage

Cone

Detector

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LC-MS

Electrospray (ionization)

Capillary, high voltage

Cone

Quadrupole 1 (mass filter)

Quadrupole 2 (Collision cell)

Quadrupole 3 (mass filter)

Sample, HPLC eluent

Detector

Mass filter

Photo: waters.com

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Detection - Multi-mycotoxin analysis

- 32 fungal metabolites
- Size: 150-700 Da

- Strong acid: Mycophenolic acid
- Weak acid: Alternariol monomethylether
- Base - amine: Roquefortine A

- Water solubility changes with pH
  - Fungal hot-spot, pH = 7
  - Unspoiled silage pH = 3-4
  \[\rightarrow\] pH buffered extraction
Detection – *sample extraction*

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

1) Anastassiades et al. (2003) *J. AOAC Int.* 86, 412-431
Detection – LC-MS/MS validation

Spiked on 3 levels, repeated 3 days

<table>
<thead>
<tr>
<th>Compound</th>
<th>ESI</th>
<th>MS-MS</th>
<th>REC&lt;sub&gt;mean&lt;/sub&gt;</th>
<th>RSD&lt;sub&gt;r&lt;/sub&gt;</th>
<th>RSD&lt;sub&gt;IR&lt;/sub&gt;</th>
<th>LOD&lt;sub&gt;spike&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycophenolic acid</td>
<td>-</td>
<td>319.1 » 190.8, 178.8</td>
<td>90</td>
<td>11</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>-</td>
<td>317.1 » 130.8, 174.7</td>
<td>90</td>
<td>12</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Alternariol</td>
<td>-</td>
<td>257 » 214.9, 146.8</td>
<td>78</td>
<td>9</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Cyclopiazonic acid</td>
<td>+</td>
<td>337.2 » 195.9, (181.9)</td>
<td>63</td>
<td>22</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>Enniatin B</td>
<td>+</td>
<td>657.4 » 314, 527</td>
<td>60</td>
<td>21</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>-</td>
<td>341.1 » 265, (295)</td>
<td>83</td>
<td>17</td>
<td>18</td>
<td>739</td>
</tr>
<tr>
<td>Marfentone B</td>
<td>+</td>
<td>464 » 436, 419</td>
<td>61</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Citrinin</td>
<td>+</td>
<td>251.1 » 233, 190.9</td>
<td><strong>Unstable LC-MS-MS sensitivity</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fumonisn B1</td>
<td>+</td>
<td>722.5 » 334, 528</td>
<td>6</td>
<td>13</td>
<td>18</td>
<td>544</td>
</tr>
</tbody>
</table>

LOD (1-739 µg/kg)

Reproducibility (7-35% RSD)
Summery - Detection

- Both pre- and post-harvest fungal metabolites
- One extract
- No clean-up
- Buffering of pH
- Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) method
- LC-MS/MS
- 27 validated: LOD (1-739 μg/kg) & Reproducibility (7–35% RSD)
- Rinsing the HPLC system between samples ensures performance
## Experiments – mycotoxins and other fungal metabolites

<table>
<thead>
<tr>
<th>Cytotoxicity</th>
<th>Detection</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparison of</td>
<td>Method development &amp; validation</td>
<td>27 fungal metabolites</td>
</tr>
<tr>
<td>- Mycotoxins</td>
<td>- Silage extraction</td>
<td>- Hot-spots</td>
</tr>
<tr>
<td>- Fungal agar</td>
<td>- LC-MS/MS</td>
<td>- Fresh &amp; ensiled maize silage</td>
</tr>
<tr>
<td>- Maize silage</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Occurrence – maize silage

- Visibly mouldy maize silage from all over Denmark
  10 hot-spots infected with
  *M. ruber, A. fumigatus, B. nivea, P. paneum* and *P. roqueforti*

- Unspoiled fresh and ensilage maize collected 2007 – 2009
  from all over Denmark

  17 whole fresh maize plants taken at field level

  82 ensiled maize samples collected
  - from the cutting face of the silage stack
  - with a silage drill approximately 1 meter behind the cutting face of the silage stack
## Occurrence - visible mouldy silage

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fungal hot-spots (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n&lt;sub&gt;pos&lt;/sub&gt;</td>
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<tr>
<td><strong>Penicillium</strong></td>
<td></td>
</tr>
<tr>
<td><em>B. nivea</em></td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td></td>
</tr>
<tr>
<td>Roquefortine C</td>
<td>3</td>
</tr>
<tr>
<td>Andrastin A</td>
<td>6</td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>6</td>
</tr>
<tr>
<td>Gliotoxin</td>
<td>2</td>
</tr>
<tr>
<td><strong>Alternaria</strong></td>
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</tr>
<tr>
<td>Alternariol</td>
<td>1</td>
</tr>
<tr>
<td>Alternariol monomethyl ether</td>
<td>1</td>
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<tr>
<td><strong>Fusarium</strong></td>
<td></td>
</tr>
<tr>
<td>Nivalenol</td>
<td>2</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>2</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>4</td>
</tr>
<tr>
<td>Enniatin B</td>
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<tr>
<td><strong>Penicillium</strong></td>
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<tr>
<td><em>A. fumigatus</em></td>
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<tr>
<td>Roquefortine A</td>
<td>3</td>
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<tr>
<td>Marcfortine B</td>
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<td><strong>Phoma</strong></td>
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<tr>
<td><em>A. fumigatus</em></td>
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<tr>
<td>Citreoisocoumarin</td>
<td>5</td>
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<tr>
<td>Fumigaclavine A</td>
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</table>

- National Food Institute, Technical University of Denmark
### Occurrence – 99 unspoiled silages

<table>
<thead>
<tr>
<th>Analyte</th>
<th>n&lt;sub&gt;pos&lt;/sub&gt;</th>
<th>µg/kg fresh weight</th>
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<tbody>
<tr>
<td></td>
<td>mean&lt;sub&gt;pos&lt;/sub&gt;</td>
<td>max</td>
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<tr>
<td>Roquefortine C</td>
<td>2</td>
<td>173 189</td>
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<td>43 52</td>
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<tr>
<td>Alternariol</td>
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<td>18 24</td>
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<td>9 11</td>
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<td>Marcfortine A</td>
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# Occurrence – 99 unspoiled silages

<table>
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<tr>
<th>Analyte</th>
<th>( n_{\text{pos}} )</th>
<th>( \mu g/kg ) fresh weight</th>
<th>mean(_{\text{pos}})</th>
<th>max</th>
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<tr>
<td><strong>Penicillium</strong></td>
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<td>Andrastin A</td>
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<td><strong>Gliotoxin</strong></td>
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<td><strong>Fusarium</strong></td>
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<tr>
<td>Deoxynivalenol</td>
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<td>1841</td>
<td>2974</td>
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<tr>
<td>Zearalenone</td>
<td>34</td>
<td>71</td>
<td>666</td>
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<td>Enniatin B</td>
<td>28</td>
<td>75</td>
<td>365</td>
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<td><strong>Quantitative</strong></td>
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<tr>
<td>Roquefortine A</td>
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<td>Marcfortine B</td>
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<tr>
<td>Marcfortine A</td>
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<tr>
<td>Citreoisocoumarin</td>
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<tr>
<td><strong>Phoma</strong></td>
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<tr>
<td><em>A. fumigatus</em></td>
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<tr>
<td>Fumigaclavine A</td>
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</table>

Only in hot-spots

National Food Institute, Technical University of Denmark
Occurrence – 99 unspoiled silages

- 35 samples was without detectable residues
- 30 samples contained only one fungal metabolite
- 8 samples contained 4 different analytes

National Food Institute, Technical University of Denmark
Summary – mycotoxin occurrence

- Low levels of multiple secondary metabolites is common
- High occurrence of *Fusarium* toxins
- *Fusarium* toxins < values recommended by the European Commission (growth season 2006-2008)
- High content of fungal metabolites with visible fungal growth
- PR-toxin and patulin (highly cytotoxic) were not detected in field samples
Conclusion

Cytotoxicity
• The assay applicable to standards and fungal agar extracts – not silage
• 8 fungal species associated with maize and silage produce cytotoxic metabolites
• Fungal toxicity depends on growth media, species, isolate
• Interactions or other metabolites than tested here often contributed to cytotoxicity
• PR-toxin an important toxic metabolites from P. roqueforti

LC-MS/MS detection method
• 27 metabolites in maize silage, QuEChERS extraction → successfully validated
• Rinsing the HPLC system between samples ensures performance

Occurrences of fungal secondary metabolites
• 15 metabolites detected
• High content of fungal metabolites with visible fungal growth
• Low level of multiple-mycotoxins is common in unspoiled silage
• Fusarium toxins dominated, < levels recommended by the European Commission
Perspectives
– mycotoxin occurrence and toxicity

• No indication that mycotoxins in maize silage have caused the general health problems observed at Danish dairy cattle farms

• Immunosuppressive (e.g. DON, NIV, GLI, MPA)
  – Continuous exposure to low levels → ? infectious diseases ?
  – Long term in vivo studies are sparse

• Human exposure, transport to milk
  – Grain and not animal products a significant source for the pre-harvest toxins deoxynivalenol, zearalenone and fumonisins
  → Genotoxic Alternaria toxins might be relevant
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