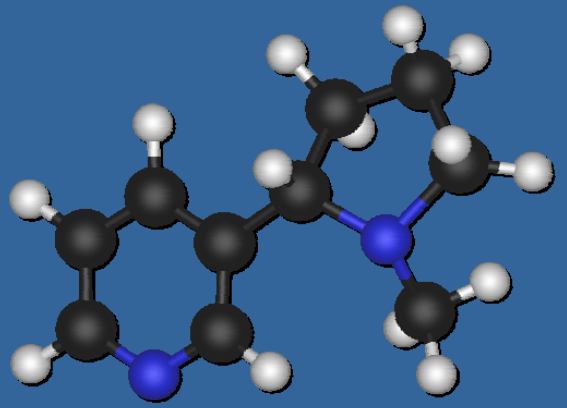
Analysis of Nicotine in Mushrooms



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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

NICOTINE-TOXICOLOGY

- 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)

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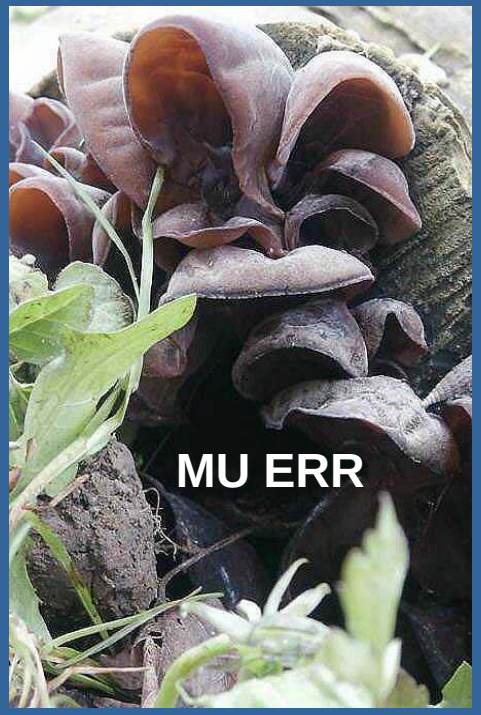
Nicotine in Mushrooms

Background Information



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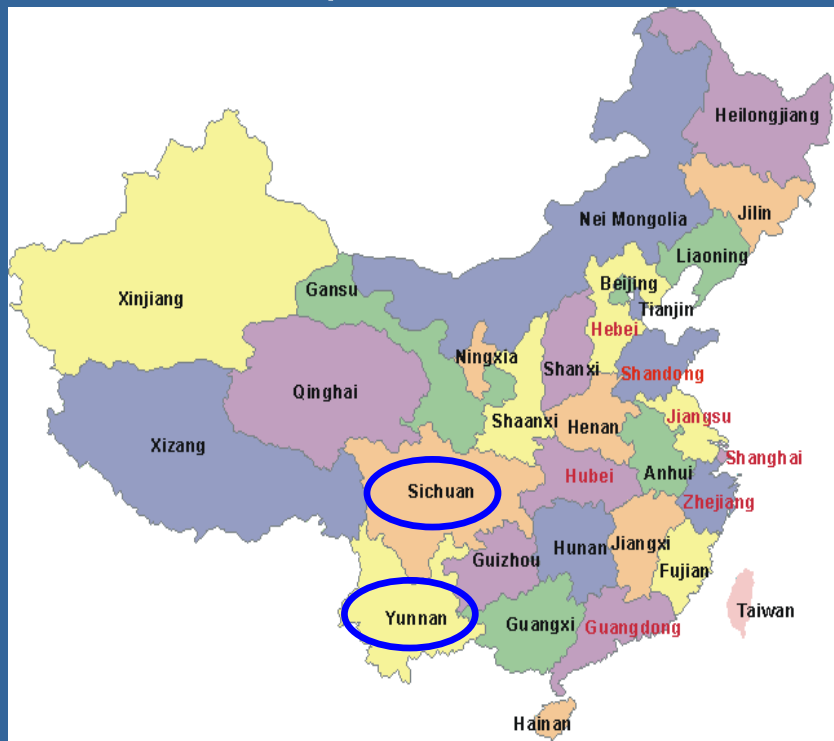
Nicotine in Mushrooms - Background Information:

- 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

Processing Practices in China

Source: http://www.sinohost.com/yunnan_pages/mushrooms/boletuspreparing.html

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.


Drying board


Sorting

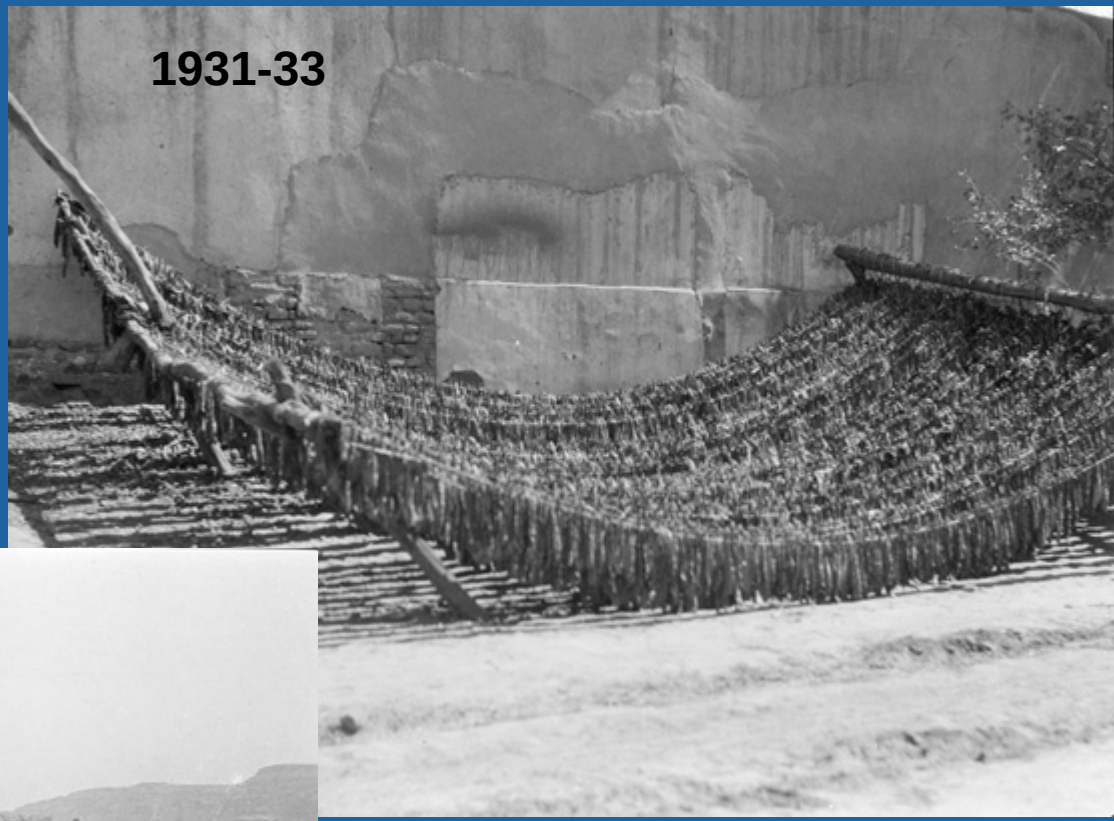

Final product

Chinese Commodity Inspection Bureau (CCIB) issues Quality Certificates

Other drying practices…

Traditional tobacco drying in China

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



Tobacco Crop

Crop duration: 9 months

Accumulation in soil (mg/kg)	
Nicotine	2,6633
Cotinine	0,0102

Concentration of Nicotine in tobacco leaves → ~ 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
http://www.efsa.europa.eu/cs/BlobServer/Statement/Statement_nicotine_mushrooms_ej286_en.pdf?ssbinary=true

Nicotine in Mushrooms – Risk Management

Nicotine Monitoring:

EU-wide monitoring program for Nicotine in wild mushrooms (imported + domestic)

- ca. 1000 samples (= 1 sample/10 tons in 2009)

Temporary measures:

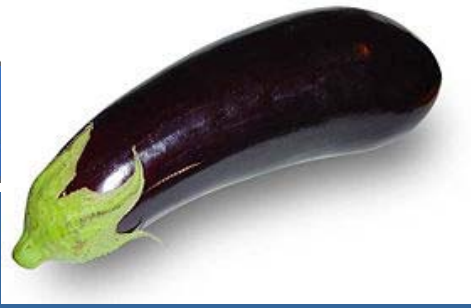
Mushrooms containing Nicotine levels above the following values should be withdrawn from the market and safely disposed of (as a precautionary step):

- *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

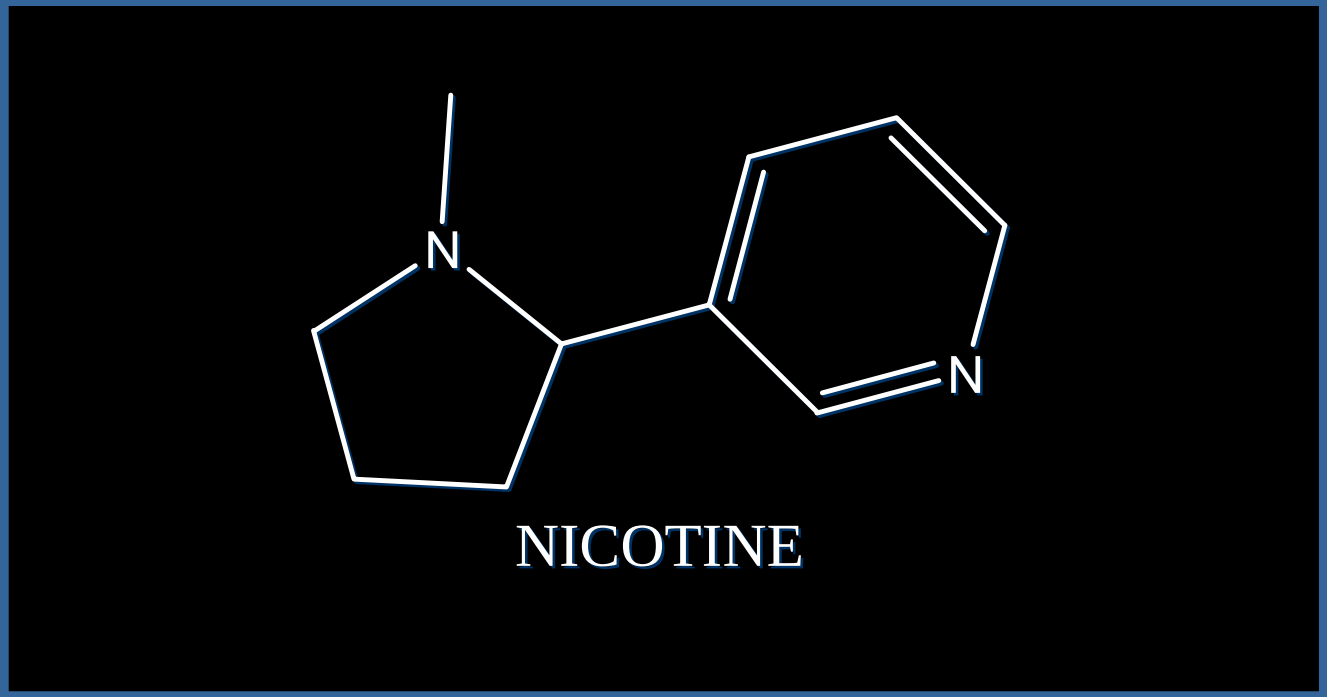
Nicotine levels were also reported in **Tea** (up to 1.6 mg/kg)
Thought to be a result of nicotine spraying

More Info:

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

Nicotine in Mushrooms

Analysis



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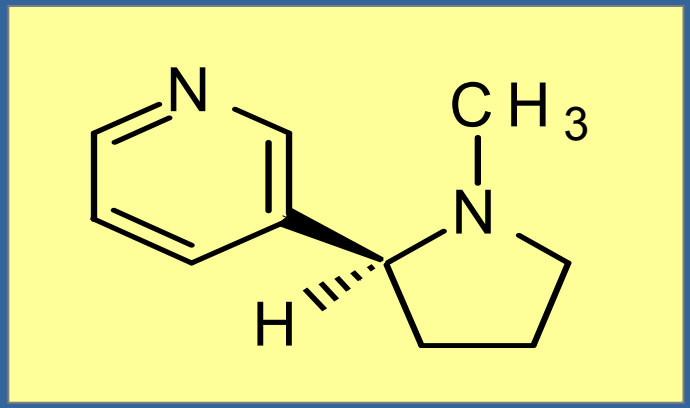
NICOTINE PROPERTIES



- **Basic:**

pKa_1 = 3.1; pKa_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:**

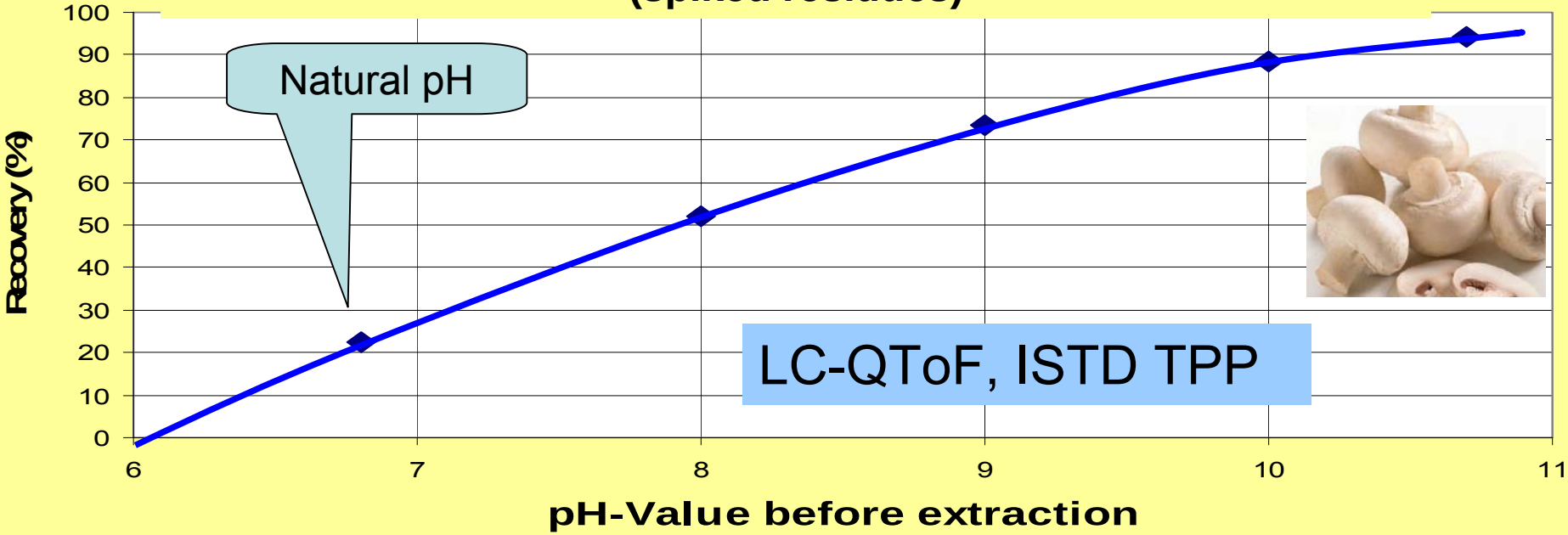
Pvap = 5.6 Pa (25 °C). Evaporation losses reduced at low pH (ionized)

Volatility Comparison:

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

(K. Chamberlain et al., Pestic. Sci., 47, 265 (1996)

QuEChERS – Optimisation of pH at extraction/partitioning step



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

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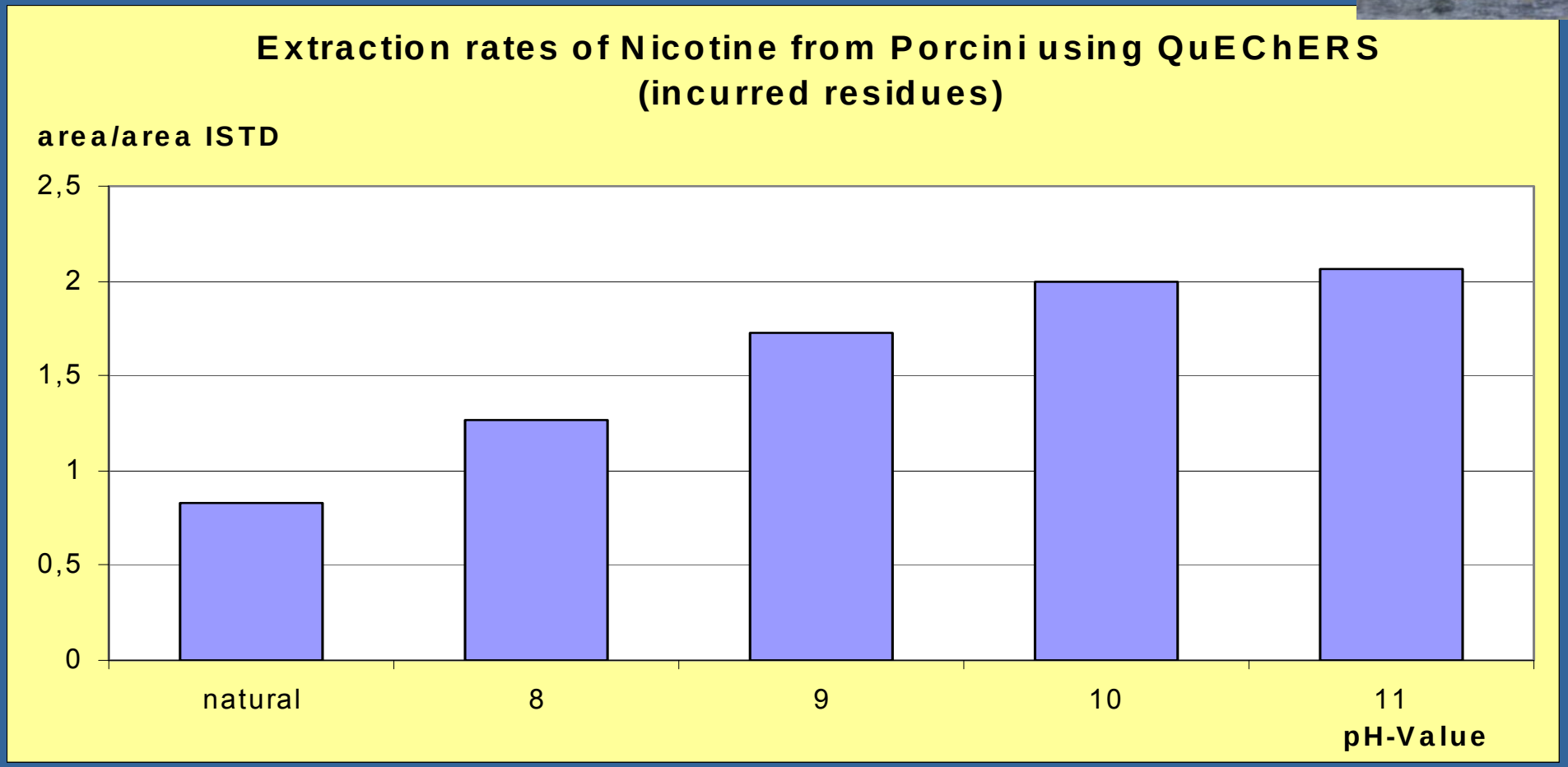
Extraction of incurred residues from Dried Boletus

(QuEChERS, variation of pH)



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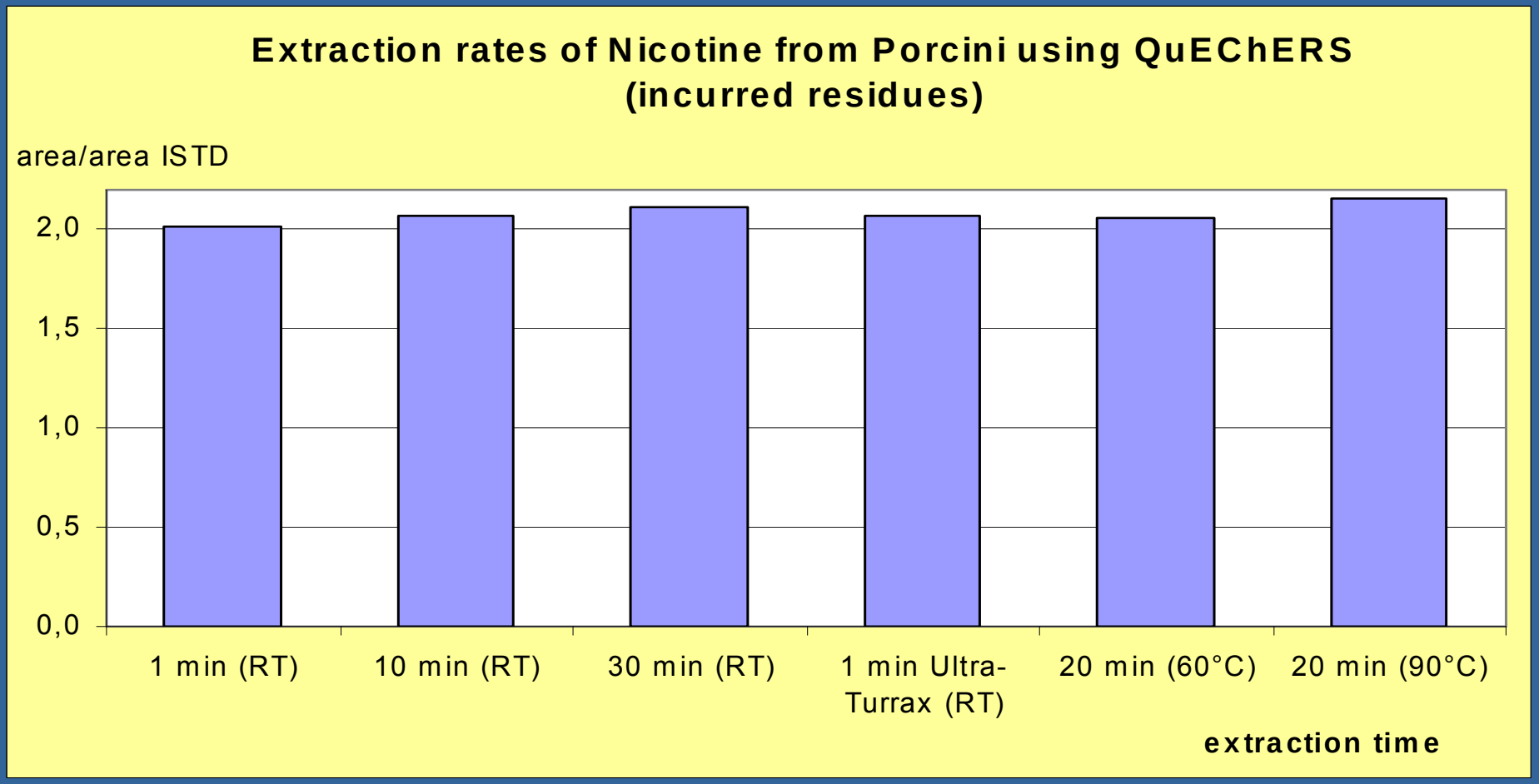
Extraction of incurred residues from Dried Porcini

(QuEChERS pH 10, variation of Temperature and Time)



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Nicotine w. modif. QuEChERS (CRL-SRM version)

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

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

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

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

Add 10 mL acetonitrile

Shake 1 min by hand

Add 4 g $MgSO_4$+ 1g NaCl

Shake 1 min by hand

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

Take aliquot

Add 150 mg $MgSO_4$ anh. + 50 mg PSA per mL extract

Shake for 30s

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

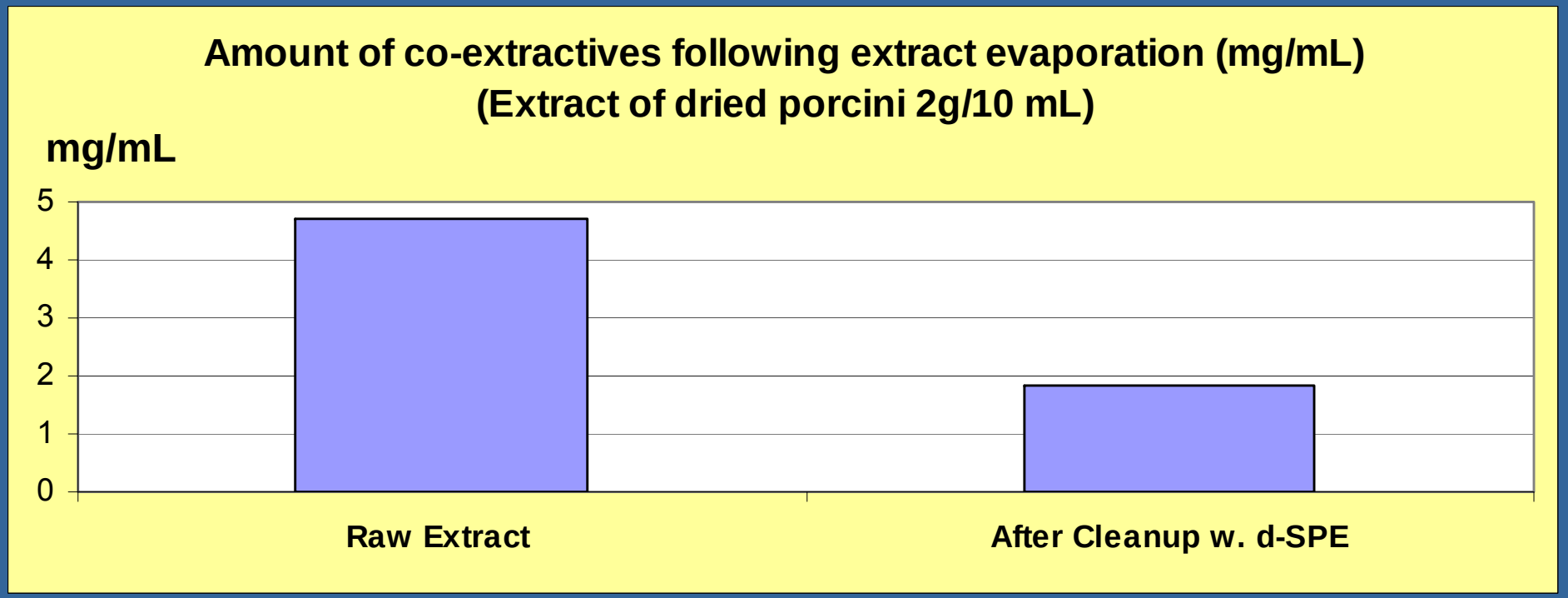
<u>If not added earlier</u> isotop.-labelled nicotine can also be added here assuming 1g sample per mL extract **(will match for matrix effects only)**

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

Mean Recovery: 106%

RSD: 4.1%

Impact of d-SPE Cleanup with PSA



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Nicotine with modif. QuEChERS (CRL-FV version)

15 g Sample

Adjust pH to 9 with NH3

+ 15 mL MeCN

+ 6 g $MgSO_4$ anhydrous

Shake automatically in Agitax

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

Take 5 ml aliquot

Add 750 mg $MgSO_4$ anh. + 250 mg PSA

Shake 20s in Vortex

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

Evaporation following addition of formic acid (pH=3-4)

Evaporate to dryness and reconstitute with AcN:H_2O (1:9)

Analysis

Extraction at basic pH (pH=9)

Reconstitution 100% H_2O milliQ

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Nicotine via EtAc Method

10 g Sample
plus mL ethylacetate

Shake vigorously 3 s by hand

Extraction at basic pH (pH=10)

Add 1.5 g NaCl + 8 g $MgSO_4$ anhydrous

Shake 15 minutes automatically in Agitax

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

Analysis in GC/MS

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Method Validation

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

Method Validation

		LC-TOF-MS Mushroom spiked @ 10 µg/kg		GC-MS/MS(IT) Mushroom spiked @ 20 µg/kg	
		QuEChERS	**Nicotine QuEChERS**	**EtAc Method**	**Nicotine EtAc Method**
RECOVERIES R(±RSD)% (n=4)	Quantification with **external standard**	**54 (±10)**	**105 (±6)**	**25 (±11)**	**80 (±7)**
	Quantification with **internal standard** (Nicotine methyl-d_3)	**110 (±8)**	**No**		**No**
LOD (µg/kg) Limit Of Determination		**3**		**4**	

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GC-IT-MS/MS

Compound name	Retention Time (min)	Ionization mode	Precursor Ion (m/z)	Excitation Storage Level (m/z)	Excitation Amplitude (Volts)	Fragments Ions	Waveform Type
Nicotine	10.03	CI	163	71.8	53.00	**106**, 163, 132	Non-resonant
Nicotine-d_3	10.06	CI	166	73.1	55.00	**106**, 107, 132,166	Non-resonant
TPP	30.9	EI	326	100.0	82.00	**169**, 289, 228	Non-resonant

In bold quantification ion

Oven Program

Temp (ºC)	Rate (ºC/min)	Hold (min)	Total (min)
80		1.00	1.00
200	8.0	0.00	16.00
290	30.0	0.00	19.00
300	30.0	5.00	24.33

Column flow: 1 ml/min

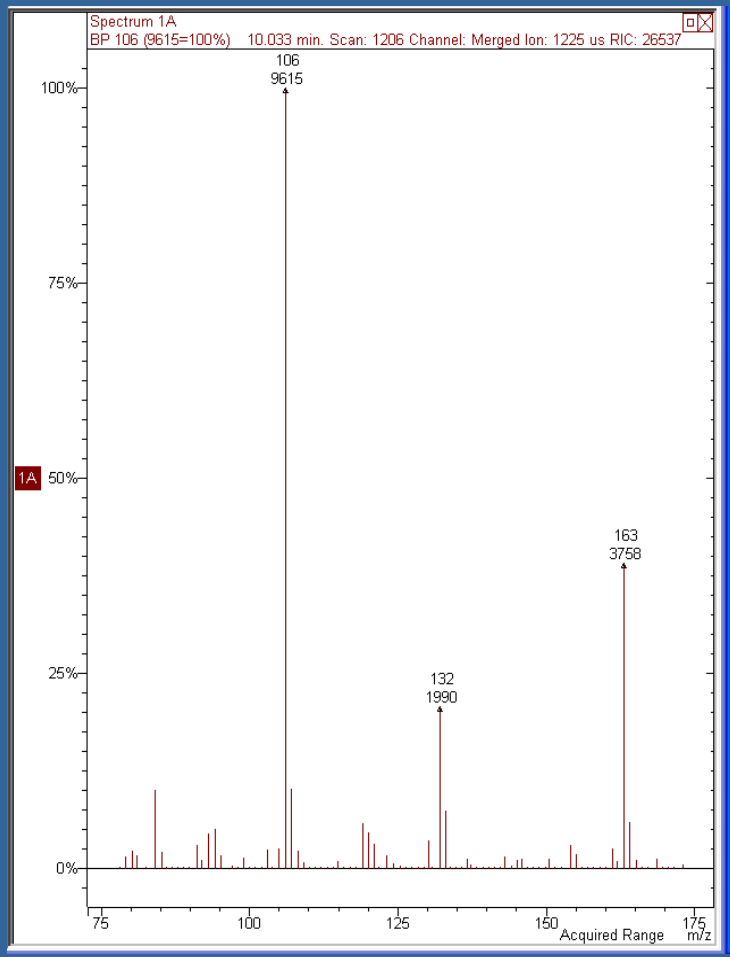
Column used: HP-5MSI 30x 0.250x 0.25

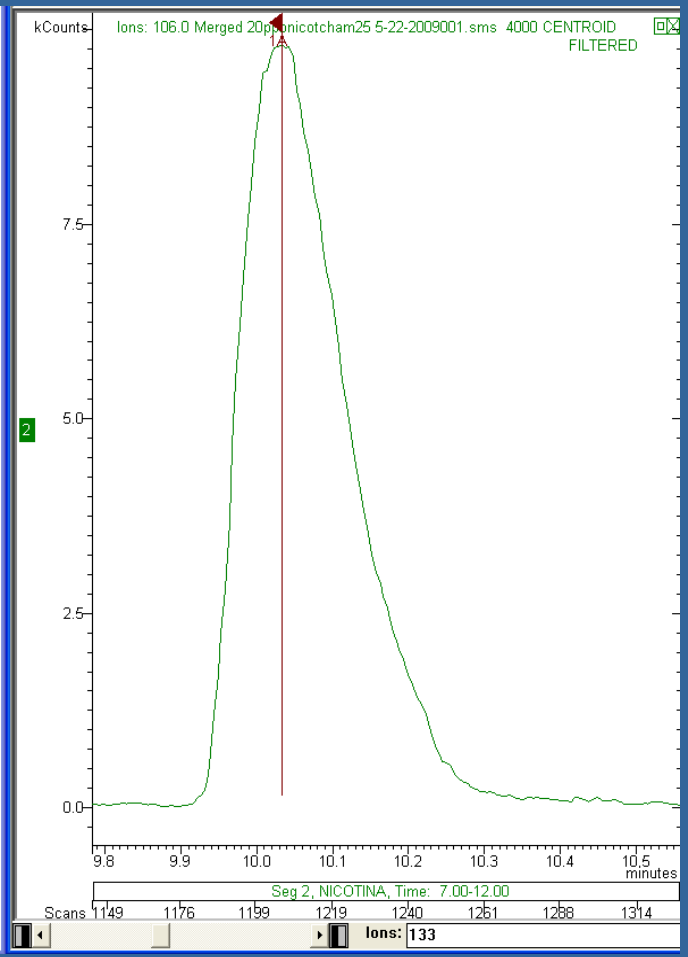
GC-IT-MS

PTV Program

Temp (ºC)	Rate (ºC/min)	Hold (min)	Total (min)
70		0.50	0.50
300	80	10.00	13.38

NICOTINE CHROMATOGRAM AND SPECTRUM (GC-MS/MS)





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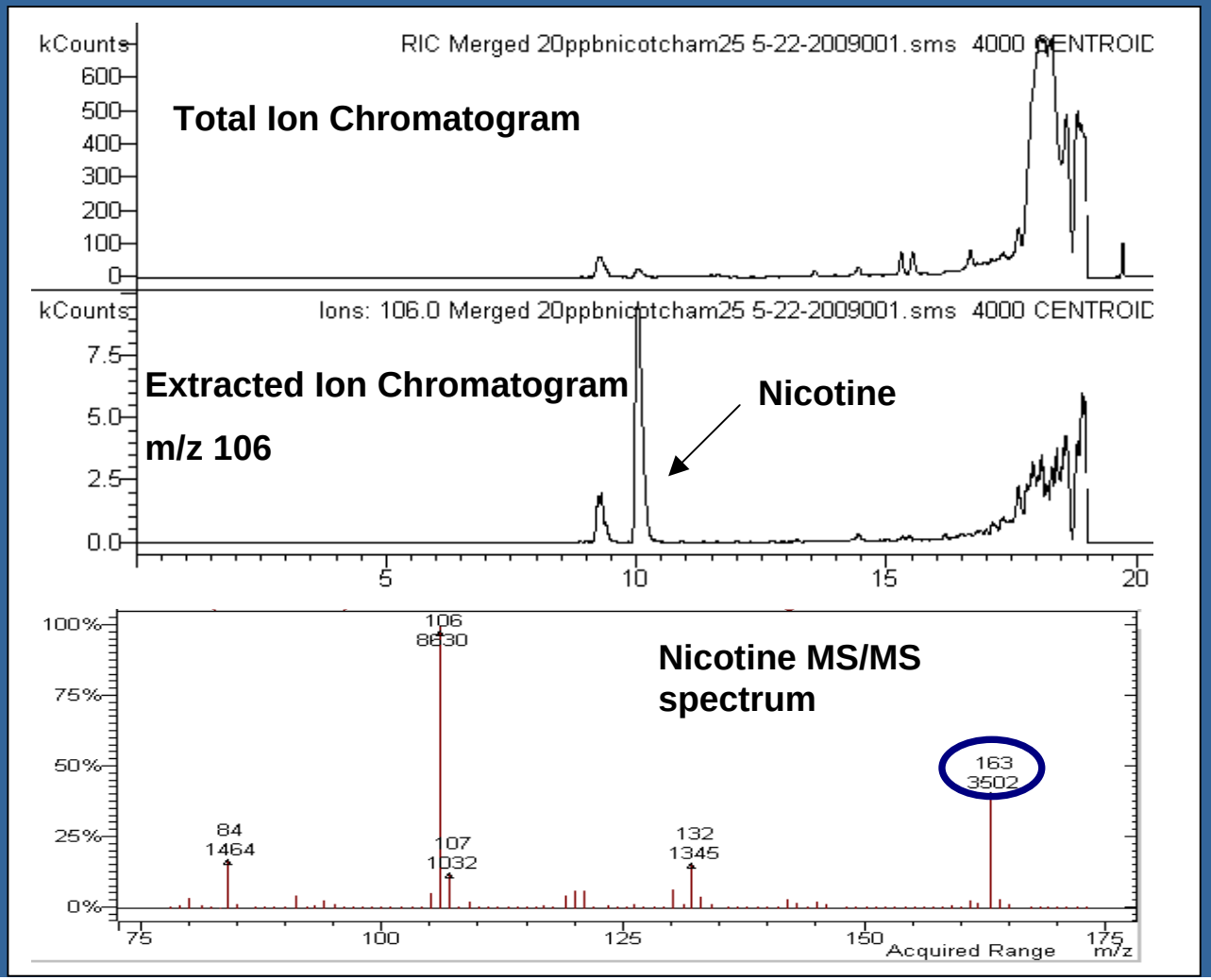
GC-MS/MS (IT)- PCI with methanol

Identification of nicotine in a mushroom extract at 20 µg/kg



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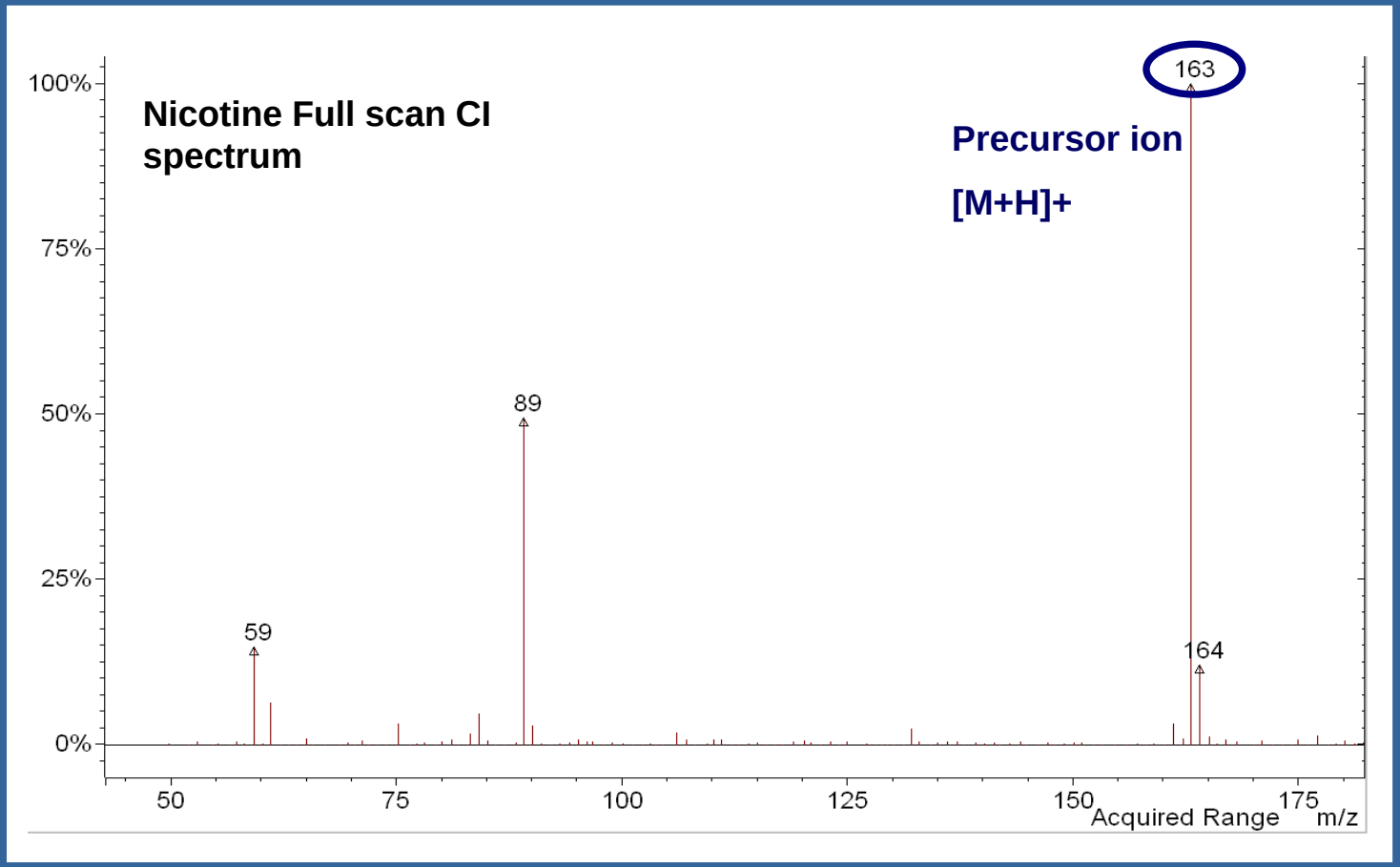
GC-MS/MS (IT)- PCI with methanol

Identification of nicotine in a mushroom extract at 20 µg/kg



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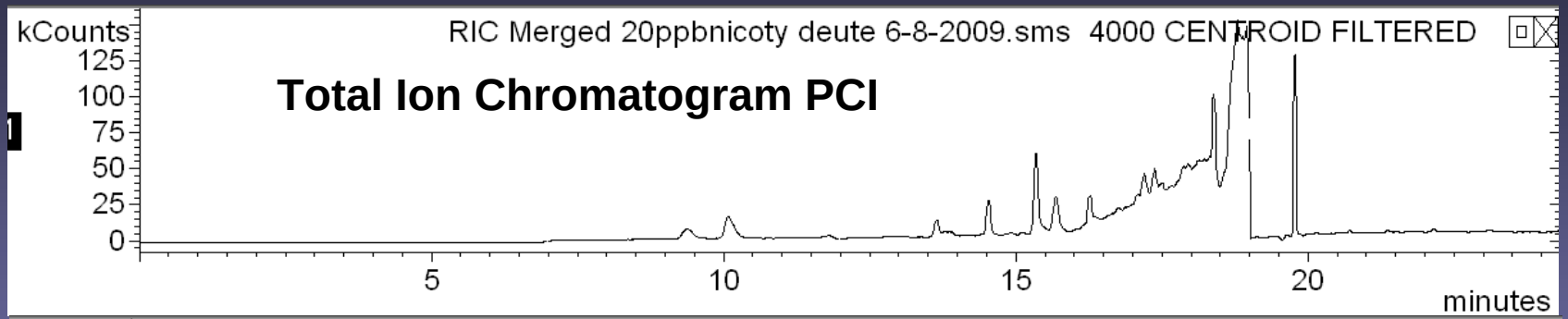
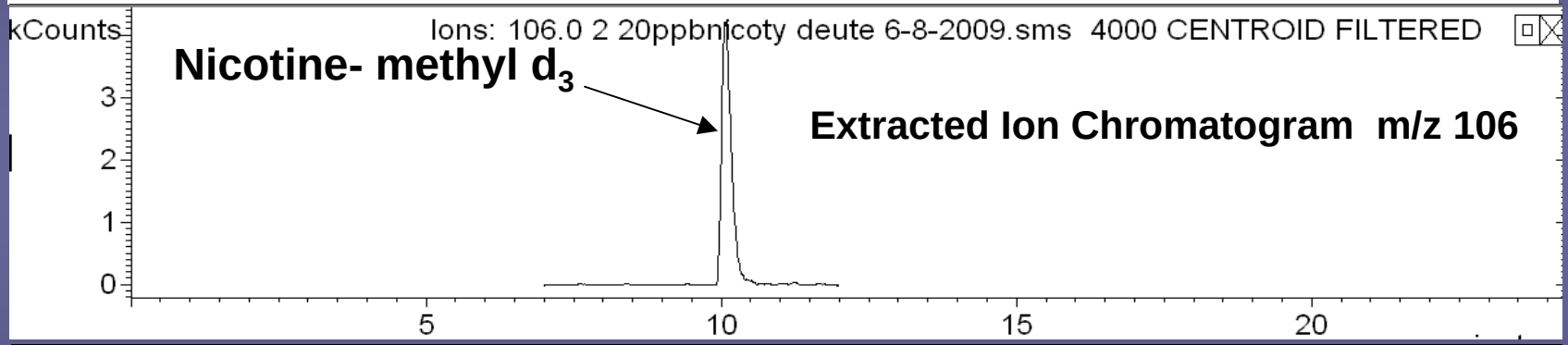
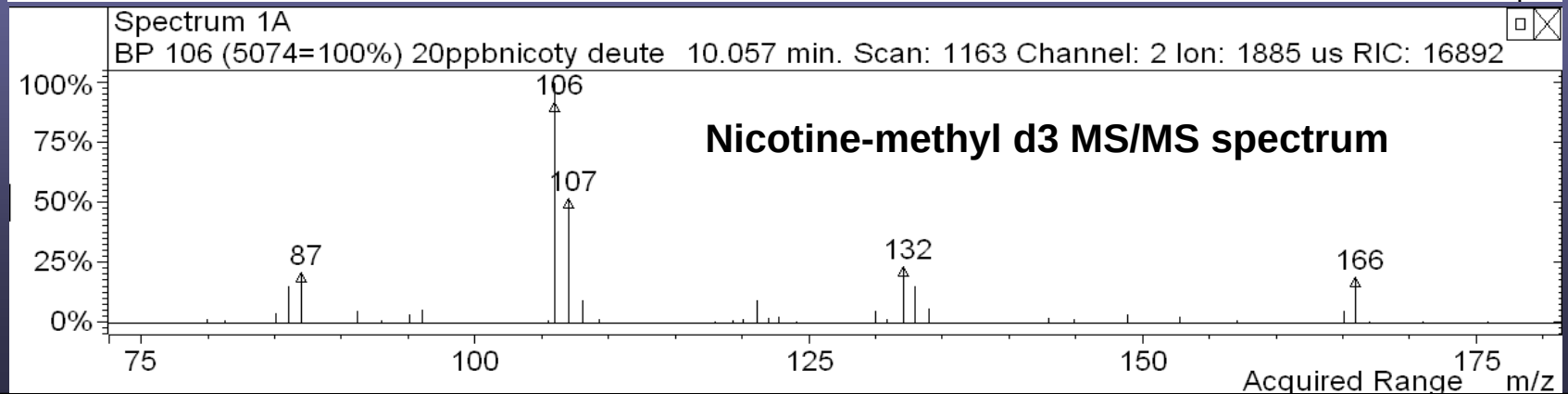
GC/MS/MS (IT)

Identification of Nicotine- methyl d_3 in mushroom extract spiked at 20 µg/Kg







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:

Time (min)	A% AcN 0.1% formic acid	B % MilliQ Water 0.1% formic acid
0	10	90
2	10	90
20	100	0
35	100	0

TOF-MS Parameters

Ion Source: Dual ESI
Polarity: Positive

Gas Temp: 300 ºC

Drying Gas: 9 L/min

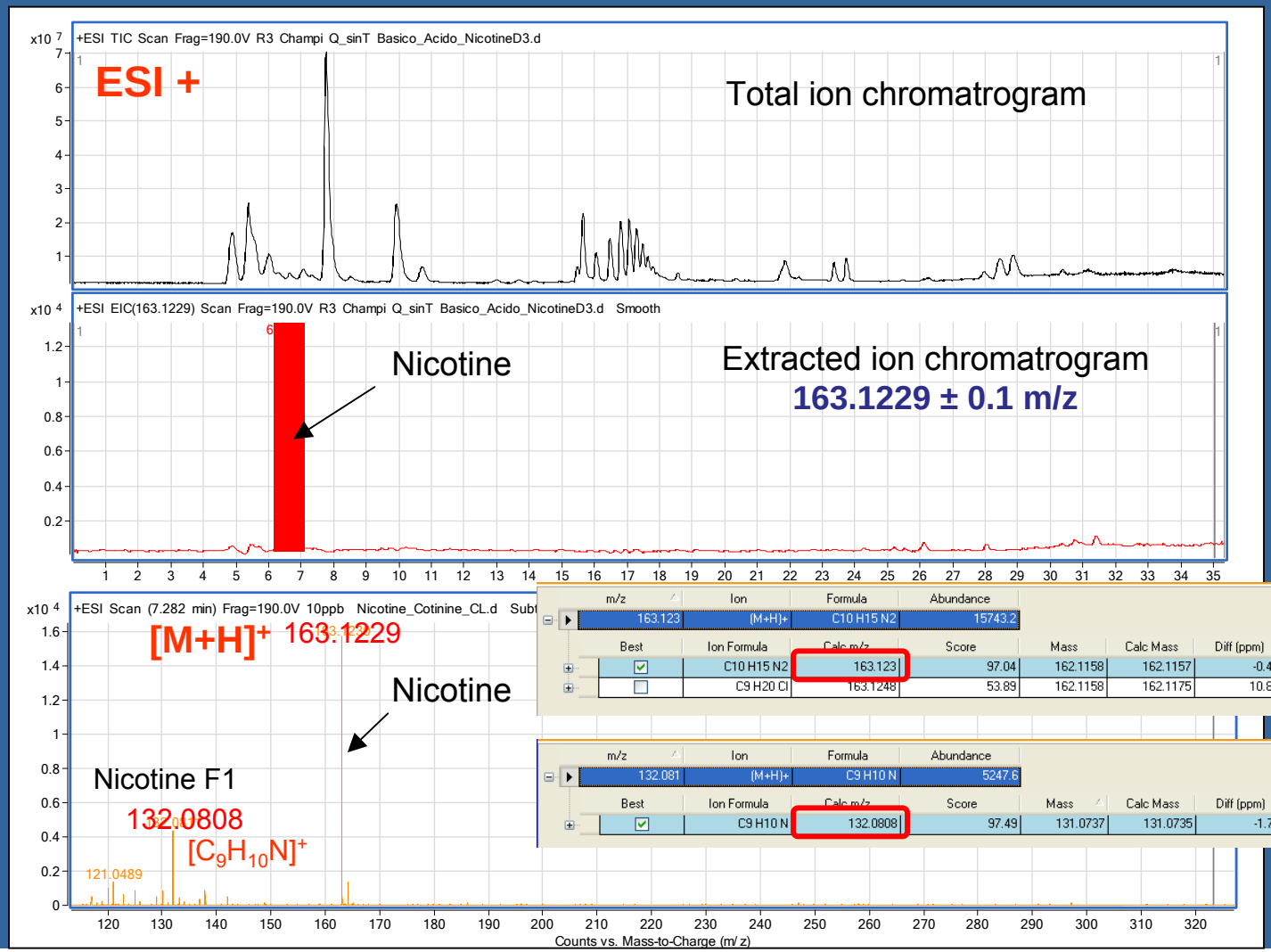
Nebulizer: 40 psig

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

LC-TOF-MS

Identification of Nicotine in mushroom extract spiked at 10 µg/kg



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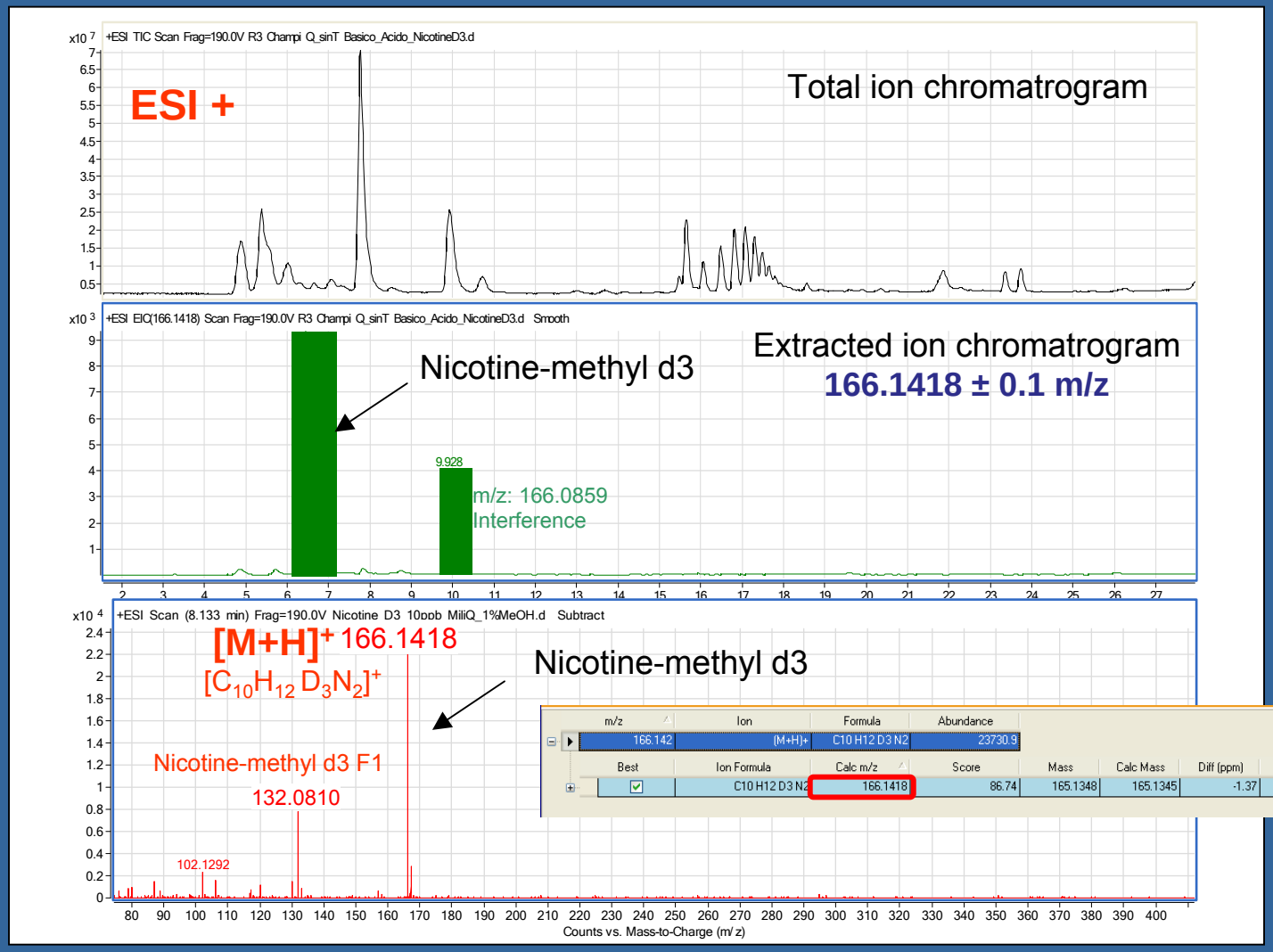
LC-TOF-MS

Identification of Nicotine-methyl d3 in mushroom extract spiked at 10 µg/kg



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Ad-hoc Mini-PT on Nicotine in Mushrooms

(in collaboration with COOP CH)

Final report in CIRCA, go to:
http://www.crl-pesticides.eu/docs/public/tmplt_article.asp?LabID=200&CntID=706&Theme_ID=1&Pdf=False&Lang=EN

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,

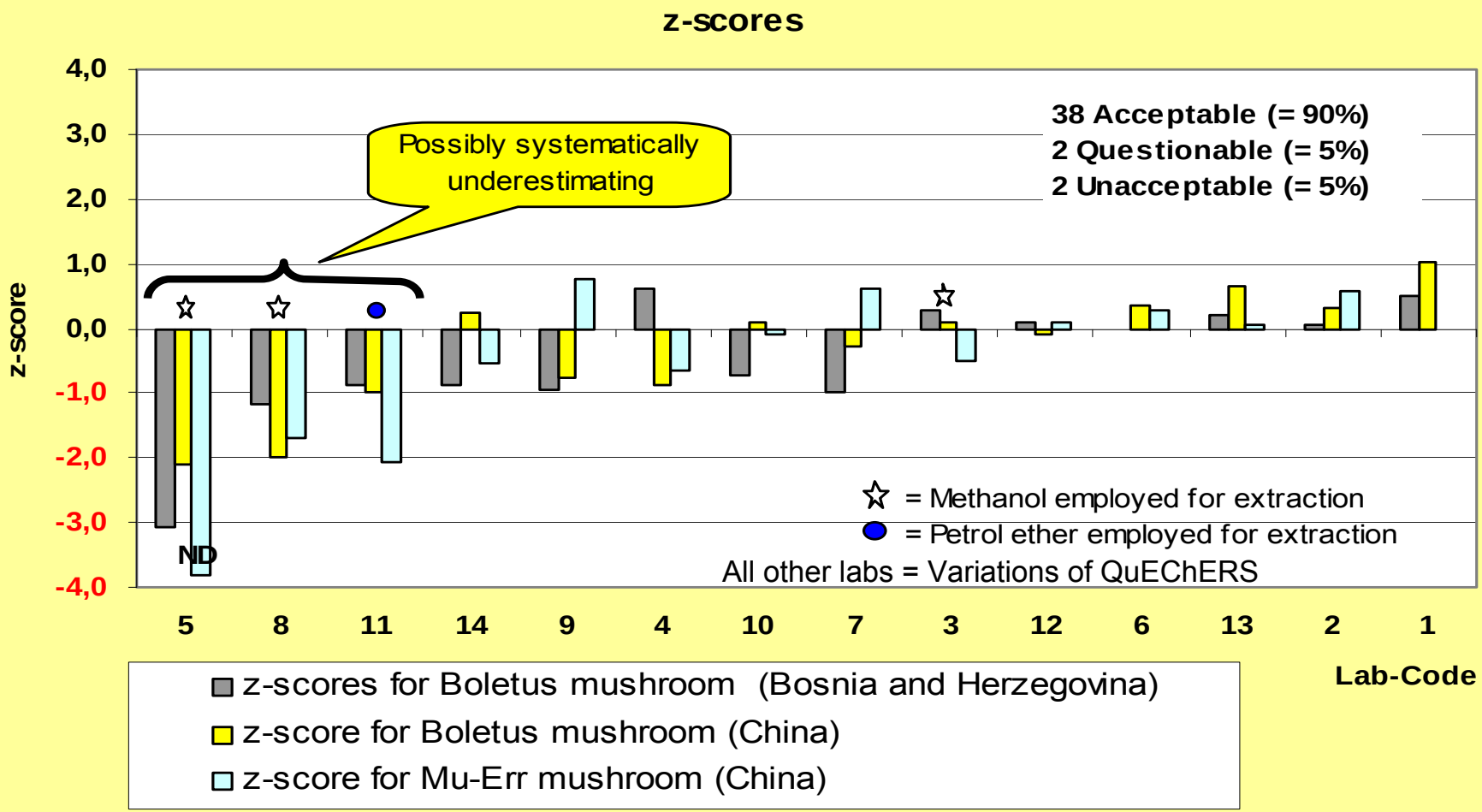
thus only 14 Laboratories could participate

PT on Nicotine in dried mushrooms- Overall performance

	Boletus BiH	Boletus CN	Mu-Err CN	Overall
Qn-RSD (%)	*23.6*	*29.4*	*23.6*	*26 %*
Median (mg/kg)	*1.01*	*0.72*	*2.56*	
Acceptable	13	13	12	**38 (90%)**
Questionable	0	1	1	**2 (5%)**
Unacceptable	1	0	1 (false neg.)	**2 (5%)**

Acceptable: $|z| \leq 2$,
Questionable $2 < |z| \leq 3$,
Unacceptable $|z| > 3$

PT on Nicotine in dried mushrooms - Z-scores



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Findings in boletus (data from pesticides-online)

Compound	No. of findings
Nicotine	24 (max.: 6.03 mg/kg)
Bromide (inorg.)	11 (max.: 590 mg/kg)
Piperonyl Butoxide	5
Propoxur	5
Orthophenylphenol	4
Tetramethrin	4
Methamidophos	3
Carbendazim	2
Triadimefon/Triadimenol (sum)	2
- Biphenyl - DEET - Endosulfan (sum) - Iprobenfos - Pirimiphos-methyl - Thiophanate methyl - Triazophos - Metalaxyl (sum)	1

Other Residue findings in wild mushrooms -
CVUA Stuttgart results in 2009 of 15 dried boletus

Compound	Detections	Remarks
Nicotine	15 (15>MRL); (5>2.3 mg/kg temp. MRL)	**CN: 0,65-4.8 mg/kg; unknown 0.8-8.2 mg/kg; SB: 0.3 mg/kg**
Propoxur	13 (6>MRL)	**All from CN and „unknown“ positive max. 0.31 mg/kg**
Tetramethrin	11 (11>MRL)	**max.: 2 mg/kg; MRL=0.01 mg/kg**
Cypermethrin	13	**max.: 1 mg/kg; MRL= 1 mg/kg**
Permethrin	10 (3>MRL)	**max.: 1.4 mg/kg; MRL=0.05 mg/kg**
Prometryn	2	**2x Traces <0.01 mg/kg**
Piperonylbutoxide	10	**max.: 1.4 mg/kg; no MRL**
Triazophos	1	**Traces <0.01 mg/kg**
Fomesafen	1 (1>MRL)	**0.11 mg/kg ; MRL=0.01 mg/kg**
Clomazone	1 (1>MRL)	**0.1 mg/kg ; MRL=0.01 mg/kg**
Carbendazim	2	**max.: 0.055 mg/kg; MRL=0.1 mg/kg**
Quizalofop	1	**0.015; MRL=0.05 mg/kg**

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!!