An overview of Ochratoxin A, associated fungal species and implications for Canada's grain industry

Keith A. Seifert
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### Proposed Health Canada maximum limits (MLs):

<table>
<thead>
<tr>
<th>Item</th>
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*These levels have been established taking into consideration the reducing effect of processing or redistribution.

**For bread, pastries and other flour-based foods, Health Canada considers these guidelines to pertain to the flour portion. In the future, based on further monitoring data (ongoing HC studies), the Department may consider modifying these MLs, or introduce MLs for products not yet covered, if necessary.
Overview

• Historical background
  – The toxin and the moulds

• Biology and taxonomy of *Penicillium* and *Aspergillus*
  – Development of modern species concepts
    • Mycotoxins
  – DNA based identification

• Ochratoxin A in Canada and internationally
  – Storage issues

• Future directions
Aspergillus flavus
the aflatoxin mould
**Penicillium**
- ~300 known species
  - > 1000 expected
- Storage fungi
- Dry, electrostatic spores
- Diverse mycotoxins
  - Ochratoxin A
  - Patulin
  - Griseofulvin

**Fusarium**
- ~150 known species
  - > 750 expected
- Field fungi, plant pathogenic
- Slimy spores
- More uniform mycotoxins
  - Trichothecenes
  - Zearalenone
  - Fumonisins
Biology of *Penicillium* and *Aspergillus*

- The quintessential moulds
- Common agents of biodeterioration in soil, wood, dung and man-made commodities
- Talented producers of secondary metabolites
  - High ‘creativity index’
- Plant diseases, especially spoilage during storage
- Common in indoor air, causing allergies
- Ecological significance possibly over-estimated
  - Ability to grow on standard microbiological media

- *P. roqueforti* makes 1–10 billion spores per Petri dish
Ochratoxin A

- A fungal toxin
- Abbreviations: OTA or OA
- Regulated in North America and Europe for many years
- Kidney cancer in humans
  - Group 2B *possible* human carcinogen
    - International Agency for Research on Cancer (IARC)
- Found in grains, in particular barley, oats and beer, and other commodities
- Detectable in blood sera of all Canadians
- Terrible confusion about what fungal species produce the toxin
  - Misidentification of moulds
  - Misidentification of toxins
Penicillium as the taxonomist’s nightmare

“Alive and actively growing, they have individuality as pronounced as their capacities for evil, but that individuality, color, odor, and habit or growth are evanescent as frost designs on a window pane in winter.”

Charles Thom 1930
Penicillium chrysogenum
The penicillin mould
Penicillium expansum
Blue mould of apple
**Penicillium camembertii**

the Brie mould
Penicillium roqueforti
the blue cheese mould
*Penicillium roqueforti*

the blue cheese mould

**Major Mycotoxins**

- Mycophenolic acid
  - PR-toxins
  - Roquefortine C

Photos Rob Samson
**Penicillium history**

- **1809** Described by Link
- **1930** Monograph by Thom
- **1949** Monograph by Raper & Thom
- **1979** Monograph by Pitt
- **1980s** Secondary metabolites & isozymes
- **1990s** DNA sequencing
- **2000s** Multigene phylogenies, molecular diagnostics
- **2010s** Genomics
Modern species concepts (1)

• Mid-1980s,
  – Jens Frisvad et al., Denmark

• Hypothesis
  – Species have constant, reproducible secondary metabolite profiles

• Secondary metabolite profiling
  – Thin layer chromatography
  – then HPLC
  – then GC-MS
  – then LC-MS

• Result
  – Division of existing morphological species
  – Identification using mycotoxin profiles
**Penicillium, Aspergillus & secondary metabolites**

- Produce all major groups of secondary metabolites
- ~ 60-100 secondary metabolites per species
Ochratoxin A production in *Penicillium*

- *Penicillium viridicatum* var. *cyclopium* (PVC)
- *Penicillium viridicatum* chemotype
- *Penicillium verrucosum*
  - Divided into 2–3 species
    - *P. verrucosum* – grains
    - *P. nordicum* – meat
    - *P. lumpii* – dried fish (not described)

- All claims of ochratoxin A production in other *Penicillium* species are unproven
Modern species concepts (2)

- Early 1990s
- DNA-based taxonomy
  - Ribosomal operon sequencing (ITS, LSU)
    - Peterson, USDA, Peoria
  - Protein gene sequencing
    - Seifert lab, Canada
- Good (but not perfect) correlation of phylogenetic species with mycotoxin groups
ITS Gene Tree
subg. *Penicillium*

- Variable sites ~ 20%
- DNA barcoding

Grain species

Beta-tubulin Gene Tree
subg. *Penicillium*

- Variable sites ~ 66%

Beta-tubulin Gene Tree

Ochratoxin A group

Molecular Assay Development
from beta-tubulin sequences

**Successes**
- *P. roqueforti* complex (3 spp.)
- Citrus penicillia (4 spp.)
- *P. brevicompactum* complex (3 spp.)

**Assays design failed**
- *P. aurantiogriseum* complex (5 grain spp.)
- *P. camemberti* complex
  - *P. commune*
  - *P. camemberti*
  - *P. caseifluvum*
  - *P. palitans*
- *P. verrucosum* complex
  - ochratoxin A
- *P. chrysogenum*
  - penicillin, IAQ
- *P. expansum*
  - apples, patulin

Seifert *et al.* unpublished
The changing ochratoxin A landscape

- Historical concerns in Europe
  - Pork products
  - Beer
- High OTA human blood titers
- High endemic kidney cancers
  - Balkans
- High OTA levels in wine and coffee
  - increased scrutiny of imports
Aspergillus ochraceus complex
beta-tubulin gene tree

Aspergillus niger complex
beta-tubulin gene tree

- A. niger
  - “Generally Recognized as Safe” (GRAS) for industrial use
  - Regulated as potential human pathogen by Bill C-11

## Determination of ochratoxin A in wine and grape juice
### Source and number of samples

<table>
<thead>
<tr>
<th>Country</th>
<th>Year of analysis</th>
<th>Red wine</th>
<th>White wine</th>
<th>Red grape juice</th>
<th>White grape juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algeria</td>
<td>2001–02</td>
<td>1(1)a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>1999–2000</td>
<td>15(1)</td>
<td>16(6)</td>
<td>24(3)</td>
<td>12(1)</td>
</tr>
<tr>
<td></td>
<td>2000–01</td>
<td>6(1)</td>
<td>6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2001–02</td>
<td>15(1)</td>
<td>21(1)</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Cyprus</td>
<td>1999–2000</td>
<td>–</td>
<td>1(1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>England</td>
<td>1999–2000</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>France</td>
<td>1999–2000</td>
<td>3(1)</td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2001–02</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greece</td>
<td>1999–2000</td>
<td>7(6)</td>
<td>9(6)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2000–01</td>
<td>3(2)</td>
<td>4(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>1999–2000</td>
<td>3(3)</td>
<td>3(1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2000–01</td>
<td>6(4)</td>
<td>10(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>1999–2000</td>
<td>1(1)</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2000–01</td>
<td>6(3)</td>
<td>4(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>2000–01</td>
<td>1(1)</td>
<td>1(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>1999–2000</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2001–02</td>
<td>11</td>
<td>12</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Total number &gt; LOQ</td>
<td>1999–2002</td>
<td>25</td>
<td>22</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Grand total</td>
<td>1999–2002</td>
<td>84</td>
<td>96</td>
<td>46</td>
<td>25</td>
</tr>
</tbody>
</table>

Searching for Aspergillus carbonarius in southern Ontario
Penicillium verrucosum in Canada

*P. verrucosum* grows slowly
Overgrown by other species
Many other *Penicillium* species in grain

### Table 1. *Penicillium* species isolated from cereal grains delivered to primary elevators from farms in western Canada

<table>
<thead>
<tr>
<th><em>Penicillium</em> species</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Manitoba</td>
</tr>
<tr>
<td>Nephrotoxigenic species</td>
<td></td>
</tr>
<tr>
<td><em>P. aurantiogriseum</em></td>
<td>14</td>
</tr>
<tr>
<td><em>P. freii</em></td>
<td>18</td>
</tr>
<tr>
<td><em>P. tricolor</em></td>
<td>1</td>
</tr>
<tr>
<td><em>P. verrucosum</em></td>
<td>2</td>
</tr>
<tr>
<td>Chemotype II</td>
<td></td>
</tr>
<tr>
<td><em>P. viridicatum</em></td>
<td>3</td>
</tr>
<tr>
<td>Non-nephrotoxigenic species</td>
<td></td>
</tr>
<tr>
<td><em>P. aurantioviolens</em></td>
<td>1</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>1</td>
</tr>
<tr>
<td><em>P. corylophilum</em></td>
<td>1</td>
</tr>
<tr>
<td><em>P. crateriforme</em></td>
<td>1</td>
</tr>
<tr>
<td><em>P. griseofulvum</em></td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
</tr>
</tbody>
</table>

Ecology of *Penicillium verrucosum*

- Presently no molecular assay
- Monitored with selective medium
  - laborious
  - low sensitivity
- In Denmark
  - Irregular distribution in fields and soils (0-300 CFU/g soil)
  - Sporadic recovery from harvested grain
  - Hot spots in stored grain
    - related to moisture, temperature

# Factor affecting ochratoxin-A during storage

<table>
<thead>
<tr>
<th>Good Storage Practice</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good Store Design and Condition</td>
<td>High</td>
</tr>
<tr>
<td>Ensure stores are well designed and maintained</td>
<td></td>
</tr>
<tr>
<td>Good Harvest and Store Hygiene</td>
<td>High</td>
</tr>
<tr>
<td>Clean harvest and store machinery</td>
<td></td>
</tr>
<tr>
<td>Timely Harvest</td>
<td>High</td>
</tr>
<tr>
<td>Service and maintain equipment</td>
<td></td>
</tr>
<tr>
<td>Adequate Drying Capacity</td>
<td>High</td>
</tr>
<tr>
<td>Ensure adequate drying capacity is available</td>
<td></td>
</tr>
<tr>
<td>Rapidly Dry Grain</td>
<td>High</td>
</tr>
<tr>
<td>Rapidly dry grain to below 18% moisture content</td>
<td></td>
</tr>
<tr>
<td>Rapidly Cool Grain</td>
<td>High</td>
</tr>
<tr>
<td>Rapidly cool grain to below 15°C</td>
<td></td>
</tr>
<tr>
<td>Continued Drying</td>
<td>Medium</td>
</tr>
<tr>
<td>Dry to 15% moisture content for long-term storage</td>
<td></td>
</tr>
<tr>
<td>Continued Cooling</td>
<td>Medium</td>
</tr>
<tr>
<td>Cool grain in winter months to below 5°C</td>
<td></td>
</tr>
<tr>
<td>Monitor Temperature and Moisture Content</td>
<td>Low</td>
</tr>
<tr>
<td>Continue monitoring temperature and moisture content</td>
<td></td>
</tr>
<tr>
<td>Monitor Insect and Mite Activity</td>
<td>Low</td>
</tr>
<tr>
<td>Use traps and sieving to monitor insects and mites</td>
<td></td>
</tr>
</tbody>
</table>

Good store hygiene
Timely harvest
Ensure adequate drying capacity; rapidly dry and cool grain
Carry out continued drying and cooling
Monitor temperature, moisture content and insect activity
It is unacceptable that a mountain of grain should have been allowed to damage the health of unwitting consumers.

A statement from the Italian pasta manufacturers association said Italian pasta was "absolutely safe and innocuous."

Polluted pasta causes toxin alarm in Italy

John Hooper in Rome
The Guardian, Thursday 12 January 2006

Italians tucked into their pasta yesterday with a little less relish than usual after learning that 58,000 tonnes of wheat infected with a powerful natural toxin had been milled into flour and sold on to the market.

Police arrested Francesco Casillo, the director of the Molino Casillo Francesco, yesterday on charges that he imported the wheat from Canada last September which was contaminated with cancer-causing toxins.
HEALTH HAZARD ALERT

HEINZ MIXED CEREAL FOR BABIES MAY CONTAIN OCHRATOXIN A (OTA)

OTTAWA, December 10, 2009 - The Canadian Food Inspection Agency (CFIA) and Heinz Canada are warning the public not to consume the Heinz Mixed Cereal for babies described below as it may contain elevated levels of Ochratoxin A (OTA).

The following product is affected by this alert:

Heinz Mixed Cereal, a Baby Cereal, Stage 2, From 6 Months
227 g
UPC 0 57000 02515 8
Codes: BB/MA 10 DE 28 and BB/MA 10 DE 29

The best before date codes are located on the top left hand corner of the package.

Ochratoxin A (OTA) is a toxic fungal metabolite that causes nephrotoxic, teratogenic, immunosuppressive and carcinogenic effects in a number of animal species. It has also been implicated in the development of a chronic kidney disease in humans known as Balkan Endemic Nephropathy. OTA occurs naturally in low concentrations in many foods, such as cereal-derived staples as well as other food commodities including grapes, raisins, wine, coffee and beer.

On August 29th, 2008 Health Canada's Bureau of Chemical Safety (BCS), Food Directorate, sent pre-consultation letters to targeted stakeholder groups requesting input on Health Canada’s proposed maximum limits for ochratoxin A (OTA) in foods. On March 2, 2009, BCS webposted its Information Document on Health Canada’s proposed Maximum Limits (Standards) for the Presence of the Mycotoxin Ochratoxin A in Foods on Health Canada's Food Safety website, requesting further comments on this proposal from the larger stakeholder community. Comments were accepted until 12:00 a.m. EDT on June 1st, 2009.

On December 9, 2009, the Bureau of Chemical Safety’s Health risk assessment of ochratoxin A for all age-sex strata in a market economy was published on-line in the Journal Food Additives and Contaminants (Volume 27, Issue 2, February 2010, pages 212-240). The article was made publicly available by Health Canada through its website in April 2010.
Ochratoxin A exposure by commodity

a) Proposed Health Canada maximum limits (MLs):

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The way forward

• Robust identification of mycotoxigenic fungi and mycotoxins
  – Detection and identification of moulds without culturing
    • Real-time PCR assays from genomic studies
  – Cheaper, higher throughput, accurate mycotoxin assays

• Ecological and ‘epidemiological’ studies
  – Amplification of low incipient contamination from soil or in storage bins is probably a significant issue

• Robust Canadian surveys for the fungus and the toxin
  – Keeping in mind the possibility of a role for A. ochraceus

• Continued collaboration among the grain industry, plant breeders, and researchers
Thanks to:

• The AAFC team:
  Gerry Louis-Seize, Sarah Hambleton, André Lévesque, Peter Sholberg

• The CGC team:
  Tom Gräfenhan, Sheryl Tittlemier

• Ontario colleagues:
  David Miller, Art Schaafsma

• The CBS team (Netherlands):
  Rob Samson, Ellen Hoekstra, Jos Houbraken

• The Lyngby team (Denmark):
  Jens Frisvad, David Overy

Keith Seifert: keith.seifert@agr.gc.ca