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Report on the validation of a method for the determination of Ochratoxin A in Capsicum spp. (paprika and chilli)

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Table of contents

1. Summary

A method validation study was conducted according to the IUPAC harmonised protocol for the determination of ochratoxin A in Capsicum spp. (paprika and chilli). The method is based on the extraction of the samples with an aqueous methanol solution, followed by immunoaffinity cleanup. The determination is carried out by reversed phase high performance liquid chromatography coupled to a fluorescence detector. The study involved 21 participants representing a cross-section of research, private and official control laboratories from 14 EU Member States and Singapore.

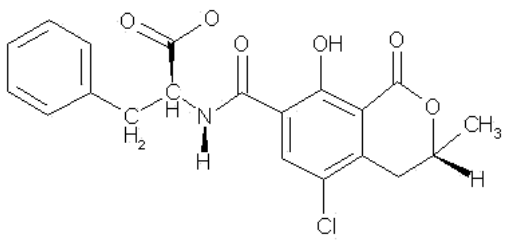
Mean recoveries reported ranged from 83.7 to 87.5. The relative standard deviation for repeatability (RSD_r) ranged from 1.7 to 14.3 %. The relative standard deviation for reproducibility (RSD_R) ranged from 9.1 to 27.5 %, reflecting HorRat values from 0.4 to 1.3 according to the Horwitz function modified by Thompson. A correction for recovery with the data generated by fortification experiments further improved the reproducibility performance of the method.

The method showed acceptable within-laboratory and between-laboratory precision for each matrix, as required by current European legislation.

2. Introduction

Ochratoxins are pentaketides made up of dihydro-isocoumarin linked to ß-phenylalanine. Ochratoxin A (OTA) [**Figure 1**] is mainly produced by *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* in tropical regions and by *Penicillium verrucosum* in temperate climates. It has been classified as a substance of Group 2B by the International Agency for Research on Cancer (IARC), meaning the existence of sufficient evidence of its renal carcinogenicity to animals and possibly to humans.

Figure 1: Structure of ochratoxin A



Cereals and their derivatives are the major contributor for ingestion of OTA but it is also found in a variety of food products ranging from coffee to nuts, wine, beer, dried fruits and spices.

The methodologies used for the determination of OTA in almost all relevant food and feed matrices range from high-performance liquid-chromatography (HPLC) with various detection systems such as fluorescence (FLD) or mass selective detection (MSD), over thin-layer chromatography (TLC) to enzyme linked immunosorbant assays (ELISA). The most common principle in EU Member States is however HPLC-FLD, which is the basis for all CEN standards for OTA. All methodologies, irrespective of their detection principle, depend on the extraction of OTA from the matrix with an aqueous-organic solvent.

Regulations (EC) No 1881/2006 [**1**] and (EC) No 105/2010 [**2**] lay down maximum limits for OTA in certain foods and methods for sampling and analysis.

In Commission Regulation 105/2010 [**2**] legislative limits have been set for OTA in liquorice and a variety of Capsicum spp. spices such as paprika and chilli. The level for spices at the moment of this project was 30 µg/kg and is indented to be lowered to 15 µg/kg in the future.

In 2010 a collaborative study was conducted at IRMM to validate an analytical method for the determination of ochratoxin A in liquorice root powder and liquorice extracts [**3**]. Several standardised methods are available by CEN/ISO and AOAC for the determination of OTA in various foodstuffs [**4**] however there is still no method available for Capsicum spp. that has proven its performance in a collaborative study.

IRMM organised proficiency tests on OTA in paprika in 2007 [**5**] and in 2010 [**6**] which indicted that methods based on immunoaffinity cleanup followed by liquid chromatography with fluorescence detection are a good basis for reliable measurements. Based on the data from these proficiency tests, a robust method principle was tested. The method was further adjusted to a scope to allow monitoring at the levels of interest. After single laboratory validation this method became the candidate for this collaborative trial.

Previous collaborative study projects have shown that, with care and attention to detail during the organisation of a collaborative trial, it is possible to achieve impressive performance characteristics for a method suitable for low limits of detection. Due to the complexity of the matrices, particular care was taken during preparation of the test

materials (blending of relevant matrix constituents and extensive homogenisation) and in demonstrating inter-unit homogeneity before undertaking the study. Furthermore the accurate determination of the contamination levels in the matrices for which the legislative limits apply, require a robust and reliable analytical method.

3. Scope

This method validation study aimed to evaluate the recovery and precision of an analytical method for the quantification of OTA in Capsicum spp. (paprika and chilli) to monitor compliance with limits set in legislation [**2**].
According to the method a test portion is extracted with a mixture of methanol and aqueous sodium bicarbonate solution. The extract is filtered, diluted with phosphate buffered saline (PBS), and OTA is purified with an immunoaffinity column containing antibodies specific to OTA. The purified extract is quantified by high performance liquid chromatography-fluorimetric detection (HPLC-FLD). [**Annex 10**]
The study was designed and evaluated according to the IUPAC Harmonised Protocol [**7**]. Statistical analysis was performed along the lines of ISO 5725 [**8**].
Precision and recovery values were compared with method performance criteria set in Regulation (EC) No 401/2006 [**9**].

4. Design of the study

4.1. Time frame

The study was open to all types of laboratories dealing with OTA determination and capable to perform the method as described. It was published on the website of the IRMM and 23 laboratories were invited to participate.

The subscription PDF form [**Annex 4**] was sent out on 8 March with a deadline set on 20 March, 2012. Together with the subscription form, participants also received the outline of the study [**Annex 3**] and the draft method description. The participants were asked to send back comments and amendments if necessary.
Parcels were dispatched on 4th of April. The reporting deadline was 5th of May 2012.
Two laboratories did not return results and were excluded from the study.

4.2. Materials and documents

The 23 laboratories that enrolled in the collaborative trial were a cross-section of research, private and official control laboratories from 14 EU Member States and Singapore (details are in [**Table 3**]).

Each participant received:

- Accompanying letter [**Annex 5**]
- Fifteen units of coded samples with unknown identity to the participants in vacuum sealed sachets
- An ampoule of ochratoxin A calibrant solution
- Four coded ampoules for spiking experiments with unknown content of OTA to the participants
- An interlaboratory study Materials Receipt Form [**Annex 6**]
- Their participation code
- A method description [**Annex 10**]
- Operational manual [**Annex 11**]
- A spiking protocol [**Annex 7**]
- Twenty immunoaffinity columns containing antibodies specific to OTA for the cleanup of the material extracts
- Safety sheets for the solvents
- A pdf form for reporting the results [**Annex 8**]
- A pdf form for questionnaire regarding general information on the laboratory, their opinion on the design of the study and on the deviations from the method description they applied, if any. [**Annex 9**]

4.3. Organisation

Upon participants' comments and amendments, the method description was changed whenever it was considered appropriate prior to the study.

Participants had to fill in a questionnaire where they were asked to report any deviations from the method description they might have applied. This information was used to identify non compliances.

5. Test materials

5.1. Description

Test materials were obtained from various sources and some paprika materials were surplus materials from previous projects. Test materials were remixed where necessary to meet the targets levels and maintain an unknown identity to the participants. [**Table 1**]

Since not all materials were available in sufficient amounts two groups of participants were formed. As a result the common set of test samples for both groups was one blank paprika for spiking *(Sample 6)* plus three different naturally contaminated paprika samples *(Samples 2, 3, 5)* and two naturally contaminated chilli materials *(Samples 1, 4)*. In addition group A (10 participants) received an additional blank paprika for spiking *(Sample 7)*, while group B (11 participants) received a low level chilli for spiking instead *(Sample 8)*. Each of the contaminated samples and the blank samples for spiking were analysed as blind duplicates.

Additionally one sachet from the blank paprika *(Sample 9)* was also sent to each participants.

Table 1: Test samples

Sample description	Test Material	Ochratoxin A(µg/kg)	Design
Sample 1	chilli	1.8	2 blind replicates
Sample 2	paprika	6.1	2 blind replicates
Sample 3	paprika	19.9	2 blind replicates
Sample 4	chilli	23.5	2 blind replicates
Sample 5	paprika	84.9	2 blind replicates
Sample 6	paprika	Sample 9 for spiking	2 blind replicates
Sample 7 received by GROUP A	paprika	Sample 9 for spiking	2 blind replicates
Sample 8 received by GROUP B	chilli	Sample 1 for spiking	2 blind replicates
Sample 9	paprika	<0.1	1 sample

5.2. Preparation

5.2.1. Test samples

The test materials were milled to a particle size < 500 µm, individually homogenized for 4 hours in a Lödige laboratory mixer (Model L20, Paderborn, Germany). Thereafter,

about 100-120 vacuum sealed packages were produced at room temperature. The amount of material in each sachet was about 30 g.

5.2.2. Common calibrant

A common calibrant was distributed, which contained OTA (OTA in the form of powder, as obtained from Sigma, code O-1877, purity 98%, lot 060M4041) - in a mixture of toluene and glacial acetic acid 99:1 (v/v).

About 150 ampoules were filled under inert atmosphere, each with 2.5 ml of calibrant and flame sealed. The ampoules were stored at -18 ºC until dispatch.

The content of the common calibrant was spectrophotometrically verified prior dispatch on three different ampoules randomly chosen in the ampouling sequence, applying Equation 1 below:

Equation 1

$$\rho_{OTA} = \frac{A_{\max} \times M \times 100}{\varepsilon \times b}$$

where

$A_{\max}$ is the absorption determined at the maximum of the absorption curve between a wavelength of 330 nm and 370 nm;

M is the molar mass, in grams per mol, of OTA (M = 403.8 g/mol);

ε is the molar absorption coefficient, in square metres per mol, of OTA in the mixture of toluene and acetic acid 99:1 v/v, (544 m^2/mol);

b is the optical path length, in centimetres, of the quartz cell.

The concentration of OTA was determined to be 9.9 µg/ml.

5.2.3. Spiking solution

Spiking solutions which contained OTA (OTA in the form of powder, as obtained from Sigma, code O-1877, purity 98%, lot 060M4041) - in a mixture of acetonitrile and glacial acetic acid 99:1 (v/v) were prepared.

Ampoules were filled under inert atmosphere, each with 1.5 ml of spiking solution, flame sealed, and stored at -18 ºC until dispatch.

The concentration of OTA was determined to be 0.6 and 2.6 µg/ml. The spiking volume was 500 µl.

5.3. Homogeneity

Sufficient homogeneity was assumed for the test solutions after mixing.

Homogeneities of the paprika and chilli test materials were evaluated according to chapter 3.11.2 of the Harmonised Protocol [**7**]. Ten sample sachets were randomly selected. The content of each sachet was split and the two sub-samples were analysed for OTA by HPLC-FLD. No trend was observed during the analysis sequence and samples were found homogeneous. The results from the homogeneity determination are included in [**Annex 1**].

5.4. Stability

The samples for stability testing were stored at room temperature. The amount of OTA in the test materials and solutions was monitored at the beginning of the study, during the study as well as after receipt of the results of the participants as it is suggested in the Harmonised Protocol. Statistically significant differences of the results of analysis obtained on the three mentioned dates were not found.

6. Results and Discussions

6.1. General

Each participant reported a full set of analytical results as listed in [**Annex 2**]. The results were subject to statistical analysis including outlier testing and the performance characteristics were calculated as shown in [**Table 2**]. HorRat values were derived from the Horwitz function modified by Thompson [**10**], leading to a constant target standard deviation of 22% for analyte levels below 120 µg/kg.

6.2. Evaluation of questionnaire – deviations from the method description

Critical points considered for possible non compliance were significant deviations from the method description and problems/abnormalities reported by the participants. [**Annex 12**]

This was the case for laboratory 106: its results were excluded from the evaluation due to application of a method different from the one required. In particular less air was pushed through the immunoaffinity column after the elution with methanol [**Table 17**] as it was required by the work instruction. Because of this, the final volume of the injection solution was less than 1.5 ml which led to significantly higher results. It proved that the correct application of this step of the method is very crucial as it is highlighted in the method description [**Annex 10**] and in the operation manual [**Annex 11**].
In no other case reported deviations from the method description were considered to be relevant for rejecting the whole set of results from the participants.

6.3. Evaluation of chromatograms

All participants sent chromatograms for analysed samples. Chromatograms were checked for consistency in the retention time of the OTA peak, for peak shape and for integration.

6.4. Evaluation of results

Table 2: Precision estimates calculated for each sample analysed during the collaborative trial study

Sample	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6 (Spiked blank, Sample 9) low level	Sample 7 (Spiked blank, Sample 9) high level GROUP A	Sample 8 (Spiked Sample 1) high level GROUP B
Test Material	chilli	paprika	paprika	chilli	paprika	paprika	paprika	chilli
Number of laboratories	21	21	21	21	21	21	10	11
Number of laboratories considered as non compliant	1	1	1	1	1	1	0	1
Number of outliers (laboratories)	0	2	0	0	1	0	0	0
Number of accepted results	20	18	20	20	19	20	10	10
Mean value, $\bar{x}$, µg/kg	1.8	6.1	19.9	23.5	84.9	11.2	45.2	45.0
Repeatability standard deviation s_r, µg/kg	0.3	0.4	1.3	1.1	7.4	0.5	2.6	0.8
Repeatability relative standard deviation, RSD_r, %	14.3	6.9	6.8	4.6	8.7	4.7	5.7	1.7
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	0.7	1.2	3.8	3.0	20.8	1.5	7.3	2.2
Reproducibility standard deviation s_R, µg/kg	0.5	0.9	2.9	2.4	10.5	1.4	4.1	5.2
Reproducibility relative standard deviation, RSD_R, %	27.5	14.0	14.5	10.4	12.4	12.2	9.1	11.6
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	1.4	2.4	8.1	6.8	29.5	3.8	11.6	14.7
Recovery, %	n.a.	n.a.	n.a.	n.a.	n.a.	87.0	87.5	83.7
HorRat value	1.3	0.6	0.7	0.5	0.6	0.6	0.4	0.5

n.a.: not applicable

The method performance parameters are reported in [**Table 2**].
Results for Sample 9 are not shown as they related to single measurement of blank paprika. Reported values for Sample 9 are in [**Table 13**].

As EU legislation for food requires to consider analyte recovery for accepting or rejection of lots in official food control: the principle of recovery correction was applied in this study. As a result, the data sets of the analytical results from naturally contaminated materials were corrected with the mean recovery value of the recovery experiments (two duplicates). The result of this treatment on the calculated method performance is shown in [**Table 3**].

Table 3: Precision estimates calculated for naturally contaminated materials after recovery correction of results

Sample	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Test Material	chilli	paprika	paprika	chilli	paprika
Number of laboratories	21	21	21	21	21
Number of laboratories considered as non compliant	1	1	1	1	1
Number of outliers (laboratories)	0	2	0	0	0
Number of accepted results	20	18	20	20	20
Mean value, $\bar{x}$, µg/kg	2.1	7.1	23.1	27.3	100.5
Repeatability standard deviation s_r, µg/kg	0.3	0.5	1.5	1.2	8.2
Repeatability relative standard deviation, RSD_r, %	14.2	7.1	6.5	4.5	8.1
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	0.8	1.4	4.2	3.4	22.9
Reproducibility standard deviation s_R, µg/kg	0.6	0.6	2.1	2.7	10.7
Reproducibility relative standard deviation, RSD_R, %	28.9	8.9	9.0	9.8	10.7
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	1.7	1.8	5.8	7.5	30.0
HorRat value	1.3	0.4	0.4	0.4	0.5

7. Interpretation of the results and conclusion

The applicability range was found to be 2 to 85 µg/kg OTA.
Reproducibility and repeatability from this study complies with legislative requirements [12] for food at levels up to 10 µg/kg: $RSD_r \leq 20\%$, $RSD_R \leq 30\%$.
The mean recoveries calculated range in the narrow window of 83.7% - 87.5%, which are within the legislatively required range (70-110%).
The HorRat values obtained ranged from 0.4 to 1.3, taking into account that the highest HorRat of 1.3 was obtained for the material with the lowest OTA content (1.8 µg/kg). All other HorRat values were below 0.7 [**Table 2**].

As a result of the recovery corrections [**Table 3**], the performance increased significantly and reproducibility showed unexpectedly low values, indicating that for this type of analysis the correction for recovery results is a drastic improvement of the method performance under the conditions of this study (use of a common calibrant, common spiking procedure). The improved reproducibility after recovery correction is very close to the calculated repeatability.

This shows a satisfactory performance of the method and that it meets the requirements for precision and recovery as laid down in Regulation 401/2006.

As a result the method will be submitted to CEN TC 275 for consideration as basis for a future CEN standard.

8. Acknowledgements

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The organisers thank Pedro A. Burdaspal (Centro Nacional De Alimentacion, Madrid Spain) and John Keegan (Public Analyst's Laboratory, Dublin, Ireland) for supplying the materials used for this study.
We are grateful to Franz Ulberth for accurate revision of this report and the Reference Materials Unit at IRMM, in particular Håkan Emteborg, for ampouling of the test samples.
The authors would also like to thank Katrien Bouten and Carsten Mischke for assistance with preparing, sealing and shipping the samples.
We wish to acknowledge the help of Silvana Albanese for the grammar corrections in this report.

The laboratories participating in this exercise, listed in [**Table 4**], are also kindly acknowledged.

Table 4: Participating laboratories

Organisation	Country
OLEOTEST n.v.	Belgium
FAVV	Belgium
Agricultural Research Centre, Lab for Residues and Contaminants	Estonia
Finnish Customs Laboratory	Finland
Laboratoire SCL de Rennes	France
Bavarian Health and Food Safety Authority	Germany
Eurofins WEJ Contaminants GmbH	Germany
General Chemical State Laboratory	Greece
NFCSO FFSD Feed Investigating NRL	Hungary
NFCSO FFSD Food Investigating NRL	Hungary
ARPA Piemonte	Italy
Public Analyst's Laboratory, Dublin	Ireland
Institute "BIOR"	Latvia
National Institute of Public Health - National Institute of Hygiene	Poland
Health Sciences Authority	Singapore
Laboratorio Normativo de Salud Pública	Spain
Laboratori Salut Pública a Tarragona. ASPC. Departament de Salut.	Spain
National Veterinary Institute (SVA)	Sweden
Kent Scientific Services	United Kingdom
Food & Environment Research Agency	United Kingdom
Edinburgh Scientific Services	United Kingdom

9. Abbreviations, definitions

CEN	European Committee for Standardisation
EC	European Commission
ELISA	Enzyme linked immunosorbant assays
EU	European Union
EU-RL	European Reference Laboratory
FLD	Fluorescent detection
HPLC	High-performance liquid chromatography
IAC	Immunoaffinity column
IRMM	Institute for Reference Materials and Measurements
ISO	International Organisation for Standardisation
IUPAC	International Union for Pure and Applied Chemistry
JRC	Joint Research Centre
OTA	Ochratoxin A

Repeatability: Precision under repeatability conditions, i.e. conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time. [ISO 3534-1]
Reproducibility: Precision under reproducibility conditions, i.e. conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment. [ISO 3534-1]
HorRat value: ratio of the reproducibility relative standard deviation to the target standard deviation (calculated by Horwitz equation modified by Thompson for the concentration below 120 ppb)
Cochran test: removal of laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material
Grubbs test: removal of laboratories with extreme averages

10. References

[**1**] Commission Regulation (EC), No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, 2006. L364: p. 5-24

[**2**] Commission Regulation (EU) No 105/2010 of 5 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Ochratoxin A http://www.fsai.ie/uploadedFiles/Legislation/FSAI_-_Legislation/2010/02_feb2010/Reg105_2010.pdf

[**3**] Lerda, D., Ambrosio, M., Kunsagi, Z., Emteborg, H., Charoud-Got, J., Stroka, J., Report on the inter-laboratory comparison organised by the European Union Reference Laboratory for Mycotoxins for the validation of a method for the determination of Ochratoxin A in liqourice roots and extracts, EUR 24778 EN: 2011

[**4**] Lerda, D., Mycotoxins Factsheet Fourth Edition – September 2011 – Joint Research Centre http://irmm.jrc.ec.europa.eu/EURLs/eurl_mycotoxins/Documents/Factsheet%20Mycotoxins.pdf

[**5**] Stroka J., Ambrosio M., Doncheva I. and Mischke C., Report on the 2007 Proficiency Test for the Determination of Ochratoxin A in Capsicum spp. (Paprika Powder), EUR 23382 EN:2008

[**6**] Kunsagi, Z., Ambrosio M., Breidbach A. and Stroka J., Report on the 2010 Proficiency Test of the European Union Reference Laboratory for Mycotoxins, for the Network of National Reference Laboratories, EUR 24621 EN: 2010

[**7**] Thompson, M., Ellison, S.L.R., and Wood, R., The International Harmonised Protocol for the Proficiency Testing of Analytical Chemistry Laboratories. Pure Appl. Chem., 2006. 78(1): p. 145–196.
http://media.iupac.org/publications/pac/2006/pdf/7801x0145.pdf

[**8**] Practical guide to ISO 5725-2:1994 — Accuracy (trueness and precision) of measurement methods and results — Part 2 : Basic method for the determination of repeatability and reproducibility of a standard measurement method. Geneva, Switzerland

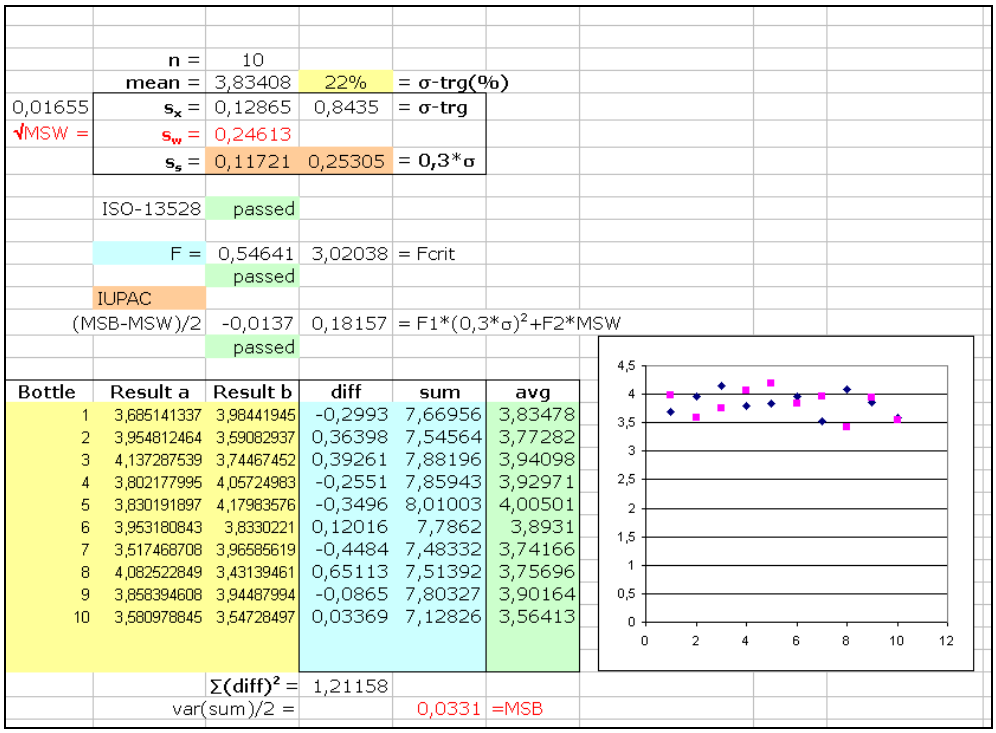
[**9**] Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, 2006. L 70: p. 12-34.

[**10**] Thompson, M., Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing, Analyst, 2000, 125, 385-386

11. Annex

Annex 1 – Homogeneity data

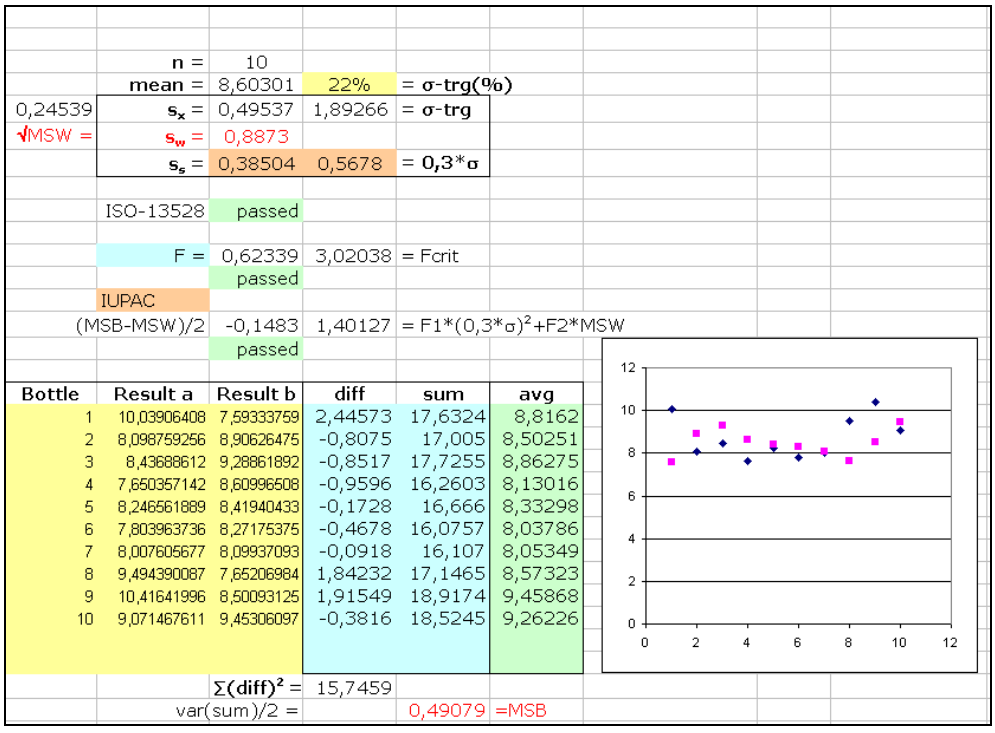
Figure 2: Homogeneity data for Sample 1 (chilli)



	n =	10			
	mean =	3,83408	22%	= σ-trg(%)	
0,01655	s_x =	0,12865	0,8435	= σ-trg	
√MSW =	s_w =	0,24613			
	s_s =	0,11721	0,25305	= 0,3*σ	
	ISO-13528	passed			
	F =	0,54641	3,02038	= Fcrit	
		passed			
	IUPAC				
	(MSB-MSW)/2	-0,0137	0,18157	= F1*(0,3*σ)²+F2*MSW	
		passed			

Bottle	Result a	Result b	diff	sum	avg
1	3,685141337	3,98441945	-0,2993	7,66956	3,83478
2	3,954812464	3,59082937	0,36398	7,54564	3,77282
3	4,137287539	3,74467452	0,39261	7,88196	3,94098
4	3,802177995	4,05724983	-0,2551	7,85943	3,92971
5	3,830191897	4,17983576	-0,3496	8,01003	4,00501
6	3,953180843	3,8330221	0,12016	7,7862	3,8931
7	3,517468708	3,96585619	-0,4484	7,48332	3,74166
8	4,082522849	3,43139461	0,65113	7,51392	3,75696
9	3,858394608	3,94487994	-0,0865	7,80327	3,90164
10	3,580978845	3,54728497	0,03369	7,12826	3,56413
		Σ(diff)² =	1,21158		
		var(sum)/2 =		0,0331	=MSB

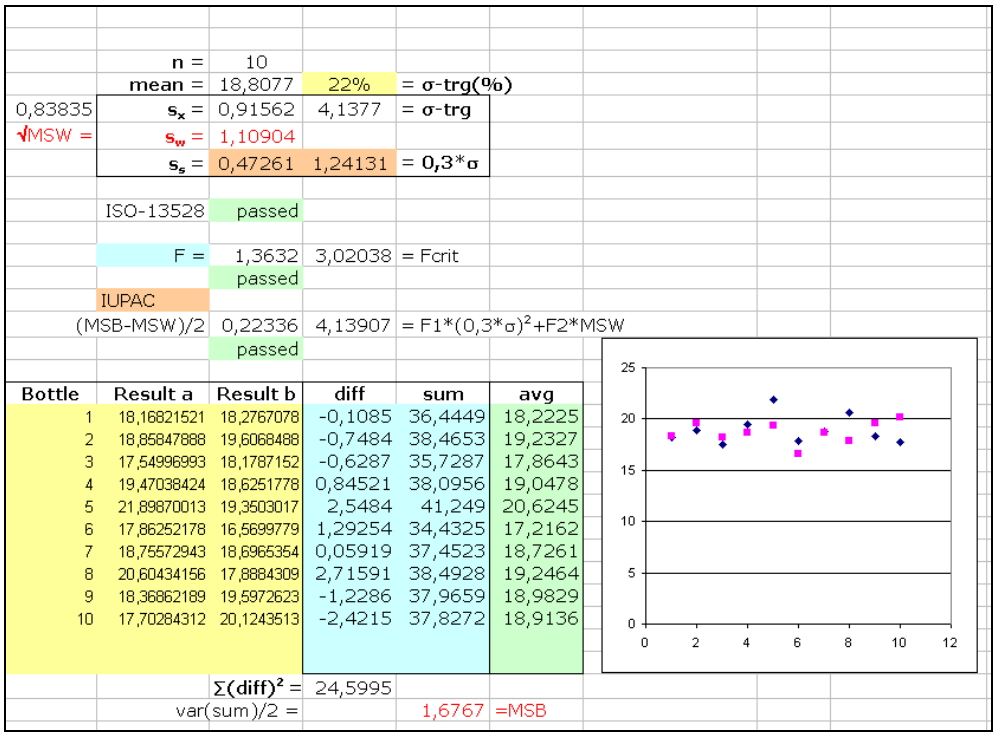
Figure 3: Homogeneity data for Sample 2 (paprika)



	n =	10			
	mean =	8,60301	22%	= σ-trg(%)	
0,24539	s_x =	0,49537	1,89266	= σ-trg	
√MSW =	s_w =	0,8873			
	s_s =	0,38504	0,5678	= 0,3*σ	
	ISO-13528	passed			
	F =	0,62339	3,02038	= Fcrit	
		passed			
	IUPAC				
	(MSB-MSW)/2	-0,1483	1,40127	= F1*(0,3*σ)²+F2*MSW	
		passed			

Bottle	Result a	Result b	diff	sum	avg
1	10,03906408	7,59333759	2,44573	17,6324	8,8162
2	8,098759256	8,90626475	-0,8075	17,005	8,50251
3	8,43688612	9,28861892	-0,8517	17,7255	8,86275
4	7,650357142	8,60996508	-0,9596	16,2603	8,13016
5	8,246561889	8,41940433	-0,1728	16,666	8,33298
6	7,803963736	8,27175375	-0,4678	16,0757	8,03786
7	8,007605677	8,09937093	-0,0918	16,107	8,05349
8	9,494390087	7,65206984	1,84232	17,1465	8,57323
9	10,41641996	8,50093125	1,91549	18,9174	9,45868
10	9,071467611	9,45306097	-0,3816	18,5245	9,26226
		Σ(diff)² =	15,7459		
		var(sum)/2 =		0,49079	=MSB

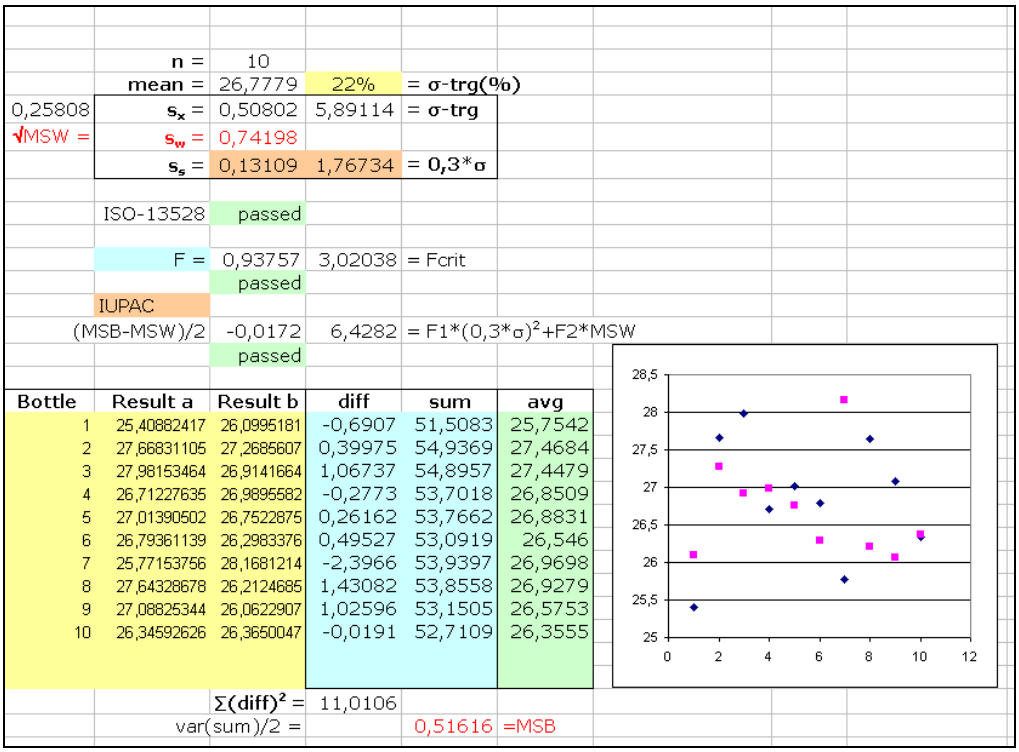
Figure 4: Homogeneity data for Sample 3 (paprika)



	n =	10			
	mean =	18,8077	22%	= σ-trg(%)	
0,83835	s_x =	0,91562	4,1377	= σ-trg	
√MSW =	s_w =	1,10904			
	s_s =	0,47261	1,24131	= 0,3*σ	
	ISO-13528	passed			
	F =	1,3632	3,02038	= Fcrit	
		passed			
	IUPAC				
	(MSB-MSW)/2	0,22336	4,13907	= F1*(0,3*σ)²+F2*MSW	
		passed			

Bottle	Result a	Result b	diff	sum	avg
1	18,16821521	18,2767078	-0,1085	36,4449	18,2225
2	18,85847888	19,6068488	-0,7484	38,4653	19,2327
3	17,54996993	18,1787152	-0,6287	35,7287	17,8643
4	19,47038424	18,6251778	0,84521	38,0956	19,0478
5	21,89870013	19,3503017	2,5484	41,249	20,6245
6	17,86252178	16,5699779	1,29254	34,4325	17,2162
7	18,75572943	18,6965354	0,05919	37,4523	18,7261
8	20,60434156	17,8884309	2,71591	38,4928	19,2464
9	18,36862189	19,5972623	-1,2286	37,9659	18,9829
10	17,70284312	20,1243513	-2,4215	37,8272	18,9136
		Σ(diff)² =	24,5995		
		var(sum)/2 =		1,6767	=MSB

Figure 5: Homogeneity data for Sample 4 (chilli)



	n =	10			
	mean =	26,7779	22%	= σ-trg(%)	
0,25808	s_x =	0,50802	5,89114	= σ-trg	
√MSW =	s_w =	0,74198			
	s_s =	0,13109	1,76734	= 0,3*σ	
	ISO-13528	passed			
	F =	0,93757	3,02038	= Fcrit	
		passed			
	IUPAC				
	(MSB-MSW)/2	-0,0172	6,4282	= F1*(0,3*σ)²+F2*MSW	
		passed			

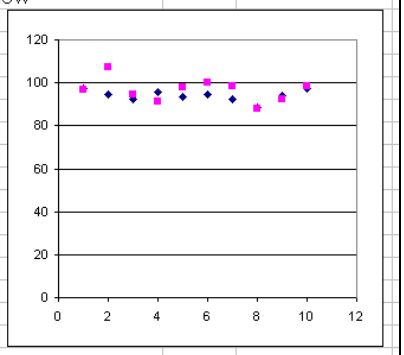
Bottle	Result a	Result b	diff	sum	avg
1	25,40882417	26,0995181	-0,6907	51,5083	25,7542
2	27,66831105	27,2685607	0,39975	54,9369	27,4684
3	27,98153464	26,9141664	1,06737	54,8957	27,4479
4	26,71227635	26,9895582	-0,2773	53,7018	26,8509
5	27,01390502	26,7522875	0,26162	53,7662	26,8831
6	26,79361139	26,2983376	0,49527	53,0919	26,546
7	25,77153756	28,1681214	-2,3966	53,9397	26,9698
8	27,64328678	26,2124685	1,43082	53,8558	26,9279
9	27,08825344	26,0622907	1,02596	53,1505	26,5753
10	26,34592626	26,3650047	-0,0191	52,7109	26,3555
		Σ(diff)² =	11,0106		
		var(sum)/2 =		0,51616	=MSB

Figure 6: Homogeneity data for Sample 5 (paprika)

	n =	10		
	mean =	95,2323	22%	= σ-trg(%)
11,4133	s_x =	3,37836	20,9511	= σ-trg
√MSW =	s_w =	3,80875		
	s_s =	2,03962	6,28533	= 0,3*σ
	ISO-13528	passed		
	F =	1,57354	3,02038	= Fcrit
		passed		
	IUPAC			
	(MSB-MSW)/2	4,16005	88,9218	= F1*(0,3*σ)²+F2*MSW
		passed		

Bottle	Result a	Result b	diff	sum	avg
1	97,23932214	96,8114718	0,42785	194,051	97,0254
2	94,3488886	107,501378	-13,152	201,85	100,925
3	92,45520084	94,8350449	-2,3798	187,29	93,6451
4	95,40686013	91,3814568	4,0254	186,788	93,3942
5	93,20777354	98,0507434	-4,843	191,259	95,6293
6	94,56777042	99,8163473	-5,2486	194,384	97,1921
7	92,15964627	98,485025	-6,3254	190,645	95,3223
8	88,66616816	88,1253752	0,54079	176,792	88,3958
9	93,92550665	92,3078329	1,61767	186,233	93,1167
10	97,13621391	98,2184804	-1,0823	195,355	97,6773

	$\Sigma(diff)^2$ =	290,132	
	var(sum)/2 =	22,8267	=MSB



Sample 6: spiked blank paprika material (Sample 9)

Sample 7: spiked blank paprika material (Sample 9)

Sample 8: spiked chilli material (Sample 1)

Sample 9: blank paprika material

Annex 2 – Results

Table 5: Sample 1 (chilli powder – low level)

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	1.61	1.97
106	1.76	3.13
109	1.79	1.73
121	2.15	2.11
124	0.498	0.784
125	1.37	1.04
128	2.06	2.21
130	1.1	1.72
133	2.1	2.26
136	2	1.8
137	2.22	2.25
148	2.04	2.16
156	1.45	1.33
159	1.66	2.03
161	1.84	1.55
164	2.61	1.66
168	2.25	1.96
172	1.88	2.04
175	1.1	0.71
186	1.87	1.33
197	2.25	2.04

Lab 106 was considered as a non compliant.

Figure 7: Distribution of individual results of replicate measurements (Sample 1)

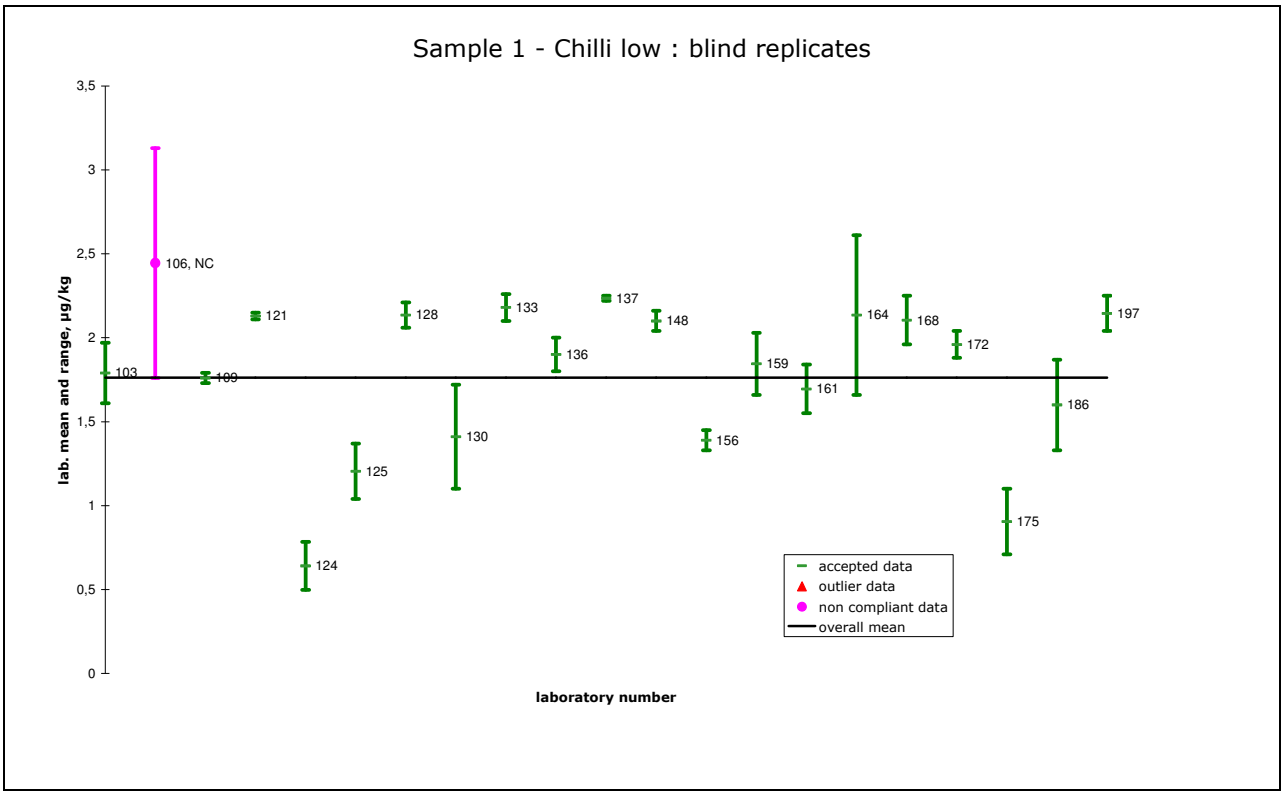


Figure 8: Youden plot (Sample 1)

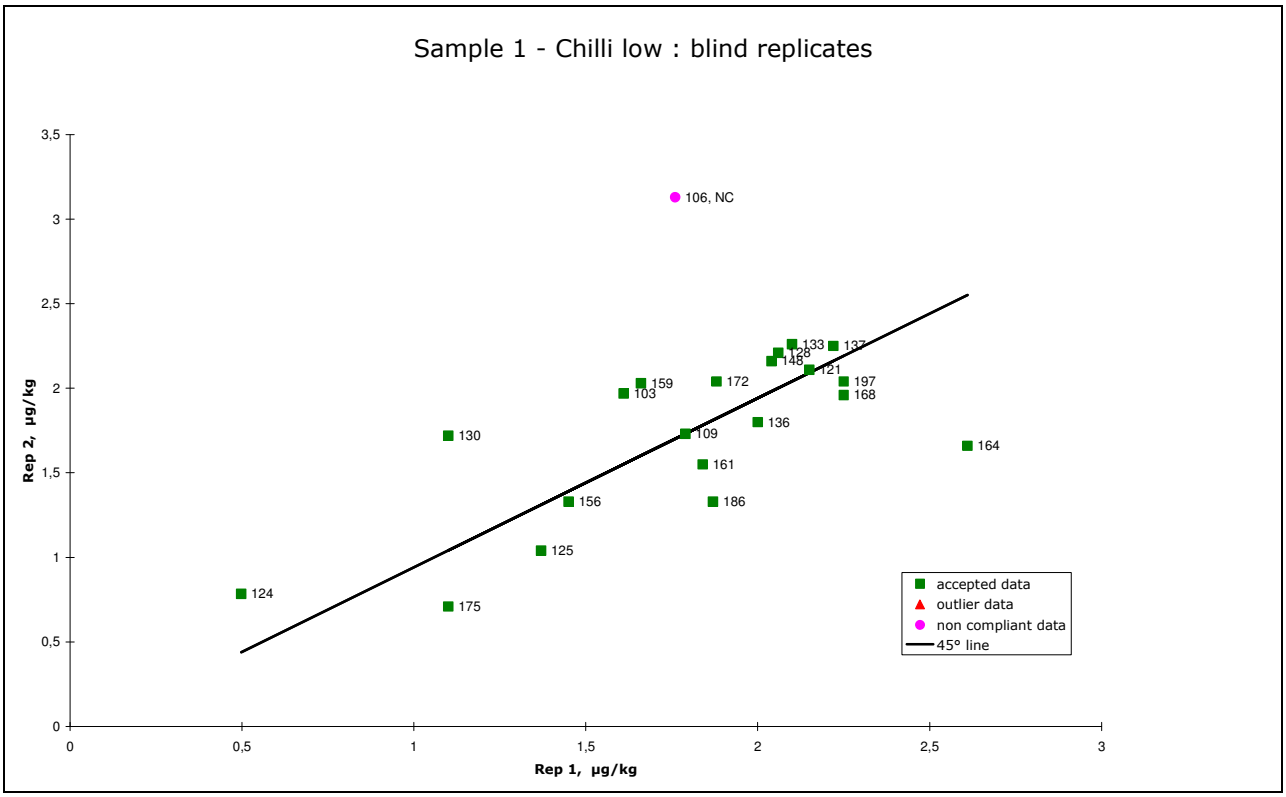


Table 6: Sample 2 (paprika powder – low level)

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	5.6	5.83
106	9.58	6.82
109	6.46	6.4
121	6.41	6.17
124	7.909	7.402
125	4.9	5.15
128	6.37	9.87
130	6.63	6.08
133	5.99	6.01
136	3.9	5.4
137	7.32	7.59
148	5.64	5.65
156	5.77	5.8
159	9.1	5.98
161	5.82	7.35
164	6.76	6.45
168	5.07	5.91
172	5.63	5.59
175	5.58	5.4
186	6.06	6.36
197	7.11	7.46

Lab 106 was considered as a non compliant.
Lab 128 was considered as an outlier applying the Cochran test.
Lab 159 was considered as an outlier applying the Cochran test.

Figure 9: Distribution of individual results of replicate measurements (Sample 2)

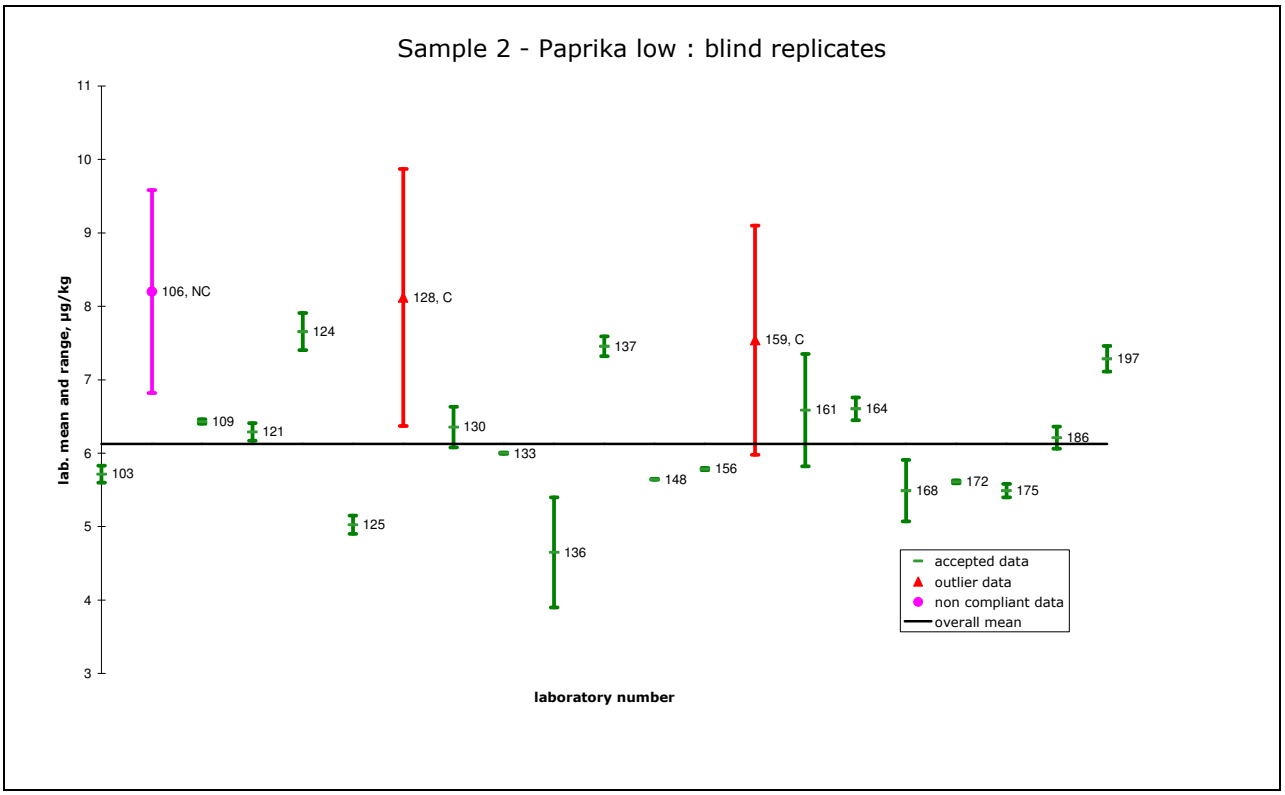


Figure 10: Youden plot (Sample 2)

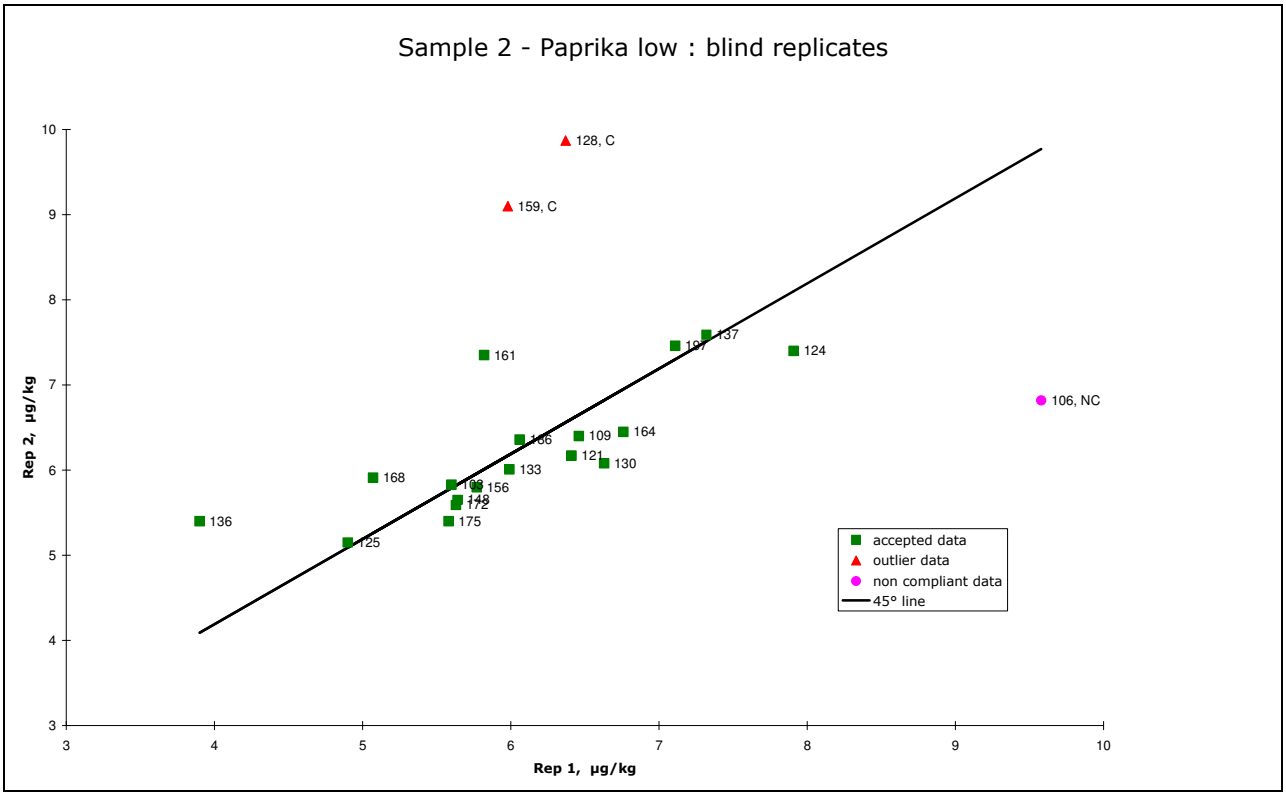


Table 7: Sample 3 (paprika powder - medium)

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	18.75	17.91
106	30.26	13.07
109	21.29	20.39
121	22.6	18.8
124	26.103	25.013
125	17.02	18.01
128	20.43	22.17
130	25.11	22.11
133	18.9	17.57
136	17.7	16.6
137	25.2	23.25
148	18.37	18.17
156	19.17	19.48
159	22.69	18.2
161	22.47	20.08
164	16.87	16.34
168	15.84	15.05
172	20.1	17.4
175	18.42	17.67
186	19.42	18.77
197	23.31	23.85

Lab 106 was considered as a non compliant.

Figure 11: Distribution of individual results of replicate measurements (Sample 3)

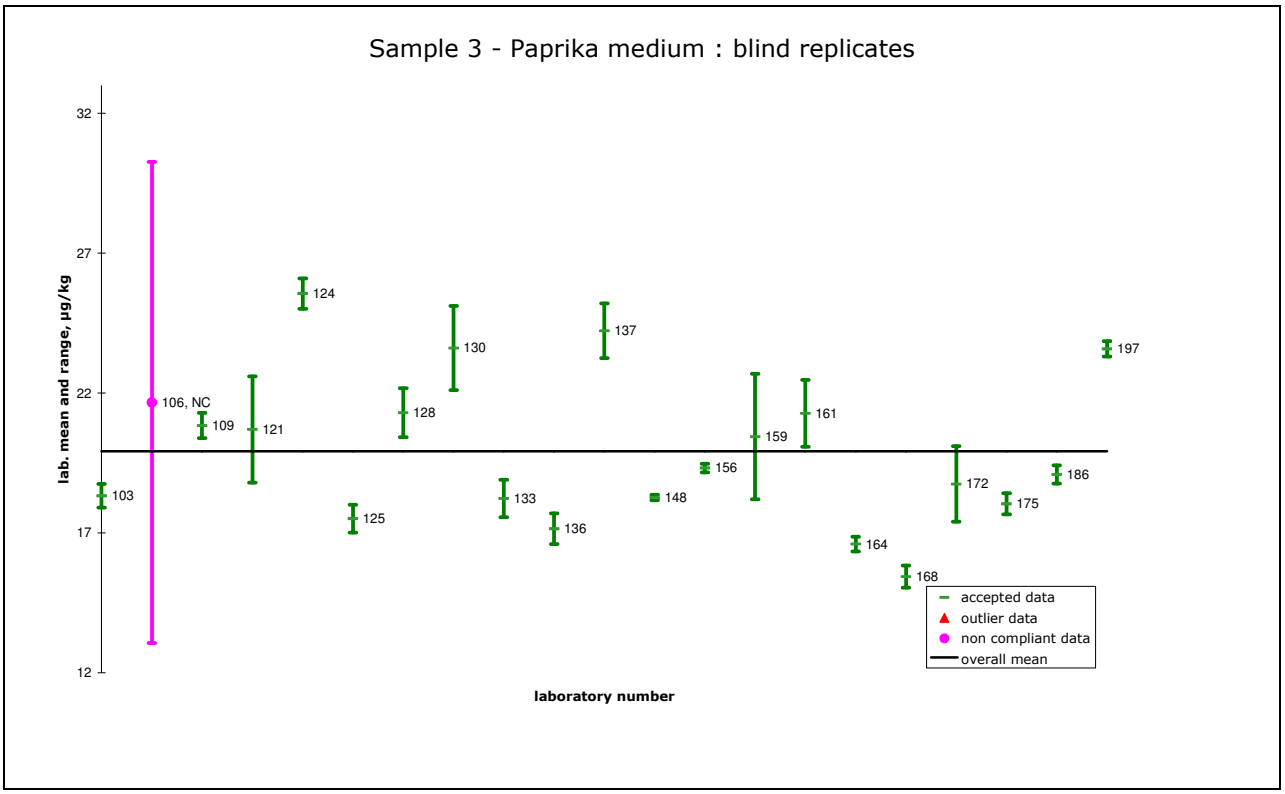


Figure 12: Youden plot (Sample 3)

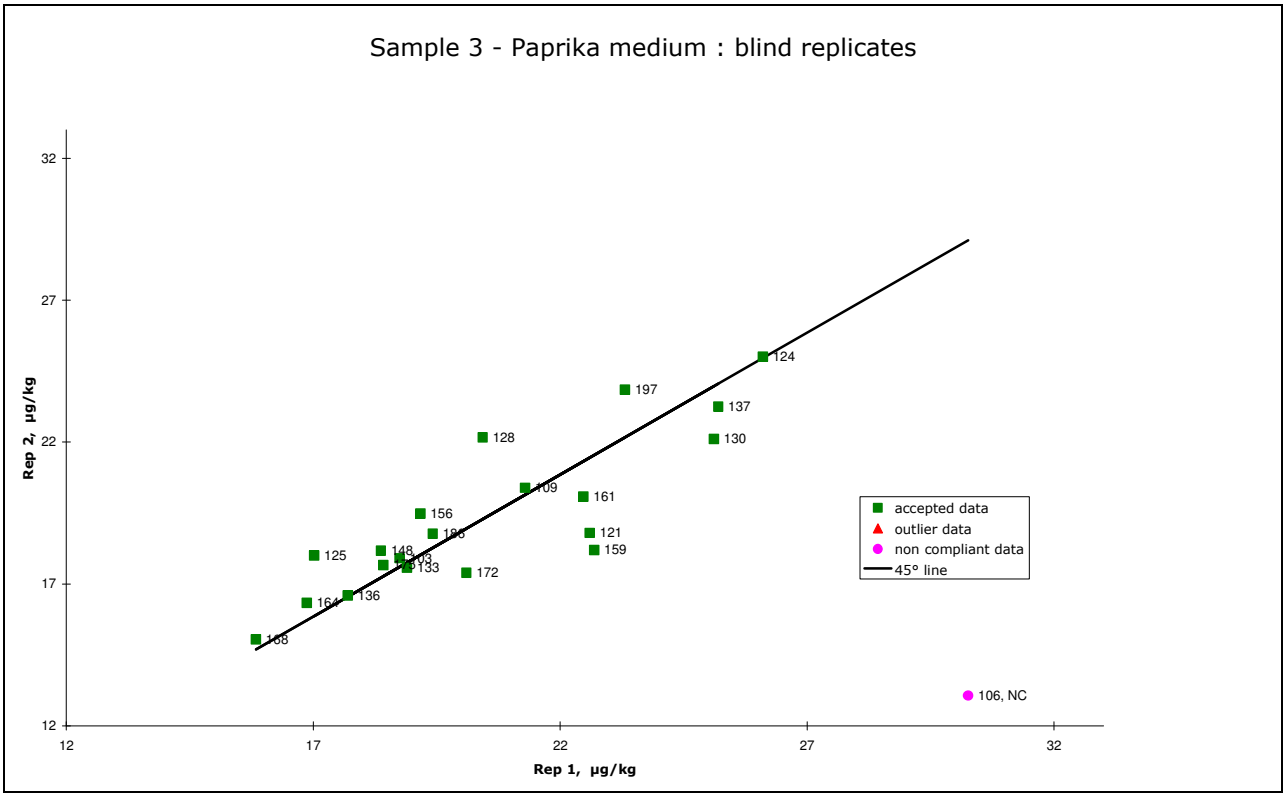


Table 8: Sample 4 (chilli powder – medium level)

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	20.73	21.05
106	26.98	29.41
109	24.15	23.72
121	23.12	24.14
124	29.096	29.888
125	21.95	22.03
128	26.91	24.61
130	26.01	26.27
133	23.02	23.24
136	23.3	23.5
137	25.68	25.92
148	21.81	20.98
156	22.49	23.15
159	21.68	19.87
161	25.17	21.36
164	19.02	22.41
168	20.94	19.53
172	22.85	22.3
175	23.68	23.33
186	24.95	22.66
197	26.39	25.91

Lab 106 was considered as a non compliant.

Figure 13: Distribution of individual results of replicate measurements (Sample 4)

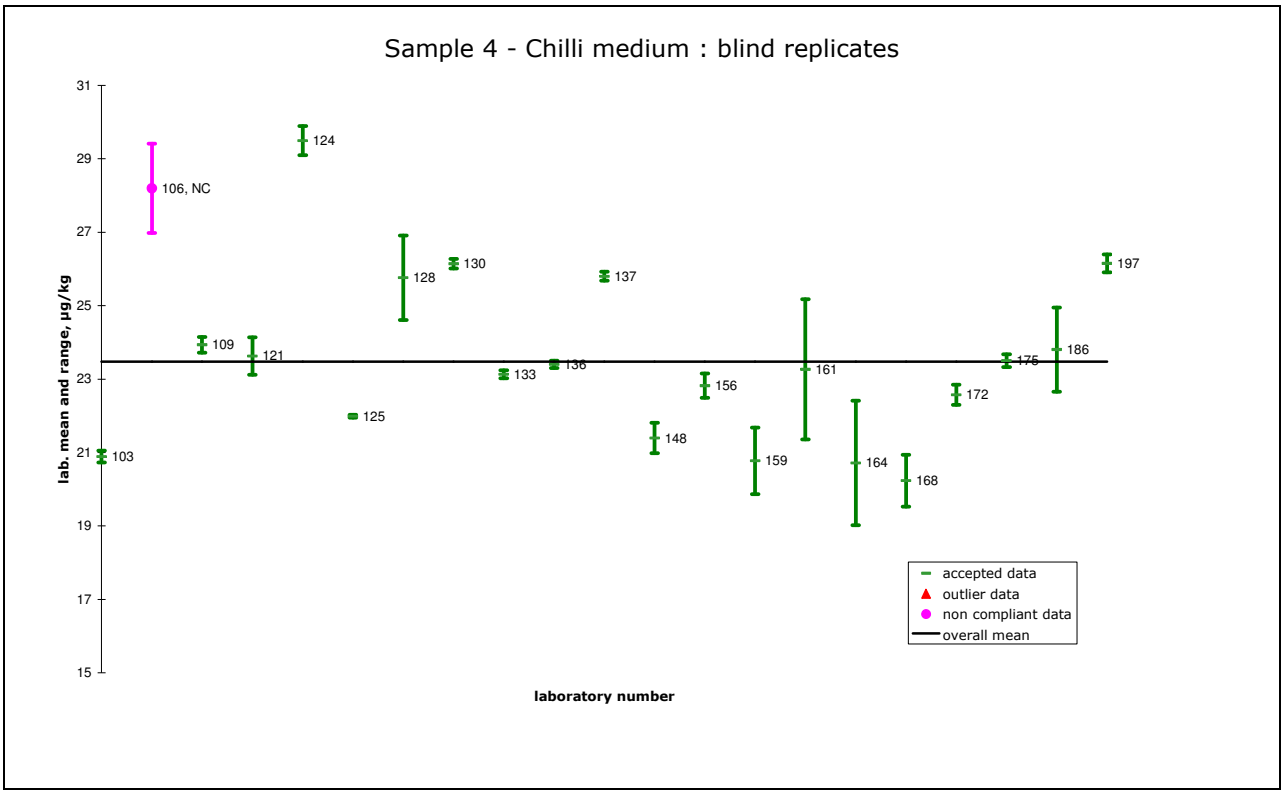


Figure 14: Youden plot (Sample 4)

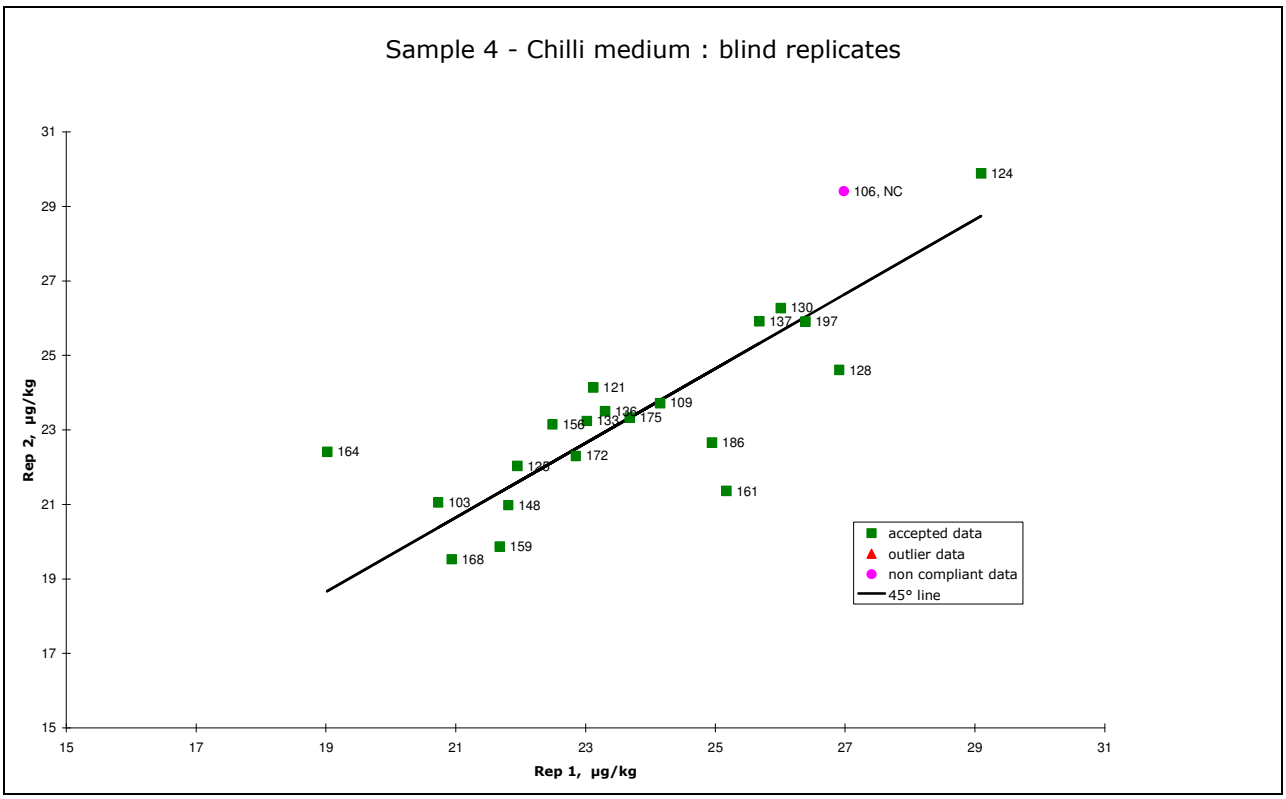


Table 9: Sample 5 (paprika powder – high level)

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	82.15	79.92
106	106.96	133.37
109	85.93	86.02
121	86.14	84.12
124	124.091	119.526
125	91.4	82.5
128	83.69	102.6
130	95.83	99.23
133	86.6	86.65
136	71.1	77.6
137	96.9	92.82
148	79.49	75.6
156	86.32	90.55
159	72.89	93.95
161	90.1	93.73
164	57.22	87.04
168	72.58	69.77
172	84.5	84.1
175	74.33	62.75
186	86.42	87.89
197	98.65	105.71

Lab 106 was considered as a non compliant.
Lab 124 was considered as an outlier applying the Grubb's single outlier test.

Figure 15: Distribution of individual results of replicate measurements (Sample 5)

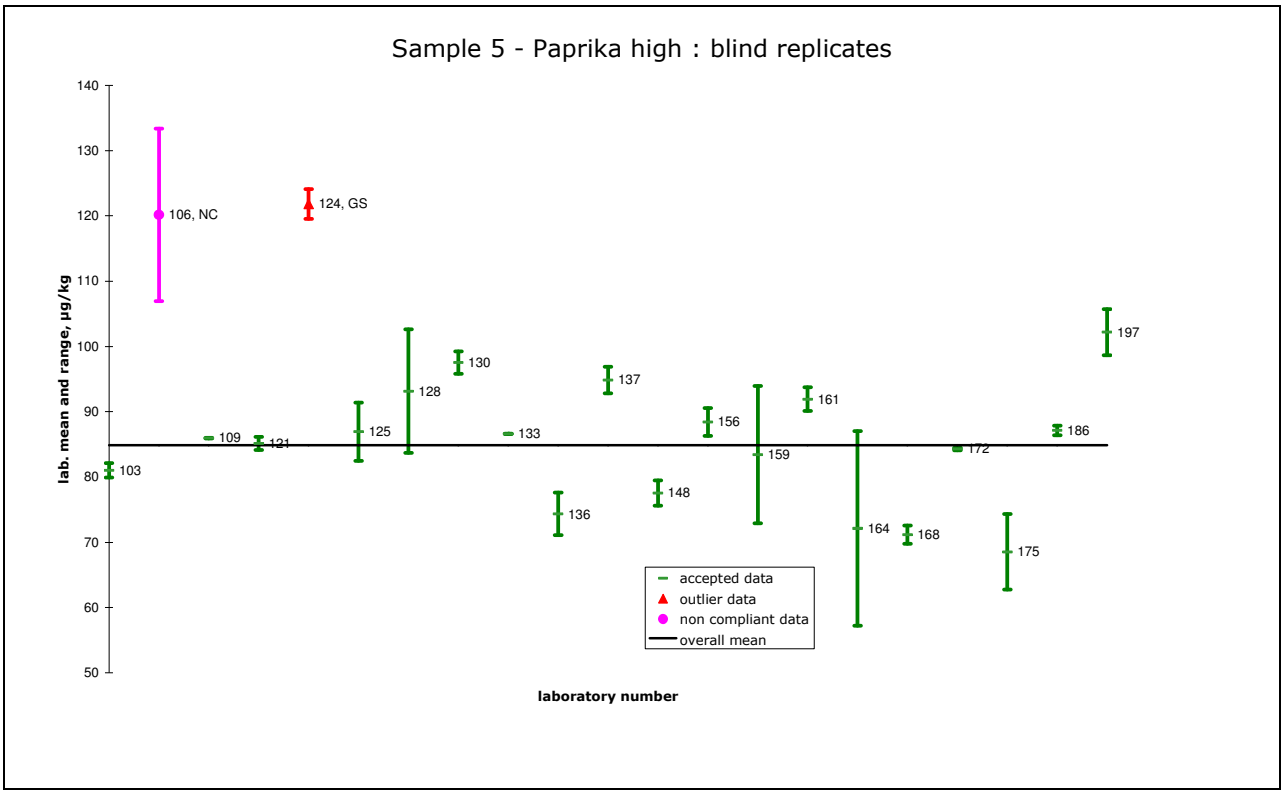


Figure 16: Youden plot (Sample 5)

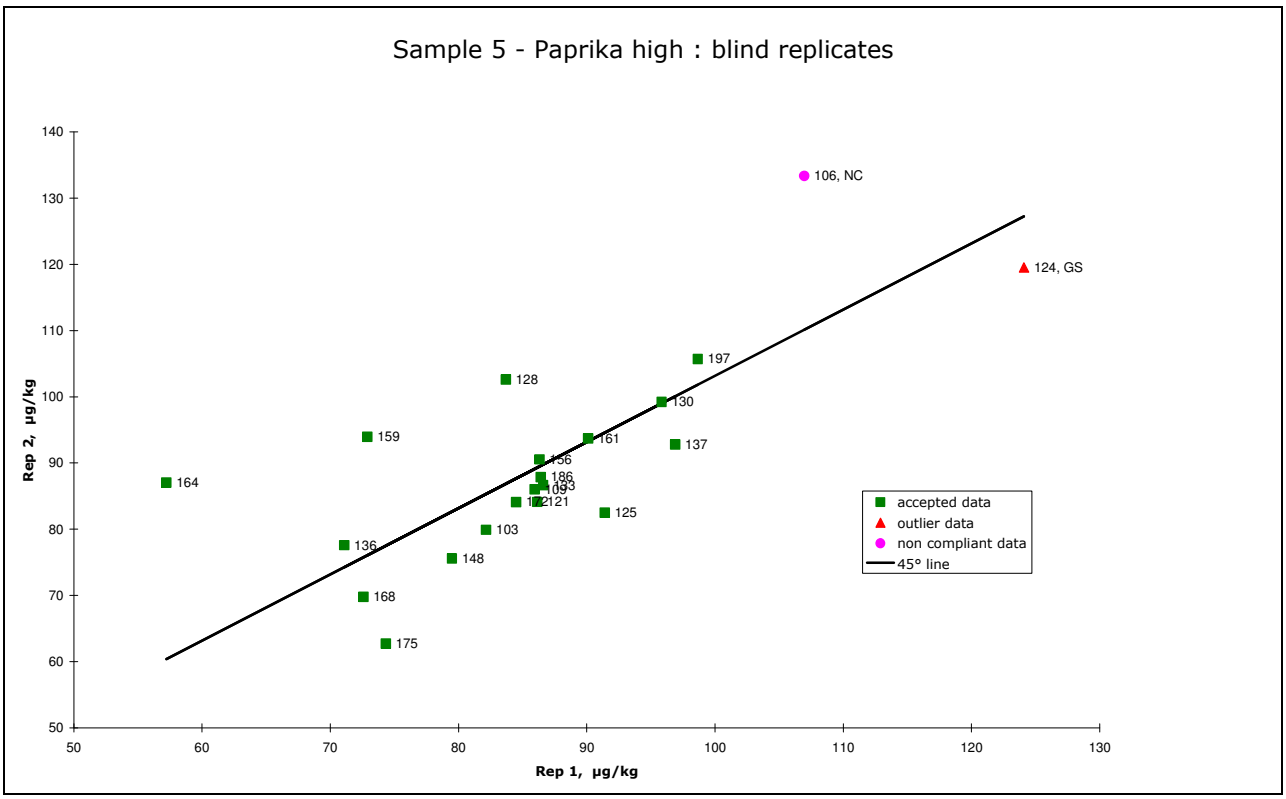


Table 10: Sample 6 - Spiked blank sample - low level (paprika powder)
Spiked with 12.9 (µg/kg) OTA. Initial OTA content prior spiking was determined as <LOD by organizer.

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	11.02	10.25
106	14.86	15.31
109	11.39	11.34
121	10.86	11.35
124	13.141	12.568
125	10.74	10.29
128	11.43	11.35
130	11.49	11.35
133	10.96	11
136	10	10.3
137	12.33	12.6
148	10.25	8.78
156	10.72	10.81
159	14.32	14.45
161	12.34	11.8
164	12.32	12.88
168	8.55	9.47
172	10.13	10.37
175	10.1	9.52
186	9.14	11.16
197	13.15	11.9

Lab 106 was considered as a non compliant.

Figure 17: Distribution of individual results of replicate measurements (Sample 6)

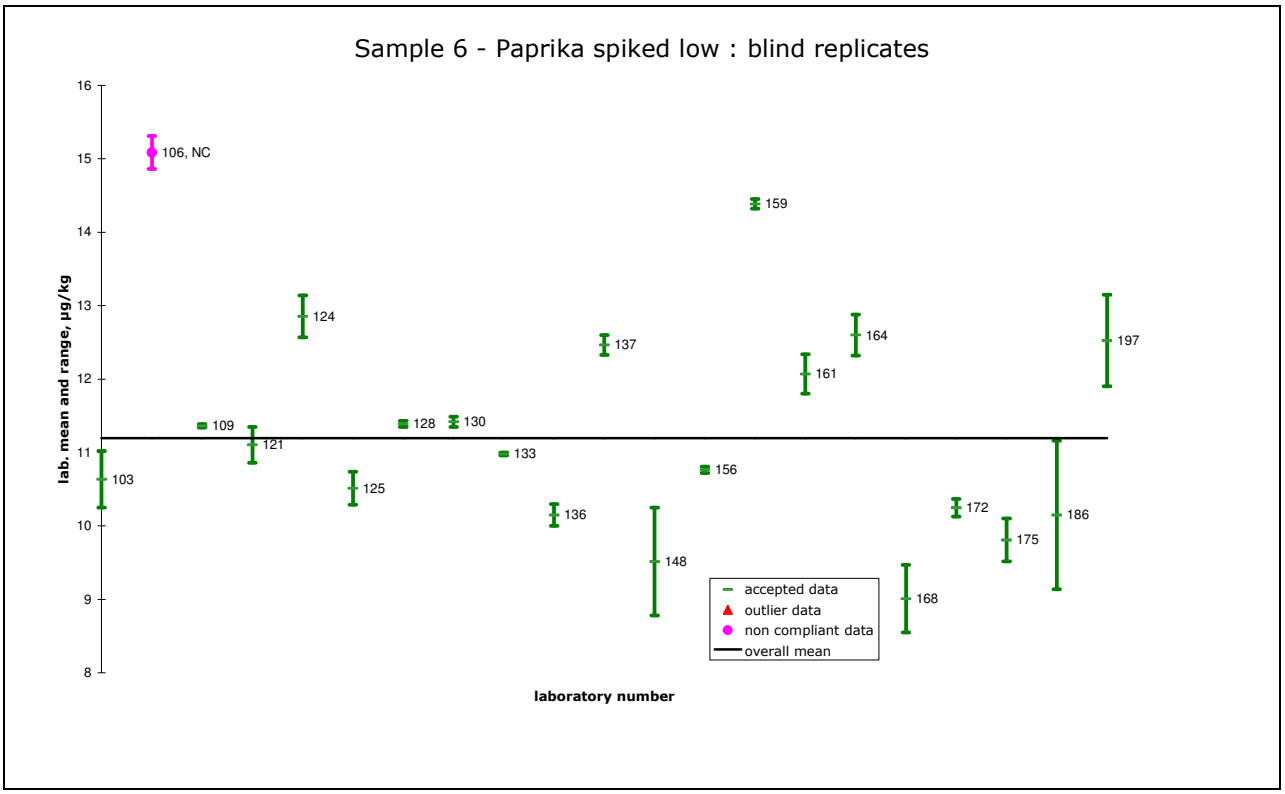


Figure 18: Youden plot (Sample 6)

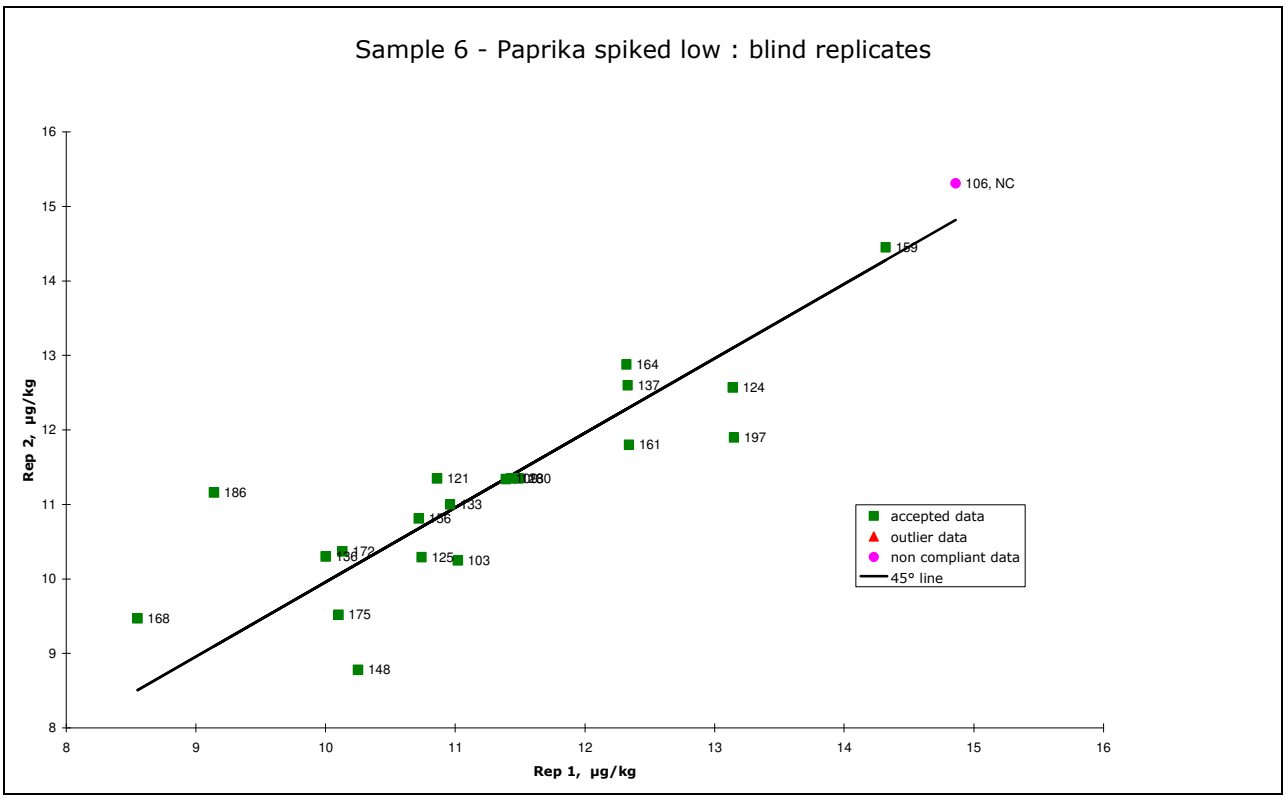


Table 11: Sample 7 - Spiked blank sample - high level - GROUP A (paprika powder)
Spiked with 51.7 (µg/kg) OTA. Initial OTA content prior spiking was determined as <LOD by organizer.

Lab code	**Result 1** (µg/kg)	**Result 2** (µg/kg)
103	42.87	44.14
109	46.18	44.7
121	46.65	43.85
125	42.49	43.13
133	43.63	43.68
137	52.2	52.5
159	46.25	41.86
161	48.64	46.57
175	42.88	34.44
197	45.93	51.16

Figure 19: Distribution of individual results of replicate measurements (Sample 7)

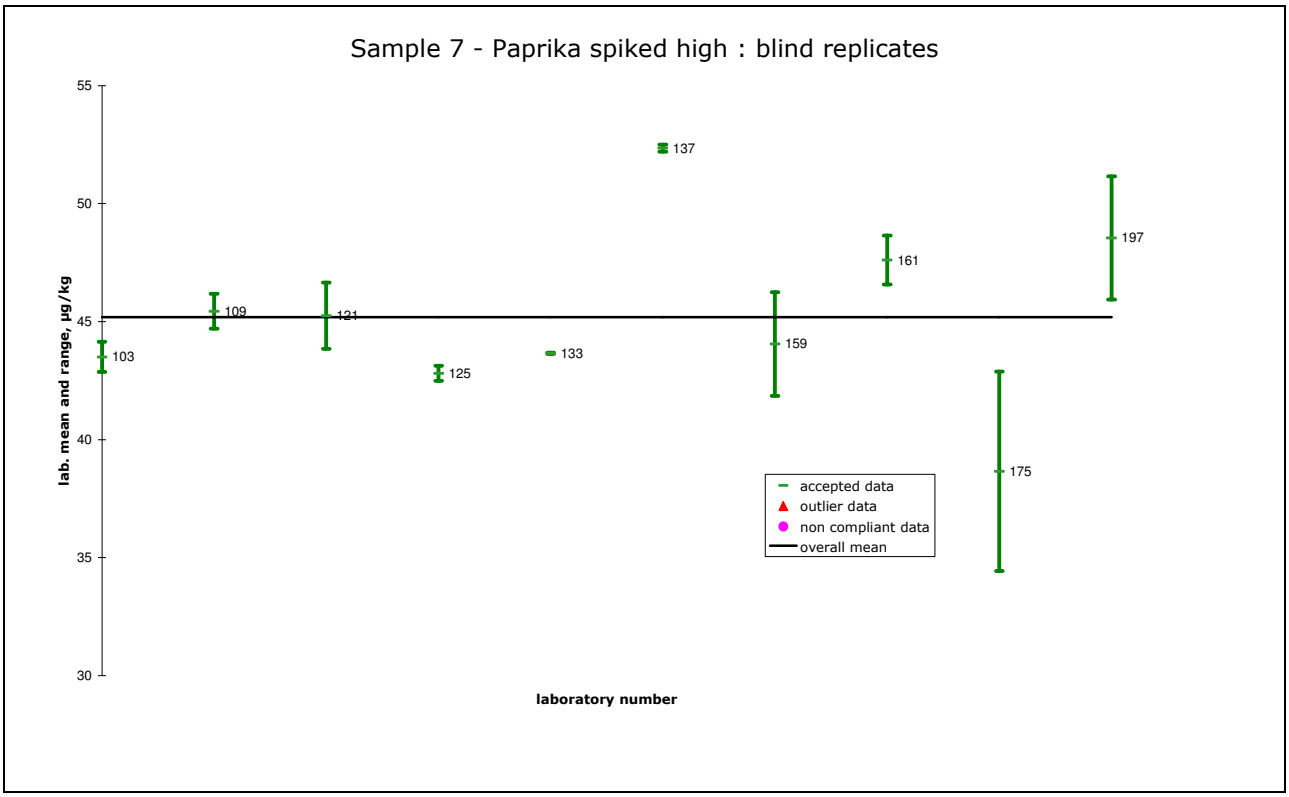


Figure 20: Youden plot (Sample 7)

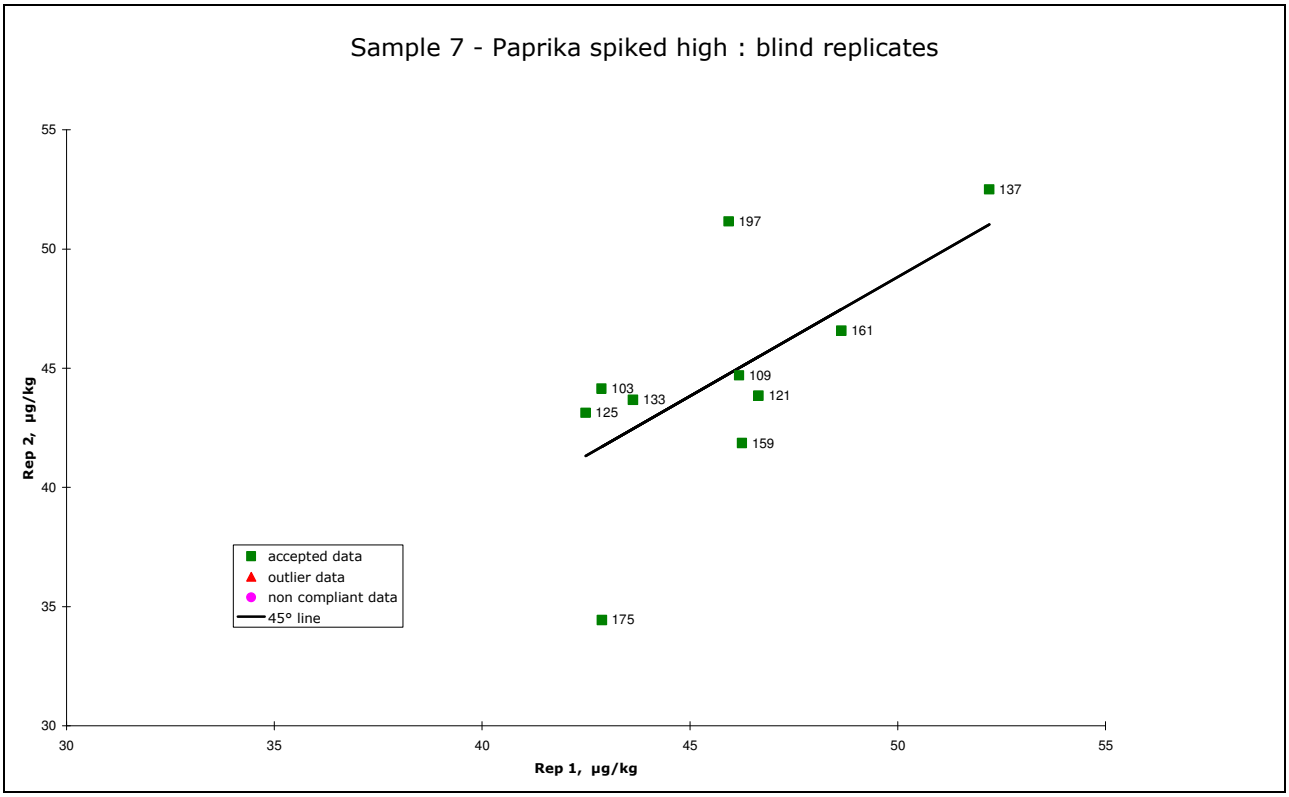


Table 12: Sample 8 - Spiked Sample 1 - high level - GROUP B (chilli powder)
Spiked with 51.7 (µg/kg) OTA. Initial OTA content prior spiking was determined as 1.8 µg/kg (mean value of Sample 1 – Table 1)

Lab code	Result 1 (µg/kg)	Result 2 (µg/kg)
106	59.2	51.08
124	55.078	56.45
128	44.19	46.35
130	51.56	50.97
136	40	41
148	42.81	41.4
156	47.71	46.64
164	43.23	43.32
168	41.45	40.92
172	44.5	44.4
186	38.84	39.11

Lab 106 was considered as a non compliant.

Figure 21: Distribution of individual results of replicate measurements (Sample 8)

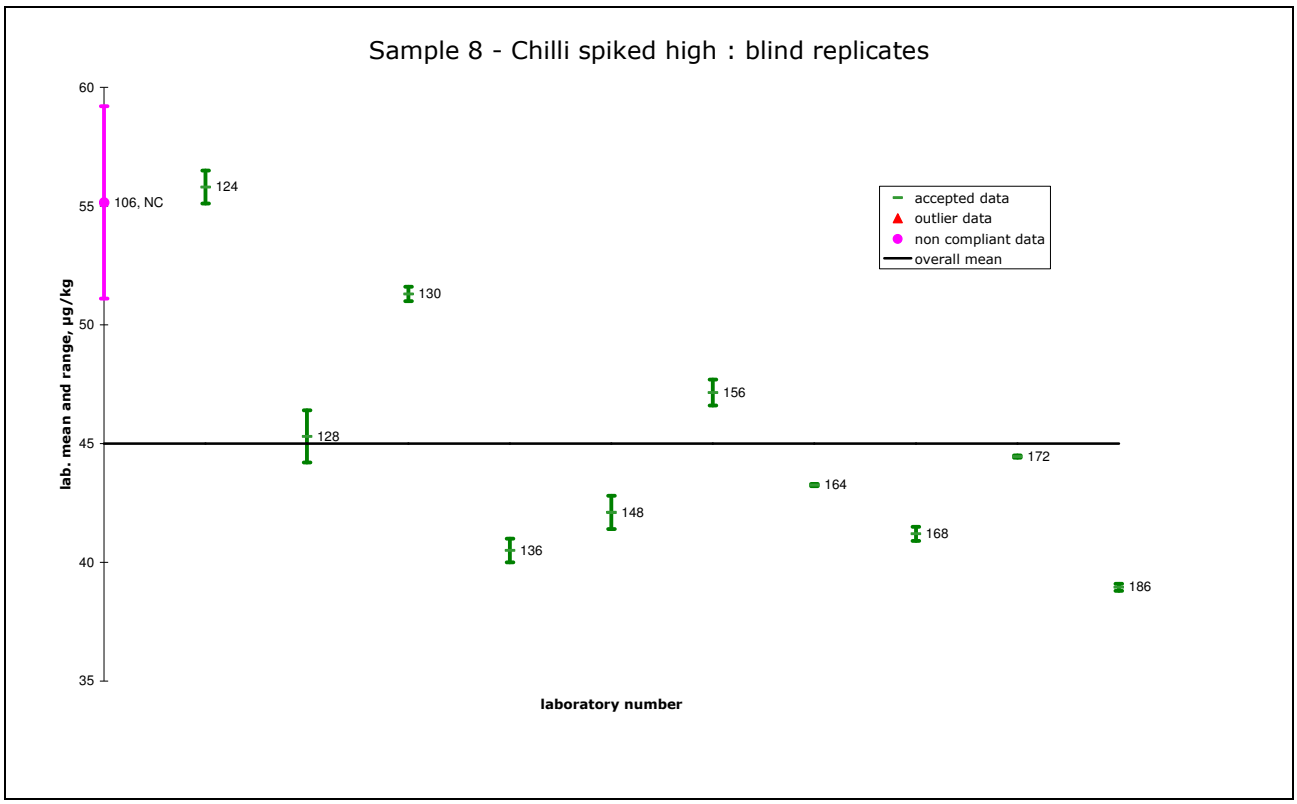


Figure 22: Youden plot (Sample 8)

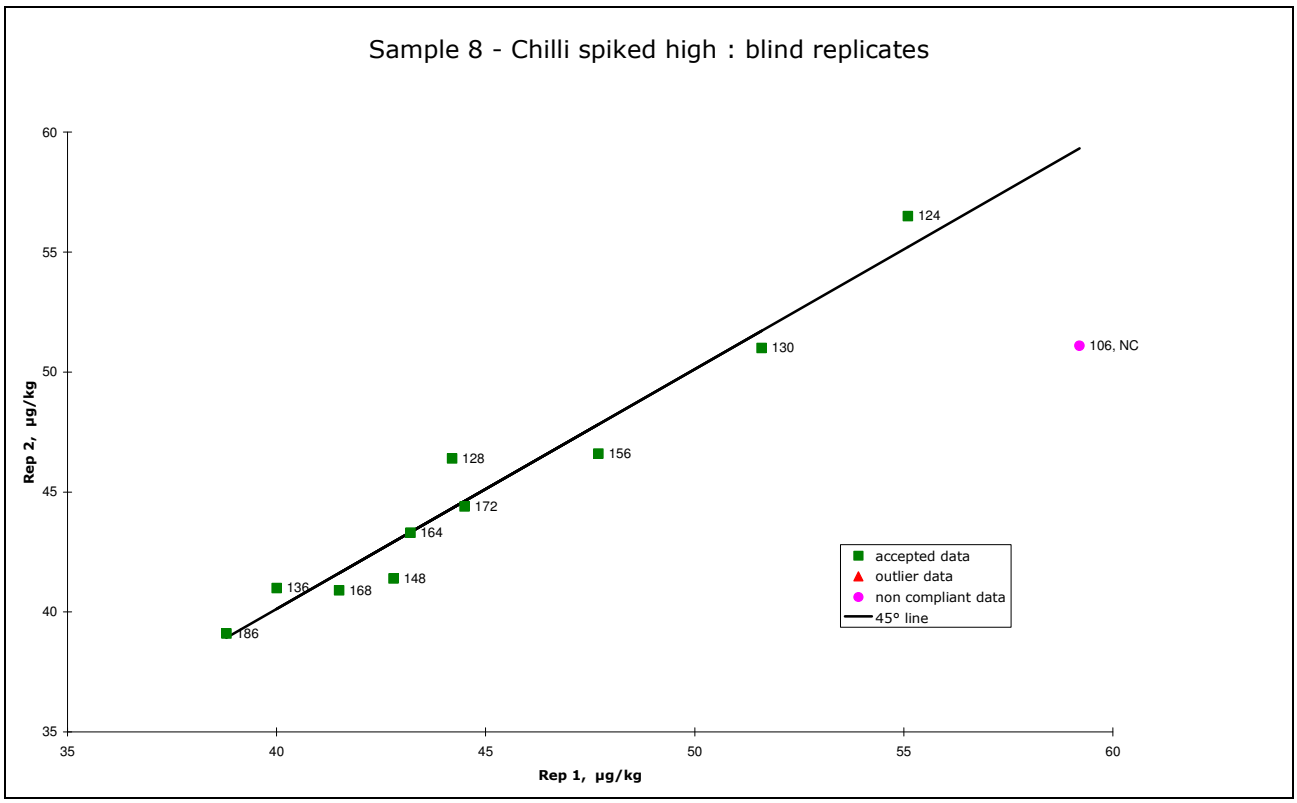


Table 13: Sample 9 - paprika powder – blank

Lab code	**Result** (µg/kg)
103	<3
106	<3
109	<0.1
121	<1
124	0.422
125	<0.5
128	<3.5
130	<0.5
133	<2.3
136	<0.7
137	<0.3
148	0.75
156	<0.6
159	<0.9
161	0.21
164	<0.6
168	0
172	<0.5
175	<0.25
186	0.43
197	2.04

Annex 3 – Outline of the study

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
European Union Reference Laboratory for Mycotoxins

European Union Reference Laboratory
Mycotoxins

Geel, 08 March 2012

Method validation study on the determination of ochratoxin A in paprika and chilli by immunoaffinity column clean-up and high performance liquid chromatography with fluorescence detection

Dear Participant,

The EU-RL Mycotoxins organises a method validation study (by inter-laboratory comparison) on the determination of ochratoxin A (OTA) in paprika and chilli. The study is foreseen to take place in **April 2012**.

Please read the following information carefully.

Timing

Participants will receive **two weeks** before the starting of the exercise a **preannouncement** of the sample dispatch.
A second reminder will be sent the **day before dispatch** of samples and participants will receive a dispatch note containing all data for tracking the shipment.

We ask you to report results back within **four weeks;** including the modalities which will be detailed in following communications.

Materials supplied for the study

Participants will receive a parcel containing the following items:

1. Their **participant code** to be used in all following communications with the organiser (the EU-RL)

2. A "**Receipt form**". If the material has been received damaged, immediately request a new material (the materials will be shipped at room temperature; storage however should be at 4° C until the analysis is performed)

3. The standard operating procedure (**SOP)** to be applied for the analysis of the samples and the spiked samples

4. The **spiking protocol**

5. The **OTA standard solution** to be used for preparing the calibration solutions

6. The necessary **immunoaffinity columns** (taking also into account possible repetition of a failed analysis)

7. The **OTA spiking standard solutions**

8. A **set of samples**, comprising:
   a. 8 samples for single analysis with different content levels of OTA (the final number of samples could slightly deviate from this number)
   b. 4 samples to be spiked for single analysis (the final number of samples could slightly deviate from this number)

Participants will also receive, after dispatch of samples, a FORM for **reporting of results** and a FORM with a **questionnaire**.

Participants will be asked to analyse each sample once and to report the requested results **in µg/kg** for both samples and spiked samples.
They will be also asked to send to the organiser the chromatograms of calibration solutions and samples as specified the SOP.

In case of questions please do not hesitate to contact us at the following address:

Zoltan Kunsagi
Institute for Reference Materials and Measurements (IRMM)
EU-RL Mycotoxins
Retieseweg 111
B-2440 Geel, Belgium
Tel: +32-14-571 313
FAX: +32-14-573 015
E-mail: Jrc-irmm-crl-mycotox@ec.europa.eu

With kind regards,

Zoltan Kunsagi

Cc: Joerg Stroka, Frans Verstraete, Franz Ulberth, Beatriz De La Calle

Annex 4 – Subscription form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Union reference laboratory for mycotoxins

European Union Reference Laboratory
Mycotoxins

Geel, 08 March 2012

Subscription questionnaire for inter-laboratory study

Determination of **ochratoxin A in paprika and chilli** by immunoaffinity column clean-up and high performance liquid chromatography coupled with fluorescence detection.

Participants data (contact person and affiliation details):

Title:	
Name + SURNAME:	
Institute:	
Department:	
Street, number:	
City:	
Post code:	
Country:	
Phone:	
Fax:	
e-mail:	

Please read carefully the following before signing

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 313. Fax: (32-14) 573 015.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

1. Having read the attached method and the outline of the study, we understand that:

   a. All essential apparatus, chemicals and other requirements specified in the method protocol attached to this form must be available in our laboratory when the programme begins;

   b. Timing requirements, such as starting date, order of testing specimens and time for reporting will be respected;

   c. The method must be strictly followed;

   d. Samples must be handled according to instructions;

   e. A qualified operator must perform the measurements;

   f. Laboratories, which don't submit results, will be asked to send back the immunoaffinity columns and the test materials.

2. Comments you wish to address before participation:

3. Our Laboratory is willing to participate in this method validation study (*collaborative trial*).

YES ○ **NO** ○

Signature:_____

Once you filled-in the form, print it (use the print button), sign the hardcopy and fax it or mail: JRC-IRMM FSQ, Zoltan Kunsagi, Retieseweg 111, B-2440 Geel, Belgium**; Fax: +32 14 573 015**

Print form

Furthermore, using the email button, submit the filled-in form to us via email. You may also save it to your computer.

Submit by Email

Annex 5 – Instructions to the participants

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
EU reference laboratory for mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

Dear Participant,

On behalf of the EU-RL for Mycotoxins, I announce the opening of the inter-laboratory comparison for the validation of the method for the determination of ochratoxin A in paprika and chilli.

I thank you for joining the study and ask you, in order to obtain consistent results, to please follow all instructions included in the documents you received.

In particular, you should note the following:

1. Please check that the content of the parcel is **complete and undamaged** (and fill out and fax/e-mail the enclosed receipt form).
2. Please store goods at appropriate conditions (**+4°C for immunoaffinity columns and -18°C for solutions and test materials**) until the analysis. Let materials reach ambient temperature before use.
3. In the parcel you will find your participation code (**Lab ID**): please use it in all following communications.
4. Read **all** accompanying documents prior starting with the analysis. **THE METHOD PROTOCOL MUST BE FOLLOWED.** In particular the following points should be remarked:
   - If more than one sequence is necessary to analyse all received samples (e.g. overnight stops, preparation of samples in different days), than a calibration curve is to be obtained for each sequence.
   - The amount of sample to be extracted should not deviate from the one indicated in the SOP (paragraph 7.1 of the method protocol). This is of crucial importance due to the material homogeneity requirements.
   - All samples should be homogenised before taking the test portion for performing the analysis.
5. Make sure that all required instruments and consumables are at hand before starting the analysis.
6. Each sample is identified with a **three digits code.** This should help in identifying the samples and in coupling them with the respective spiking solutions (when they are to be spiked). The numerical codes must be used for reporting of all results.

Retieseweg 111, B-2440 Geel - Belgium. Telephone: (32-14) 571 211. http://irmm.jrc.ec.europa.eu
Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 783.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

7. **Analyse each sample only once**. In case you should encounter any problem during the analysis, please contact us for a replacement of the lost sample
8. **A report sheet and a questionnaire** are attached to this same **email**. Please use them to report your results and notes to us.
9. Please also send back the **chromatogram** for each sample. They can be sent back either by e-mail (jrc-irmm-mycotox@ec.europa.eu) or by FAX (0032-14-571783).

The deadline for this collaborative trial is **04/05/2012** which gives a time period of **three weeks** for all experiments. We are looking forward to hear from you and hope the method suits your needs for future use.

A detailed outline of the study is included in the MVS sample parcel together with the spiking protocol and the method protocol (SOP); in addition, in this document you find further details. Anyhow we would like to encourage you to contact us, in case you seek further clarification, at the following address:

MVS coordinator

Zoltan KUNSAGI
Fax: 0032-14-571313
e-mail: jrc-irmm-crl-mycotox@ec.europa.eu

With kind regards,

Joerg Stroka
(Operating Manager of the European Union Reference Laboratory for Mycotoxins)

Cc: Frans Verstraete, Franz Ulberth, Beatriz De La Calle, Zoltan Kunsagi

Annex 6 – Materials receipt form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Community Reference Laboratory for Mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

RECEIPT FORM

Name of Participant	
Affiliation	
Lab ID	
Country	

NOTE: UPON RECEIPT STORE THE IMMUNOAFFINITY COLUMNS IN A FRIDGE (AT 4 °C) AND THE CALIBRANT AND THE TEST MATERIALS IN A FREEZER (AT -18 °C)

Please ensure that the items listed below have been received undamaged, and then check the relevant statement in the **table at next page**:

Contents of parcel

a) A copy of the instructions
b) The SOP of the method
c) The spiking protocol
d) Your participation code (LAB ID)
e) 11 coded test materials for direct analysis
f) 4 test materials identified for spiking
g) Ochratoxin A stock solution
h) 4 spiking solutions
i) 20 immunoaffinity columns
j) Safety sheets for solvents and Ochratoxin A

Date of the receipt of the test materials	
All items have been received undamaged	YES / NO
If NO, please list damaged items according to the letters associated at each item in the list above Please write one item per row	
Items are missing	YES / NO
If YES, please list missing items according to the letters associated at each item in the list above Please write one item per row	
Serial numbers of the samples you received	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
	__ __ __
Codes of the samples to be spiked/spiking solutions you received	A
	B
	C
	D

SIGNATURE: _____

Please fax or email the completed form to:

Zoltan Kunsagi
European Commission - DG Joint Research Centre
Institute for Reference Materials and Measurements
B-2440 Geel, Belgium
Fax No: 0032-14-571 783
Email: jrc-irmm-crl-mycotox@ec.europa.eu

Annex 7 – Spiking protocol

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
Community reference laboratory for mycotoxins

European Union Reference Laboratory
Mycotoxins

SPIKING PROTOCOL FOR THE METHOD VALIDATION STUDY ON THE DETERMINATION OF OCHRATOXIN A IN PAPRIKA AND CHILLI

This box contains four ampoules labelled Spiking solution A to D with correspondingly labelled blank and naturally contaminated materials for spiking.

The solvent for the Ochratoxin A (OTA) spiking solutions is acetonitrile/acetic acid (99/1, v/v).

For spiking experiments, proceed as follows:

- Weigh **25.0 g**, to the nearest 0.1 g, of the test sample into a. 500 ml conical flask or similar.
- Add **exactly 500 µL** of each "Spiking solution" to the respective (same code) test material. You will end up with four (4) spiked samples, each spiked with one spiking solution.
- Let stand for at least 1 h at room temperature to allow the solvent of the spiking solution to evaporate and the OTA to migrate into the matrix.
- Analyse the spiked test material according to the method protocol.

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Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 783.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

Annex 8 – Results form

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Union reference laboratory for mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

Reporting of results for the participants to the Inter-laboratory comparison for the validation of a method to determine Ochratoxin A in paprika and chilli

This FORM has to be filled and submitted electronically by all participants. For this we need your collaboration in processing this FORM in the way we propose.

Please fill all fields using Adobe Acrobat Reader. At the end of this FORM you will find two buttons for sending the created FORM Submit by Email and to Print form. Please make use of these features and follow carefully the instructions at the end of this form.

We need the "PDF" file generated by the above suggested procedure to collate the data.

We also need a signed proof of the results report: you can send it by FAX or by e-mail by scanning it and sending the so obtained PDF to the e-mail address: jrc-irmm-mycotox@ec.europa.eu.

Please remember also to send chromatograms from samples and STD 2 (see Table 1 in method protocol). This can be send electronically (f.i. pdf file).

>> Read carefully before filling-in the FORM <<

1. The fields marked with a * are mandatory: you will not be able to send the FORM if you have not filled in all the mandatory fields.

2. When the description of the field includes an indication of the format, please follow exactly the indication (e.g. Frank MILLER), you should write only your surname in capital letters.

3. Please always report in the first column of the table the code of the sample, also when you do not report any results for it.

1

4. For all samples where the result obtained should be below the LOQ, please mark YES in the table.

5. All the fields in the second and fourth column of the Table are numeric fields: **you can only enter numbers**.

6. Results obtained shall be reported as expressed in **µg/kg** and with **two decimals** (e.g. 12.13). Please enter ONLY ONE result for each field.

NOTE: please remember to mix the sample before taking the test portion for performing the analysis

Participant details

1. Your Laboratory ID (3 digits number)*:
2. Your Country:
3. Your Title (Mr. / Ms. / Mrs. / Dr. / Prof.):
4. Your Name (First name + SURNAME)*:
5. Your Affiliation (Institute / Company)*:
6. Your phone number*:
7. Your FAX number*:
8. Your e-mail address*:
9. Second contact (First name + SURNAME) (if applicable)
10. Second contact e-mail address (if applicable):

Table of Results

Line #	Sample code (3 digits number)	OTA content (µg/kg)	Below LOQ (YES)	If YES, please report the LOQ you estimated (µg/kg)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12	SPIKE A			
13	SPIKE B			
14	SPIKE C			
15	SPIKE D			

ATTENTION

The EU-RL Mycotoxins thanks you for answering to this results report.

Please, send back this FORM before the 04/05/2012

Once you filled-in the form, use the email button and submit the filled-in form to us via email. You may also save it to your computer.

Submit by Email

Furthermore, print it (use the print button), sign the hardcopy and fax it: JRC-IRMM FSQ, Zoltan Kunsagi, Retieseweg 111, B-2440 Geel, Belgium**; Fax: +32 14 571 783**
(you can also scan the signed FORM and send the PDF file by e-mail at the mail address: jrc-irmm-crl-mycotox@ec.europa.eu)

Print form

YOUR Signature: _____

Questionnaires not transmitted both by e-mail as PDF Forms and by FAX (or signed PDF by e-mail) as signed Forms cannot not be included in the report

5

Annex 9 – Questionnaire

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Union reference laboratory for mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

Questionnaire for the participants to the Inter-laboratory comparison for the validation of a method to determine Ochratoxin A in paprika and chilli

This FORM has to be filled and submitted electronically by all participants to the exercise. For this we need your collaboration in processing this questionnaire in the way we propose.

Important!
Please fill all fields using Adobe Acrobat Reader. **Send the filled FORM by email**
We need the "PDF" file generated by the above suggested procedure to collate the data.

We also need a signed proof of the questionnaire: you can send it by FAX or by e-mail by scanning it and sending the so obtained PDF to the e-mail address jrc-irmm-mycotox@ec.europa.eu.

At the end of the questionnaire you will find two buttons for sending the created FORM Submit by Email and to Print form. Please make use of these features and follow carefully the instructions at the end of this form.

Please remember also to send a printout (or its PDF) of one chromatogram obtained by injecting STD 2 (see Table 1) and one chromatogram for each test sample where the separation of OTA peak from the matrix peaks is evidenced.

1

>> Read carefully before filling-in the FORM <<

1. The fields marked with a * are mandatory: you will not be able to send the FORM if you have not filled in all the mandatory fields.

2. When numeric values are required, please **do not try to enter other formats**.

Participant details

1. Your Laboratory ID (3 digits number)*:

Participant background

1. For how long (years) your laboratory has been analysing food or feed for the determination of Ochratoxin A (OTA)?*

2. Is your laboratory accredited for the determination of OTA?*

YES	NO

If YES, please write in the following field for which matrix (matrices) is your laboratory accredited

3. How many samples does your laboratory analyse for Ochratoxin A per year?*

Less than 5	5-49	50-149	150-500	More than 500

4. Which of the following matrices does your laboratory analyse for the determination of Ochratoxin A on a routine basis?*

☐	Spices
☐	Unprocessed cereals
☐	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption
☐	Dried vine fruit (currants, raisins and sultanas)
☐	Roasted coffee beans and ground roasted coffee - soluble coffee
☐	Wine - Aromatised wine
☐	Grape juice
☐	Baby food and dietary food for infants and young children
☐	Liquorice and liquorice extracts
☐	Feed
☐	Other

If OTHER, please specify

Questions on the organisation of this exercise

1. Did you find the instructions distributed for this MVS adequate?

○	○
YES	NO

If NO, which parts do you think could be improved?

2. What do you think about the reporting by electronic forms?

3. Did you have any problems in using the forms?

○	○
YES	NO

If YES, which were these problems?

4. Any other comments you wish to address?

General questions on the method

1. Did you find the method description adequate?

○	○
YES	NO

If NO, in which part(s) could it be improved?

2. Were you able to follow the method in all details?*

○	○
YES	NO

If NO, which part(s) required deviations from the protocol?*

Method paragraph	Description of the deviation applied

3. Did you encounter any problem during the analysis?*

○	○
YES	NO

If YES, what were the specific problems and to which samples do they apply?*

4. Did you notice any abnormality, which however seem to had no effect on the result? (please list also any fast or slow running IACs)*

○	○
YES	NO

If YES, please describe and report for which samples (codes) they occurred.*

5. Were you familiar with all the steps performed during the analysis?*

○	○
YES	NO

If NO, please describe and report for which step(s). (Refer to the respective paragraph number in the SOP*)

6. Any other information you wish to add

Specific details on the method

1. Did you need to include any "over night" stops in the analysis of the MVS samples without performing a new calibration when resuming the sequence?*

YES	NO

If YES, please state for which samples and at what stage of the analysis.*

2. How did you integrate the signals (automatically or manually)?*

Automatically	Manually

If AUTOMATICALLY, did you visually check the correctness of integration?*

○	○
YES	NO

If YES, for how many chromatograms was it necessary to re-integrate the OTA peak? (Numeric value)*

3. Which global settings did you use for automatic integration (e.g. valley-to-valley or horizontal baseline or tangential, etc.)?

ATTENTION

The EU-RL Mycotoxins thanks you for answering to this questionnaire.

Please, send back this FORM before the 04/05/2012

Once you filled-in the form, use the email button and submit the filled-in form to us via email. You may also save it to your computer.

Submit by Email

Furthermore, print it (use the print button), sign the hardcopy and fax it: JRC-IRMM FSQ, Zoltan Kunsagi, Retieseweg 111, B-2440 Geel, Belgium**; Fax: +32 14 571 783**
(you can also scan the signed FORM and send the PDF file by e-mail at the mail address: jrc-irmm-crl-mycotox@ec.europa.eu)

Print form

YOUR Signature: _____

Questionnaires not transmitted both by e-mail as PDF Forms and by FAX (or signed PDF by e-mail) as signed Forms cannot not be included in the report

10

Annex 10 – Method Description

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
European Union reference laboratory for mycotoxins

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Mycotoxins

Determination of Ochratoxin A in paprika and chilli by immunoaffinity column clean-up and High Performance Liquid Chromatography with fluorescence detection

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Telephone: direct line (32-14) 571 229. Fax: (32-14) 571 783.

E-mail: jrc-irmm-crl-mycotox@ec.europa.eu

Contents

Page

Foreword

THIS IS A STUDY FOR THE EVALUATION OF THE METHOD, NOT FOR ASSESSING THE PERFORMANCE OF THE LABORATORY. THE METHOD MUST BE FOLLOWED AS CLOSELY AS PRACTICABLE, AND ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM.

WARNING — the use of this protocol involves hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This protocol specifies a candidate method for the determination of Ochratoxin A (OTA) in paprika and chilli using liquid-chromatography with fluorescence detection. This candidate method will be validated for the determination of OTA via the analysis of fortified and naturally contaminated samples.

2 Normative reference

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987).*

ISO 1042/1998, *Laboratory glassware -- One-mark volumetric flasks.*

Commission regulation (EC) No 401/2006, *of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (Text with EEA relevance)*

3 Principle

A test portion is extracted with a mixture of methanol and aqueous sodium bicarbonate solution. The extract is filtered, diluted with phosphate buffered saline (PBS), and applied to an immunoaffinity column containing antibodies specific to Ochratoxin A (OTA). The OTA is isolated, purified and concentrated on the column then released using methanol. The purified extract is quantified by reverse-phase high performance liquid chromatography (RP-HPLC) coupled with fluorescence detection.

4 Reagents

Use all solvents and solutions in a fume hood. Wear safety glasses, protective clothing and avoid skin contact.

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696, unless otherwise specified. Solvents shall be of quality for HPLC analysis, unless otherwise specified. Commercially available solutions with equivalent properties to those listed may be used.

WARNING — Dispose of waste solvents according to applicable environmental rules and regulations. Decontamination procedures for laboratory wastes have been reported by the International Agency or Research on Cancer (IARC), see [1].

4.1 Nitrogen purified compressed gas (purity equivalent to 99.95% or better)

4.2 Methanol, Technical grade

4.3 Methanol, HPLC grade

4.4 Acetonitrile, (CH_3CN) HPLC grade

4.5 Glacial acetic acid, (CH_3COOH) mass fraction $w \geq 96\%$

4.6 Toluene, UV grade

4.7 Sodium hydrogen carbonate, ($NaHCO_3$) minimum 99% purity

4.8 Sodium chloride, (NaCl) minimum 99% purity

4.9 Disodium hydrogen orthophosphate, (Na_2HPO_4 x 12 H_2O) minimum 99% purity

4.10 Potassium dihydrogen phosphate, (KH_2PO_4) minimum 99% purity

4.11 Potassium chloride, (KCl) minimum 99% purity

4.12 Sodium hydroxide, (NaOH) minimum 99% purity

4.13 Hydrochloric acid solution, the mass fraction w(HCl) = 37 % in water

4.14 Hydrochloric acid solution, the substance concentration c(HCl) = 0,1 mol/l

Dilute 8.28 ml of hydrochloric acid solution (4.13) to 1 l with water.

4.15 Sodium hydroxide solution, c(NaOH) = 0.2 mol/l

Dissolve 8 g NaOH (4.12) in 1 l of water

4.16 Phosphate buffered saline (PBS), pH = 7,4

Dissolve 8 g NaCl (4.8), 2.9 g Na_2HPO_4 x $12H_2O$ (4.9), 0.2 g KH_2PO_4 (4.10) and 0.2 g KCl (4.11) in 900ml of water. After dissolution, adjust the pH to 7,4 with hydrochloric acid solution (4.14) or sodium hydroxide solution (4.15) as appropriate, then dilute to 1 l with water.

Alternatively, a PBS solution with equivalent properties can be prepared from commercially available PBS material.

4.17 Sodium hydrogen carbonate solution, c($NaHCO_3$) = 30 g/l (3%)

Add 30 g of sodium hydrogen carbonate (4.7) to 1000 ml of grade 3 water.

4.18 Extraction solvent

Mix 50 parts per volume of methanol (4.2) to 50 parts per volume of sodium hydrogen carbonate solution (4.17). Mix well.

4.19 HPLC mobile phase

Mix 35 parts per volume of methanol (4.3), and 35 parts per volume of acetonitrile (4.3) with 29 parts per volume of grade 1 water and with 1 part per volume of glacial acetic acid (4.5).

Degas mobile phase solvent with for example helium or equivalent methods.

4.20 Immunoaffinity column

The immunoaffinity column contains antibodies raised against ochratoxin A. The column shall have a capacity of not less than 100 ng of OTA and shall give a recovery of not less than 85 % when applied as a standard solution of ochratoxin A in a mixture of 15 parts per volume of methanol (4.2) and 85 parts per volume of PBS solution (4.16) containing 3 ng of ochratoxin A. Immunoaffinity columns are to be stored in the refrigerator and must be allowed to equilibrate at room temperature before use.

4.21 OTA stock solution

A solution in toluene/acetic acid 99:1(≈ 10 µg/ml) will be provided for this study.

WARNING – Ochratoxin A is a potent nephrotoxin with immunotoxic, teratogenic and potential genotoxic properties. The International Agency for Research on Cancer (IARC) has classified ochratoxin A as a possible human carcinogen (group 2B). Protective clothing, gloves and safety glasses should be worn at all times, and all standard and sample preparation stages should be carried out in a fume cupboard.

4.22 OTA standard solution, α(Ochratoxin A) ≈ 1 µg/ml

Prepare OTA standard solution by diluting 10 times the OTA stock solution (4.21) with mobile phase:

Pipette 100 µl OTA stock solution (4.21) into a 1 ml volumetric flask (5.15), bring it to dryness by applying a gentle flow of nitrogen (4.1), then dilute to 1 ml (up to the mark) with the mobile phase (4.19) and shake it vigorously. This gives a standard solution containing ≈ 1000 ng/ml of OTA (the exact concentration depending on the concentration of the stock solution). Store this solution in a freezer at approximately -18 ºC.

5 Apparatus

5.1 General

Usual laboratory glassware (such as graduated cylinders, glass funnels, beakers, pipettes, screw-cap flasks, screw-cap amber vials, etc) and equipment and, in particular, the following:

5.2 Conical flasks, with screw cap, 500 ml capacity, or similar recipients

5.3 Laboratory balance, with a mass resolution of 0.01 g

5.4 Analytical balance, with a mass resolution of 0.0001 g

5.5 Adjustable vertical or horizontal shaker

5.6 Calibrated volumetric pipettes

5.7 Displacement pipettes, of 100 µl and 1000 µl capacity, with appropriate tips

5.8 Calibrated microsyringes or variable capacity pipettes, of various capacities (e.g. 100 µl up to 2000 µl)

5.9 Disposable syringe barrels, to be used as reservoirs, of 50 ml capacity, luer locks and attachments to fit to immunoaffinity columns.

5.10 Glass microfibre filter paper, 1.6 µm retention size, 150 mm diameter, or equivalent[1]. As an alternative, paper filters (**5.11**) can be used as they have been proven to give equivalent results.

5.11 Cellulose filter paper, 11 µm retention size, 150 mm diameter.

5.12 Automated SPE Vacuum System

5.13 Vortex mixer

5.14 Glass vials, ~ 2 ml capacity and crimp caps or equivalent

5.15 Volumetric flasks, of various capacities (e.g. 1 ml, 5 ml, 10 ml, 25 ml)

5.16 Conical flasks, e.g. 100 ml with screw cap

5.17 HPLC apparatus, comprising the following:

5.17.1 Injector system, capable of injecting e.g. 20 µl

5.17.2 Mobile phase pump, gradient, capable of maintaining a volume flow rate of 1 ml/min pulse free

5.17.3 Fluorescence detector, suitable for measurements with excitation wavelengths of λ = 332 nm, and emission measurement at a wavelength of λ = 476 nm. The bandwidth should be 16 nm or below.

5.17.4 Recorder, integrator or computer based data processing system

5.17.5 Analytical reverse-phase HPLC separating column, C18 RP-column suitable to allow a sufficient separation of OTA from other interfering components. Fully end capped with column dimensions preferably 250 mm x 4.6 mm I.D. stationary phase with particles of size 5 µm.

5.17.6 Pre-column, with the same stationary phase material as the analytical column, and dimension of 12.5 mm x 4.6 mm ID

5.17.7 Degasser, optional, for degassing HPLC mobile phase (4.19)

5.17.8 Column oven, capable to operate at 22 ºC ± 1 ºC

6 Sampling

Sampling is not part of this method.

[1] In alternative a filter of the same typology with 47mm diameter can be used. See 7.1.

7 Procedure

7.1 Extraction

Weigh 25 g [W] (recorded to 2 decimal places) of test portion into a conical flask or similar recipient of 500 ml (5.2).

Add 200 ml [V_1] of extraction solvent (4.18). Mix shortly by hand for a few seconds to obtain a homogeneous suspension, and then shake 40 minutes in a shaker (5.5).

Transfer at least 10 ml of the extract so obtained onto the 150 mm glass fibre (or cellulose) filter paper (5.10, 5.11), conically folded. Collect the filtered extract in a screw cap flask (5.16) for further analysis. Proceed immediately with the immunoaffinity column clean-up procedure (7.2)

7.2 Immunoaffinity column cleanup

Connect the immunoaffinity column (4.20) to the vacuum manifold (5.12), and attach a reservoir of 50 ml capacity (5.9) to the immunoaffinity column.

Place the immunoaffinity column on a suitable support.

Columns should be allowed to reach room temperature prior to using.

Add in the reservoir (5.9) 50 ml of PBS (4.16), transfer 4 ml [V_2] of the filtered extract and mix. Draw the mixture (extract + PBS) through the column by gravity at a steady flow rate (the flow rate should result in a dropping speed of 1 drop/sec, which is about 3 ml/min) until all extract has passed the column and the last solvent portion reaches the frit of the column.

NOTE 1: If necessary, the process could be accelerated by applying slight pressure to the IAC by a syringe or by applying little vacuum (e.g. by using the vacuum system described in 5.12). In both cases, attention should be paid not to exceed the flow rate of 3 ml/min (1 drop /sec).

CAUTION — If using a vacuum manifold, extra care is necessary to avoid increasing the flow rate through the column to the point where recovery is adversely affected.

After the extract has passed through the column, wash it with 10 ml of water at a rate not exceeding 3 ml/min. Dry the column by pushing 50 ml air through it with a syringe. Then discard all the eluent from this stage of the clean-up procedure.

Finally, place a 2 ml vial (5.14) under the column and pass 1.50 ml of methanol (4.3) through the column, collecting the eluate. Carefully push 50 ml air through the column with a syringe in order to collect any final drops without spilling.

Close the vial and shake it vigorously. This 1.5 ml of eluate will be analysed directly.

CAUTION – Since this 1.5 ml eluate will be used for the quantitative analysis directly it is very important to dry the immunoaffinity column effectively by air after the washing step <u>and</u> after the elution by methanol. Shaking the vials before injection is also critical.

(Alternatively, evaporate the methanolic eluate to dryness applying either gentle stream of nitrogen at about 30-35°C or vacuum centrifugation and re-dissolve the purified sample residues in 1.5 ml mobile phase.)

<u>Make note of columns with exceptionally fast or slow flow rates</u>

8 HPLC Analysis

8.1 HPLC operating conditions

- Mobile phase: as in 4.19
- Flow rate: 0.8 ml/min
- Column: as in 5.17.5 and 5.17.6
- Column oven temperature (including the guard column) is 22 ºC ± 1 ºC;
- injection volume is 20 µl;
- autosampler (optional) temperature is 15 ºC to 20 ºC;
- Detector wavelength: excitation 332 nm, emission 476 nm

Ochratoxin A elutes with retention of approximately 6.5 min. Other column dimensions may be used, provided that the required resolution is achieved. This shall be demonstrated (Ochratoxin A should be baseline resolved from any interfering substances, if present). The flow rate may be adjusted according to the column dimension. A typical chromatogram is enclosed in Annex A.

Participants are asked to send a printout (or its PDF) of one chromatogram obtained by injecting STD 2 (see Table 2) and one chromatogram for each test sample where the separation of OTA peak from the matrix peaks is evidenced.

8.2 Preparation of calibration solutions for HPLC

Prepare six HPLC calibration solutions from the standard solutions prepared (4.22).

With appropriate calibrated pipettes or microsyringes (5.8) the volumes of the ochratoxin A standard solution (4.22) listed in Table 1 are to be distributed separately into a set of volumetric flasks (5.15). After having added the standard solution (4.22), add the mobile phase (4.19) up to the mark, close and mix manually. This will result in 6 OTA solutions with approximately the concentrations listed in Table 1.

These six solutions cover a range from 3 µg/kg to 150 µg/kg for ochratoxin A under the conditions of this protocol.

The solutions should be protected from light and can be stored in the freezer at -18 ºC. Peak areas corresponding to the same calibration solution injected at regular intervals should be within ± 3 %.

These solutions shall be used directly for injection into the HPLC system, after transferring them in the vials (5.14).

Table 1: Preparation of HPLC calibration solutions

STD	µL Std (4.22)	Final Volume (ml)	Nominal concentration (ng/ml)	Corresponding contamination level (µg/kg)
STD 1	25	25	1	3
STD 2	25	5	5	15
STD 3	50	5	10	30
STD 4	100	5	20	60
STD 5	150	5	30	90
STD 6	250	5	50	150

NOTE 4: The exact concentrations of OTA in the OTA standard solution (4.22) and in the calibration standard solutions (8.2) have to be derived from the concentration of the reference standard material (4.21) and the volumes used.

8.3 Calibration curve

Prepare a calibration curve by injecting 20 µl of the six ochratoxin A calibration solutions (8.2) at the beginning of every day of the analysis. Plot the peak area against the concentration of ochratoxin A in the calibration solutions injected and check the curve for linearity.

8.4 Determination of ochratoxin A in test solutions

Inject 20 µl aliquots of the test solutions into the chromatograph using the same conditions used for the preparation of the calibration curve.

The sequence of injections will be performed in single injection and has to include, in the order reported below:

- Mobile phase (4.19)
- The six calibration standards obtained from the standard solution (4.22) at the concentrations included in Table 1 from the lowest to the highest level
- Mobile phase (4.19)
- Test solutions (from samples and from spiking experiments)

Every 10 test solutions, one injection of STD 2 (Table 1) has to be performed (control standard). If the area of the standard deviates for more than ±10% from the area found in the calibration performed in the first part of the analytical sequence, the possible source of failure has to be identified and fixed and all test solutions injected after the previous control standard have to be re-injected.

8.5 Peak identification

Identify the ochratoxin A peak of the test solution by comparing the retention time in the test solution with that of the standard solutions. When this protocol is followed, the retention time of OTA peak in STD 2 (Table 1) being considered as a reference, the retention time of the OTA peak in the test solution is not expected to deviate more than ± 2.5% from the reference retention time.

9 Calculation

9.1 Preparation of the calibration graph

Plot the peak signals as area or height (y-axis) against the concentration of the OTA calibration standard solutions (8.2) [ng/ml] (x-axis) and calculate the calibration curve using linear regression.

Calculate the concentration of OTA expressed in ng/ml in the injected sample purified extract (the test solution) by using the resulting function ($y = ax + b$) using Equation 1.

Equation 1

$$C_{OTA} = \frac{S_{OTA} - b}{a}$$

Where:

C_{OTA} [ng/ml]	is the concentration of ochratoxin A, in nanograms per milliliter, in the aliquot of test solution injected and corresponding to the area of the ochratoxin A peak;
S_{OTA}	is the signal of OTA peak obtained from the chromatogram of the test solution;
a	is the value of the slope of the linear function;
b	is the value where the calibration function intercepts the y-axis.

9.2 Calculation of OTA content in the sample

Calculate the mass fraction, w_{OTA}, of ochratoxin A in micrograms per kilogram, using **Equation 2**

Equation 2

$$w_{OTA} = \frac{C_{OTA} \times V_1 \times V_3}{W \times V_2}; \qquad \text{(i.e. } w_{OTA} = C_{OTA} \times 3\text{)}$$

Where:

w_{OTA}	is the mass fraction of ochratoxin A, in micrograms per kilogram, in the aliquot of test test sample;
C_{OTA}	is the concentration of ochratoxin A, in nanograms per milliliter, in the aliquot of test solution injected and corresponding to the area of the ochratoxin A peak;
V_1	is the volume, in milliliters, of the extraction solvent used for the extraction of the test sample;
V_2	is the volume, in milliliters, of the test sample extract aliquot applied onto the immunoaffinity column;

V_3 is the final volume, in milliliters, of the test solution;

W is the weight, in grams, of the test sample extracted.

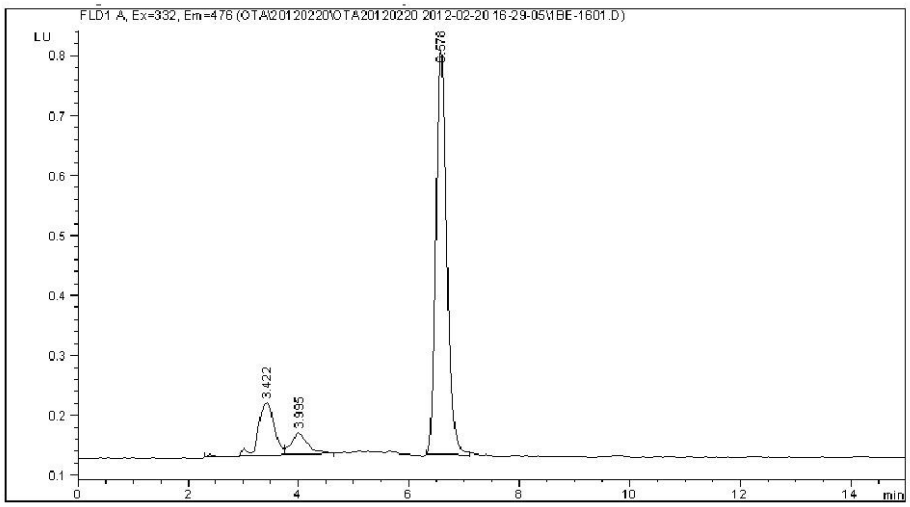
10 Reporting of results

Results for OTA in the samples and in the spiked samples will have to be reported in µg/kg and to the nearest 0.10 µg/kg.

Annex A
(informative)

Typical chromatogram

Figure 1



Key:
LU: fluorescence
Ochratoxin A: peak at 6.6 min

Figure 1 — Paprika powder (Ochratoxin A concentration ~ 30 µg/kg)

Operating conditions for Figure 1 and 2:

Column:	RP-C18 with column dimensions of 250 mm x 4.6 mm I.D. stationary phase with particles of size 5 µm.
Flow rate:	0.8 ml/min
Mobile phase:	see 4.19
Column	22 ° C controlled
Injection volume:	20 µL
Detection:	Fluorescence, 332 nm excitation, 476 nm emission, bandwidth ≤16 nm

Annex 11 – Operation manual

Operation manual for the method

"Determination of Ochratoxin A in paprika and chilli by immunoaffinity column clean-up and High Performance Liquid Chromatography with fluorescence detection"

- Weigh 25 g of test portion into a 500 ml conical flask
- Add 200 ml extraction solvent (50 parts per volume of methanol and 50 parts per volume of $NaHCO_3$ solution)
- Shake it for 40 minutes
- Filtration with filter paper
- Immunoaffinity clean-up: 4 ml filtrate + 50 ml PBS
- Wash the IAC with 10 ml H_2O
- Dry the column by pushing 50 ml air through it with a syringe
- Pass 1.50 ml of methanol through the column and collect the eluate in a vial
- Carefully push 50 ml air through the column with a syringe in order to collect any final drops without spilling
- Close the vial and shake it vigorously
  This 1.5 ml of eluate will be analysed directly!

} **Critical steps!**

HPLC conditions

- Mobile phase
  - 35 part per volume of methanol
  - 35 part per volume of acetonitrile
  - 29 parts per volume of grade 1 water
  - 1 part per volume of glacial acetic acid
- Flow rate: 0.8 ml/min
- Column: C18 RP-column, 250 mm x 4.6 mm, 5 µm
- Column oven temperature: 22 °C ± 1 °C
- Injection volume: 20 µl
- Autosampler temperature: 15 °C to 20 °C
- Fluorescence detector wavelength
  - excitation 332 nm
  - emission 476 nm

Preparation of HPLC calibration solutions

OTA stock solution (10 µg/ml) – ***provided***

↓ Pipette 100 µl to 1 ml volumetric flask
Dry it with N_2
Add 1 ml mobile phase (up to the mark)
Shake it vigorously

OTA standard solution (1 µg/ml)

↓ Prepare them according to Table 1

Calibration solution 1 (1 ng/ml)
Calibration solution 2 (5 ng/ml)
Calibration solution 3 (10 ng/ml)
Calibration solution 4 (20 ng/ml)
Calibration solution 5 (30 ng/ml)
Calibration solution 6 (50 ng/ml)

Annex 12 – Experimental details

Table 14:
For how long (years) your laboratory has been analysing food or feed for the determination of Ochratoxin A (OTA)?
Is your laboratory accredited for the determination of OTA?
If YES, please write in the following field for which matrix (matrices) is your laboratory accredited.
How many samples does your laboratory analyse for Ochratoxin A per year?

	Years of experience	Accredited	Accredited matrices	Samples per year
103	10	Yes	cocoa and products derived, roasted and green coffee, cereal and products derived, dried fruits and products derived, baby food.	150-500
106	6	Yes	Cereals, Dried fruits, coffee, drinks (alcoholic and non-alcoholic), meat and meat products, entrails, milk, fat, fruits and vegetables, honey and sugar	150-500
109	10	Yes	Feed and feedstuffs	150-500
121	9	Yes	Coffee, Cereal, Dried Fruit, Wine, Beer, Baby Food, Chocolate, Paprika, Chilli, Liquorice, Black Pepper, White Pepper, Nutmeg, Ginger, Turmeric, White Grape Juice, Red Grape Juice	150-500
124	6	Yes	feed and food from plant origin	150-500
125	10	Yes	Animal feed, cereals, nuts and spices	50-149
128	7	Yes	liquid samples (wine - beer), cereals, coffee, dried fruits	150-500
130	20	Yes	food & feed	More than 500
133	17	Yes	Cereals, cereal products, cereal based foods, dried vine fruit and some other dried fruits, raw coffee, roasted coffee, soluble coffee, grape and some other juices, some spices, liquorice	More than 500
136	15	Yes	Beer, infant products, coffee beans	150-500
137	15	Yes	In foods of plant origin (coffee, cereals, spices, baby food)	50-149
148	14	Yes	Food - flexible scope of accreditation	5-49
156	20	No		150-500
159	22	Yes	Cereals & cereal products, dried fruit, wine & grape juice, coffee (green, instant & roasted), cocoa & chocolate products, beer, baby & infant foods, beans/pulses, spices, nuts & nut butters, coconut, duplicate diets, pork & pork products	More than 500
161	15	Yes	Basically all usual food and feed (inclusive materials) matrixes.	50-149
164	1	No		5-49
168	15	Yes	cereals, cereals products, feedingstuff dried fruits (figs, currants, raisins etc.) coffee (green, roasted, instant)	5-49
172	15	Yes	feed	50-149
175	9	Yes	Wine, Beer, Spices, Cereals, Fruit and Coffee	150-500
186	5	Yes	Food generally	150-500
197	12	Yes	Feed, kidneys Food: coffee, raisins, wine and juices, fish, cereals	150-500

Table 15:
Which of the following matrices does your laboratory analyse for the determination of Ochratoxin A on a routine basis?

	Spices	Unprocessed cereals	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	Dried vine fruit (currants, raisins and sultanas)	Roasted coffee beans and ground roasted coffee - soluble coffee	Wine - Aromatised wine	Grape juice	Baby food and dietary food for infants and young children	Liquorice and liquorice extracts	Feed	Other (Specify)
103		√	√	√	√	√		√			cocoa, dried fruits (nuts, figs, hazelnuts, ...), dried legumes (soy beans, chickpeas, lentils, ...) and products derived
106			√								Meat and entrails
109	√	√	√		√					√	
121	√		√	√	√	√	√	√	√		
124		√	√	√	√	√				√	
125										√	
128	√	√	√	√	√	√	√				Beer
130	√	√	√	√	√	√	√	√	√	√	meat, feed additives, dairy products, herbs, plant extracts, other dried fruits than specified, nuts / treenuts
133	√	√	√	√	√		√	√	√		some cocoa products
136					√			√			
137	√		√	√	√			√			

148	√		√								
156	√	√	√	√	√			√	√	√	
159	√	√	√	√	√	√		√		√	
161	√	√	√	√	√	√	√			√	Various feed materials.
164		√	√		√			√			
168		√	√	√	√					√	dry yeast
172		√								√	
175	√	√	√	√	√	√				√	Beer
186	√	√	√	√	√	√	√	√	√		
197	√	√	√	√	√	√	√	√	√	√	kidneys, fish

Table 16:
Did you find the instructions distributed for this MVS adequate?
What do you think about the reporting by electronic forms?
Did you have any problems in using the forms?
Did you find the method description adequate?
If NO, in which part(s) could it be improved?

T

	Instructions	**Electronic forms**	**Problems**	**Method description**	**Proposed improvements**
103	Yes	really helpful and easy to use	No	Yes	
106	Yes	It is user friendly	No	Yes	The shaker speed was not specified.
109	Yes	Perfect!	No	Yes	
121	Yes	It is convenient and user friendly	No	Yes	
124	Yes	Very nice	No	Yes	
125	Yes	Easy to use. Saves paper. Easy storage of results.	No	Yes	
128	Yes	It's excellent	No	Yes	
130	Yes		No	Yes	
133	Yes	Easy. Fax is not in use in our lab, only so called virtual fax, we prefer e-mail.	No	Yes	
136	Yes	Clear and easy to follow	No	Yes	
137	Yes	It is OK.	No	Yes	
148	Yes	OK	No	Yes	
156	Yes	It's easy and clear	No	Yes	
159	Yes	OK so far	No	Yes	
161	Yes	OK	No	Yes	
164	Yes	It's great and easy.	No	Yes	
168	Yes	OK	No	Yes	
172	Yes	OK	No	Yes	
175	Yes		No	No	Instructions following the elution step at 7.2 in the method are confusing - analysing the methanol extracts does not work. Samples have to be dried and taken up in mobile phase to give the expected chromatography. Methanol only extracts generate poor peak shapes.
186	Yes	In case of detailed comments the fields may be too small.	No	Yes	
197	Yes	easy	No	Yes	

Table 17:
Were you able to follow the method in all details?
If NO, which part(s) required deviations from the protocol?

	Following the method	Deviations
103	No	4.22 - we don't have 1ml volumetric flask than we use a calibrated syringe
106	No	7.1 - Cellulose filter paper used: 8um retention size, 240mm 7.2 - When drying the IAC after the elution with methanol, negligible spattering occurred with samples 123, 183, 225 and 273. Only 10mL air were pushed through the rest of IAC to avoid spattering. It seemed enough air to collect final drops in the vial.
109	No	We have no volumetric flask of 1 ml. We redissolved the residue of the OTA-stock in the vial with 1 ml added with a electronic multi pipette.
121	No	The flow rate on the HPLC was set at 1.0ml/min, not 0.8ml/min. Hamilton autodilutor was used instead of pipettes and volumetric flasks to prepare the 6 calibration solutions.
124	Yes	
125	Yes	
128	Yes	
130	Yes	
133	No	4.22 - All calculations were based on given standard concentration 10 microgram/ml, STD Ampoule 0087. 5.17.5 - HPLC-column: particle size 4 um, I.D. 3.9 mm 5.17.6 - no pre-column 5.17.8 - No column oven, ambient temperature was + 22C +/- 1C 7.2 - Purified samples were evaporated and re-dissolved in mobile phase 8.4 - All test solutions were analysed by isocratic method, between injections a short wash with 95 % acetonitrile
136	No	4.9 – NaH2PO4.2H2O 5.10 – Glass microfiber filter paper, 1.5um retention size, 110 diameter 8.1 – Column oven temperature = 30 C
137	Yes	
148	Yes	
156	No	4.22 - We did 5 ml OTA standard at 1 µg/ml : 500 µl 4.21 in 5 ml volumetric flask. 5.18.5 - We used a C18 column 150mm x 4 mm ID 5.18.8 - Column oven we operated at 30°C 8.1 - We worked at 0.3 ml/min
159	No	8.1 - Mobile phase flow rate used was 1.0ml/min not 0.8ml/min 8.1 - Column oven was not available so column was not thermostated. Laboratory temperature is controlled at constant temperature of 21 oC 8.1 (optional) - Autosampler not temperature controlled (at room temperature)
161	Yes	
164	Yes	

168	No	5.17 - HPLC column - Lichrosorb RP-18, 200 mm x 4,6 mm, particales of size 5 um 5.15 - We had not volumetric flask 1 ml. We used 2 ml glass vial and did the operations as described in 4.22 7.2 - We used the same apparatus for MVS as in routin analysis. Syringe barrel was 10 ml, capisity. We transfer 10 ml diluted, filtred extract to IAC. 10 ml was taken from (5 ml extract + 20 ml PBS) 8.1 - Flow rate: 1,0 ml/min Injection volume: 50 ul Detector wavelength: exitation 333 nm, emission 460 nm
172	Yes	
175	No	7.2 - Following the confusion surrounding the methanol extraction (see above) I had to take 1ml of the eluate, evaporate it to dryness at 40 degrees C and then reconstitute the residue in 1ml of mobile phase.
186	Yes	
197	No	7.2 - used a test tube for collecting the eluate (1.5 ml methanol out of the column)

Table 18:
Did you encounter any problem during the analysis?
If YES, what were the specific problems and to which samples do they apply?
Did you notice any abnormality, which however seem to had no effect on the result? (please list also any fast or slow running IACs)
If YES, please describe and report for which samples (codes) they occurred.

	Problem	Description	Abnormality with no effect on the result	Description
103	No		No	
106	Yes	During filtration step (7.1) two filters broke and samples "spiked B and D" had to be filtered again.	No	
109	No		No	
121	No		No	
124	No		No	
125	No		No	
128	No		No	
130	No		No	
133	No		Yes	Sample 288 was difficult to homogenize, it was like a stone
136	Yes	Problem: interfering peak residing at Ochratoxin retention time. Strategy: 10 blank injections were performed.	Yes	Spike D and Sample 460 were slow to elute
137	No		No	
148	No		No	
156	No		Yes	a IAC ran twice slower than all the others. It was the sample 325. A second IAC analysis were performed and this time, worked perfectly. The results are similar : 22,489 µg/kg and 22,785 µg/kg
159	No		No	
161	Yes	The material was too hard vacuumed so it was not easy to take the laboratory portions.	No	
164	Yes	The extraction part. We're using an Ultraturrax for the extraccion the OTA from the samples (2 minutes/11000rpm). It was a surprise for us, you decided to use this shaking method to extract the toxin (40 minutes). Is there any reason, maybe crossed-contamination?	No	
168	No		No	
172	No		No	
175	Yes	See above, plus stoppers exploded from the extraction flasks during shaking at 7.1 - no sample was lost however.	Yes	Fast running 173 and 234. Slow running 115 and 375.
186	Yes	see supplement	No	
197	No		Yes	Spike A extract had a slower flow rate than the others

Table 19:
Were you familiar with all the steps performed during the analysis?
If NO, please describe and report for which step(s). (Refer to the respective paragraph number in the method description)
Any other information you wish to add
Did you need to include any "over night" stops in the analysis of the MVS samples without performing a new calibration when resuming the sequence?
If YES, please state for which samples and at what stage of the analysis.

	Familiarity	Paragraph	Additional information	Stops	Samples and stage
103	No	The procedure used for routine analysis is very similar to the method proposed. I list below the differences, probably not relevant: §4.20 - we normally use narrow bore immunoaffinity column §7.1 - we centrifuge samples before filtering and than we filter all the extract §7.2 - normally we filter (syringe-filter) the eluate before HPLC injection		No	
106	No	We never used shaking in conical flasks as extraction procedure before (7.1).	We used two different shakers in the extraction step (7.1). We chose similar speeds, but one of them kept the speed constant whereas the other made cycles (speed up and slow down, each 30s approx.). The samples were distributed as follows: Constant shaker: 123, 183, 225, 273, 278, 300, 348, 360, 396 Cyclic shaker: 398, 473, spiked A, B, C and D	No	
109	Yes			No	
121	Yes			No	
124	Yes			No	
125	Yes			No	
128	Yes		Minor deviation from method: method paragraph 8.4 : every 10 test solutions the 6 calibration standards were injected again - the calibration curve was calculated taking into account all calibration standards before and after the test solutions.	No	
130	Yes			No	
133	Yes			Yes	Samples were weight on previous evening, spiking was also made and left to evaporate overnight
136	Yes			No	

137	Yes			No	
148	Yes		Retention time of OTA in our column (Symmetry C18 4,6 X 250 5 um, Waters) was longer (9.8 min) than given in SOP	No	
156	Yes		During the IAC eluting, we leaved methanol in contact for around one minute.	No	
159	Yes			No	
161	Yes			No	
164	Yes		We've analysed the samples in 4 days. Every day, we followed the sequence as described in the method. For this reason, we're sending you 4 chromatograms of STD2.	No	
168	Yes			No	
172	Yes			No	
175	Yes		All samples analysed by duplicate injection on the HPLC on a single overnight run. Std 2 used as a check - please note contradiction between 8.2 and 8.4. The former allows for a 3% variation in the standard area, whilst 8.4 allows for a 10% variation. Note also the difference in weights stipulated on the spiking protocol (Weigh 25.0g to the nearest 0.1g) to that stipulated in the SOP (Weigh 25g to 2 decimal places).	No	
186	Yes			No	
197	Yes			No	

Table 20:
How did you integrate the signals (automatically or manually)?
If AUTOMATICALLY, did you visually check the correctness of integration?
If YES, for how many chromatograms was it necessary to re-integrate the OTA peak?
Which global settings did you use for automatic integration (e.g. valley-to-valley or horizontal baseline or tangential, etc.)?

	Integration	**Visual check**	**Chromatograms #**	**Integration mode**
103	Automatically	Yes	0	horizontal baseline
106	Automatically	Yes	0	Valley-to-valley
109	Automatically	Yes	0	tangential
121	Manually			
124	Manually			
125	Automatically	Yes	8	Horizontal baseline
128	Manually			
130	Automatically	Yes	4	horizontal baseline
133	Manually			
136	Manually			Manual integration only. Tangential.
137	Manually			valley to valley
148	Automatically	No		valley to valley
156	Automatically	Yes	1	
159	Automatically	Yes	1	Report by area Min area report: 1000 Peak sensitivity: 5 (initial default) (set to 10 for samples 309, 361) Peak width: 1.5 min (initial default) (set to 1.0 for samples 309, 361) Default baseline Integration enabled 5-10.0 minutes
161	Automatically	Yes	0	
164	Automatically	Yes	6	It depends, but most of the times we used tangencial.
168	Manually			
172	Automatically	Yes	0	Inhibit integration between 0-4 min and 5,2-10 min
175	Manually			
186	Manually			horizontal baseline
197	Automatically	Yes	2	width: 0.2

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Abstract
A method validation study was conducted according to the IUPAC harmonised protocol for the determination of ochratoxin A in Capsicum spp. (paprika and chilli). The method is based on the extraction of the samples with an aqueous methanol solution, followed by immunoaffinity cleanup. The determination is carried out by reversed phase high performance liquid chromatography coupled to a fluorescence detector. The study involved 21 participants representing a cross section of research, private and official control laboratories from 14 EU Member States and Singapore.
Mean recoveries reported ranged from 83.7 to 87.5. The relative standard deviation for repeatability (RSD_r) ranged from 1.7 to 14.3 %. The relative standard deviation for reproducibility (RSD_R) ranged from 9.1 to 27.5 %, reflecting HorRat values from 0.4 to 1.3 according to the Horwitz function modified by Thompson. A correction for recovery with the data generated by fortification experiments further improved the reproducibility performance of the method.
The method showed acceptable within-laboratory and between-laboratory precision for each matrix, as required by current European legislation.

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