PROFICIENCY TEST

Nicotine Residues in Dried Mushrooms

FINAL REPORT

CRL for Pesticide Analysis
using Single Residue Methods
(CRL-SRM)

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Organizer

Dr. Michelangelo Anastassiades

CRL for Single Residue Methods

Schaflandstrasse 3/2

D-70736 Fellbach

Germany

Phone: +49-711-3426-1124

Fax: +49-711-588176

E-Mail: **Michelangelo.Anastassiades@cvuas.bwl.de**

Co-Organizer

Dr. Marc Lutz

Central Laboratory and Quality Centre

COOP

Gottesackerstrasse 4

CH-4133 Pratteln,

Switzerland

Phone: +41 61 825 45 56/Fax 45 50

Fax: +41 61 825 45 50

E-Mail: **Marc.Lutz@coop.ch**

http://www.crl-pesticides.eu

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INTRODUCTION

Following frequent detections of nicotine in dried mushrooms, mainly of Chinese origin, , at levels of toxicological concern in 2008 and 2009, DG-SANCO and the European Food Safety Authority (EFSA) assessed the situation, and guideline temporary limits for nicotine levels in fresh and dried wild mushrooms were set (0.04 mg/kg in fresh wild mushrooms, 1.2 mg/kg in dried wild mushrooms other than boletus (ceps), 2.3 mg/kg in wild dried boletus)[1][2]. It was tated that, as a precautionary step, mushrooms containing nicotine above these levels should be withdrawn from the market and safely disposed of. It was furthermore decided to put a monitoring program for nicotine in wild mushrooms in place with the EU Member States having to test 1000 samples of EU and imported wild mushrooms. The information collected should be used to establish official MRLs for this substance in mushroom but also to assess the consumer risks and to gain a better understanding of the sources of the high nicotine levels (physiological contamination, cross contamination during processing, or intentional pesticide use).

In the private sector there has also been considerable movement with the EU food and drink industries confederation (CIAA) putting in place quality control provisions for nicotine in wild mushrooms as well as an action plan to identify the source of nicotine

Nicotine is analytically very challenging with special attention being required at various steps of the analytical procedures such as extraction (pH-issue), evaporation (losses at high pH) and measurement (adsorption problems etc.). As nicotine is ubiquitous, cross-contamination is also an issue to take care of.

As a reaction to the massive nicotine findings reported by European laboratories and with the risk of an imminent breakdown of the Chinese export market of mushrooms to Europe in the 2009 season, Chinese export control laboratories have intensified their controls. At the same time efforts to elucidate the sources of the high nicotine levels in mushrooms were initiated

[1] 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

[2] 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

by the interested parties. However, as the results reported by Chinese laboratories were generally lower than those reported by the European ones, concerns were expressed on behalf of the Chinese side as regards the plausibility of the European results and the validity of the methods used. Following a CODEX meeting in China in May 2009, it was thus decided to start a dialogue at technical level to elucidate the reasons behind the deviating results.
As the 2009 harvesting season in China was on the way and with the start of the EU-monitoring program approaching, quick actions were necessary in order to clarify the differences in the analytical methodologies and to ensure the comparability and mutual recognizability of results, thus avoiding unnecessary conflicts at technical level.
Within this aim the Commission has requested the CRL for Fruits and Vegetables (CRL-FV) and the CRL for Single Residue Methods (CRL-SRM) to elaborate methods for the analysis of nicotine in mushrooms and to distribute these methods among the laboratories as well as to start a dialogue with the Chinese side. In addition, the need to perform a proficiency test using real samples was recognized in order to compare the results generated by the different laboratories, to identify problems and possibly elucidate method deficiencies. In view of the beginning summer holiday season, the time was deemed as too short to launch any big scale PT. Luckily, however, the opportunity arose to collaborate with a Swiss industrial laboratory (of the COOP supermarket chain) that was on the way to launch a small-scale interlaboratory comparison within the framework of the Swiss Manual of Food Analysis (Schweizerisches Lebensmittelbuch, SLMB) using real samples of dried mushrooms containing incurred nicotine. A limited amount of test material was still available and could be used for the present PT. The following test samples were provided to the laboratories in form of a highly homogeneous powder:

- Dried Boletus mushrooms homogenate from Bosnia and Herzegovina
- Dried Boletus mushrooms homogenate from China and
- Dried Mu-Err mushrooms homogenate from China

As the amount of the material available was limited, only 14 laboratories could participate at this exercise. The participating laboratories included 10 government and 4 private laboratories from 6 countries, namely: Germany (5), Switzerland (4), China (2), Spain (1), Austria (1) and France (1). In July 2009 the laboratories were sent the three test-samples and were asked to submit their results by the 31st of August 2009. Two variations of the QuEChERS-method were distributed among the labs for informative purposes in advance to the test, but the laboratories were free to employ any method they liked. An excel sheet was provided that the labs could use to submit the nicotine results and the details on the methodology used. The medians of the analytical data submitted were used to obtain the assigned concentrations for each sample. A fit-for-purpose target relative standard deviation (FFP RSD) of 25% was chosen to calculate the target standard deviations (σ) as well as the z-scores for each sample. For informative purposes, the robust standard deviations (Qn-RSDs) were also calculated and were found to be close to the 25% figure on average.

1. TEST MATERIALS

1.1 Preparation of the treated test material

A portion of 200 - 400 g sample material each from a single lot was pre-homogenized in a Retsch Grindomix GM 200 blender. The resulting powder was further homogenized using a Retsch ZM100 centrifugal grinding mill with a 1 mm sieve.

1.2 Homogeneity test

Ten portions of each test material were analyzed using the QuEChERS-method involving addition of 10 mL water to 2 g sample, extraction with acetonitrile, partitioning after addition of salts, centrifugation and cleanup by dispersive SPE-with PSA. The sequence of injections of the extracts was chosen randomly. The quantification was performed using 4-point calibration curves but without the use of internal standard for calculation. Thus no recovery-correction was performed.

The individual results of the homogeneity tests are shown Table 1.

Table 1: Statistical test to demonstrate homogeneity of the test material

Sample	Boletus (Bosnia and Herzeg.)	Boletus (China)	Mu-Err (China)
	[mg/kg]		
01	0.376	1.665	0.375
02	0.332	1.760	0.391
03	0.346	1.650	0.335
04	0.335	1.600	0.360
05	0.302	1.750	0.336
06	0.308	1.780	0.337
07	0.302	1.680	0.332
08	0.310	1.865	0.320
09	0.335	1.615	0.324
10	0.314	1.585	0.311
Mean	**0.326**	**1.695**	**0.342**
RSD	**0.024**	**0.091**	**0.025**
%RSD	**7.2%**	**5.4%**	**7.4%**

The test material was considered to be sufficiently homogenous and suitable for the use in the PT.

1.3 Stability tests

The tests were performed on two occasions with the test samples being stored at room temperature.

The dates of testing were as follows:

Day 1: on 30 March 2009.

Day 2: on 6 July 2009

The analytical method described above was used, however this time with recovery correction by means of standard additions to portions of the matrix in Day 1 and by means of the isotopically labeled ISTD in Day 2.

The individual results are shown in Table 2.

The tests did not show any significant decrease in the nicotine levels, which suggests that at these storage conditions nicotine remained stable for the entire duration of the Proficiency Test.

Table 2: Test to demonstrate the stability of nicotine, storage at RT

	Boletus mushrooms (Bosnia and Herzeg.)	**Boletus mushrooms (China)**	**Mu-Err mushrooms (China)**
	[mg/kg]		
Day 1	0.69	2.86	1.08
Day 2	0.73	2.73	1.15
% deviation (Day 2 vs. 1)	+5.8%	-4.5%	+6.5%
	Passed	**Passed**	**Passed**

1.4 Organizational details

One plastic bag of each of the three test materials containing 15-20 g material was shipped to the laboratories in April 2009 (participants of the first round) and July 2009 (participants of the second round).

An excel submission file was distributed for the participants to submit their results as well as details about the analytical procedures used.

2. STATISTICAL METHODS

The median concentrations of all the reported results for each of the three test materials, excluding the outliers, were used as the assigned values.
Based on previous experience from EU proficiency tests on fruit and vegetables and cereals a fixed fit-for-purpose relative standard deviation (FFP RSD) of 25 % was used. The target standard deviation (σ) was calculated by multiplying this FFP-RSD by the assigned value. In addition, the robust relative standard deviations Qn was calculated for informative purposes.

A z-score for each laboratory/ test sample/ nicotine result combination was calculated according to the following equation:

$$z = (x-X) / \sigma$$

Where:
x is the result reported by the participant or the specific reporting limit (MRRL) for those labs not having detected the nicotine present in the test material
X is the assigned value
σ is the target standard deviation and equals 25% of the assigned value.

The z-score classification is as follows:

$\|z\| \leq 2$	acceptable
$2 < \|z\| \leq 3$	questionable
$\|z\| > 3$	unacceptable

Z-scores for false negatives are calculated using the reporting level of the laboratory.

3. RESULTS

14 laboratories from 6 countries agreed to participate in this proficiency test. The participating laboratories are listed in Table 3.

Table 3: List of laboratories participating at the PT for nicotine

Sector	Country	Laboratory name
Government	AT	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	CH	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	CH	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	CN	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	DE	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	DE	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	DE	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	DE	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	ES	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Government	FR	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Industry	CH	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Industry	CH	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Industry	CN	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
Industry	DE	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

In general the results submitted by the laboratories were excellent with 90% being within the acceptable range of $|z| \leq 2$. Only two results were in the questionable range $2 < |z| \leq 3$ and another two unacceptable with one of them being a false negative result. Table 4 gives an overview of the results.

Table 4: Classification of the results

	Boletus (BiH)	Boletus (CN)	Mu-Err (CN)	Sum
Acceptable	13	13	12	**38 (90%)**
Questionable	0	1	1	**2 (5%)**
Unacceptable	1	0	1 (false negative)	**2 (5%)**

Table 5 shows the individual results submitted by the laboratories as well as the median value that was considered as the assigned value for this PT. The robust standard deviation (Qn-RSD) was also calculated and was found to be 26% on average, which is very close to the 25% RSD level that was used to calculate the z-scores.

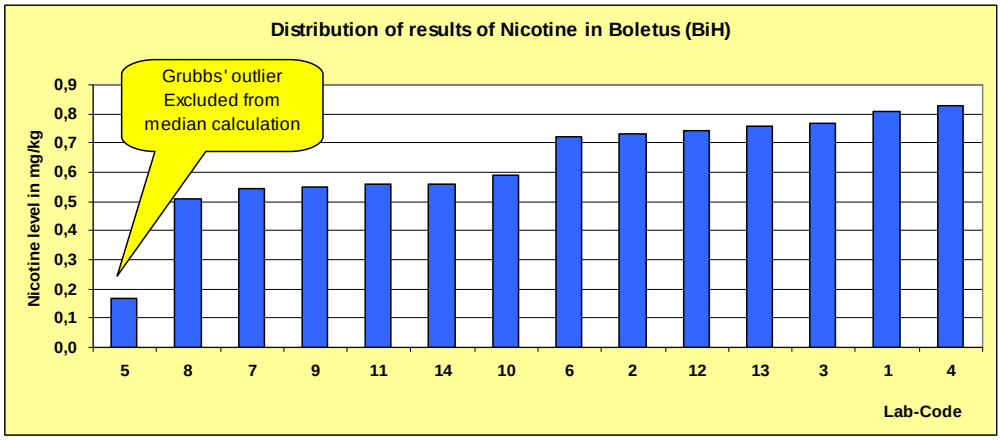
Table 5: Overview of the reported nicotine results and the z-scores

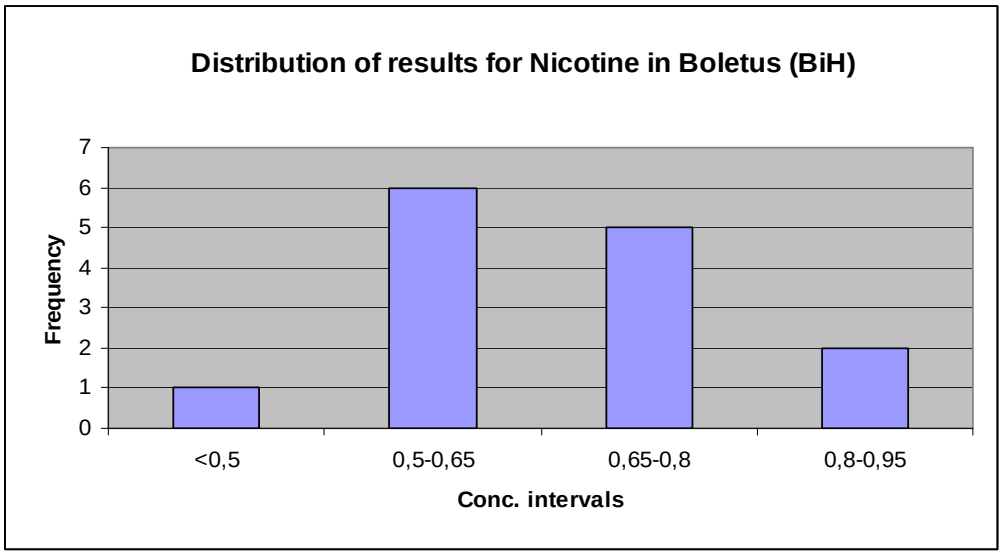
Lab.-code	Boletus mushroom (Bosnia and Herzeg.)		Boletus mushroom (China)		Mu-Err mushroom (China)	
	mg/kg	z-score	mg/kg	z-score	mg/kg	z-score
01	0.81	0.50	3.18	1.02	1.01	0.00
02	0.73	0.07	2.73	0.31	1.15	0.57
03	0.77	0.28	2.6	0.10	0.88	-0.51
04	0.83	0.61	1.99	-0.86	0.85	-0.63
05	0.17	**-3.06**	1.21	**-2.09**	**ND (= RL=0.05)**	**-3.80**
06	0.72	0.00	2.77	0.37	1.08	0.28
07	0.55	-0.97	2.36	-0.28	1.17	0.61
08	0.51	-1.17	1.28	-1.98	0.58	-1.70
09	0.55	-0.94	2.06	-0.75	1.2	0.75
10	0.59	-0.72	2.59	0.09	0.99	-0.08
11	0.56	-0.89	1.91	-0.99	0.49	**-2.06**
12	0.74	0.11	2.48	-0.09	1.03	0.08
13	0.76	0.22	2.94	0.64	1.02	0.04
14	0.56	-0.89	2.7	0.26	0.87	-0.55
Median	**0.72**		**2.56**		**1.01**	
Qn RSD	**23.6**		**29.4**		**23.6**	

One laboratory (Code 05) reported ND ("not detected") for nicotine in Chinese Mu-Err mushroom test-sample giving a reporting level (RL) of 0.05 mg/kg. As all other laboratories reported positive findings with an average concentration of 1,01 mg/kg this result was considered to be a false negative and the z-score was calculated using the reporting level provided by the lab.

The distributions of the results reported by the laboratories were plotted as histograms and can be seen in the following Figures 1-3.

Figure 1: Histograms showing the distribution of results and z-scores for Nicotine in Boletus mushrooms from Bosnia and Herzegovina (BiH)





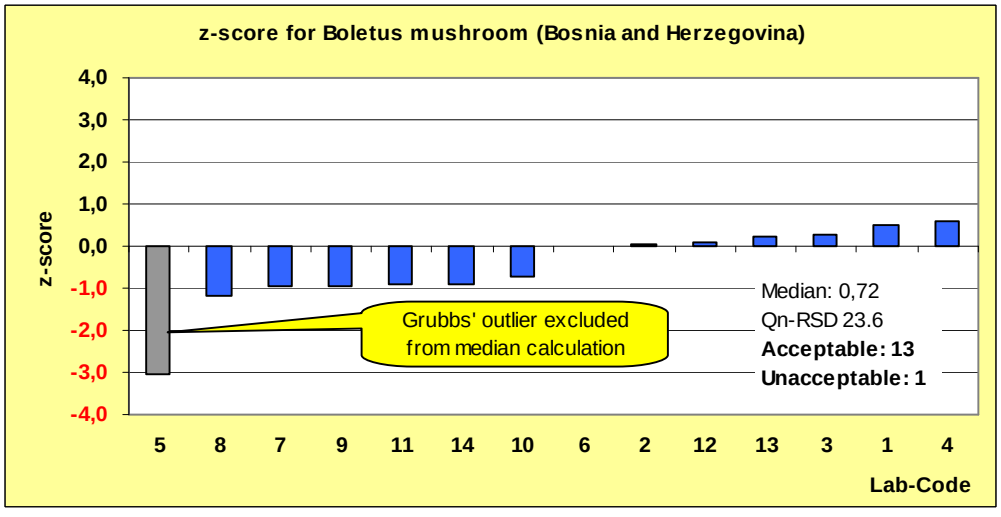
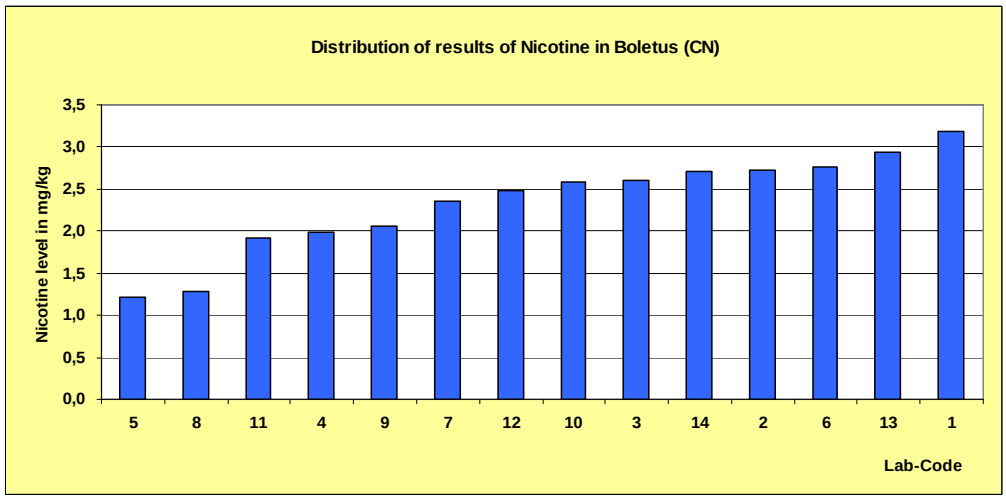
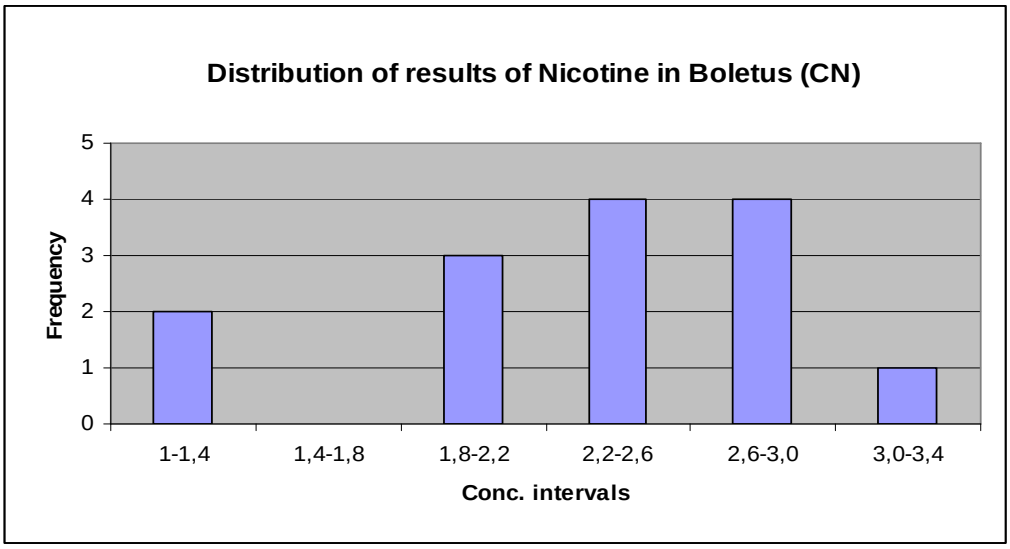


Figure 2: Histograms showing the distribution of results and z-scores for Nicotine in Boletus mushrooms from China (CN)





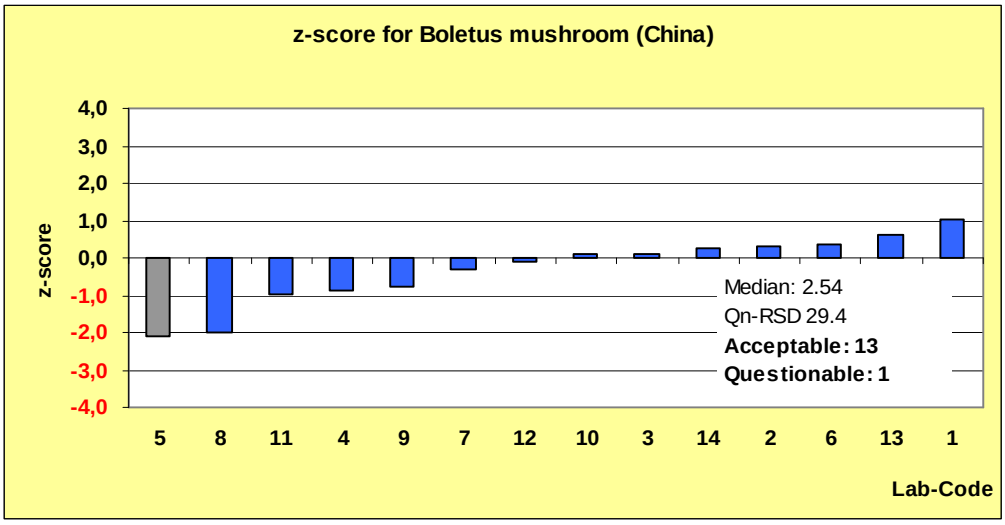
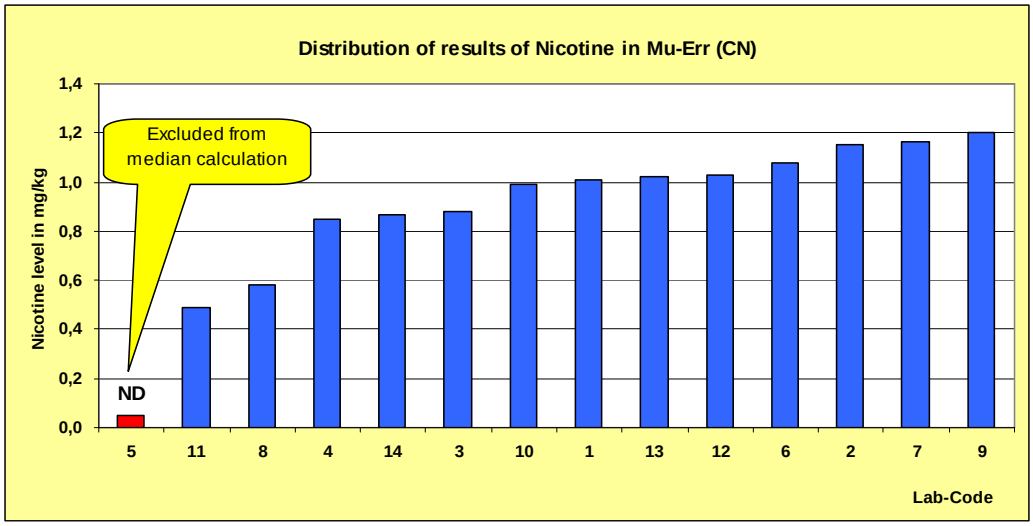
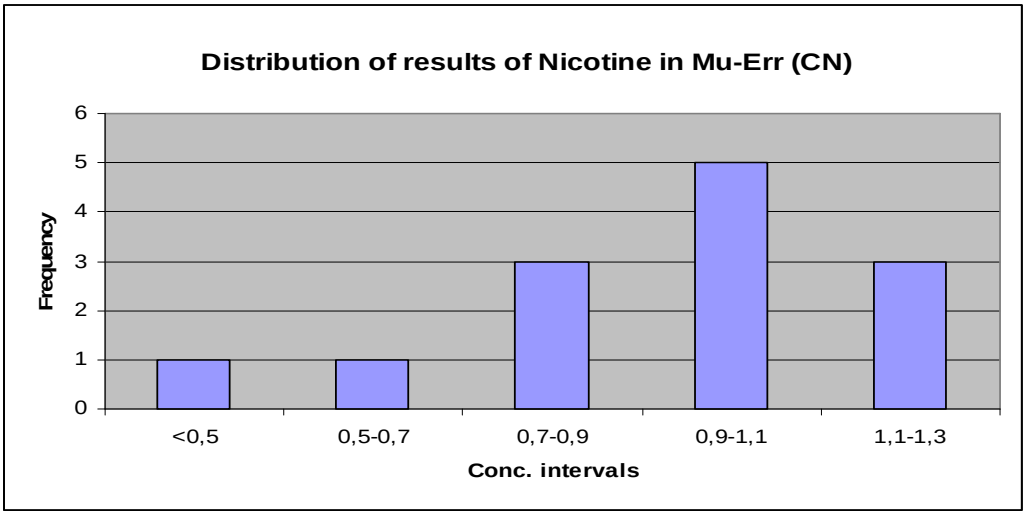
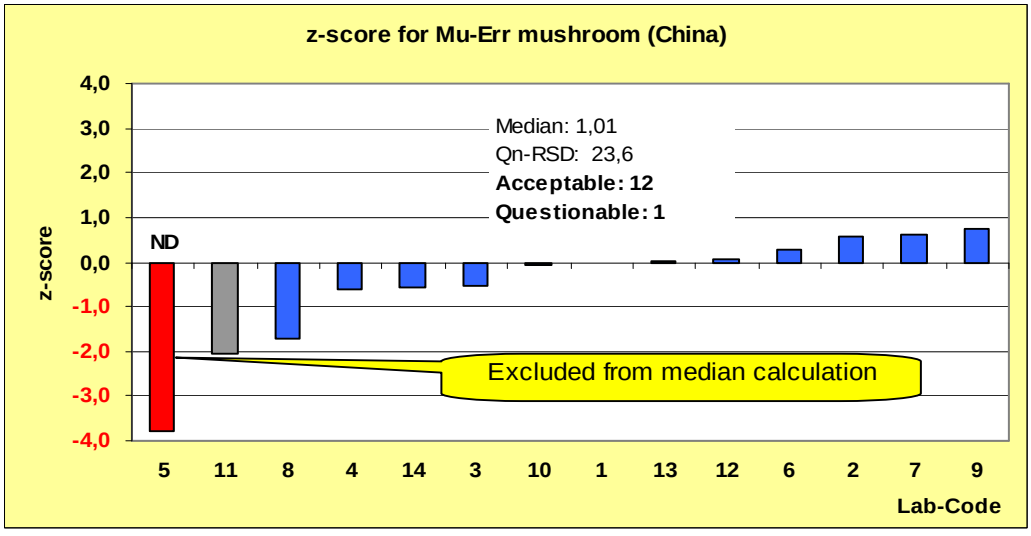


Figure 3: Histograms showing the distribution of results and z-scores for Nicotine in Mu-Err mushrooms from China (CN)







3.1 Assessment of laboratory performance

Figure 4 shows the average z-scores achieved by the laboratories considering all three samples.

Figure 4: Average z-scores of the laboratories

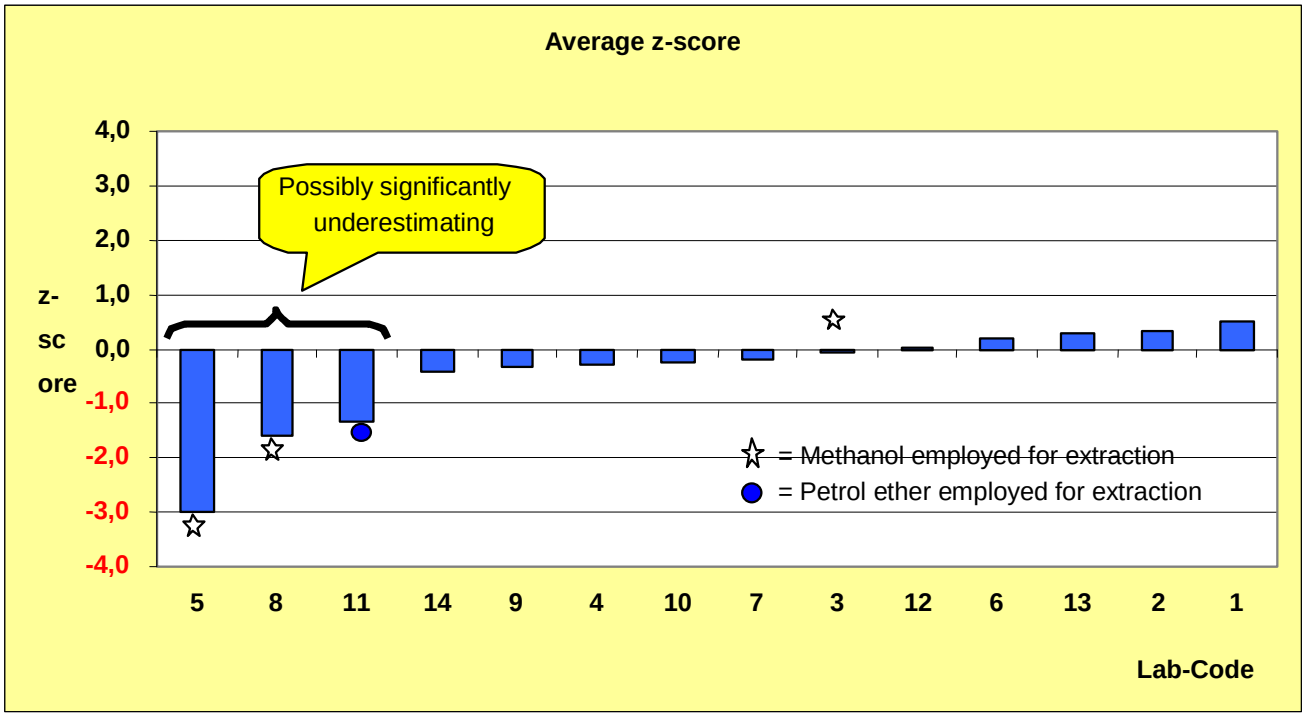
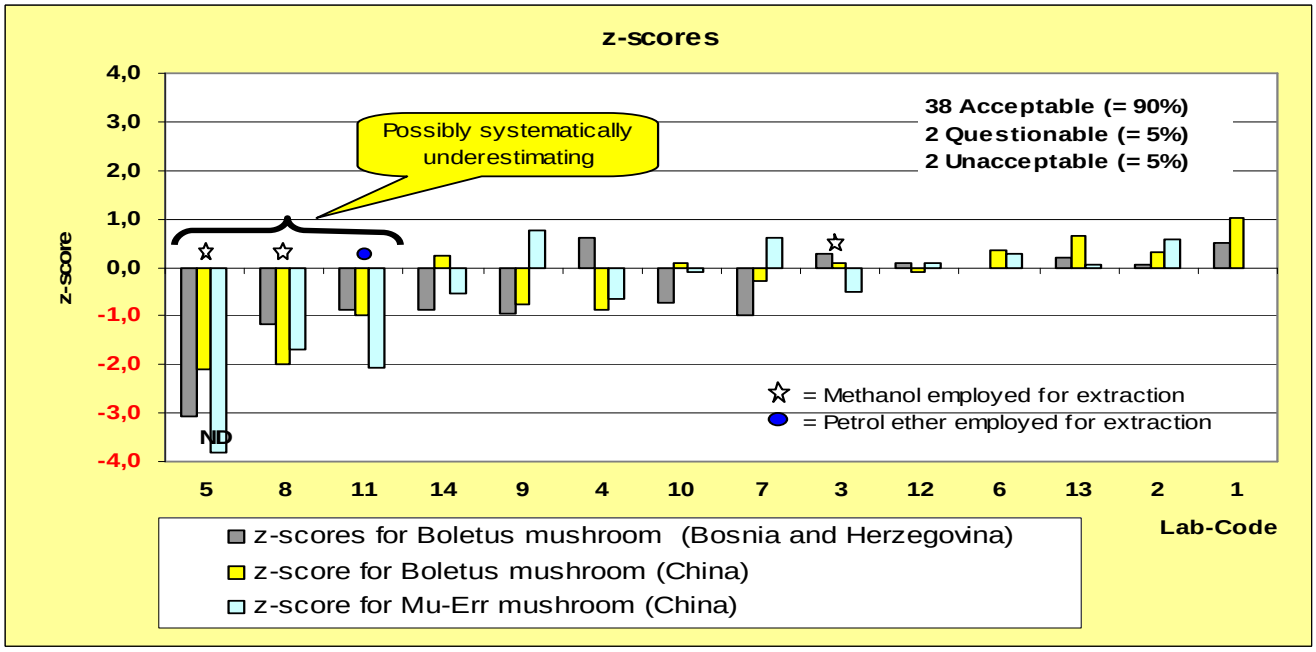


Figure 5 shows the individual z-scores submitted by each laboratory for all three samples with the laboratories being ordered according to the average z-scores.

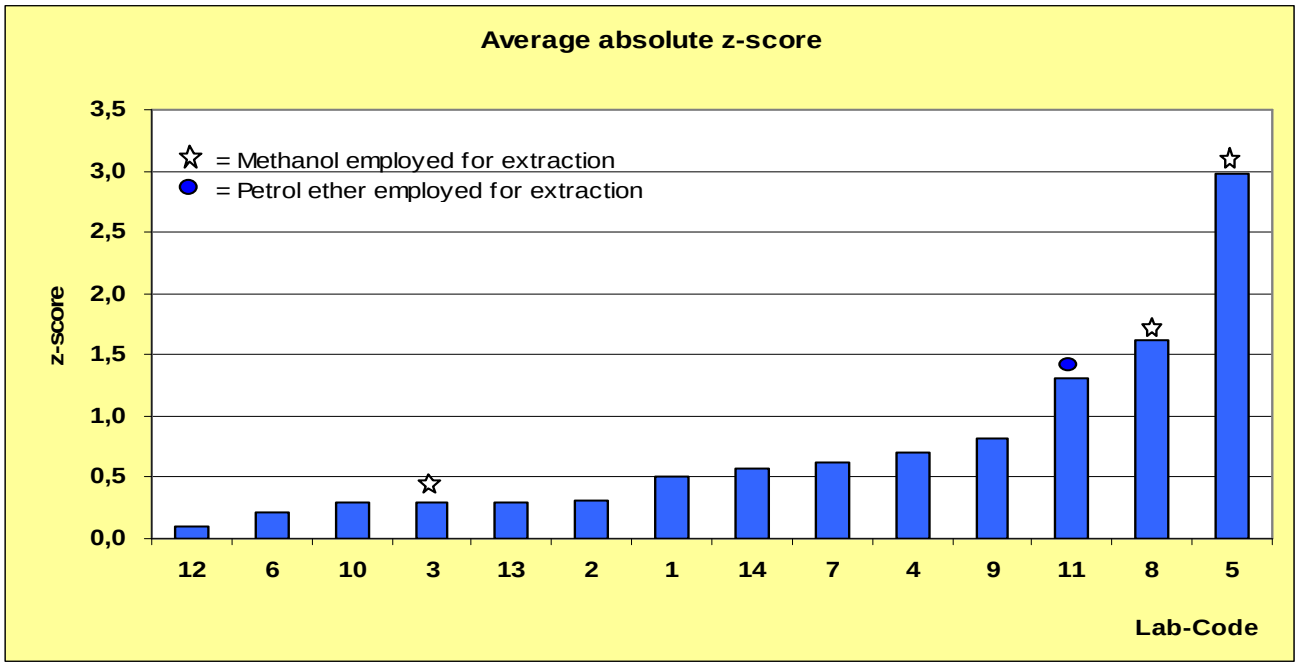
The results suggest that the laboratories with the codes 05, 08 and 11 systematically underestimate the nicotine concentration to a significant degree. None of the laboratories seems to systematically overestimate the concentration at a significant degree.

Figure 5: Individual z-scores of the laboratories



In order to rank the laboratories according to their overall performance in this exercise, the average absolute z-score of all results was calculated for each laboratory. All but three laboratories achieved a figure well below 1 and all but one laboratory a value well below 2. This indicates an overall good performance.

Figure 6: Ranking of laboratories according to the Average of Absolute z-Scores of the laboratories



3.2 Analytical methods

Detailed information about the analytical methods used by the laboratories can be seen in the Tables 6, 7 and 8. Table 6 summarizes the main differences of the methods employed with the main deviations highlighted. Tables 7 and 8 show the detailed information provided by the labs.

Due to the small number of participating laboratories and the large number of different methodologies employed it is difficult to draw any clear conclusions as regards the-suitability or not of certain analytical steps or methodologies. In any case laboratories that have employed variations of the QuEChERS method (9 out of the 14) have delivered results showing a narrow distribution with average z-scores between -0.39 and +0.51 and individual z-scores ranging between -0.97 and +0.75. The narrow distribution of the results generated employing QuEChERS-based techniques is also reflected by the small standard deviations of the submitted results being 17% for the boletus (BiH), 13% for the boletus (China) and 12% for the mu-err (China) test-samples. One of the laboratories (Code 01) employing acetonitrile for extraction did not perform a liquid-liquid partitioning step. 7 out of 10 laboratories that have employed acetonitrile for extraction have raised the pH of the extraction step by base addition. The three laboratories (Codes: 01, 02 and 06) that did not perform this step have corrected their results for recovery. In total 8 out of the 14 laboratories have corrected for recovery (Codes 01, 02, 04, 06, 08, 09, 13 and 14) without necessarily performing better than the other labs. Six of those labs (Codes 02, 04, 06, 09, 13 and 14) have employed isotopically labeled nicotine as internal standard.

Two of the three laboratories that have employed methanol for extraction (Code 05 and 08) have reported results that were lower than the median in all samples. The laboratory with the Code 05, that did not correct for recovery, reported two unacceptable and one questionable result (average z-score -2.98). The laboratory with the Code 08, that reported three acceptable results (average z-score -1.62) all with a negative z-score, indicated a correction for recovery via recovery figures of the ISTD. However, as the labeled ISTD was only added to the final extract, this correction probably only compensated for measurement effects, such as matrix-induced signal suppressions, but not for losses in the extraction step. The other labo-

ratory employing methanol (Code: 03) performed very well (average z-score -0.04) with the main methodological differences to the other two being the use of a high-speed mixer.
One laboratory employing ASE with petrol ether (Code 11) also delivered lower results than the median in all three test samples with only one of them being slightly unacceptable and two acceptable (average z-score -1.31).
12 out of the 14 laboratories have employed LC-MS/MS for analysis. The remaining two labs (Codes 12 and 10) have performed very well employing GC-MS. All but two laboratories (Codes 11 and 05) have added water to the sample to assist extraction. These two labs show systematically lower results (Note: Laboratory with Code 11 has added some water via the ammonium hydroxide addition).
The test sample amounts employed by the laboratories ranged between 0.25 and 10 g with 2 g being the most frequent figure (8 laboratories).
Although some trends as regards the methodologies are visible, it should be noted that the small population of data and the fact that the assigned value is too much influenced by the results submitted by laboratories using QuEChERS-type approaches, does not allow us to draw any robust conclusions as regards the suitability or not of the various analytical approaches. Nevertheless, some trends can be recognized suggesting the importance of water addition as well as of pH-adjustment especially if no recovery correction is performed (e.g. via isotopically labeled ISTD added at the beginning of the procedure or via standard additions on sample portions).

Table 6: Some main characteristics of the methods used by the laboratories

Lab-Code	Average absolute z-score	Average z-score	Solvent	Water addition	Basification	LLP performed	Recovery correction			ISTD used	ISTD Addition step (not necessarily isotope labeled)	Ratio sample/ water/ solvent	Extraction Means
							Via Isotope labeled ISTD	Via standard addition to sample portions	Via recovery figure				
12	0,09	0,03	ACN	Yes	**Yes, NaOH**	yes, single	No	No	No	**other**	after water addition	1/5/5	By hand
06	0,22	0,22	ACN	Yes	No	yes, single	**Yes**	No	No	Isotope labelled	to test sample portion	1/5/5	By hand
10	0,30	-0,24	ACN	Yes	**Yes, NaOH**	yes, single	No	No	No	**other**	after water addition	1/5/5	By hand
03	0,30	-0,04	**MeOH**	Yes	No	yes, single	No	No	No	**other**	to test sample portion	1/1/**20**	**High speed dispenser**
13	0,30	0,30	ACN	Yes	**Yes, NaOH**	yes, single	**Yes**	No	No	Isotope labelled	after water addition	1/5/5	By hand
02	0,31	0,31	ACN	Yes	No	yes, single	**Yes**	No	No	Isotope labelled	to test sample portion	1/5/5	?
01	0,51	0,51	ACN	Yes	No	**No**	No	**Yes**	No	**none**	-	1/10/**40**	By autom. shaker
14	0,57	-0,39	ACN	Yes	**Yes, NH4OH**	yes, single	**Yes**	No	No	Isotope labelled	to test sample portion	1/20/**20**	By autom. shaker
07	0,62	-0,21	ACN	Yes	**Yes, NaOH**	yes, single	No	No	No	**none**	-	1/5/5	By hand
04	0,70	-0,29	ACN	Yes	**Yes, NH4OH**	yes, single	**Yes**	No	No	Isotope labelled	to test sample portion	1/5/7.5	By autom. shaker
09	0,82	-0,31	ACN	Yes	**Yes, NH4OH**	yes, single	**Yes**	No	No	Isotope labelled	to test sample portion	1/20/**20**	By hand
11	1,31	-1,31	**PE**	**Yes (small amount via NH4OH)**	**Yes, NH4OH**	Yes (in cell)	No	No	No	**other**	**to final extract**	1/**0**/**30**	**ASE**
08	1,62	-1,62	**MeOH**	Yes	No	**No**	No	No	**Yes ?**	Isotope labelled	**to aliquot of final extract.**	1/8/**100** 1/4/**50**	By autom. shaker
05	2,98	-2,98	**MeOH**	**No**	No	**No**	No	No	No	**none**	-	1/**0**/5	By autom. shaker

Table 7: Methods used by the participating Laboratories - Part1

Lab code	Test sample amount [g]	Water addition, amount [mL]	Extraction pH adjusted?	if yes, how ?	Solvent used for initial extr.	Extraction details	LLP	if yes, how was LLP induced	cleanup step ?	if yes, description	Was ISTD used?	ISTD addition step
01	0,25	Yes, 2,5 mL	No		ACN 10 mL	Agitation with automatic shaker at ambient temp.	No		Yes	Dilution 1/5 with 80 % Acetonitril	No	
02	2	Yes, 10 mL	No		ACN 10 mL		Yes single	4 g MgSO4 und 1 g Sodium acetate added	Yes	D-SPE: 8 ml solvent extract to 1.2 g MgSO4/ 200 mg PSA, shake for 30 sec	Yes isotope labeled	to test sample portion
03	10	Yes, 10 mL	No		MeOH 100-200 mL	Agitation by high-speed dispenser (e.g. ultra turrax) at ambient temp.	No		No		Yes other	to test sample portion
04	2	Yes, 10 mL	Yes, pH9	NH3 addition	ACN 15 mL	Agitation with automatic shaker at ambient temp.	Yes single	MgSO4 (6 g)	Yes	SPE: MgSO4 (750 mg) and PSA (250 mg)	Yes isotope labeled	In the test sample portion
05	2 g	No	No		MeOH 10 mL	Agitation with automatic shaker at ambient temp.	No		No		No	
06	2	Yes, 10 mL	No		ACN 10 mL	Agitation by hand at ambient temp.	Yes single	QuEChERS salt mix, Citrate buffered tube Supelco, 55227-U	Yes	d-SPE, PSA SPE Clean-Up Tube 1, Supelco, 55228-U, 6 ml	Yes isotope labeled	to test sample portion
07	2	Yes, 10 mL	yes pH 10-11	NaOH (5N)	ACN 10 mL	Agitation by hand at ambient temp.	Yes single	Add. of MgSO4 4g and NaCl 1g shaking & centrifugation	Yes	SPE with 750mg MgSO4 and 250mg PSA for 5ml supernatant	No	
08	0.25-0.50	Yes, 2 mL	No		MeOH 25 mL	Agitation with automatic shaker at ambient temp.	No		Yes	SPE clean up (Waters Oasis MCX).The residue is taken up in 2.0% aqueous formic acid and eluted with 12 ml 5% ammonia NH3 in methanol.	Yes isotope labeled	D4-nicotine is added to an aliquot of final extract.

Lab code	Test sample amount [g]	Water addition, amount [mL]	Extraction pH adjusted?	if yes, how ?	Solvent used for initial extr.	Extraction details	LLP	if yes, how was LLP induced	cleanup step ?	if yes, description	Was ISTD used?	ISTD addition step
09	0,5	Yes, 10 mL	Yes pH 9	Ammonia	ACN 10 mL	Agitation by hand at ambient temp.	Yes single	addition of sodium chloride: separation into water and acetonitrile phase	No		Yes isotope labeled	to test sample portion
10	2	Yes, 10 mL	Yes pH 10	NaOH 5N	ACN 10 mL	Agitation by hand at ambient temp.	Yes single	Add. of MgSO4 4g and NaCl 1g shaking & centrifugation	Yes	D-SPE	Yes other	after water addition
11	1g	No	Yes pH 10	500 µl NH4OH in cell	PE 30 mL	ASE	Yes, in cell		No		Yes other	ISTD added to the final extract
12	2	Yes, 10 mL	Yes pH 10-11	NaOH (5N)	ACN 10 mL	Agitation by hand at ambient temp.	Yes single	Add. of MgSO4 4g and NaCl 1g shaking & centrifugation	Yes	SPE (25mg PSA + 150mg MgSO4 /mL extract)	Yes other	after water addition
13	2g	Yes, 10 mL	Yes pH 10	NaOH	ACN 10 mL	Agitation by hand at ambient temp.	Yes single	Add. of MgSO4 4g and NaCl 1g shaking & centrifugation	Yes	D-SPE , 200mg PSA + 1,2 g MgSO4 in 8ml extract	Yes isotope labeled	after water addition
14	0,5	Yes, 10 mL	Yes	by addition of NH3	ACN 10 mL	Agitation with automatic shaker at ambient temp.	yes single	by NaCL	No		yes isotope labeled	after weighing the sample, before adding anything else

Table 8: Methods used by the participating Laboratories - PART2

Lab co-de	Evaporation step	Chr/phy	Separation column	LC/GC Inj. Vol.	LC-Gradient	Detec-tion	Calibration Info	recovery exp.; recovery	Recovery cor-rection
01	No	UPLC/UHPLC	ACQUITY UPLC BEH HILIC Column, 1.7 µm, 2.1 x 100 mm, Waters	20 µl	Solvent A: 10 mmol/l Ammonium formate in MeCN; Sol-vent B: 10 mmol/l Ammonium formate in Water; Flow: 0.4 ml/min / 0-1.6 min 80 % A / 2.7-4.4 min 20 % A / 4.97 - 6.5 min 80 % A	MS/MS	Std additions to portions of test sample	No	Yes, autom. via std add to por-tions of test sample
02	No	LC	WATERS Atlantis HILIC Silica 3 µ, 2.1 x 100 mm / w. 3 µ, 2.1 x 10 mm Guard Column same type	1 µl	A) ACN/H2O (90:10) 10mMol NH4-Formate B) 10 mMol NH4-Formate in H20 (pH 3.7) Flow : 0.2 ml/min Gradient: 0 -2 min 80 % A; 2 -4 min 20%A; 4 - 7 min 20 %A; 7 -8 min 80 %A; 8 - 15 min 80 %A.	MS/MS	External using std in pure solvent	No	No
03	No	UPLC/UHPLC	Synergi 2.5 u Hydro-RP 100A von Phenomenex	5 µl	A: Water + 5 mM NH4-Formate; B: Methanol 0.00 min 80 % A ; 12.00 min 5 % A ; 27.00 min 5 % A ; 28.00 min 80 % A ; 32.00 min Stop	MS/MS	External using std in pure solvent	Yes 80%	No
04	Yes; pH=4-5 (with formic acid) reconst in MilliQ water	LC	AGILENT. Zorbax SB-C18 5 µm, 3 x 250 mm Pas Nº: 880975-302	20 µl	Solvent A: ACN with 0.1% de formic acid Solvent B: MilliQ water with 0.1% formic acid Gradient: linear 10% to 100% A in 20 min, stay for 15 min. Re-equilibrate 12 min., Flow: 0,2 ml/min	ToF	Std additions to aliquots of ex-tract	Yes Mushroom 99% (C.V:9%)	Yes, autom. via isot. labeled ISTD
05	No	LC	Phenomenex Luna C18, 150x3mm, 3µm	5 µl	A: 5 mmol NH4-formiat in H2O / MeOH (100:0) B: 5 mmol NH4-formiat in H2O / MeOH (0:100) gradient yes / flow 0,4 ml/min	MS/MS	Std additions to aliquots of ex-tract	No	No
06	No	LC	Waters Atlantis HILIC, 2.1x100 mm, 3µm	10 µl	Solvent A: ACN + NH4-Formate, Solvent B: Water + NH4-Formate pH 3.7, Flow 0.2 ml/min, Gradient: 0 min 80% A, 2 min 80% A, 4 min 20% A, 7min 20% A, 8 min 80% A.	MS/MS	External using std in pure solvent	Yes	Yes, autom. via isot. labeled ISTD
07	No	LC	Phenomenex Luna Hilic 3µ 50x2 mm	50 µl	Solvent A: 10mM NH4-Formate in ACN, Solvent B: 10mM NH4-Formate in Water - pH adjusted to 3.7 with Formate ; Gradient: 0 min 80%A, 2min 80%, 4min 20%, 4.1min 80%A, 10min 80%A; flow 200µl/min	MS/MS	Std additions to aliquots of ex-tract	Yes 92% in Shiitake	No

Lab co-de	Evaporation step	Chr/phy	Separation column	LC/GC Inj. Vol.	LC-Gradient	Detec-tion	Calibration Info	recovery exp.; recovery	Recovery cor-rection
08	Yes below 40 °C ,add 25 ul 2.0% aq. formic acid + 100 uL H2O.;reconst. in MeCN	UPLC/UHPLC	2.1X50mm 1.7um BEH HILIC column	10 µl	solvent A:10mmol NH4OH solution; solvent B:ACN, gradi-ent, flow:0.25ml/min	MS/MS		Yes Nicotine:83.0%; d4-nicotine : 60.3%	Yes, using the recovery figure
09	No	LC	phenomenex synergi 4 u polar RP 80 A 150 x 3 mm	20 µl	A: Methanol/water 1:1 (10 mm NH4Ac in water); B: ACN, gradient, 600 uL/min	MS/MS	External using std in pure solvent	No	Yes, autom. via isot. labeled ISTD
10	No	GC	Agilent, HP5MS, 0,25 mm ID, 30 m, 0,25 µm	3 µl PTV		MS	External using std in blank matrix extract	Yes 101 %, Shiitake	No
11	Yes ; pH adj. w. 5% formic acid in water and evapor. under vacuum at 40°C; reconst in acidif. water	LC	Uptisphere 3 ODB , 100 x 4,6, 3µm	10 µl	Solvent A acidified water (0,05%), solvent B acidified MeOH (0,05%), gradient : 80A/20B during 3 mn then 20A/80B during 5 mn , total: 20mn	MS/MS	External using std in blank matrix extract	Yes 92 % dried mushr.	No
12	no	GC	J&W Scientific, DB-5MS +Duraguard,Film:0,25um; 30m (+10m Durguard),I.D.:0,250mm	3 µl PTV		MS	Std additions to aliquots of ex-tract	Yes 107%, champig-non	no
13	No	LC	Phenomenex, Gemini 5µ, C18, 2mm inner, 150 mm length, 110 A	20 µl	A= 0,025% NH4COOH, B= ACN, Gradient A 30% B 70%, 10 Min, 300 µl	MS/MS	External using std in blank matrix extract	Yes 97%, dried mushr.	Yes, autom. via isot. labeled ISTD
14	No	LC	Thermo Hyppurity C18, 150x3 mm, 5µm	20 µl	A: Water/ACN, 9/1, 0.1% formic acid, B: ACN, 0.1% formic acid	MS/MS	External using standards in pure solvent	Yes 70% (n=12, recov. of ISTD)	Yes, autom. via isot. labeled ISTD

3.3 Conclusions

In general, the participants have performed very well in this exercise delivering acceptable result in 90% of the cases and just two unacceptable results. Laboratories employing QuEChERS-type methodologies involving extraction with acetonitrile and addition of a base, have reported results within a narrow concentration range. One laboratory employing petroleum ether and ASE technique for extraction and two laboratories employing methanol showed a trend for underestimating the results. Another laboratory employing methanol reported good results. However, the small population of results and the fact that the assigned value (median) is greatly influenced by the great population of laboratories employing QuEChERS-type methodologies does not allow to draw any robust conclusions as regards the suitability or not of the various methodologies. Nevertheless, some trends can be recognized suggesting the importance of water addition as well as pH-adjustment especially if no recovery correction is performed (e.g. via isotopically labeled ISTD added at the beginning of the procedure or via standard additions on sample portions).