Analysis of Nicotine in Mushrooms

Michelangelo Anastassiades and Amadeo R. Fernández-Alba
NICOTINE-USES

Has been used as a pesticide at least since the 15th century.

- Insecticide with predominantly respiratory action, but also slight contact and stomach action

- Was used on a variety of crops, including fruit, vines, vegetables, and ornamentals to control aphids, thrips, whitefly, and other insects

- Its main use was in greenhouses or enclosed spaces, where a smoke canister was set and allowed the nicotine to fill the space

- In the past approved in EU (also for organic farming).

- Non-inclusion in Annex I (10 January 2009) (phased out by June 2010)

- In US registrations for use in food cancelled in 1994
High levels of nicotine, when ingested, are very toxic and consuming greater than 40-60 mg (the amount contained in 4 cigarettes) is potentially lethal for adults (http://www.inchem.org/).

Note: The smoke of one cigarette contains ca. 0.9-1.7 mg Nicotine.

NICOTINE-MRLs (396/2005/EC):
Default at 0.01 mg/kg for all commodities
(However, 99% of dried wild mushrooms do not meet this level)
Nicotine in Mushrooms

Background Information

BOLETUS EDULIS = PORCINI = CEPS

MU ERR
Nicotine in Mushrooms - Background Information:

- In 2008 high levels of Nicotine detected in mushrooms at CVUA Sigmaringen.
- CVUA Stuttgart consulted for confirmation (LC-TOF, -MS/MS, GC-MS).
- Since then many findings by various labs in dried mushrooms mainly from CN.
- Most affected dried Porcini (Boletus edulis) but also Truffles and Chanterelles.
- Porcini are reported to be not cultivable.
- China (Yunnan and Sichuan Regions) largest mushroom producers.
- 80% of EU-imports from CN.
- Tobacco also widely cultivated in Yunnan.
- Nicotine is a naturally occurring alkaloid in tobacco (*Nicotiana tabacum*) where it occurs at concentrations ranging from 2% to 8%.
- CN-Authorities and EU food and drink industries confederation (CIAA) have started actions to elucidate the source of Nicotine in dried mushrooms.
Source of contamination

not clarified yet,…

...many possibilities...

- Cross-contamination in drying/packing sites may be an issue
- Intentional use as pesticide in growing areas or during storage
- Natural occurrence in mushrooms
- Natural formation during drying process
- Contamination during harvest via contact with smokers’ hands
Harvest season for fresh boletus: mid May - mid October

Boletus are picked, cleaned, selected and dried by home-based units run by the farmers (fresh boletus quickly spoils and must be processed ASAP)

Fresh boletus is dried using home made drying equipment (an open-bottom board with many bamboo drawers). Boletus are put fresh into the drawers and wood fire is lighted beneath. (are residuals of tobacco plants also disposed this way?)

The drying process for a batch of 200 kg fresh boletus takes around 6 hours and is controlled by experienced humans. 8-10 kg fresh boletus give ca. 1 kg dried product with ca. 11% residual water

Post-harvest treatments before exporting are common a. with fumigants to kill the worms. b. by $^{60}$Co-irradiation to avoid worm breeding.
Other drying practices...
Traditional tobacco drying in China

Source: http://library.duke.edu/digitalcollections
Tobacco Crop

Crop duration: 9 months

<table>
<thead>
<tr>
<th>Accumulation in soil (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
</tr>
<tr>
<td>Cotinine</td>
</tr>
</tbody>
</table>

Concentration of Nicotine in tobacco leaves $\rightarrow$ $\sim$ 20,000 mg/kg (Dry weight)
Nicotine in Mushrooms - Risk assessment

**EFSA:**
ARfD: 0.0008 mg/kg body weight; ADI: 0.0008 mg/kg bw per day

- Safe concentration in fresh mushrooms 0.036 mg/kg (=highest level not exceeding ARfD for Italian consumer)

Evaluation of data submitted by EU food and drink industries confederation (CIAA) and official labs: 99% of dried mushroom samples contained Nicotine (conc. often above 1 ppm)

"Any effects of eating contaminated wild mushrooms are likely to be mild and would be short term, possibly including increased heart rate, dizziness and headaches"

**Source:**
Statement of EFSA on the risks for public health due to the presence of nicotine in wild mushrooms, 7 May 2009
Nicotine Monitoring:
EU-wide monitoring program for Nicotine in wild mushrooms (imported + domestic)
- ca. 1000 samples (= 1 sample/10 tons in 2009)

Temporary measures:
Mushrooms containing Nicotine levels above the following values should be withdrawn from the market and safely disposed of (as a precautionary step):
- Fresh wild mushrooms : 0.04 mg/kg
- Dried wild mushrooms other than Ceps (=porcini=boletus): 1.2 mg/kg
- Dried Ceps (Boletus): 2.3 mg/kg

MRL-Setting:
It is intended to use the monitoring results for setting an MRL for N. under the provisions of Reg. 396/2005/EC (foreseen at the end of 2009)

Source: Guidelines as regards measures to be taken as regards the presence of nicotine in wild mushrooms agreed by the Standing Committee of the Food Chain and Animal Health (SCoFCAH) on 11 May 2009
Other Crops with Nicotine levels

Nicotine naturally occurs at low levels in various solanaceous crops:

• Potatoes, tomatoes, eggplants, sweet pepper (<10µg/kg)
• Egg-plants (<100 µg/kg)

Intake of nicotine via:
- solanaceous crops: ca. 1-2 µg/d on average
- passive smoking e.g. 80 µg/d,
- smoking= No of cigarettes X 1 mg /d)

Also, intestinal absorption is poor compared to absorption through lungs

Nicotine levels were also reported in Tea (up to 1.6 mg/kg )
Thought to be a result of nicotine spraying

More Info:
http://www.norden.org/da/publikationer/publikationer/2003-531/at_download/publicationfile
www.foodstandards.gov.au/_srcfiles/P278_Nicotine_FAR_Final.pdf

Michelangelo Anastassiades and Amadeo R. Fernández-Alba
Nicotine in Mushrooms

Analysis

NICOTINE
NICOTINE PROPERTIES

• **Basic:**
  pK\(_a\)_1 = 3.1; pK\(_a\)_2 = 8.2
  (i.e. predominantly protonated at pH<8.2 and double protonated at pH<3.1)

• **Polar:**
  logP = 0.93 (25 °C/unionised), the lower the pH the lower the logP

• **Volatile:**
  P\text{vap} = 5.6 Pa (25 °C). Evaporation losses reduced at low pH (ionized)

Volatility Comparison:

Biphenyl: 30 Pa (20°C); Dichlorvos: 2.1 Pa (25°C); Methamidophos: 0.0023 Pa (20°C)

QuEChERS – Optimisation of pH at extraction/partitioning step

Recovery of Nicotine from fresh mushrooms at different pH (spiked residues)

Using Nicotine-D3 as ISTD, losses were compensated (rec. 93 - 120 %)
On average 106%

Michelangelo Anastasiades and Amadeo R. Fernández-Alba
Extraction of incurred residues from Dried Boletus (QuEChERS, variation of pH)

**Extraction rates of Nicotine from Porcini using QuEChERS**

*(incurred residues)*

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**Graph:**

- **Y-axis:** area/area ISTD
- **X-axis:** pH-Value (natural, 8, 9, 10, 11)

The graph shows the extraction rates of nicotine from porcini mushrooms using the QuEChERS method at different pH values. The extraction rates increase with higher pH values, with the highest rates observed at pH 11.
Extraction of incurred residues from Dried Porcini (QuEChERS pH 10, variation of Temperature and Time)

Extraction rates of Nicotine from Porcini using QuEChERS (incurred residues)

<table>
<thead>
<tr>
<th>Extraction time</th>
<th>area/area ISTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min (RT)</td>
<td>2.0</td>
</tr>
<tr>
<td>10 min (RT)</td>
<td>2.0</td>
</tr>
<tr>
<td>30 min (RT)</td>
<td>2.0</td>
</tr>
<tr>
<td>1 min Ultra-Turrax (RT)</td>
<td>2.0</td>
</tr>
<tr>
<td>20 min (60°C)</td>
<td>2.0</td>
</tr>
<tr>
<td>20 min (90°C)</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Michelangelo Anastasiades and Amadeo R. Fernández-Alba
Nicotine w. modif. QuEChERS (CRL-SRM version)

10 g fresh mushrooms or (2 g dried sample + 10 mL water)

Bring pH to 10-11 by addition of NaOH 5N

Add 10 mL acetonitrile

Add 4 g MgSO₄ + 1g NaCl

Centrifugue 5 min. at 3500 r.p.m.

Add 150 mg MgSO₄ anh. + 50 mg PSA per mL extract

Centrifugue 5 min. at 3500 r.p.m.

Take aliquot and acidify to pH~5 w. 5% formic acid in MeCN

Analysis via GC or LC

Validation (n=5) using Champignons 10 g spiked at 0.1 µg/kg

Mean Recovery: 106%

RSD: 4.1%

Optionally add isotopically labelled Nicotine (will match for recovery and matrix effects).
- if pH is not adjusted, isotope-labelled ISTD is essential to correct for the low recoveries

Typically ca. 300-400 µL for dried mushrooms (check before)

Shake 1 min by hand

Shake 1 min by hand

Shake for 30s

If not added earlier isotop.-labelled nicotine can also be added here assuming 1g sample per mL extract (will match for matrix effects only)
Impact of d-SPE Cleanup with PSA

Amount of co-extractives following extract evaporation (mg/mL)
(Extract of dried porcini 2g/10 mL)

<table>
<thead>
<tr>
<th>mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

Raw Extract | After Cleanup w. d-SPE
15 g Sample
Adjust pH to 9 with NH3
+ 15 mL MeCN
+ 6 g MgSO₄ anhydrous
Centrifuge 5 min. at 3700 r.p.m.
Add 750 mg MgSO₄ anh. + 250 mg PSA
Centrifuge 5 min. at 3700 r.p.m.
Evaporation following addition of formic acid (pH=3-4)
Reconstitution 100% H₂O milliQ
Evaporate to dryness and reconstitute with AcN:H₂O (1:9)
Analysis

Extraction at basic pH (pH=9)
Nicotine via EtAc Method

10 g Sample plus mL ethylacetate

Shake vigorously 3 s by hand

Add 1.5 g NaCl + 8 g MgSO₄ anhydrous

Shake 15 minutes automatically in Agitax

Centrifuge 5 min. at 3700 r.p.m.

Extraction at basic pH (pH=10)

Analysis in GC/MS
Method Validation

NOTE: cross-contamination of low levels of nicotine within the lab to be considered when evaluating residues!
# Method Validation

<table>
<thead>
<tr>
<th>Method</th>
<th>Mushroom Spiked @ 10 µg/kg</th>
<th>Mushroom Spiked @ 20 µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-TOF-MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC-MS/MS(IT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QuEChERS</td>
<td>Nicotine QuEChERS</td>
<td>EtAc Method</td>
</tr>
<tr>
<td>Quantification with external standard</td>
<td>54 (±10)</td>
<td>105 (±6)</td>
</tr>
<tr>
<td>Quantification with internal standard (Nicotine methyl-d₃)</td>
<td>110 (±8)</td>
<td>No</td>
</tr>
<tr>
<td>LOD (µg/kg)</td>
<td>Limit Of Determination</td>
<td>3</td>
</tr>
</tbody>
</table>
### GC-IT-MS/MS

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Retention Time (min)</th>
<th>Ionization mode</th>
<th>Precursor Ion (m/z)</th>
<th>Excitation Storage Level (m/z)</th>
<th>Excitation Amplitude (Volts)</th>
<th>Fragments Ions</th>
<th>Waveform Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>10.03</td>
<td>CI</td>
<td>163</td>
<td>71.8</td>
<td>53.00</td>
<td>106, 163, 132</td>
<td>Non-resonant</td>
</tr>
<tr>
<td>Nicotine-d₃</td>
<td>10.06</td>
<td>CI</td>
<td>166</td>
<td>73.1</td>
<td>55.00</td>
<td>106, 107, 132, 166</td>
<td>Non-resonant</td>
</tr>
<tr>
<td>TPP</td>
<td>30.9</td>
<td>EI</td>
<td>326</td>
<td>100.0</td>
<td>82.00</td>
<td>169, 289, 228</td>
<td>Non-resonant</td>
</tr>
</tbody>
</table>

*In bold quantification ion*
**Oven Program**

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Rate (°C/min)</th>
<th>Hold (min)</th>
<th>Total (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>200</td>
<td>8.0</td>
<td>0.00</td>
<td>16.00</td>
</tr>
<tr>
<td>290</td>
<td>30.0</td>
<td>0.00</td>
<td>19.00</td>
</tr>
<tr>
<td>300</td>
<td>30.0</td>
<td>5.00</td>
<td>24.33</td>
</tr>
</tbody>
</table>

Column flow: 1 ml/min

Column used: HP-5MSI 30x 0.250x 0.25
### GC-IT-MS

#### PTV Program

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Rate (°C/min)</th>
<th>Hold (min)</th>
<th>Total (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>300</td>
<td>80</td>
<td>10.00</td>
<td>13.38</td>
</tr>
</tbody>
</table>
NICOTINE CHROMATOGRAM AND SPECTRUM (GC-MS/MS)
Identification of nicotine in a mushroom extract at 20 µg/kg

**GC-MS/MS (IT)- PCI with methanol**

- **Total Ion Chromatogram**
- **Extracted Ion Chromatogram**
  - m/z 106
- **Nicotine MS/MS spectrum**
  - m/z 183
Identification of nicotine in a mushroom extract at 20 µg/kg

Nicotine Full scan CI spectrum

Precursor ion [M+H]^+
Identification of Nicotine- methyl d₃ in mushroom extract spiked at 20 µg/Kg

**Total Ion Chromatogram PCI**

**Nicotine- methyl d₃**

**Extracted Ion Chromatogram m/z 106**

**Nicotine-methyl d₃ MS/MS spectrum**
Conditions of the LC-TOF-MS Method

HPLC Parameters

**Injection volume:** 20 µl.
**Flow:** 0.2 ml/min.

**Column:** Zorbax SB-C18 ;5 µm  3 x 250 mm (Agilent  Part Nº: 880975-302).

**Gradient LC elution:**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A% AcN 0.1% formic acid</th>
<th>B % MilliQ Water 0.1% formic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>35</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
TOF-MS Parameters

**Ion Source**: Dual ESI  
**Polarity**: Positive

**Gas Temp**: 300 °C

**Drying Gas**: 9 L/min

**Nebulizer**: 40 psig

**V Cap**: 4000 V  
**Fragmentor**: 190 V  
**Skimmer**: 60 V  
**OCT 1 RF Vpp**: 250 V

Continuous calibration- Reference mases:  
121.0509 m/z  
922.0098 m/z
Identification of Nicotine in mushroom extract spiked at 10 µg/kg

ESI +

Total ion chromatogram

Nicotine

Extracted ion chromatogram

163.1229 ± 0.1 m/z

[M+H]+ 163.1229

Nicotine

Nicotine F1

132.0808

[C₉H₁₀N]⁺
Identification of Nicotine-methyl d3 in mushroom extract spiked at 10 µg/kg

+ESI TIC Scan Frag=190.0V R3 Champi Q_sinT Basico_Acido_NicotineD3.d

Counts vs. Acquisition Time (min)

+ESI EIC(166.1418) Scan Frag=190.0V R3 Champi Q_sinT Basico_Acido_NicotineD3.d Smooth

Counts vs. Mass-to-Charge (m/z)

\[ [\text{M+H}]^+ \quad 166.1418 \]
\[ [\text{C}_{10}\text{H}_{12}\text{D}_3\text{N}_2]^+ \]

Counts vs. Mass-to-Charge (m/z)

Nicotine-methyl d3

166.1418 ± 0.1 m/z

Nicotine-methyl d3 F1

132.0810

[LC-TOF-MS]
Ad-hoc Mini-PT on Nicotine in Mushrooms
(in collaboration with COOP CH)

Final report in CIRCA, go to:
Ad-hoc PT on Nicotine in Mushrooms

Aims:
- Need to check methodologies in view of the EU-monitoring program on nicotine in mushrooms
- Need to settle the dispute with the Chinese colleagues as regards nicotine analysis
- Possibly draw some conclusions as regards the impact of certain analytical steps

Samples: 3 samples with incurred Nicotine
- **Mu Err** powder - China
- **Boletus** powder - China
- **Boletus** powder - Bosnia and Herzegovina

Only limited amount of material available, thus only 14 Laboratories could participate
## PT on Nicotine in dried mushrooms - Overall performance

<table>
<thead>
<tr>
<th></th>
<th>Boletus BiH</th>
<th>Boletus CN</th>
<th>Mu-Err CN</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Qn-RSD (%)</strong></td>
<td>23.6</td>
<td>29.4</td>
<td>23.6</td>
<td>26 %</td>
</tr>
<tr>
<td><strong>Median (mg/kg)</strong></td>
<td>1.01</td>
<td>0.72</td>
<td>2.56</td>
<td></td>
</tr>
<tr>
<td><strong>Acceptable</strong></td>
<td>13</td>
<td>13</td>
<td>12</td>
<td>38 (90%)</td>
</tr>
<tr>
<td><strong>Questionable</strong></td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2 (5%)</td>
</tr>
<tr>
<td><strong>Unacceptable</strong></td>
<td>1</td>
<td>0</td>
<td>1 (false neg.)</td>
<td>2 (5%)</td>
</tr>
</tbody>
</table>

Acceptable: $|z| \leq 2$,  
Questionable: $2 < |z| \leq 3$,  
Unacceptable: $|z| > 3$
PT on Nicotine in dried mushrooms - Z-scores

- z-scores for Boletus mushroom (Bosnia and Herzegovina)
- z-score for Boletus mushroom (China)
- z-score for Mu-Err mushroom (China)

Possibly systematically underestimating

38 Acceptable (= 90%)
2 Questionable (= 5%)
2 Unacceptable (= 5%)

= Methanol employed for extraction
= Petrol ether employed for extraction

All other labs = Variations of QuEChERS
### Findings in boletus (data from pesticides-online)

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>24 (max.: 6.03 mg/kg)</td>
</tr>
<tr>
<td>Bromide (inorg.)</td>
<td>11 (max.: 590 mg/kg)</td>
</tr>
<tr>
<td>Piperonyl Butoxide</td>
<td>5</td>
</tr>
<tr>
<td>Propoxur</td>
<td>5</td>
</tr>
<tr>
<td>Orthophenylphenol</td>
<td>4</td>
</tr>
<tr>
<td>Tetramethrin</td>
<td>4</td>
</tr>
<tr>
<td>Methamidophos</td>
<td>3</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>2</td>
</tr>
<tr>
<td>Triadimefon/Triadimenol (sum)</td>
<td>2</td>
</tr>
<tr>
<td>- Biphenyl</td>
<td></td>
</tr>
<tr>
<td>- DEET</td>
<td></td>
</tr>
<tr>
<td>- Endosulfan (sum)</td>
<td>1</td>
</tr>
<tr>
<td>- Iprobenfos</td>
<td></td>
</tr>
<tr>
<td>- Pirimiphos-methyl</td>
<td></td>
</tr>
<tr>
<td>- Thiophanate methyl</td>
<td></td>
</tr>
<tr>
<td>- Triazophos</td>
<td></td>
</tr>
<tr>
<td>- Metalaxyl (sum)</td>
<td></td>
</tr>
</tbody>
</table>
## Other Residue findings in wild mushrooms - CVUA Stuttgart results in 2009 of 15 dried boletus

<table>
<thead>
<tr>
<th>Compound</th>
<th>Detections</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>15 (15&gt;MRL); (5&gt;2.3 mg/kg temp. MRL)</td>
<td>CN: 0.65-4.8 mg/kg; unknown 0.8-8.2 mg/kg; SB: 0.3 mg/kg</td>
</tr>
<tr>
<td>Propoxur</td>
<td>13 (6&gt;MRL)</td>
<td>All from CN and „unknown“ positive max. 0.31 mg/kg</td>
</tr>
<tr>
<td>Tetramethrin</td>
<td>11 (11&gt;MRL)</td>
<td>max.: 2 mg/kg; MRL=0.01 mg/kg</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>13</td>
<td>max.: 1 mg/kg; MRL= 1 mg/kg</td>
</tr>
<tr>
<td>Permethrin</td>
<td>10 (3&gt;MRL)</td>
<td>max.: 1.4 mg/kg; MRL=0.05 mg/kg</td>
</tr>
<tr>
<td>Prometryn</td>
<td>2</td>
<td>2x Traces &lt;0.01 mg/kg</td>
</tr>
<tr>
<td>Piperonylbutoxide</td>
<td>10</td>
<td>max.: 1.4 mg/kg; no MRL</td>
</tr>
<tr>
<td>Triazophos</td>
<td>1</td>
<td>Traces &lt;0.01 mg/kg</td>
</tr>
<tr>
<td>Fomesafen</td>
<td>1 (1&gt;MRL)</td>
<td>0.11 mg/kg ; MRL=0.01 mg/kg</td>
</tr>
<tr>
<td>Clomazone</td>
<td>1 (1&gt;MRL)</td>
<td>0.1 mg/kg ; MRL=0.01 mg/kg</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>2</td>
<td>max.: 0.055 mg/kg; MRL=0.1 mg/kg</td>
</tr>
<tr>
<td>Quizalofop</td>
<td>1</td>
<td>0.015; MRL=0.05 mg/kg</td>
</tr>
</tbody>
</table>

**Note:** the MRLs apply to fresh products. A processing factor of ~9 from fresh to dried mushrooms is to be considered, however not for pesticides used for stock preservation of dried mushrooms (e.g. pyrethroids and carbamates are typical).

Results by the Confederation of the Food and Drink Industries of the EU (CIAA) in 2008: 176 samples with values between 0.21 and 9.9 mg/kg dried mushrooms.
Thank you very much for your Attention!!