JRC Scientific and Technical Reports

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 liquorice roots and extracts

Method based on immunoaffinity column clean-up with high performance liquid chromatography and fluorimetric detection method

Donata Lerda, Massimo Ambrosio, Zoltan Kunsagi, Håkan Emteborg, Jean Charoud-Got and Joerg Stroka

EUR 24778 EN - 2011

JRC EUROPEAN COMMISSION

irmm Institute for Reference Materials and Measurements

EURL European Union Reference Laboratory Mycotoxins

The mission of the JRC-IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Address: Retieseweg 111, B-2440 Geel
E-mail: jrc-irmm-crl-mycotox@ec.europa.eu
Tel.: +32 (0)14 571 320
Fax: +32 (0)14 571 783

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC 64399

EUR 24778 EN
ISBN 978-92-79-19831-1
ISSN 1018-5593
doi:10.2787/39928

Luxembourg: Publications Office of the European Union

Printed in Belgium

European Union Reference Laboratory
Mycotoxins

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 liquorice roots and extracts

Method based on immunoaffinity column clean-up with high performance liquid chromatography and fluorimetric detection method

Donata Lerda, Massimo Ambrosio, Zoltan Kunsagi, Håkan Emteborg,
Jean Charoud-Got and Joerg Stroka

EC-JRC-IRMM
March 2011

Table of Contents

1 Summary

The European Union Reference Laboratory for Mycotoxins (EU-RL Mycotoxins), operated by the Institute for Reference Materials and Methods (IRMM) of the Joint Research Centre (JRC), organised a method validation study (MVS) for evaluating the effectiveness of a method for the determination of Ochratoxin A (OTA) in liquorice root and liquorice extracts.

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 dried, reconstituted and quantified by high performance liquid chromatography-flourometric dectection (HPLC-FLD).

Twenty laboratories from 13 EU Member States, a laboratory in Uruguay, one in Turkey and one in US participated in this study. Contents of OTA ranged from 26 to 141 µg/kg and from 8 to 52 µg/kg for liquorice extracts and root material respectively.

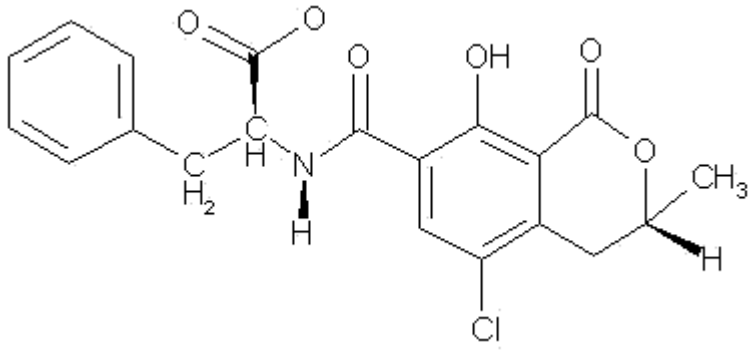
Mean recoveries were calculated as 87 % for liquorice root, and 84 to 88 % for liquorice extracts.

Based on results for the spiked and naturally contaminated samples the relative standard deviations for reproducibility (RSD_R) ranged from 10 to 17 % and from 11 to 22 % in liquorice extracts and liquorice root respectively. Standard deviations for repeatability (RSD_r) ranged from 4 to 9 % and from 6 to 9 % in liquorice extracts and liquorice root respectively.
The Commission Regulation (EC) No 401/2006 lays down performance criteria that must be met by a method for official control of OTA in food at levels up to 10 µg/kg: $RSD_r \leq 20$ %, $RSD_R \leq 30$%, and Recovery from 70 to 110 %. These criteria have been extended by analogy to the levels of interest for this study and were met by this method for both the liquorice root and the liquorice extracts.

2 Introduction

Ochratoxins are pentaketides made up of dihydro-isocoumarin linked to phenylalanine.
Ochratoxin A (OTA) (Figure 1) is a secondary metabolite of several species of fungi, notably Penicillium verrucosum (in temperate climates) and Aspergillus ochraceus, but also A. carbonarius and A. niger aggregate species [1] (in particular in tropical regions). OTA contaminates foods such as cereals, coffee, dried fruit, grapes, cocoa and wine, and it can also occur in animal products. OTA is a mycotoxin with carcinogenic (IARC class2B [2] - meaning the existence of sufficient evidence of its renal carcinogenicity to animals and possibly to humans), nephrotoxic, teratogenic and immunotoxic properties, and it has been linked to nephropathy in humans [3].

Figure 1: Structure of ochratoxin A



Commercially available liquorice products are the part of the plant containing the active principles (the root) and its derivate products (the extracts).
Extracts are produced as paste or as powder, depending on the processing and on the final content of water (powder is obtained by spray drying the liquid extract), and are traditionally obtained via infusion of the ground roots in water in stainless steel equipment along with the support of low-temperature technology in order to fully maintain the organoleptic properties and not burn the natural sugars and the thermo-labile substances which form its aroma.
As demonstrated by Ariño et al. [4], the OTA level in liquorice extracts is stable to heat treatment at 150 °C for 60 min. The OTA concentration is also unaffected by sorting or washing, but it is much reduced by peeling (about 50 % reduction). When performing a small scale preparation of liquorice extract and block liquorice, Ariño et al. could measure a strong reduction of the initial OTA content of about 10 µg/kg (by, respectively about 80 % and about 90 %, when including the peeling of the root).
Therefore, industrial products (extracts) prepared by extracting the active components from the unpeeled roots, are contaminated to an extent which depends on the initial level of contamination of the roots, and on the enrichment achieved by the preparation process of the extracts (about 4:1). Extracts are used for the preparation of candies, pastilles, elastic liquorice and flavours, whilst liquorice root can be consumed as such, or as finely grinded products, for herbal infusions.

The European Food Safety Authority (EFSA) considered that it would be prudent to reduce exposure to OTA as much as possible, establishing a tolerable weekly intake (TWI) of 120 ng kg^{-1} body weight [5]. Liquorice and its derived products may also contribute to human exposure to OTA, particularly for high consumers of these foods, as noted in a recent international conference [6]. New limits were recently set in European legislation (Commission Regulation (EC) No 1881/2006 [7]), including OTA in liquorice (as root) and liquorice extracts (in the form of both paste and powder). The maximum levels are set taking into account the above mentioned considerations about OTA levels in extracts, the average levels found in the roots and the toxicological / occurrence / intake aspects; they are 20 µg/kg for root and 80 µg/kg for extracts. No maximum levels are set for OTA in liquorice in other jurisdictions.

As a follow-up of these last updates, the Directorate General for Health and Consumers asked the support of the European Union Reference Laboratory for Mycotoxins (EU-RL Mycotoxins), hosted by the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Joint Research Centre, to develop and validate by an inter-laboratory comparison a method for the quantitation of Ochratoxin A (OTA) in liquorice and liquorice extracts.

3 Scope

This method validation study aimed to evaluate the recovery and precision of an analytical method, for the quantification of OTA in liquorice and liquorice extracts to monitor compliance with limits set in legislation [7].
The study was designed and evaluated according to the IUPAC Harmonised Protocol (8, 9). Statistical analysis was performed along the lines of ISO 5725 [10-11].
Found values for the precision and recovery were compared with the method performance criteria set in Regulation (EC) No 401/2006 [12] for levels of OTA in food up to 10 µg/kg and extended by analogy to the levels of interest for this study: $RSD_r \leq 20$ %, $RSD_R \leq 30$ %, and recovery from 70 to 11 0%.

4 Participating Laboratories

Some of the National Reference Laboratories of the EU Member States, some public and private laboratories, and other institutions, like universities and public research laboratories joined the study.

Table 1: List of participants to the ILC for the validation of a method on OTA in liquorice

Institute	***Country***
SGS Belgium NV – Chromatography department	Belgium
Federal Agency for the Safety of the Food Chain (FASFC) - Federal Laboratory for Feed and Food Safety of Tervuren (FLVVT)	Belgium
CODA-CERVA - Toxines and Natural Substances	Belgium
Health Canada - Food Lab	Canada
State General Laboratory - Food Contamination Laboratory	Cyprus
National Food Administration - Chemical Division 2	Sweden
Universität Hohenheim (University of Hohenheim) - Landesanstalt für Landwirtschaftliche Chemie (state institute for agricultural chemistry)	Germany
Public Health Laboratory - Environmental Health Authority	Malta
GBA Geselleschaft fur Bioanlytik Hamburg mbH	Germany
AGRICULTURAL RESEARCH CENTRE - LABORATORY FOR RESIDUES AND CONTAMINANTS	Estonia
CENTRO NACIONAL DE ALIMENTACIÓN - Chemical Area - Unit of Toxins and PAHs	Spain
Finnish Customs Laboratory	Finland

Institute	*Country*
GENERAL CHEMICAL STATE LABORATORY - ENVIRONMENT	Greece
Public Analyst's Laboratory Dublin - LC-MS	Ireland
National Research Council (CNR) - Institute of Sciences of Food Production (ISPA) -	Italy
Institute of Food Safety, Animal Health and Environment "BIOR" - Instrumental Analysis Division	Latvia
MSM Food Control Laboratory	Turkey
Food & Environment Research Agency (Fera) - Consumer Protection & Health	United Kingdom
Trilogy Analytical Laboratory	United States
Technological Laboratory of Uruguay – Natural Toxins Department	Uruguay

All participants reported back results, calibration and chromatograms and filled in a questionnaire,.

5 Design of the study

5.1 Time frame

The study was announced via the EU-RL Mycotoxins web-page and via CEN TC275/WG 5 on 28 July 2010. Thirty-nine laboratories expressed their interest in participating in the study and 20 of them were invited to subscribe based on the aim of having different laboratory typologies, with different levels of experience, and different countries included, beside the "first come first served" criterion.
The subscription PDF form was sent out on 15 of September with a deadline set on 1 October 2010.
Together with the subscription form, participants also received the outline of the study and the draft standard operating procedure (SOP). They were asked to send back to the organiser comments and amendments to the SOP if necessary.
Parcels were dispatched on 15/11/2010 and on 18/11/2010 participants received the reporting forms by e-mail. The last laboratory reported back the PDF forms on 01/02/2011 (deadline 10/01/2011).
All the above mentioned communications are included in ANNX 7.

5.2 Materials and documents

Each participant received:

a) One inter-laboratory comparison sample receipt form to be sent back to the organiser upon reception of the parcel

b) The ILC-MVS outline

c) The instructions regarding materials storage, samples treatment, requirements, deadlines.

d) The final version of the method SOP

e) The spiking protocol

f) The participation code (LAB ID)

g) 18 coded test materials for direct analysis (about 12 grams each)

h) 10 coded test materials for spiking before analysis (about 12 grams each)

i) A standard solution for the preparation of the calibration curve

j) 10 spiking standard solutions

k) 35 immunoaffinity columns

l) Safety sheets for solvents and OTA

m) A PDF form for reporting of results

n) A PDF form for reporting the calibration curve(s)

o) A PDF form with a questionnaire regarding general information on the participating laboratory, on their opinion on the design of the study, and on the deviations from the SOP they applied at their laboratory, if any.

The documents sent to the participants are found in the Annex 6, 7 and 8.

5.3 Organisation

Upon participants' comments and amendments, the SOP was changed whenever it was considered appropriate prior the study. In particular, the SOP proposes a specific HPLC analytical column; however participants could apply any other analytical column, given that it was demonstrated as capable of baseline separating the OTA peak from the matrix peaks. Participants were asked to verify that a resolution (Rs) of at least 1.5 is obtained for the OTA peak.
In addition participants had to fill in a questionnaire, where they were asked, in particular, to report any deviation from the SOP they might have applied at their laboratory. This information was used to identify non compliances and when evaluating outliers detected from the statistical tests.

6 Test materials

6.1 Description

Different materials were obtained from different suppliers, covering all categories included in legislation [7] and at levels of interest (below, above and around the maximum levels); however only one blank material was available: a decorticated (peeled) root in form of finely ground powder.

As a result, spiking experiments were made on blank as well as on contaminated materials. According to the recommendations on standard additions proposed by Ellison and Thompson [13], spiking levels were selected to obtain at least four times the initial contamination level.
The final contents of OTA were within the calibration range for all samples and spiked samples.
The test materials of this study are listed in Table 2. The OTA contents reported in this table are based on in-house experiments preliminary to the study. These contents might differ from the mean of the study results (Tables 3 to 5).

Table 2: Test samples and spiking solutions

Sample name/ description	Sample code	Amount available kg	OTA µg/kg	OTA in the spiking solution µg/ml	OTA in the spiked sample µg/kg	Design
Extract (Powder) 2007 TM070024	Extract powder medium low	< 2	27.1	n.a.	n.a.	2 blind replicates
Extract (Powder) 2008 TM080019	Extract powder low	5	6.5	n.a.	n.a.	2 blind replicates
				0.6	36.5	2 blind replicates for spiking level A
				1.5	81.5	2 blind replicates for spiking level B
Extract (Powder) 2010 TM100028	Extract powder medium high	3	59.2	n.a.	n.a.	2 blind replicates
Extract (Powder) 2010 TM100029	Extract powder high	3	90.3	n.a.	n.a.	2 blind replicates
Extract (Paste) 2007 TM070025	Extract paste high	< 2	78.5	n.a.	n.a.	2 blind replicates
Extract (Paste) 2008 TM080020	Extract paste low	5	32.0	n.a.	n.a.	2 blind replicates
				2.6	162.0	2 blind replicates for spiking
Root powder decorticated 2010 TM100034	Root blank	4	Blank (< LOD/ <2 µg/kg)	n.a.	n.a.	2 blind replicates
				1.2	30.0	2 blind replicates for spiking level A
				2.4	60.0	2 blind replicates for spiking level B
Root powder 2010 TM100035	Root high	4	20.1	n.a.	n.a.	2 blind replicates
Root powder 2008 TM080021	Root low	2.5	6.5	n.a.	n.a.	2 blind replicates

n.a.: not applicable

The spiking amount was 500 µl. Following the recommendations of Burns et al [14] regarding the risk of dilution-modification of the matrix, the concentrations of OTA in the spiking solutions were high enough to allow using a sufficiently low volume.

6.2 Preparation and verification

Test samples

The test materials were used as obtained by the supplier, with the exception of the Root powder 2010TM100035 (Root high) sample.

This material was found to be not sufficiently homogeneous. However, due to its OTA content, of about 20 µg/kg (maximum level set in legislation), the material was considered of interest for the study.

Therefore, it was treated at the Reference Material Unit at IRMM. It was milled to eliminate most of the fibres originally present; than particle size analysis was performed on Root high and Root low in parallel to see if they were comparable (Root low was found to be sufficiently homogeneous for the ILC-MVS). They were measured with laser diffraction before and after the milling of Root high finding that after the milling, the two diffraction curves were closer. The two materials were also checked with manual sieving: each material was placed on sieves and manually shaken. After milling, most of the fibres present in the Root High before milling, disappeared on the 500 µm sieve.

Since the extract pastes tend to stratify during storage. Participants were asked to mix paste samples before analysis.

Common calibrant

A common calibrant was distributed for the preparation of the calibration curve, which contained OTA (OTA in the form of powder, as obtained from Sigma, code O-1877, purity 98%, lot 125K4063)- in a mixture of toluene and glacial acetic acid (both supplied by VWR) 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;

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, see [15]);

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

The concentration of OTA was determined to be 13.46 µg/ml.

The content level was demonstrated to be stable for the whole duration of the study.

Spiking solutions

Spiking solutions were obtained by dilution. An intermediate solution was prepared with a target concentration of 50 µg/ml. The concentration was verified spectrophotometrically to be 48.10 µg/ml. From this solution spiking solutions were prepared as listed in Table 2. Ampoules were filled under inert atmosphere, each with 1.5 ml of spiking solution, flame sealed, and stored at -18 ºC until dispatch.

The content levels were demonstrated to be stable for the whole duration of the study.

6.3 Homogeneity

Homogeneity of the naturally contaminated test samples was tested according to the IUPAC Harmonised Protocol for Proficiency Testing [16].
The criterion to consider the material sufficiently homogeneous is given in Equation 1.

Equation 1 $\sigma^2_{sam} \leq 0.07C$

Where
σ^2_{sam}: sampling variance
C: content of analyte in the sample as estimated by the organiser prior to materials dispatch
0.07: =0.3X0.22 (0.22 C is the reproducibility standard deviation as defined in the Horwitz equation as modified by Thompson for concentrations below 120 ppb [17]:

The samples were chosen at regular intervals along the packing order of each test material to check for possible trends in composition. All test materials were rated sufficiently homogeneous (see ANNEX 1). No homogeneity assessment was carried out for sample Root powder – blank (2010).

7 Evaluation of results

7.1 General

Calibration
Each participant was asked to report peak areas and corresponding OTA concentrations in ng/ml of the calibration curve. In case of an interruption of the analytical process, a calibration curve was required for each sequence and peak areas obtained for all calibration curves had to be reported in the respective PDF form.
Calibrations where checked for linearity applying the Mandel test. The test was carried out by applying MVA version 1.1software developed by © Novia.

Questionnaire
Each participant was asked to answer several questions regarding the organisation of the study, the method, and the analytical process as carried out at their laboratory.
In particular, they were asked to report possible deviations from the methods at different stages of the analysis.
In case of reported relevant deviations, the different procedure(s) applied were taken into account when identifying possible outliers.

Chromatograms
Laboratories were asked to send back to the organiser the chromatogram obtained from the analysis of each sample.
They represented an additional indication, together with the resolution (R_S) between the OTA peak and neighbouring peaks. Validity of results was subject to sufficient R_S values.

Results
Method precision was calculated with the Excel Template CLSTD.XLT version 3.6- 2/12/98, CSL, Food Science Laboratory, Norwich, UK.

The Horwitz ratio, HorRat, was calculated applying both the Horwitz equation [18] and the Horwitz equation as modified by Thompson for the concentrations below 120 ppb [17].

For each of the 14 test samples (8 naturally contaminated, 1 blank, and 5 spiked) analysed as blind duplicates (for a total of 28 expected results from each laboratory) the set of results were evaluated as reported by participants.

Results were checked for deviations from the method protocol and insufficient R_S. Resulting non compliant data were removed prior statistical evaluation. Remaining results were checked for outliers applying Cochran's and single and double Grubbs' tests.
Non compliances, together with the outliers, are detailed in the Annexes 2 and 3, where data reported for all materials are listed. Precision estimates were obtained according to the IUPAC Harmonised Protocol [8, 9].

Recoveries were obtained from the values reported for the 5 duplicate spiked samples by applying the Equation 3. This type of recovery is also called "surrogate recovery" (the added analyte acts as a surrogate for the native analyte) or marginal recovery [19].

Equation 3

$$\%R = \frac{(OTA_{SP+N} - OTA_N)}{OTA_{SP}} \times 100$$

Where

OTA_{SP+N}: mean result of the spiked sample
OTA_N: mean result of non spiked corresponding sample
OTA_{SP}: concentration of OTA added to the sample

OTA_N was considered null for the Root blank material.

7.2 Evaluation of calibrations

All calibrations reported were found to be linear in the working range.
Laboratory 7103 reported constantly negatively biased results; however, as bias was not constant for all samples, this problem could not be solely attributed to mistakes in the preparation of the calibration curve. This laboratory was asked to report about possible problems occurring during the analysis. Their answer is summarised in paragraph 7.4.

7.3 Evaluation of questionnaire

All answers were compiled in the tables in the Annexes 4 and 5.
Critical points considered for possible non compliance were either a deviation from the SOP and/or an incorrect analytical procedure (e.g. incorrect peak integration).
No reported deviation from the SOP was considered to be relevant for rejecting the whole set of results from a participant.

7.4 Evaluation of chromatograms

All participants sent the chromatograms for all the samples. Chromatograms were checked for consistency in the retention time of the OTA peak, for peak shape, R_S values (see Annex 5), and for integration.

Laboratory 6658 reported chromatograms with non acceptable peak shape and integration. Upon communication to the participant, it was decided to mark their results as non-compliant.

7.5 Evaluation of results

The method parameters, calculated as described in 7.1, are reported for liquorice extracts in form of powder and paste in Tables 3 and 4 respectively

Table 3 — Precision data ILC-MVS for OTA in liquorice extracts - powder

Sample	Extract powder medium low	Extract powder low - spA	Extract powder low - spB	Extract powder medium high	Extract powder high
Number of laboratories	20	20	20	20	20
Number of laboratories considered as non compliant	1	1	1	1	1
Number of outliers (laboratories)	0	0	0	2	0
Number of accepted results	19	19	19	17	19
Mean value, $\bar{x}$, µg/kg	25.7	34.2	71.8	59.6	96.8
Repeatability standard deviation s_r, µg/kg	1.5	2.6	5.8	2.3	5.8
Repeatability relative standard deviation, RSD_r, %	6	8	8	4	6
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	4.3	7.2	16.2	6.4	16.2
Reproducibility standard deviation s_R, µg/kg	3.1	5.0	9.4	6.6	12.4
Reproducibility relative standard deviation, RSD_R, %	12	15	13	11	13
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	8.6	14.0	26.3	18.6	34.7
Recovery, %	n.a.	86	84	n.a.	n.a.
HorRat value	0.54	0.66	0.60	0.51	0.58

n.a.: not applicable

Table 4 — Precision data ILC-MVS for OTA in liquorice extracts - paste

Sample	Extract paste high	Extract paste low	Extract paste low - sp
Number of laboratories	20	20	20
Number of laboratories considered as non compliant	1	1	1
Number of outliers (laboratories)	2	0	1
Number of accepted results	17	19	18
Mean value, $\bar{x}$, µg/kg	64.3	27.4	141.4
Repeatability standard deviation s_r, µg/kg	3.6	2.4	6.3
Repeatability relative standard deviation, RSD_r, %	6	9	4
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	10.1	6.9	17.6
Reproducibility standard deviation s_R, µg/kg	8.4	4.6	13.7
Reproducibility relative standard deviation, RSD_R, %	13	17	10
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	23.5	12.9	38.4
Recovery, %	n.a.	n.a.	88
HorRat value	0.59	0.77	0.45

n.a.: not applicable

Data for paste and powder have been splitted for readability in two sets; however, both are to be considered as “extract” materials for the deduction of method performances data.
In Table 5 are reported the method parameters for liquorice root powder.

Table 5 — Precision data ILC-MVS for OTA in liquorice - root powder

Sample	Root blank - spA	Root blank - spB	Root low	Root high
Number of laboratories	20	20	20	20
Number of laboratories considered as non compliant	1	2	4	1
Number of outliers (laboratories)	0	1	1	2
Number of accepted results	19	17	15	17
Mean value, $\bar{x}$, µg/kg	26.1	51.9	7.7	22.0
Repeatability standard deviation s_r, µg/kg	1.6	2.9	0.7	2.0
Repeatability relative standard deviation, RSD_r, %	6	6	9	9
Repeatability limit r [$r = 2{,}8 \times s_r$], µg/kg	4.4	8.1	1.9	5.7
Reproducibility standard deviation s_R, µg/kg	4.0	5.5	1.7	3.3
Reproducibility relative standard deviation, RSD_R, %	15	11	22	15
Reproducibility limit R [$R = 2{,}8 \times s_R$], µg/kg	11.2	15.3	4.8	9.2
Recovery, %	87	87	n.a.	n.a.
HorRat value	0.69	0.48	1.02	0.67

n.a.: not applicable

Reproducibility and repeatability from this study complies with legislative requirements [12] for food at levels up to 10 µg/kg: $RSD_r \leq 20\%$, $RSD_R \leq 30\%$ and HorRat values are in all cases below 2. Recoveries too are within the legislatively required range (70-110%).

Laboratory 7103 reported negatively biased results identified as outliers and rejected in several cases. However, the laboratory did not report any deviation from the method. Abnormalities were reported for 4 samples but only in one case coinciding with outlier detection. Upon request of the organiser, participant re-checked their results and reported that, due to a fault in the autosampler, samples had to be re-injected starting from frozen extracts, purified with IAC columns of a different brand than that those dispatched by the organiser. This might have caused the bias observed.

Laboratory 6426 reported results which were, in the case of materials Extract paste high and Root high, detected as outliers and rejected, but no cause for this was identified. Chromatograms showed no lack of resolution and integration was correct. However, for Root high material the chromatogram corresponding to the anomalous result showed a peak width about the double than what found for the standard. This indicates the co-elution of an interfering peak. For Extract Paste high no cause could be identified.

Laboratory 6926 reported results which were, in the case of material Extract powder low, identified as outliers and rejected, but no cause for this was identified.

Laboratory 6631 reported results which were, in the case of materials Root low and Root high, detected as outliers and rejected, but no cause for this was identified. Chromatograms showed no lack of resolution and integration was correct.

Laboratory 6381 reported results which were, in the case of material Extract powder medium high, identified as outliers and rejected. No cause for this was identified.

Finally, laboratory 6595 reported results which were, in the case of material Extract powder medium high, identified as outliers. One of the chromatograms showed lack of resolution/peak asymmetry.

In this study, the material Root blank was not used for the evaluation of method performances but only for the preparation of spiking materials; however participants were asked to report results to verify whether the OTA content in the material was consistently found below the LOQ of the method, as in fact it was.
Also for the material Extract powder low, participants were asked to report the data not only for the two derived spiked material but also for the native material (OTA content estimated by the organiser was 6.5 µg/kg). However, as the European legislation sets limits for OTA in extracts at 80 µg/kg [7], this material was not considered as relevant for the definition of method performances for the scope of official controls. Results reported by participants were considered to verify whether the method is applicable at low levels of OTA (below 10 µg/kg). As a matter of fact this extension of the method scope was considered to be possible with some additional analytical effort.

The method is to be considered as validated by this study in the following concentration ranges, as defined from the OTA contents in the tested materials:

- Liquorice extracts: from 26 to 141 µg/kg
- Liquorice (root): from 8 to 52 µg/kg

7.6 Deviations from the SOP

Participants were asked to report in the questionnaire deviations from the SOP. List of deviations is reported in the Annex. None of those deviations were considered to justify a non compliance.
In particular it has to be noted that other HPLC columns than the one indicated in the SOP where applied in many laboratories. For laboratory 6658, incorrect integration of poorly separated peaks obtained with a different analytical column was considered as cause for non compliance.

8 Conclusions

All 20 invited participants reported their analysis results. The performance of the method showed to be satisfactory, in particular as regards legislative requirements for recovery and precision, for all materials of interest to confirm the scope of the method.
The JRC will submit this fully validated method to CEN TC 275/WG 5 and suggest it for formal standardisation.

9 Acknowledgements

The organisers would like to thank NOREVO GmbH - NOREVO GmbH (Schnackenburgallee 50, D-22525 Hamburg – Germany, www.norevo.de), AURELIO MENOZZI & R. DE ROSA S.A.S. (C.P. 83; 65015 Montesilvano (PE) – Italy, www.menozziderosa.com), and Martin Bauer GmbH & Co. (KG 91487 Vestenbergsgreuth - Germany, www.martin-bauer-group.com/en) for supplying the materials used for the study.
We acknowledge that without the positive co-operation of all participants, this study would not have been possible.
We would like to thank also Ms Helena Ernst for her precious co-operation in the bottling of materials and preparation of parcels, and Ms Beatriz de la Calle and Mr Franz Ulberth for their accurate revision of this report.

10 References

1 Esteban A, Abarca ML, Bragulat MR, Caban˜ es FJ. 2006. Effect of water activity on ochratoxin A production by Aspergillus niger aggregate species. International Journal of Food Microbiology 108:188–195.

2 Castegnaro M., Barek J., Fremy J.M., Lafontaine M., Sansone E.B. and Telling G.M. Laboratory decontamination and destruction of carcinogens in laboratory wastes: some mycotoxins. IARC Scientific Publication No. 113, International Agency for Research on Cancer, Lyon (France), 1991, p. 63.

3 Creppy EE. 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters 127:19–28.

4 Ariño A., Herrera M., Langa E., Raso J., Herrera A. *Ochratoxin A in liquorice as affected by processing methods.* Food Addit.Contam., 2007. **24**(9): p. 987-992

5 European Food Safety Authority (EFSA). 2006. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to ochratoxin A in food. EFSA Journal 365:1–56.

6 Fink-Gremmels J. 2005. Conclusions from the workshops on Ochratoxin A in Food: Recent developments and significance, organized by ILSI Europe in Baden (Austria), 29 June–1 July 2005. Food Additives and Contaminants 22(Suppl. 1):1–5.

7 EU, *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. **L 364**: p. 5-24.
Available from (consolidated version):
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2006R1881:20100701:EN:PDF

8. Horwitz, W. (IUPAC) (1995), *Pure & Applied Chem.*, **67**, 331-343

9. Horwitz, W. and Albert, R. (1991) *J.AOAC Int.*, **74**: 718-744

10 ISO5725-2 (1996) 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

11 ISO5725-1 (1996) Accuracy (trueness and precision) of measurement methods and results - Part 1: General principls and definitions. Geneva, Switzerland

12 EU, *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.
Available from (consolidated version):
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2006R0401:20100313:EN:PDF

13 Ellison S.L.R., Thompson M. Standard additions: myth and reality. The Analyst, 2008. 133: p. 992-997

14 Burns D.T., Danzer K. and Townshend A., 2002, Use of the terms "recovery" and "apparent recovery" in analytical procedures, Pure Appl. Chem., Vol. 74, No. 11, pp. 2201–2205, 2002.

15 Wood, G. M., Patel, S., Entwisle, A. C. and Boenke, A., 1996, Ochratoxin A in wheat: a second intercomparison of procedures, Food Additives and Contaminants, 13, 519 - 539.

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

17 Thompson M., (2000), Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing, Analyst, vol. 125: 385 – 386

18 Horwitz W. and Albert R, (2006), The Horwitz Ratio (HorRat): A useful Index of Method Performance with Respect to Precision, Journal of AOAC International, vol. 89, 1095 – 1109

19 Thompson M., Ellison S.L.R., Fajgelj A., Willets P. (1996), Harmonised Guidelines for the Use of Recovery Information in Analytical Measurement, Pure Appl. Chem., 1999, 71, 337-348

11 Annex

List of Annexes

ANNEX 1 – Homogeneity data for OTA in all materials

ANNEX 2 - Individual data reported by the participants - Data for spiked materials (recovery estimation)

ANNEX 3 - Individual data reported by the participants -Data for naturally contaminated materials

ANNEX 4 - Questionnaire output: general

ANNEX 5 - Questionnaire output: deviations from SOP and the analytical process

ANNEX 6 - Supporting documents: Method and spiking protocols

ANNEX 7 - Supporting documents: communication

ANNEX 8 - Supporting documents: FORMS

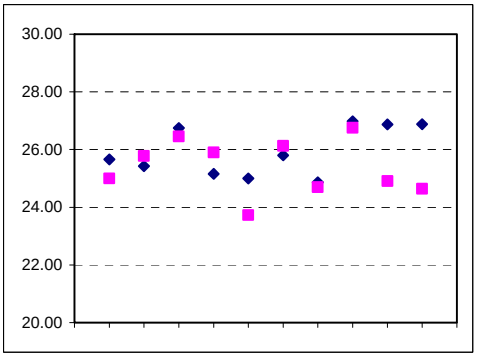
ANNEX 1 – Homogeneity data for OTA in all materials

Extract powder medium low

	n =	10		
	mean =	25.67	22%	**= σ-trg(%)**
0.5648	**s_x =**	0.7515	5.6474	**= σ-trg**
$MSW^{\wedge-2}$ =	**s_w =**	0.7697		
	s_s =	0.5182	1.6942	**= 0,3*s**

IUPAC
(MSB-MSW)/2 0.2686 5.9948 = $F1*(0,3*s)^2+F2*MSW$
passed

Bottle	Result a	Result b	diff	sum	avg
1	25.66	25.00	0.66	50.66	25.33
2	25.42	25.78	-0.35	51.20	25.60
3	26.75	26.45	0.30	53.20	26.60
4	25.16	25.90	-0.74	51.06	25.53
5	25.00	23.73	1.27	48.73	24.37
6	25.80	26.13	-0.33	51.93	25.97
7	24.86	24.70	0.16	49.56	24.78
8	26.98	26.76	0.23	53.74	26.87
9	26.88	24.91	1.96	51.79	25.89
10	26.88	24.64	2.24	51.52	25.76



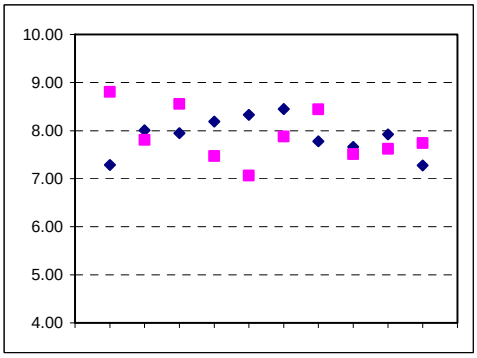
$\sum(diff)^2$ = 11.8501
var(sum)/2 = 1.1297 =MSB

Extract powder low

	n =	10		
	mean =	7.89	22%	**= σ-trg(%)**
0.0634	**s_x =**	0.2518	1.7352	**= σ-trg**
$MSW^{\wedge-2}$ =	**s_w =**	0.5443		
	s_s =	0.2910	0.5206	**= 0,3*s**

IUPAC
(MSB-MSW)/2 -0.0847 0.8086 = $F1*(0,3*s)^2+F2*MSW$
passed

Bottle	Result a	Result b	diff	sum	avg
1	7.29	8.80	-1.52	16.09	8.04
2	8.01	7.81	0.20	15.82	7.91
3	7.95	8.55	-0.61	16.50	8.25
4	8.19	7.47	0.71	15.66	7.83
5	8.33	7.06	1.27	15.39	7.70
6	8.45	7.88	0.57	16.33	8.16
7	7.78	8.44	-0.67	16.22	8.11
8	7.66	7.51	0.15	15.17	7.59
9	7.92	7.62	0.30	15.54	7.77
10	7.27	7.74	-0.47	15.02	7.51



$\sum(diff)^2$ = 5.9247
var(sum)/2 = 0.1268 =MSB

Extract paste low

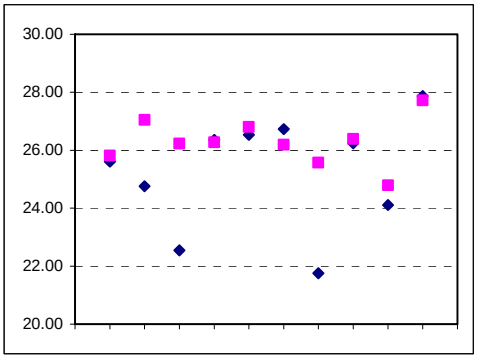
	n =	10		
	mean =	25.77	22%	**= σ-trg(%)**
1.5637	**s_x =**	1.2505	5.6690	**= σ-trg**
MSW$^{\wedge-2}$ =	**s_w =**	1.3107		
	s_s =	0.8395	1.7007	**= 0,3*s**

IUPAC

(MSB-MSW)/2 0.7048 7.1729 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	25.61	25.82	-0.21	51.42	25.71
2	24.76	27.04	-2.28	51.81	25.90
3	22.55	26.23	-3.69	48.78	24.39
4	26.36	26.27	0.08	52.63	26.32
5	26.53	26.81	-0.28	53.34	26.67
6	26.73	26.19	0.55	52.92	26.46
7	21.75	25.58	-3.82	47.33	23.67
8	26.24	26.39	-0.15	52.63	26.32
9	24.11	24.79	-0.68	48.91	24.45
10	27.87	27.72	0.15	55.59	27.80



∑(diff)^2 = 34.3584

var(sum)/2 = 3.1275 =MSB

Root low

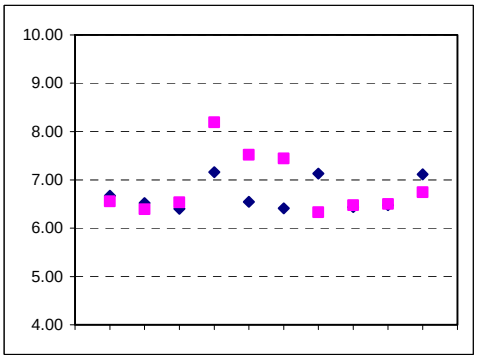
	n =	10		
	mean =	6.78	22%	**= σ-trg(%)**
0.1484	**s_x =**	0.3853	1.4910	**= σ-trg**
MSW$^{\wedge-2}$ =	**s_w =**	0.4422		
	s_s =	0.2251	0.4473	**= 0,3*s**

IUPAC

(MSB-MSW)/2 0.0507 0.5736 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	6.67	6.55	0.11	13.22	6.61
2	6.52	6.39	0.13	12.91	6.45
3	6.40	6.54	-0.13	12.94	6.47
4	7.16	8.19	-1.04	15.35	7.68
5	6.54	7.52	-0.97	14.06	7.03
6	6.41	7.44	-1.03	13.85	6.93
7	7.13	6.33	0.80	13.46	6.73
8	6.44	6.48	-0.04	12.91	6.46
9	6.47	6.50	-0.03	12.97	6.49
10	7.12	6.75	0.37	13.86	6.93



∑(diff)^2 = 3.9103

var(sum)/2 = 0.2969 =MSB

Extract paste high

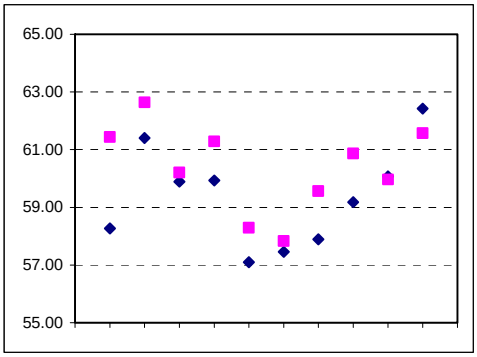
	n =	10		
	mean =	59.87	22%	**= σ-trg(%)**
2.3072	**s_x =**	1.5189	13.1704	**= σ-trg**
MSW$^{\wedge-2}$ =	**s_w =**	1.0373		
	s_s =	1.3301	3.9511	**= 0,3*s**

IUPAC

(MSB-MSW)/2 1.7691 30.4363 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	58.27	61.44	-3.17	119.70	59.85
2	61.40	62.64	-1.24	124.04	62.02
3	59.89	60.21	-0.32	120.10	60.05
4	59.93	61.29	-1.36	121.22	60.61
5	57.10	58.29	-1.19	115.40	57.70
6	57.46	57.84	-0.38	115.29	57.65
7	57.89	59.57	-1.68	117.46	58.73
8	59.18	60.87	-1.69	120.05	60.03
9	60.08	59.97	0.11	120.04	60.02
10	62.43	61.57	0.85	124.00	62.00



∑(diff)^2 = 21.5212

var(sum)/2 = 4.6143 =MSB

Extract powder medium high

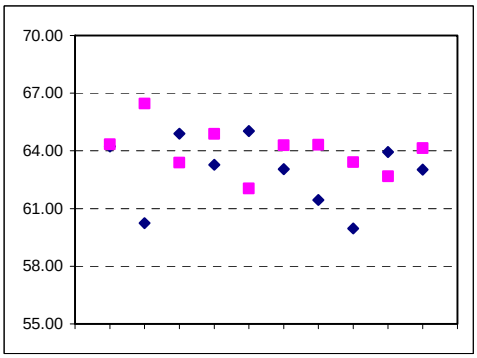
	n =	10		
	mean =	63.45	22%	**= σ-trg(%)**
0.5683	**s_x =**	0.7539	13.9599	**= σ-trg**
MSW$^{\wedge-2}$ =	**s_w =**	1.9625		
	s_s =	1.1651	4.1880	**= 0,3*s**

IUPAC

(MSB-MSW)/2 -1.3574 36.8634 = F1*(0,3*s)^2+F2*MSW

passed

Bottle	Result a	Result b	diff	sum	avg
1	64.24	64.34	-0.11	128.58	64.29
2	60.24	66.47	-6.23	126.71	63.35
3	64.89	63.39	1.51	128.28	64.14
4	63.28	64.89	-1.61	128.16	64.08
5	65.03	62.05	2.98	127.09	63.54
6	63.05	64.29	-1.24	127.34	63.67
7	61.45	64.32	-2.87	125.77	62.88
8	59.96	63.41	-3.45	123.37	61.69
9	63.94	62.68	1.26	126.62	63.31
10	63.03	64.14	-1.11	127.17	63.59



∑(diff)^2 = 77.0291

var(sum)/2 = 1.1367 =MSB

Extract powder high

	n =	10		
	mean =	91.38	22%	**= σ-trg(%)**
6.2759	**s_x =**	2.5052	20.1044	**= σ-trg**
$MSW^{\wedge-2}$ =	**s_w =**	4.6288		
	s_s =	2.1065	6.0313	**= 0,3*s**

IUPAC

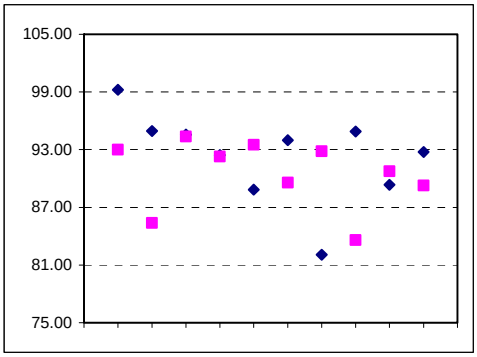
(MSB-MSW)/2 -4.4371 90.0286 = $F1*(0,3*s)^2+F2*MSW$

passed

Bottle	Result a	Result b	diff	sum	avg
1	99.24	93.01	6.23	192.24	96.12
2	94.94	85.38	9.56	180.32	90.16
3	94.57	94.35	0.22	188.92	94.46
4	92.42	92.29	0.13	184.72	92.36
5	88.85	93.52	-4.67	182.37	91.18
6	94.00	89.58	4.42	183.58	91.79
7	82.08	92.84	-10.76	174.92	87.46
8	94.88	83.61	11.28	178.49	89.24
9	89.34	90.74	-1.40	180.08	90.04
10	92.75	89.28	3.47	182.04	91.02

$\sum(diff)^2$ = 428.5215

var(sum)/2 = 12.5518 =MSB



Root high

	n =	10		
	mean =	23.65	22%	**= σ-trg(%)**
1.9846	**s_x =**	1.4088	5.2024	**= σ-trg**
$MSW^{\wedge-2}$ =	**s_w =**	1.4147		
	s_s =	0.9919	1.5607	**= 0,3*s**

IUPAC

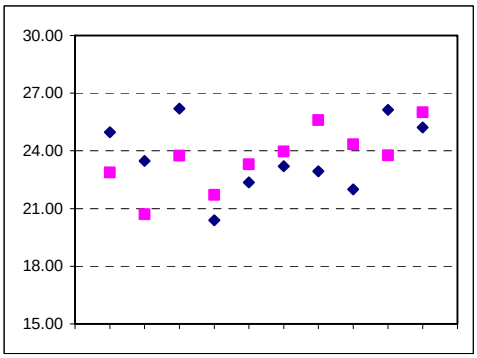
(MSB-MSW)/2 0.9839 6.6009 = $F1*(0,3*s)^2+F2*MSW$

passed

Bottle	Result a	Result b	diff	sum	avg
1	24.97	22.88	2.09	47.85	23.93
2	23.47	20.71	2.77	44.18	22.09
3	26.21	23.75	2.45	49.96	24.98
4	20.40	21.71	-1.31	42.11	21.05
5	22.36	23.30	-0.93	45.66	22.83
6	23.20	23.97	-0.77	47.17	23.59
7	22.94	25.60	-2.66	48.53	24.27
8	22.00	24.35	-2.35	46.35	23.17
9	26.13	23.77	2.36	49.90	24.95
10	25.22	26.01	-0.79	51.23	25.62

$\sum(diff)^2$ = 40.0294

var(sum)/2 = 3.9692 =MSB



ANNEX 2 - Individual data reported by the participants

Data for spiked materials (recovery estimation)

Analytical results are listed as reported by the individual participants. Values reported as <LOD excluded from statistical treatment. Grey shaded entries in the tables refer to non compliant data.

Abbreviation **NC** in the graphics refers to **N**on **C**ompliant results. Results marked with **GS**, **GD** and **C** refer to results identified as **G**rubb's **S**ingle, **G**rubb's **D**ouble and **C**ochran outliers in the statistical evaluation after **NC** removal.

Table 7: Extract Powder low spiked with 30 µg/kg of OTA; initial OTA content prior spiking was determined as 6.5 µg/kg by organiser (see Table 2)

Participant	Result 1	Result 2
3063	35.03	33.77
3084	35.1	32.79
6371	41.49	41.46
6381	37.05	36.57
6426	38.77	39.24
6482	35.18	26.09
6512	35.55	35.29
6584	37.58	33.45
6595	37.07	41.16
6600	31.41	34.62
6631	37.74	36.49
6635	28.5	30.13
6658	34.87	35.04
6696	32.93	31.81
6699	35.4	26.6
6926	35.6	39.2
6938	39.37	40.97
6942	23.8	24.22
7026	33.78	34.1
7103	28.13	23.74

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Figure 1: Distribution of individual results of replicate measurements.

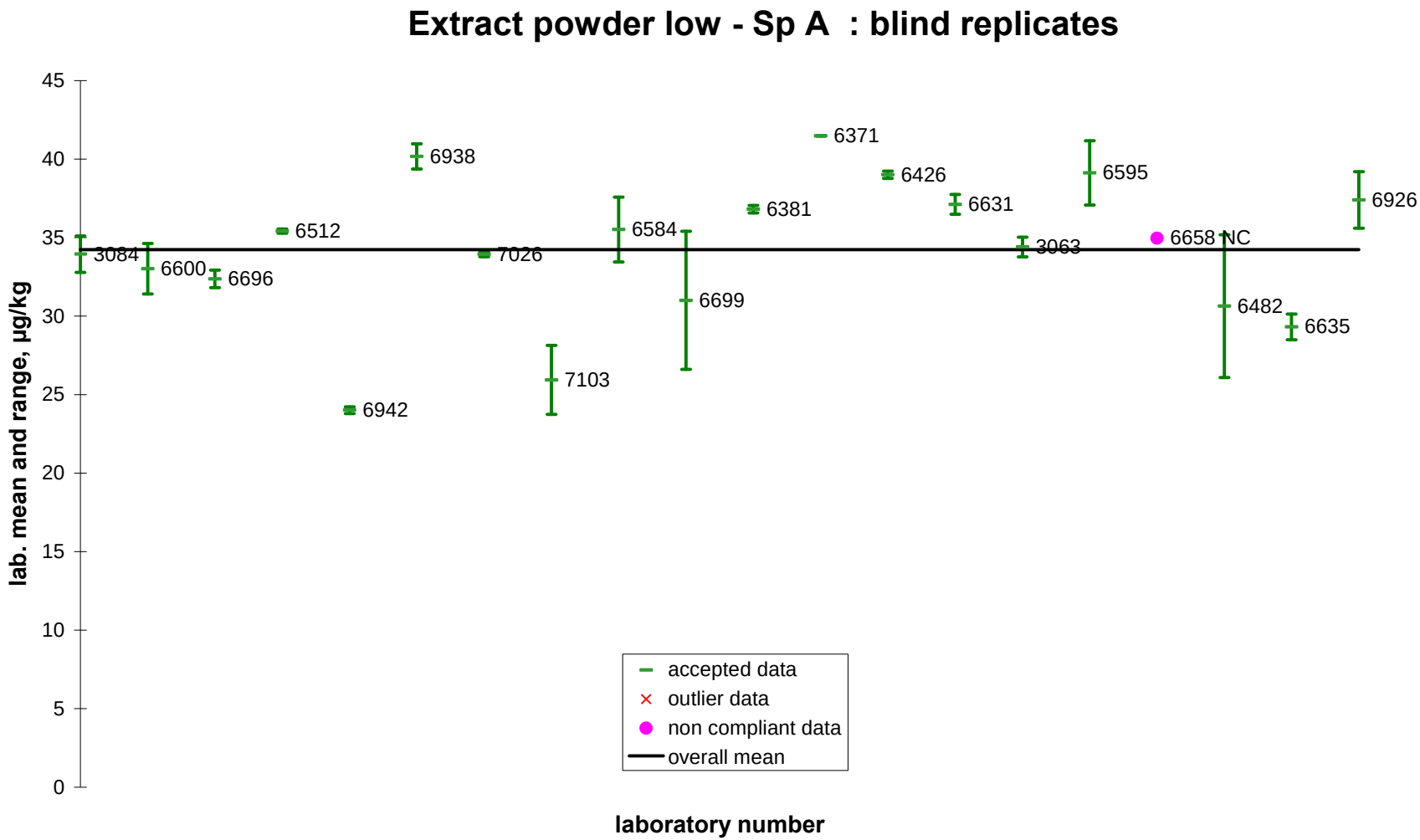


Figure 2: Youden Plot

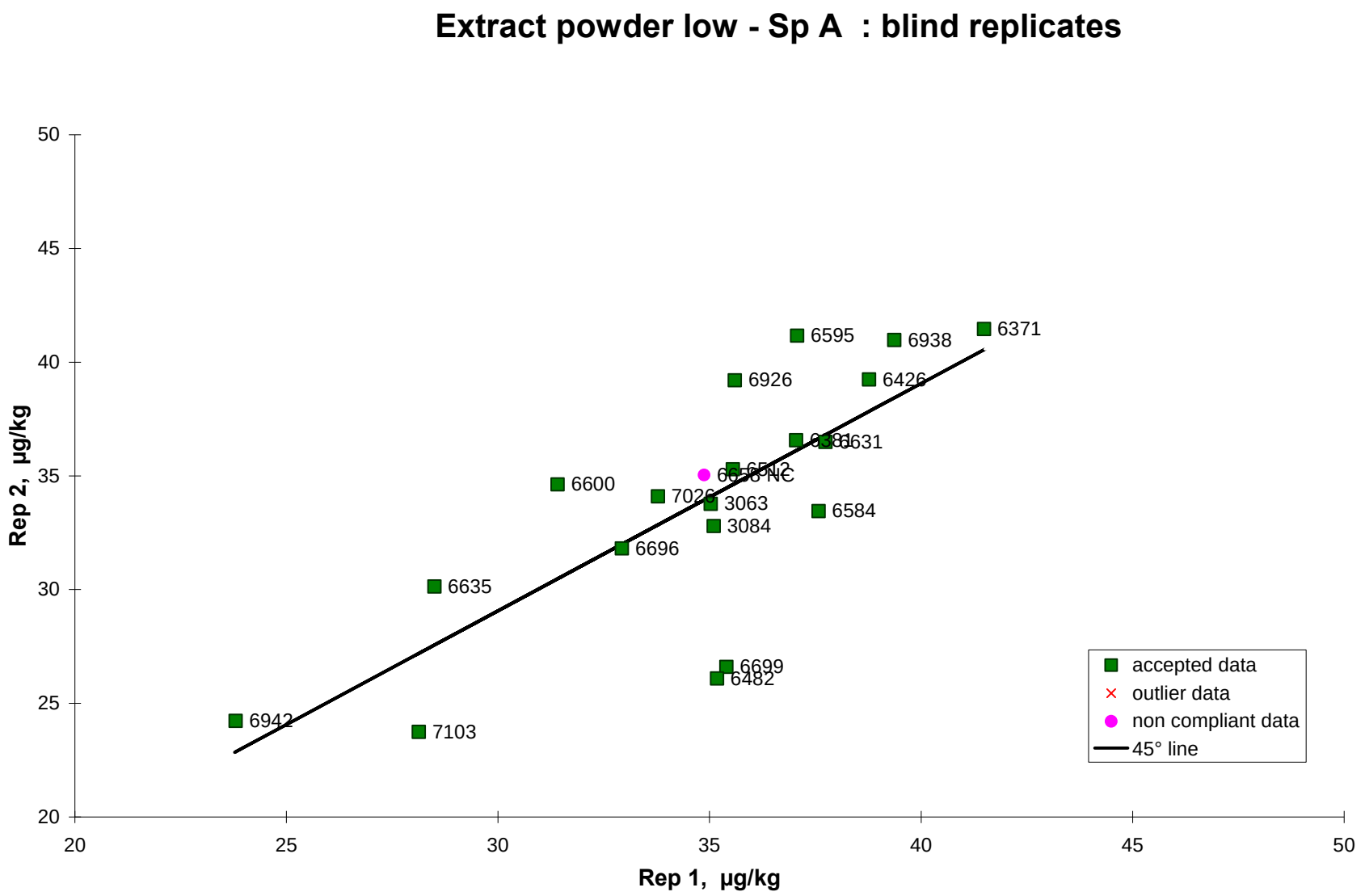


Table 8: Extract powder low spiked with 75 µg/kg of OTA; initial OTA content prior spiking was determined as 6.5 µg/kg by organiser (see Table 2)

Participant	Result 1	Result 2
3063	74.84	76.55
3084	82.01	64.19
6371	83.57	86.47
6381	67.88	58.46
6426	80.34	79.02
6482	72.07	68.03
6512	72.37	77.51
6584	74.25	76.62
6595	71.83	73.34
6600	62.78	70.43
6631	69.86	80.8
6635	61.96	64.5
6658	76.45	68.17
6696	68.39	72.24
6699	74.98	77.07
6926	79.2	76.4
6938	76.35	83.2
6942	51.29	64.61
7026	77.82	76.33
7103	59.31	40.24

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Figure 3: Distribution of individual results of replicate measurements.

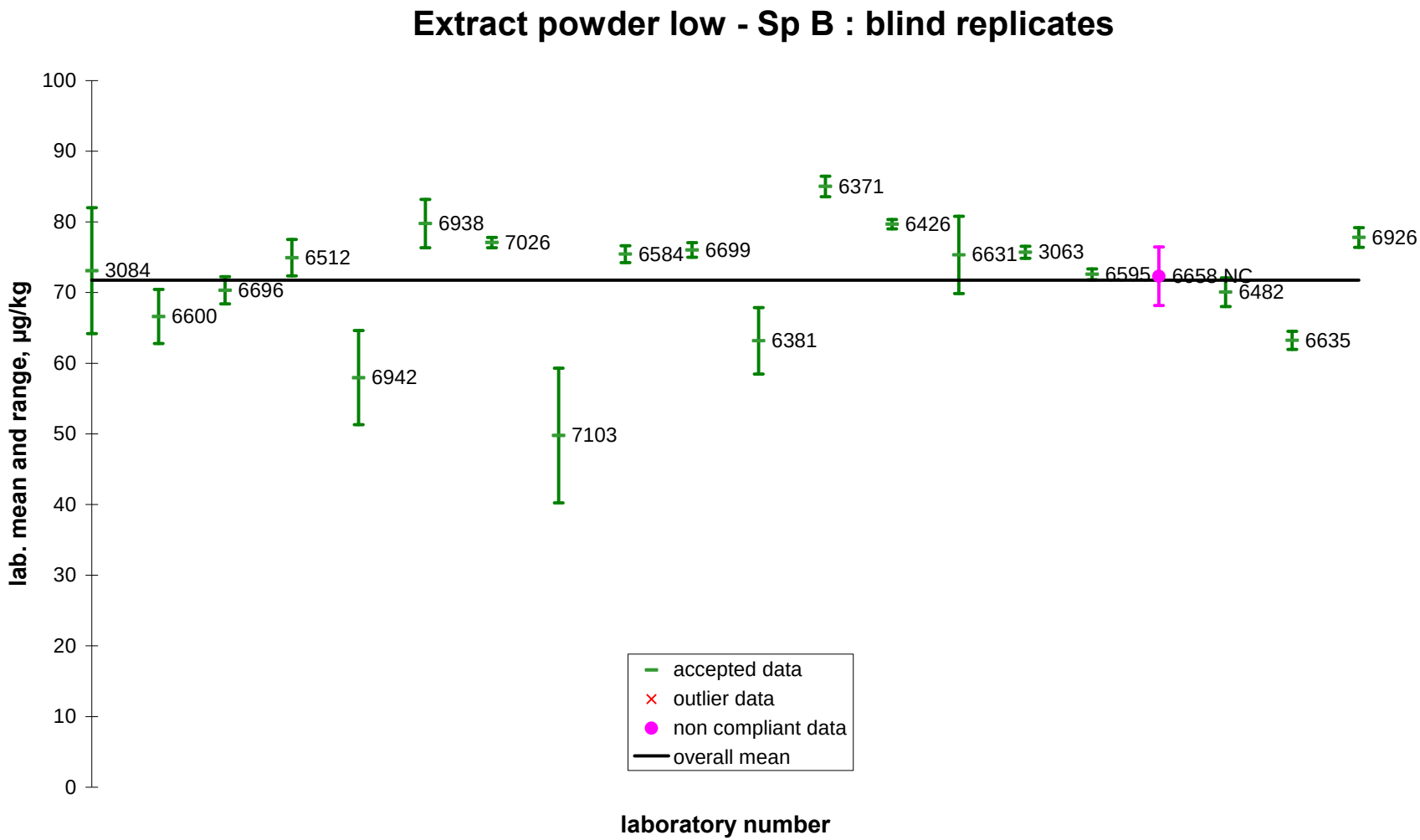


Figure 4: Youden Plot

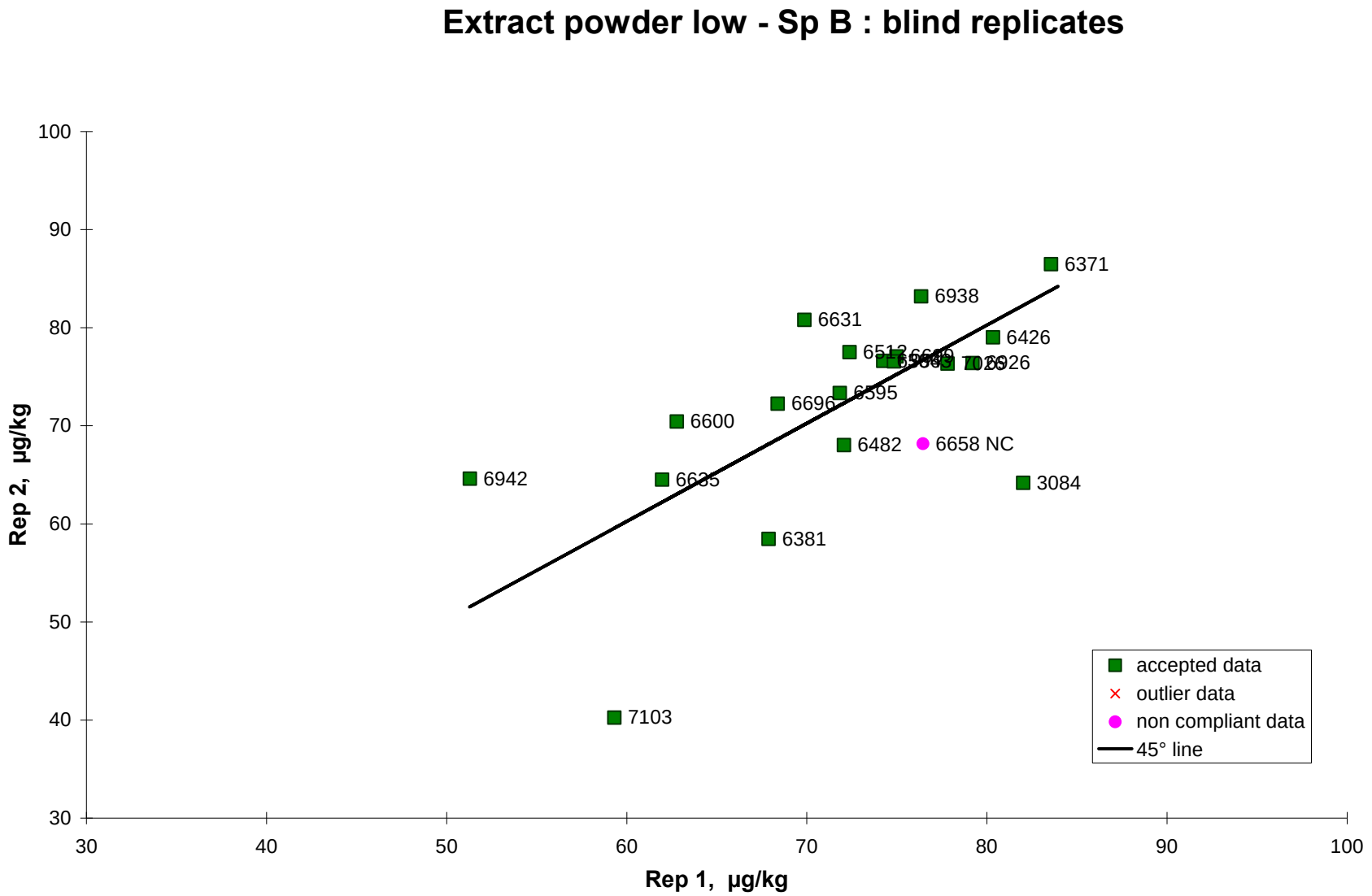


Table 9: Extract paste low, spiked with 130 µg/kg of OTA; initial OTA content prior spiking was determined as 32.0 µg/kg by organiser (see Table 2)

Participant	Result 1	Result 2
3063	157.48	146.8
3084	143.61	131.62
6371	163.43	144.63
6381	136.34	153.74
6426	162.88	149.86
6482	132.71	140.82
6512	147.94	149.45
6584	133.78	131.72
6595	139.87	149.89
6600	119.51	115.5
6631	149.99	144.81
6635	127.56	126.44
6658	149.11	148.83
6696	143.2	143.32
6699	137.54	131.04
6926	149.2	148.4
6938	149.17	147.79
6942	115.4	107.02
7026	160.06	156.77
7103	86.95	81.92

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Lab 7103 was considered as an outlier applying the Grubb's single outlier test.

Figure 5: Distribution of individual results of replicate measurements.

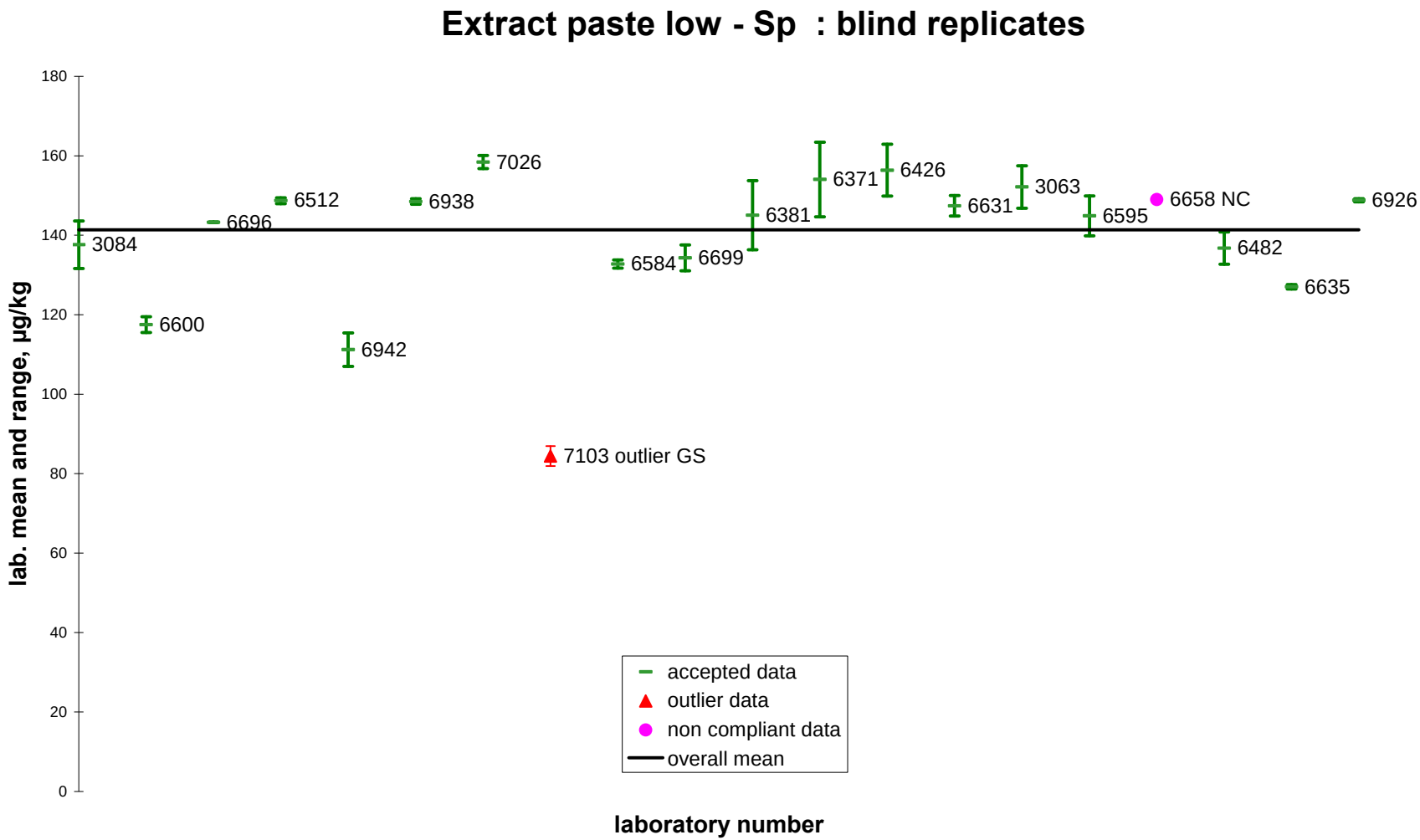


Figure 6: Youden Plot

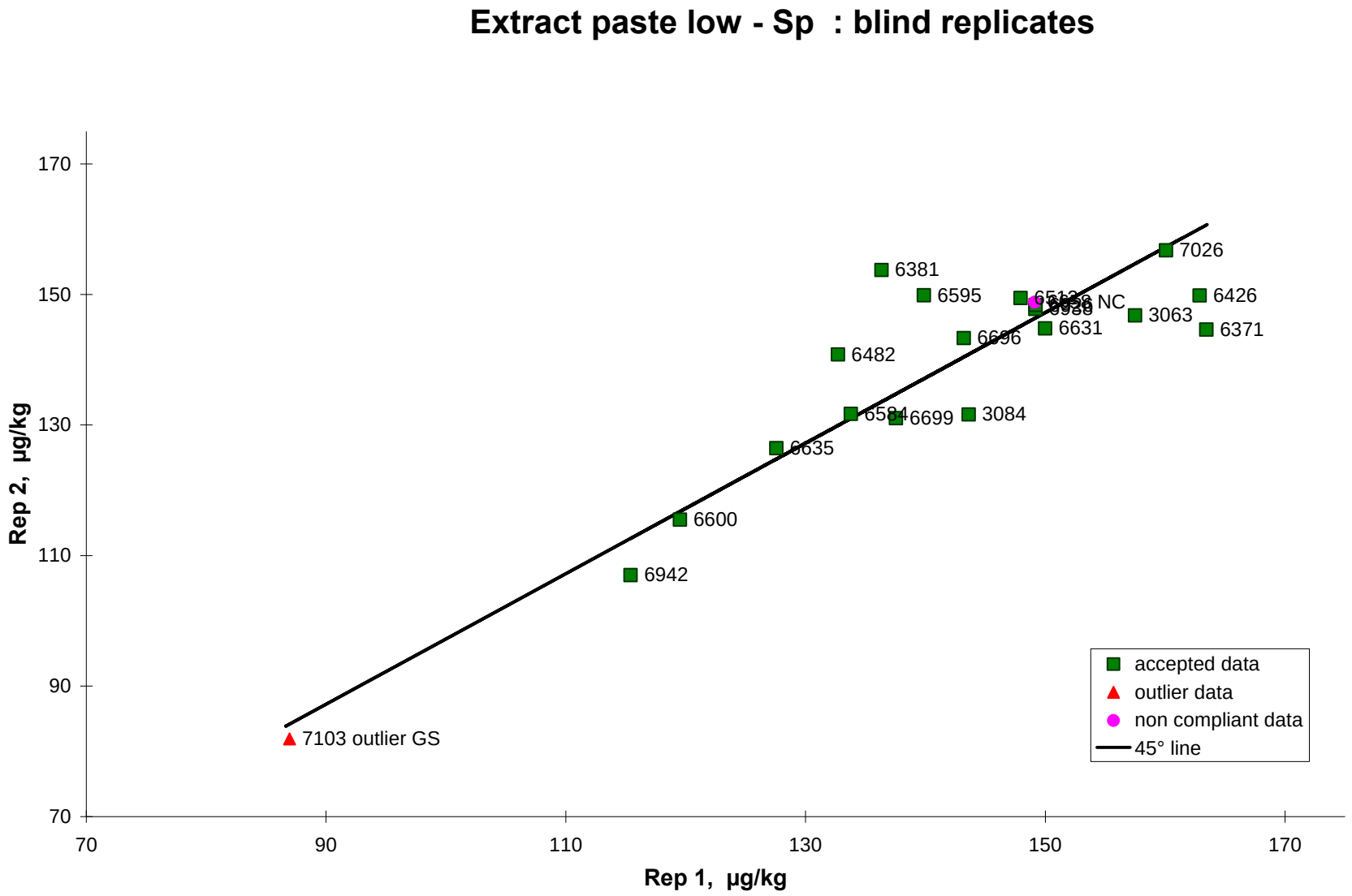


Table 10: Root blank spiked with 30 µg/kg of OTA; initial OTA content prior spiking was determined as <LOD by organiser (see Table 2)

Participant	Result 1	Result 2
3063	27.85	26.93
3084	26.91	31.16
6371	28.02	27.29
6381	26.51	23.39
6426	25.95	24.16
6482	24.84	25.52
6512	28.38	25.97
6584	24.04	24.15
6595	29.27	24.98
6600	25.48	27.56
6631	33.55	35.38
6635	22.61	23.06
6658	33.81	40.78
6696	23.02	25.39
6699	24.77	24.02
6926	33.6	35.2
6938	21.45	23.29
6942	25.38	26
7026	26.56	23.94
7103	16.08	19.35

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Figure 7: Distribution of individual results of replicate measurements.

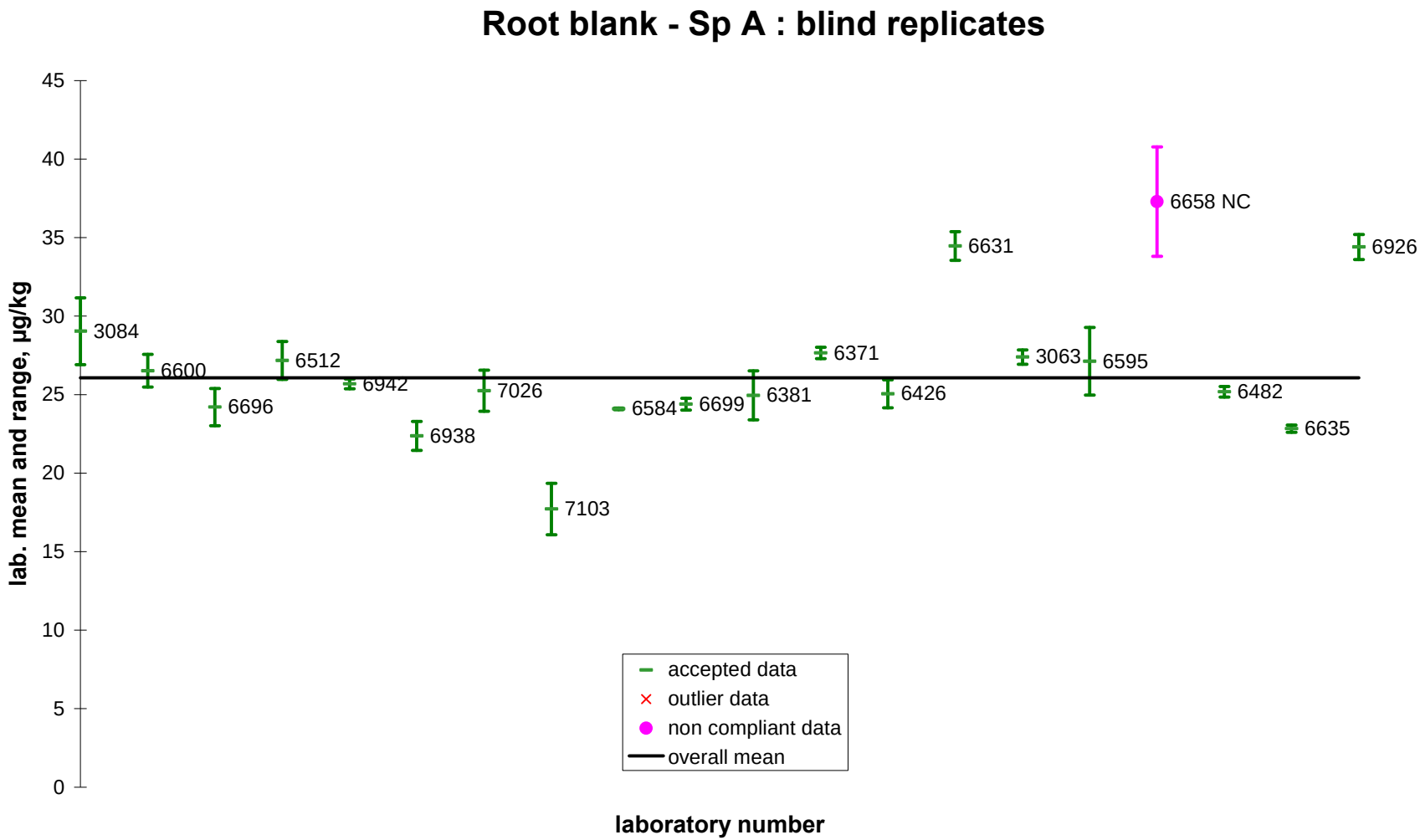


Figure 8: Youden Plot

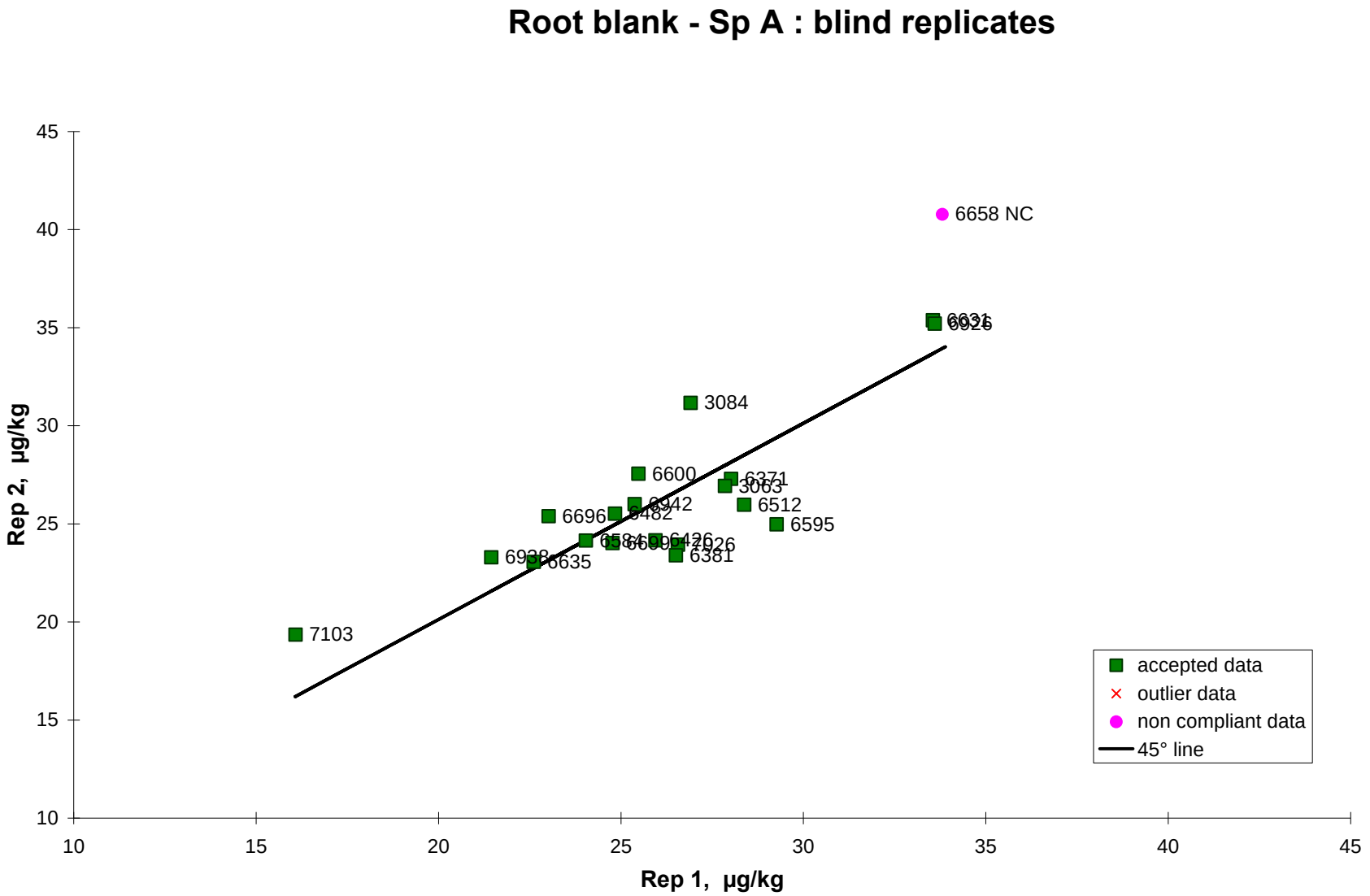


Table 11: Root blank spiked with 60 µg/kg of OTA; initial OTA content prior spiking was determined as <LOD by organiser (see Table 2)

Participant	Result 1	Result 2
3063	54.33	55.47
3084	48.32	57.68
6371	56.39	55.69
6381	57.18	
6426	51.33	49.02
6482	52.65	52.38
6512	53.01	57.6
6584	48.3	45.93
6595	52.17	48.8
6600	44.79	44.35
6631	62.63	64.58
6635	47.48	48.26
6658	65.28	58.61
6696	45.27	44.64
6699	53.47	57.16
6926	54	63.6
6938	44.71	50.33
6942	46.92	50.46
7026	51.73	52.09
7103	39.86	20.39

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.
Lab 6381 was considered non compliant as one of the results was not reported

Lab 7103 was considered as an outlier applying the Cochran test (unusually high "within-group" variation).

Figure 9: Distribution of individual results of replicate measurements.

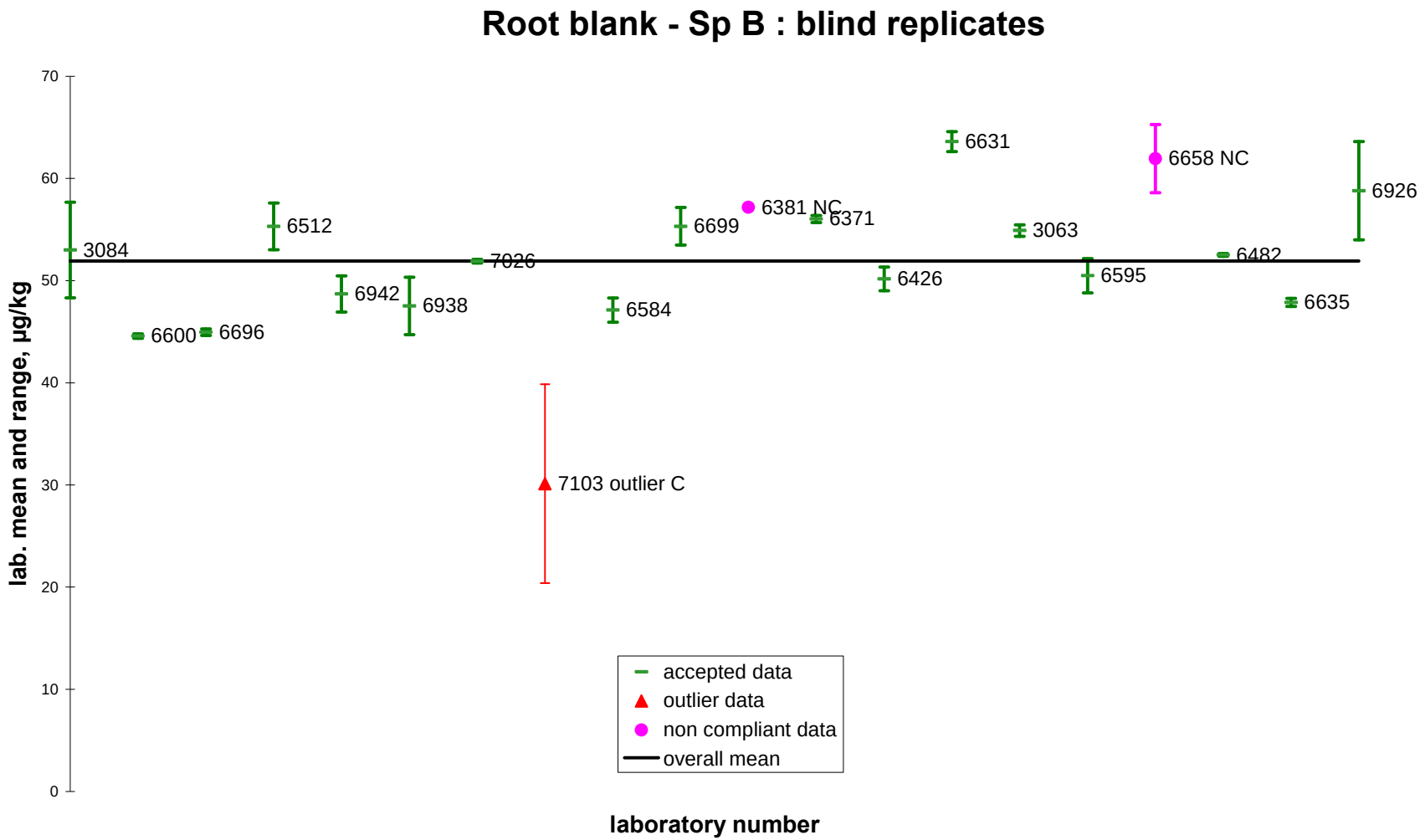
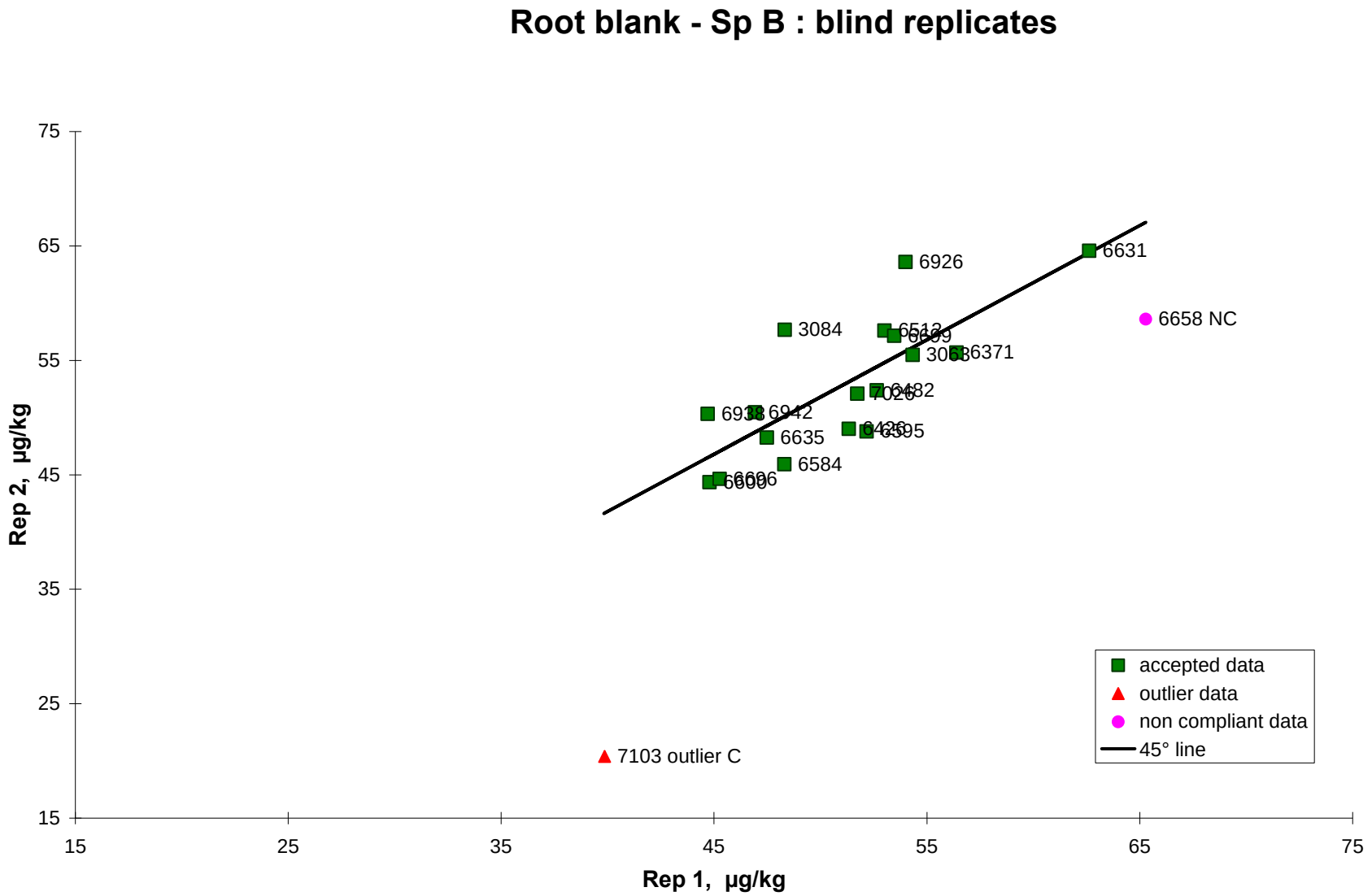


Figure 10: Youden Plot



ANNEX 3 - Individual data reported by the participants

Data for naturally contaminated materials

Table 12: Extract powder medium low

Participant	Result 1	Result 2
3063	26.97	27.2
3084	26.11	26.99
6371	28.18	29.33
6381	30.11	30.31
6426	25.41	23.5
6482	22.56	21.42
6512	27.96	28.79
6584	24.98	28.73
6595	25.41	22.43
6600	23.42	25.17
6631	26.44	28.35
6635	23.11	22.2
6658	35.68	39.82
6696	25.24	25.17
6699	29.06	29.83
6926	31.2	27.6
6938	26.78	22.03
6942	22.42	21.12
7026	24.24	26.25
7103	18.94	21.81

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Figure 11: Distribution of individual results of replicate measurements.

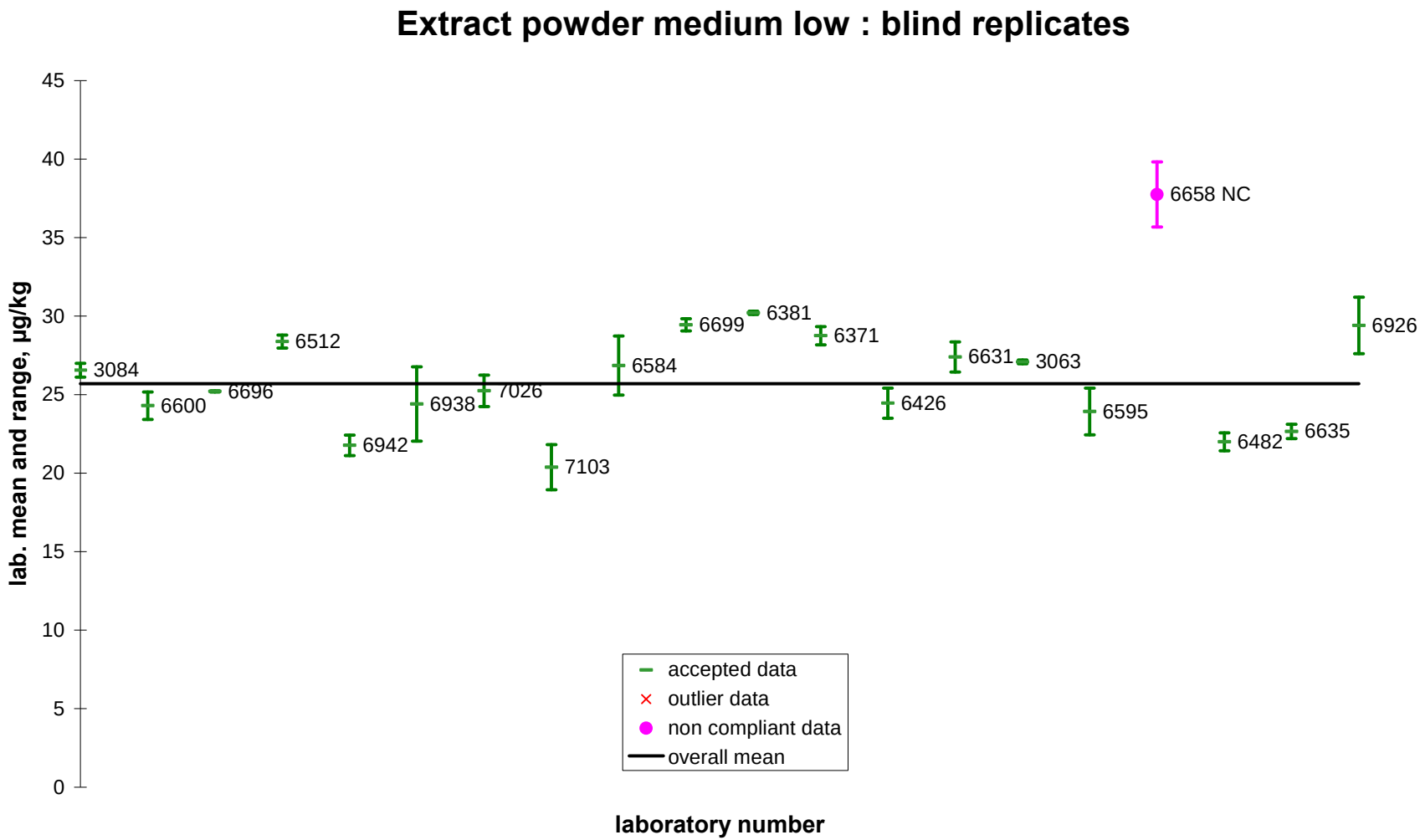


Figure 12: Youden Plot

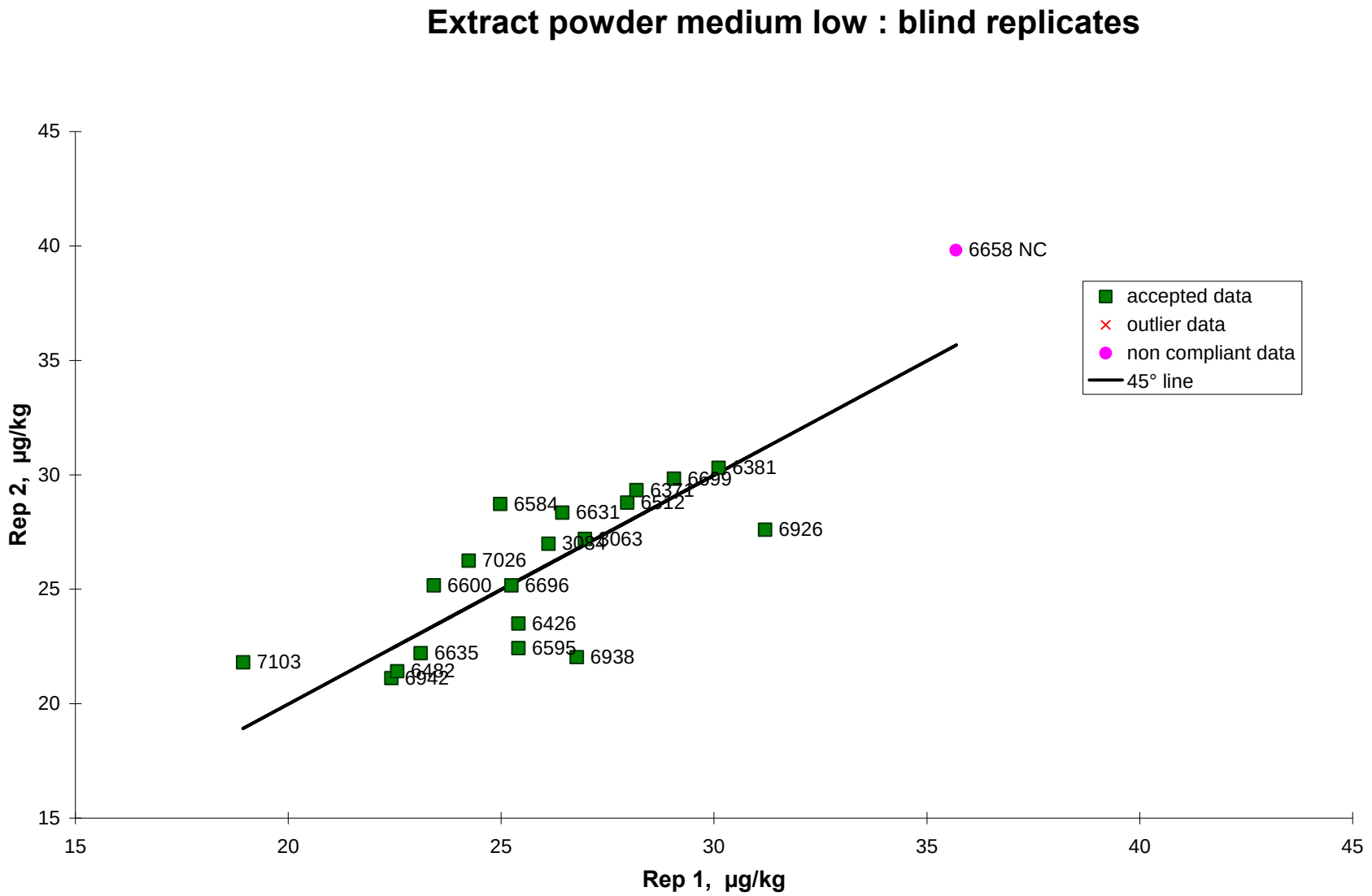


Table 13: Extract paste high

Participant	Result 1	Result 2
3063	68.28	68.78
3084	67.65	65.43
6371	72.09	71.65
6381	66.61	59.06
6426	66.18	30.23
6482	63.58	55.14
6512	63.03	61.82
6584	62.41	58.64
6595	82.9	86.95
6600	69.38	58.71
6631	68.94	68.33
6635	57.48	53.77
6658	55.02	49.52
6696	61.95	60.74
6699	59.01	69.47
6926	70	71.2
6938	64.77	61.49
6942	48.72	44.24
7026	63.13	60.12
7103	36.91	23.64

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Lab 6426 was considered as an outlier applying the Cochran test.
Lab 7103 was considered as an outlier applying the Grubb's single outlier test.

Figure 13: Distribution of individual results of replicate measurements.

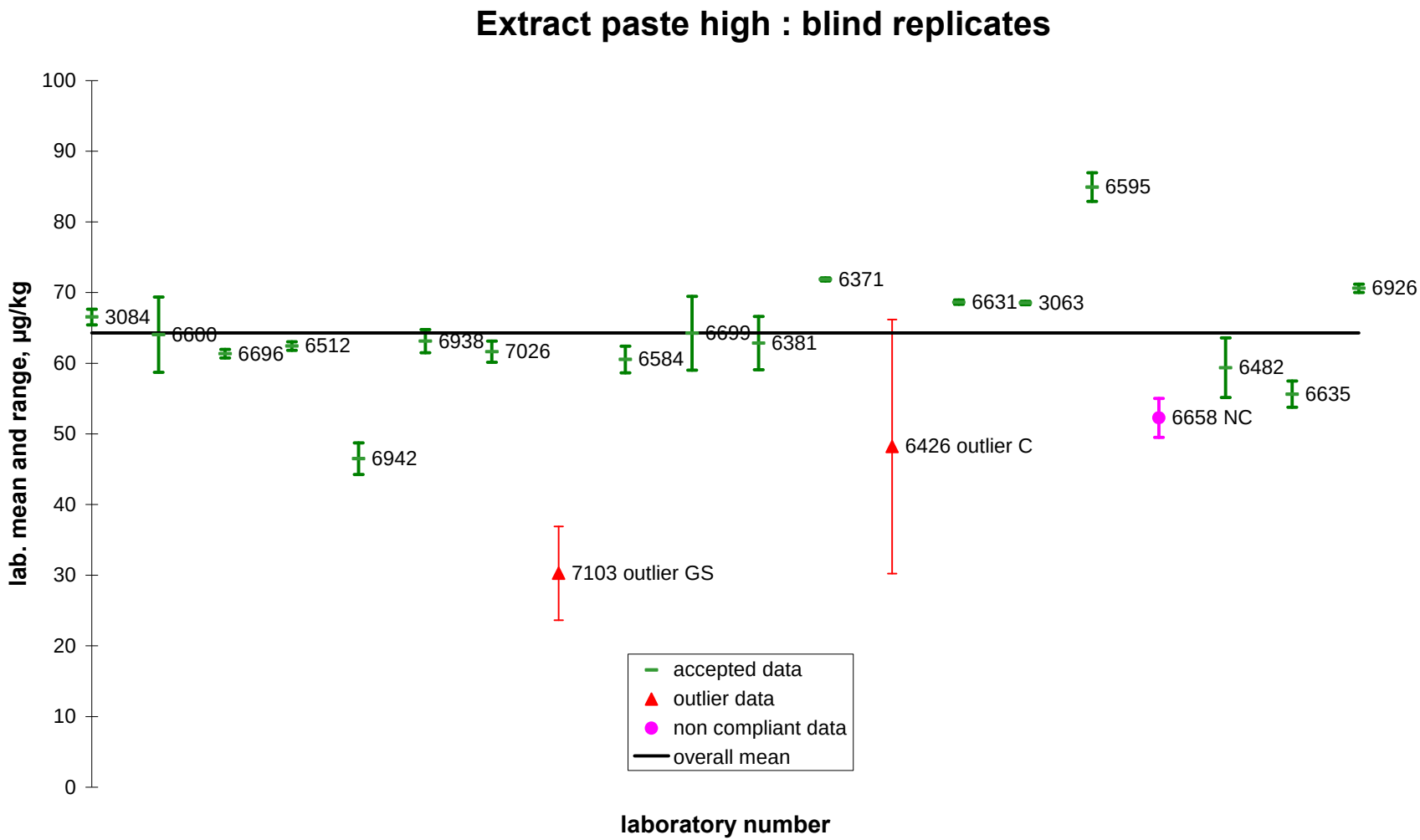


Figure 14: Youden Plot

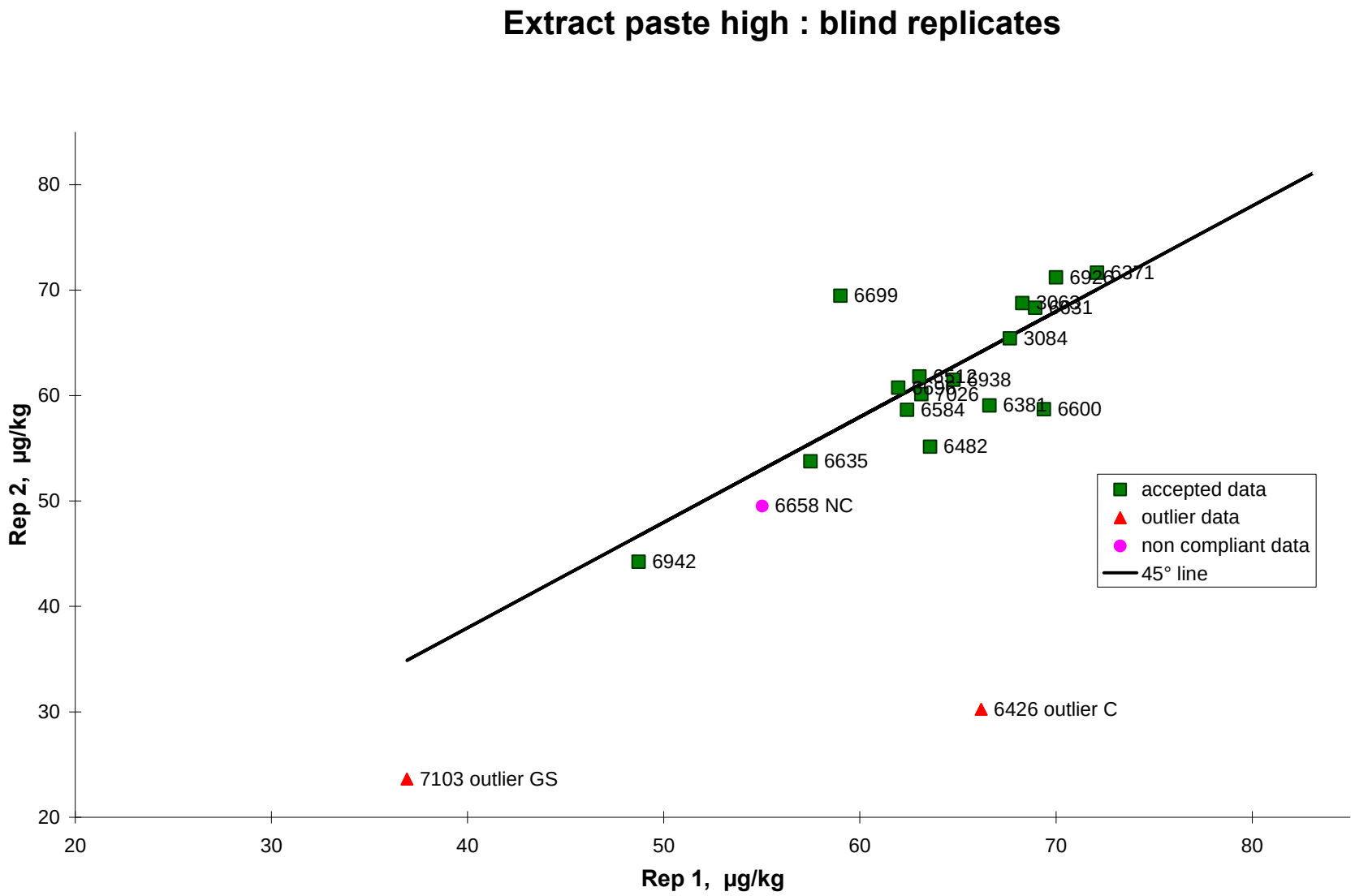


Table 14: Extract powder low

Participant	Result 1	Result 2
3063	5.84	6.1
3084	6.47	8.67
6371	16.61	11.91
6381	9.08	12
6426	<LOD	<LOD
6482	<LOD	6.26
6512	7.88	8.41
6584	14.54	10.55
6595	10.67	6.97
6600	12.7	10
6631	8.16	7
6635	5.93	6.05
6658	<LOD	<LOD
6696	5.65	5.77
6699	6.09	6.21
6926	7.2	21.6
6938	11.58	12.36
6942	0	0
7026	7.69	7
7103	4.51	6.62

Lab 6426 was considered non compliant as reporting results as < LOD (not included in statistical evaluation)
Lab 6482 was considered non compliant as reporting one of the results as < LOD (not included in statistical evaluation)
Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.
Lab 6942 was considered non compliant as reporting 0 (results were considered as < LOD, therefore not included in statistical evaluation).

Lab 6926 was considered as an outlier applying the Cochran test.

Figure 15: Distribution of individual results of replicate measurements.

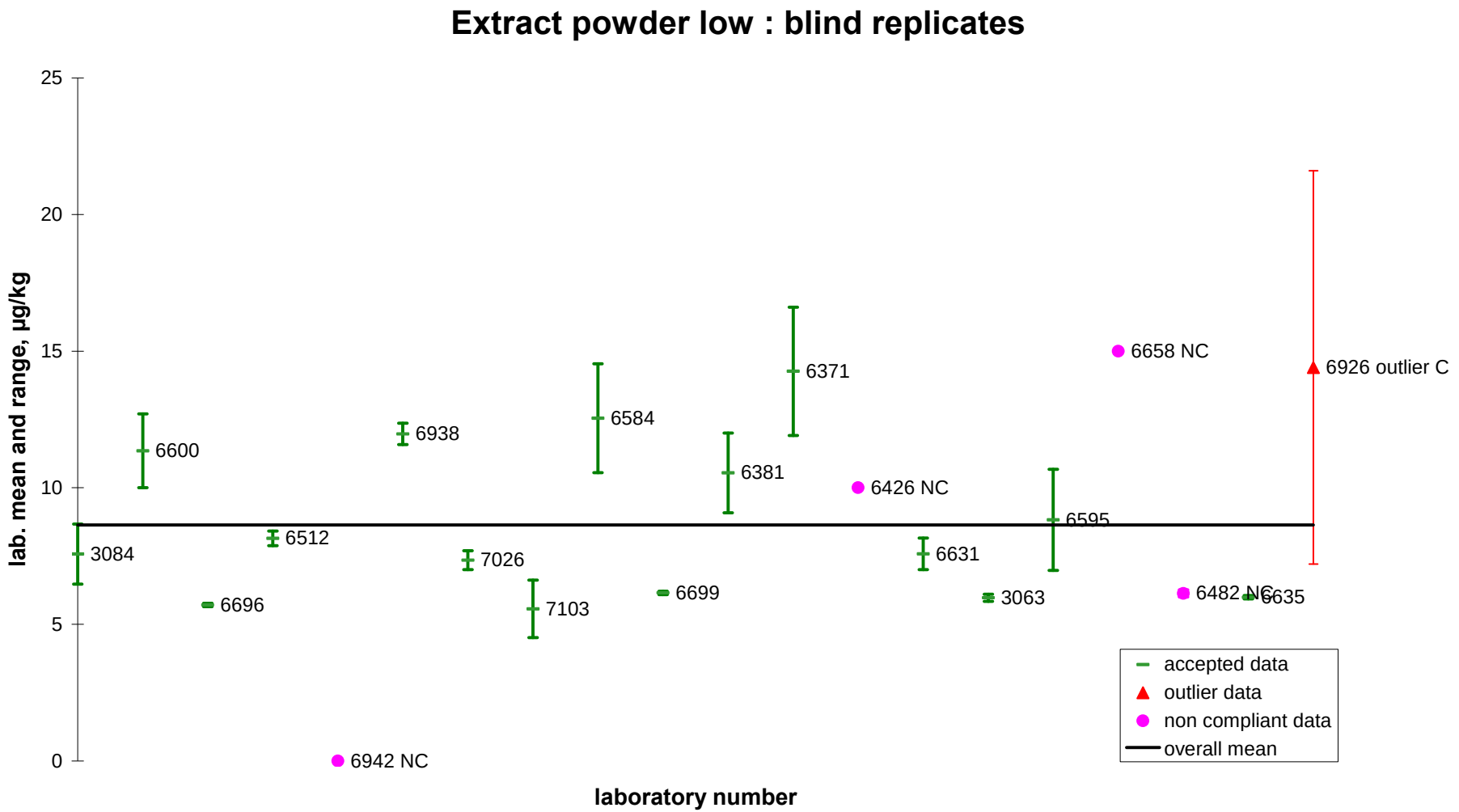


Figure 16: Youden Plot

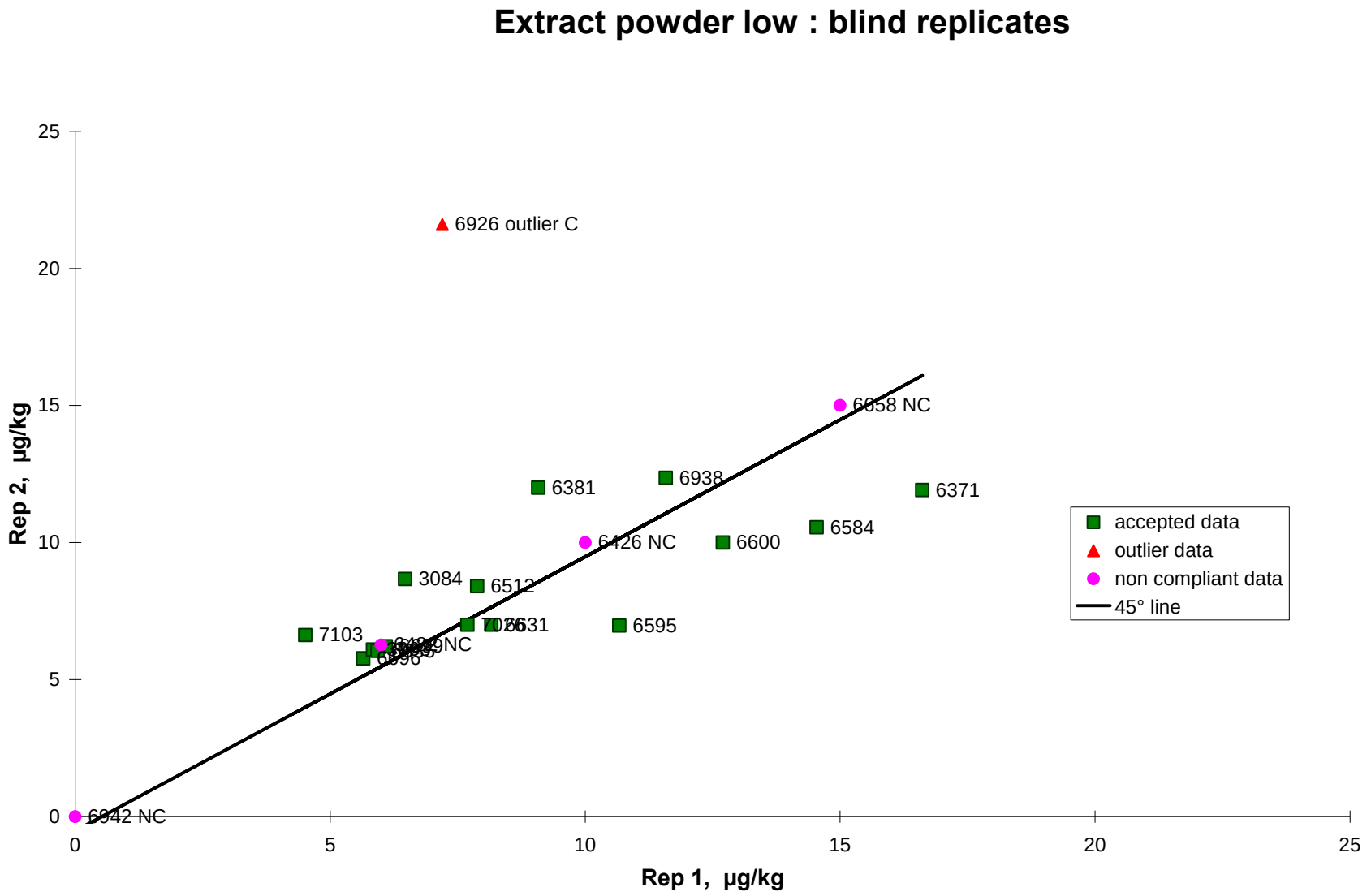


Table 15: Extract paste low

Participant	Result 1	Result 2
3063	28.68	27.18
3084	32.03	24.88
6371	29.01	26.29
6381	28.4	28.49
6426	31.17	32.03
6482	25.46	23.47
6512	30.17	31.59
6584	28.74	25.28
6595	19.61	20.53
6600	23.88	29.97
6631	31.94	29.49
6635	24.33	24.57
6658	7.4	48.01
6696	27.24	25.03
6699	26.8	23.76
6926	38.4	38.4
6938	28.79	30.98
6942	25.8	25.96
7026	26.28	28.3
7103	13.96	22.98

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Figure 17: Distribution of individual results of replicate measurements.

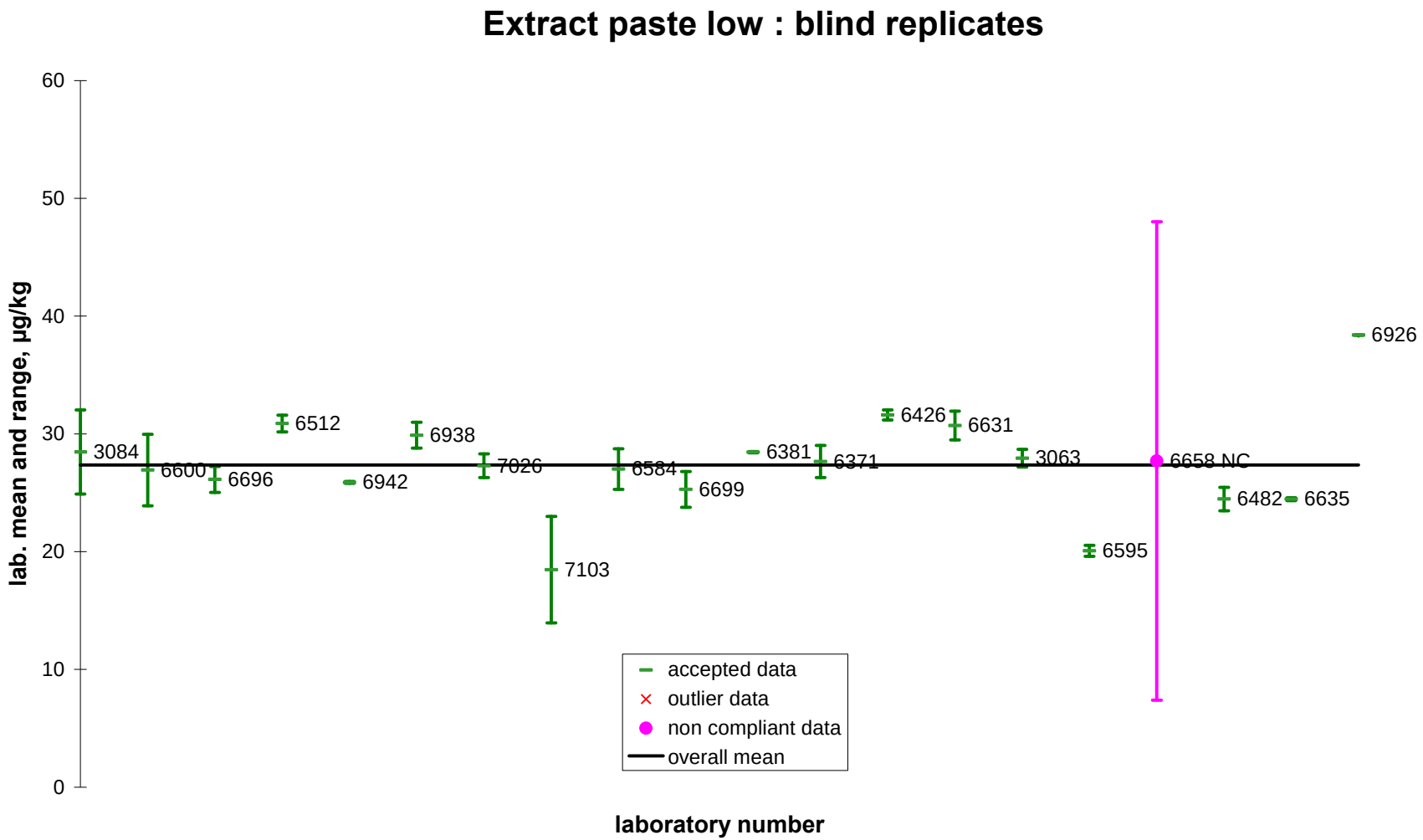


Figure 18: Youden Plot

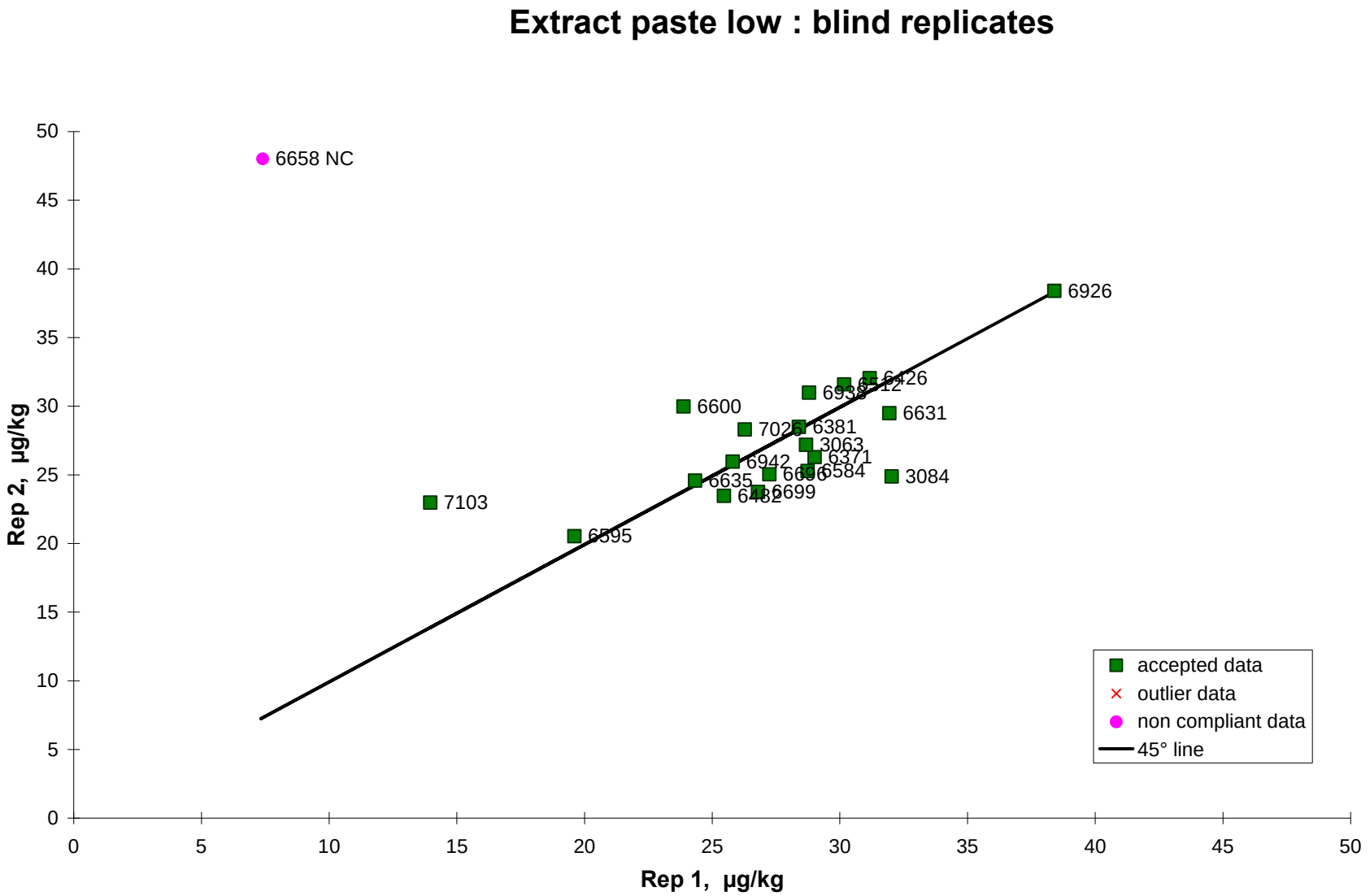


Table 16: Root low

Participant	Result 1	Result 2
3063	7.83	8.24
3084	6.67	6.9
6371	9.57	8.49
6381		6.39
6426	<LOD	<LOD
6482	<LOD	7.12
6512	7.82	7.32
6584	7.34	7.39
6595	7.97	7.39
6600	9.49	9.94
6631	11.96	19.11
6635	6.67	6.6
6658	30.58	<LOD
6696	6.65	7.29
6699	5.2	7.87
6926	12	10.4
6938	6.8	6.25
6942	10.88	9.78
7026	5.9	6.54
7103	5.32	5.59

Lab 6426 was considered non compliant as reporting results < LOD (not included in statistical evaluation)
Lab 6482 was considered non compliant as reporting one of the results as < LOD (not included in statistical evaluation)
Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.
Lab 6381 was considered as a non compliant as only one result was reported.

Lab 6631 was considered as an outlier applying the Cochran test.

Figure 19: Distribution of individual results of replicate measurements.

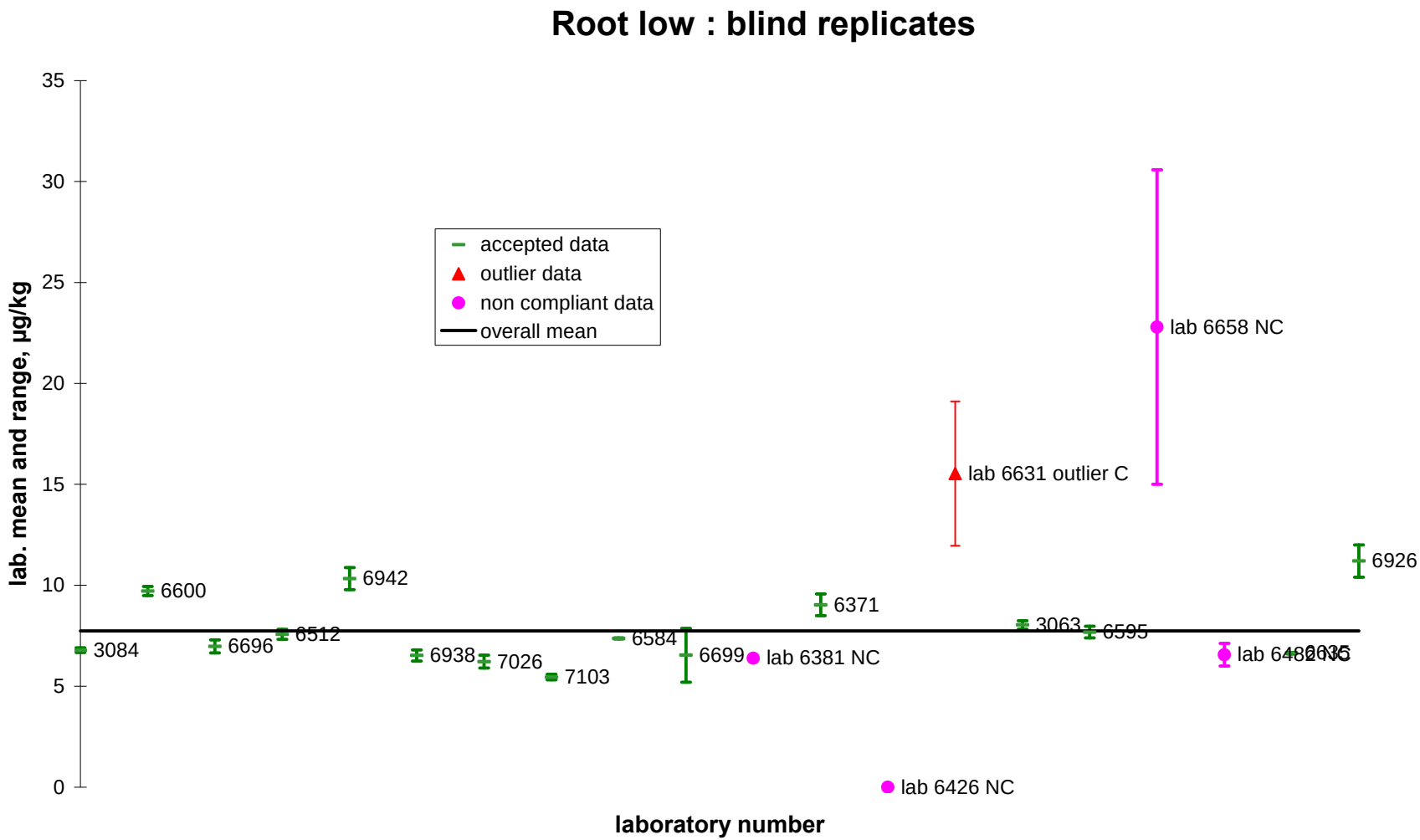


Figure 20: Youden Plot

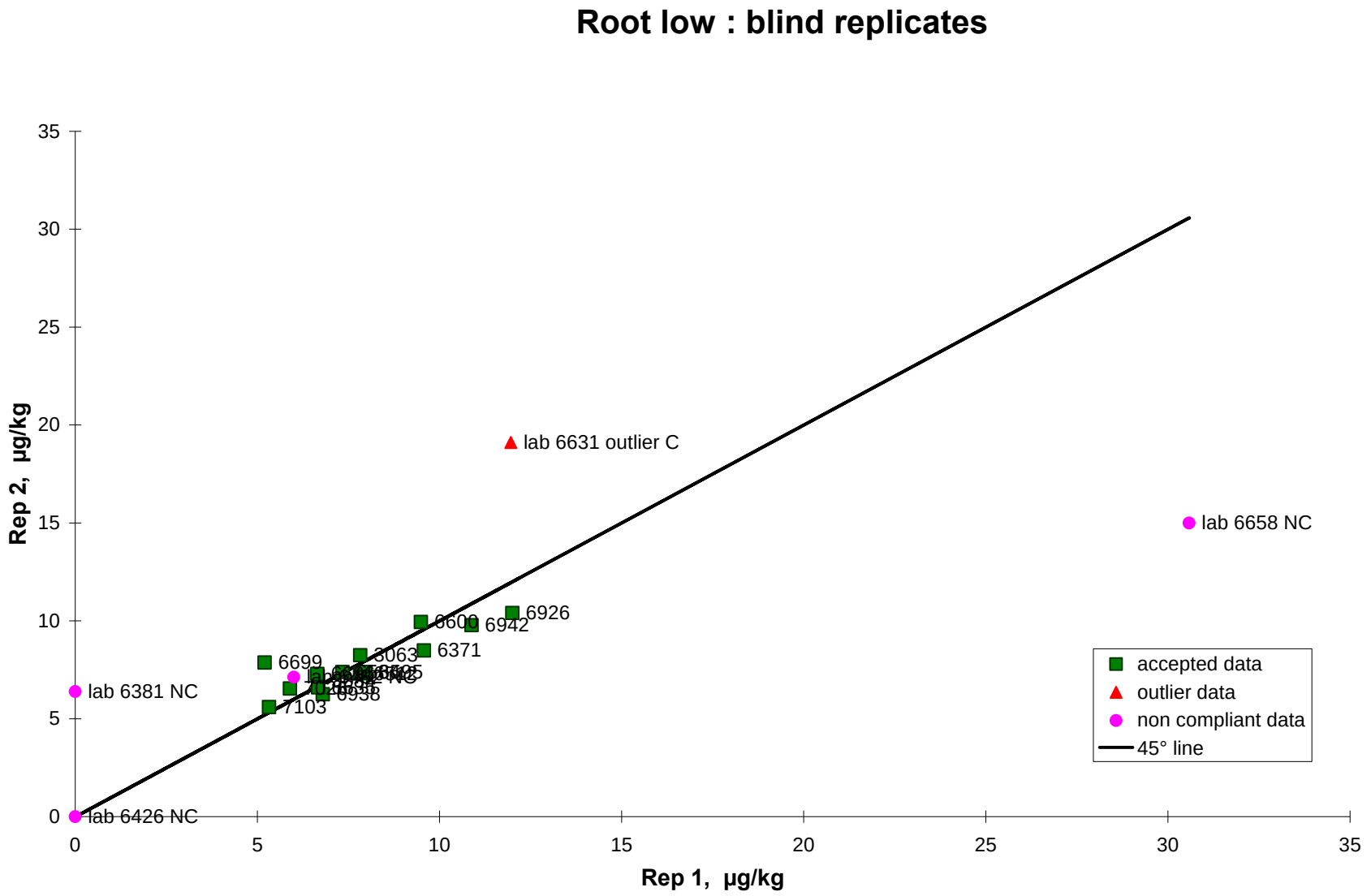


Table 17: Extract powder medium high

Participant	Result 1	Result 2
3063	67.41	65.63
3084	57.8	52.65
6371	73.87	71.88
6381	89.47	76.43
6426	69.81	67.44
6482	54.09	56.58
6512	60.87	62.35
6584	55	59.37
6595	105.36	68.43
6600	55.6	57.65
6631	64.73	63.52
6635	58	51.1
6658	66.9	55.55
6696	62.93	60.2
6699	53.65	56.83
6926	65.6	64
6938	60.84	58.57
6942	46.89	49.84
7026	57.94	62.56
7103	50.5	50.9

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Labs 6381 and 6595 were considered as outliers applying the Cochran test.

Figure 21: Distribution of individual results of replicate measurements.

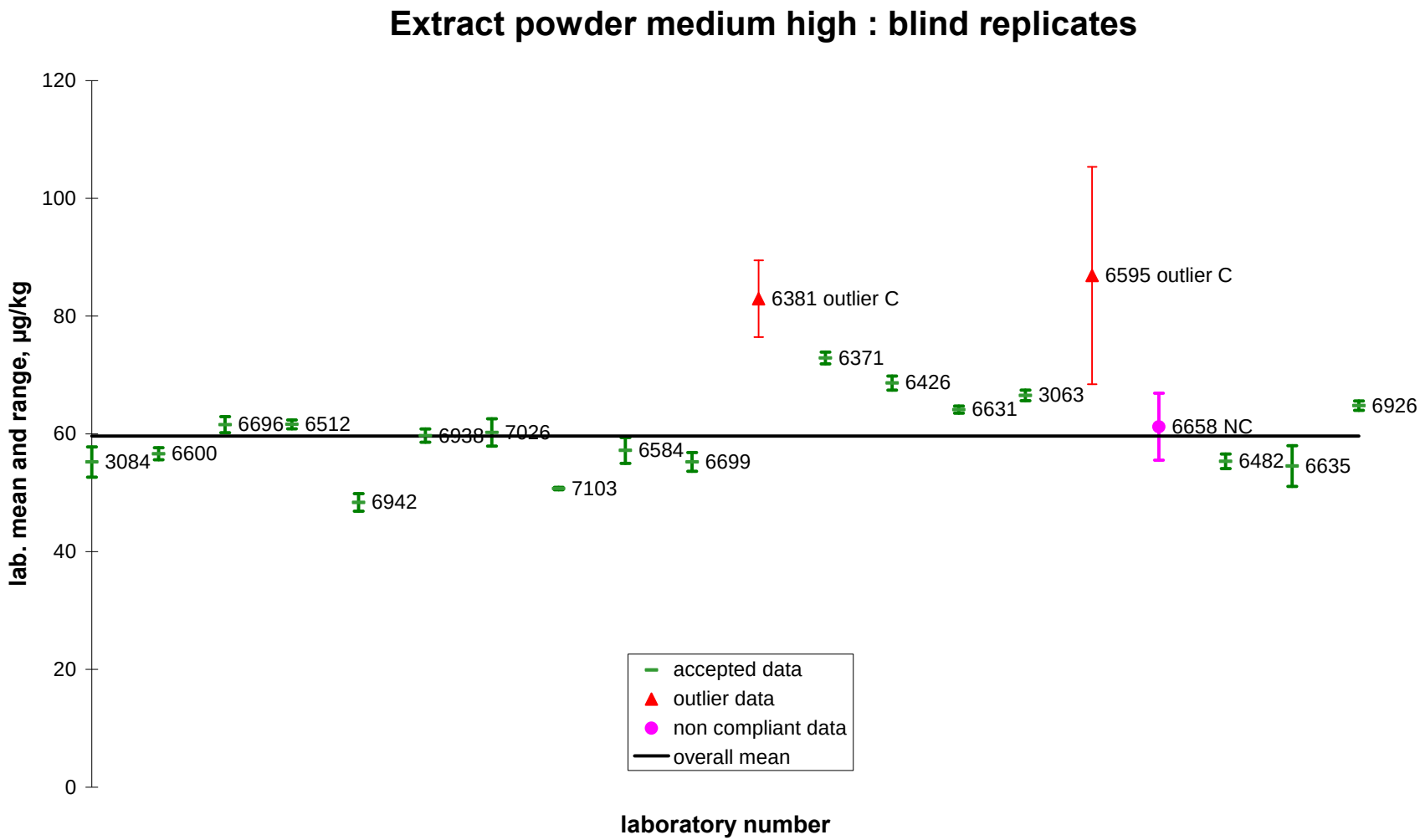


Figure 22: Youden Plot

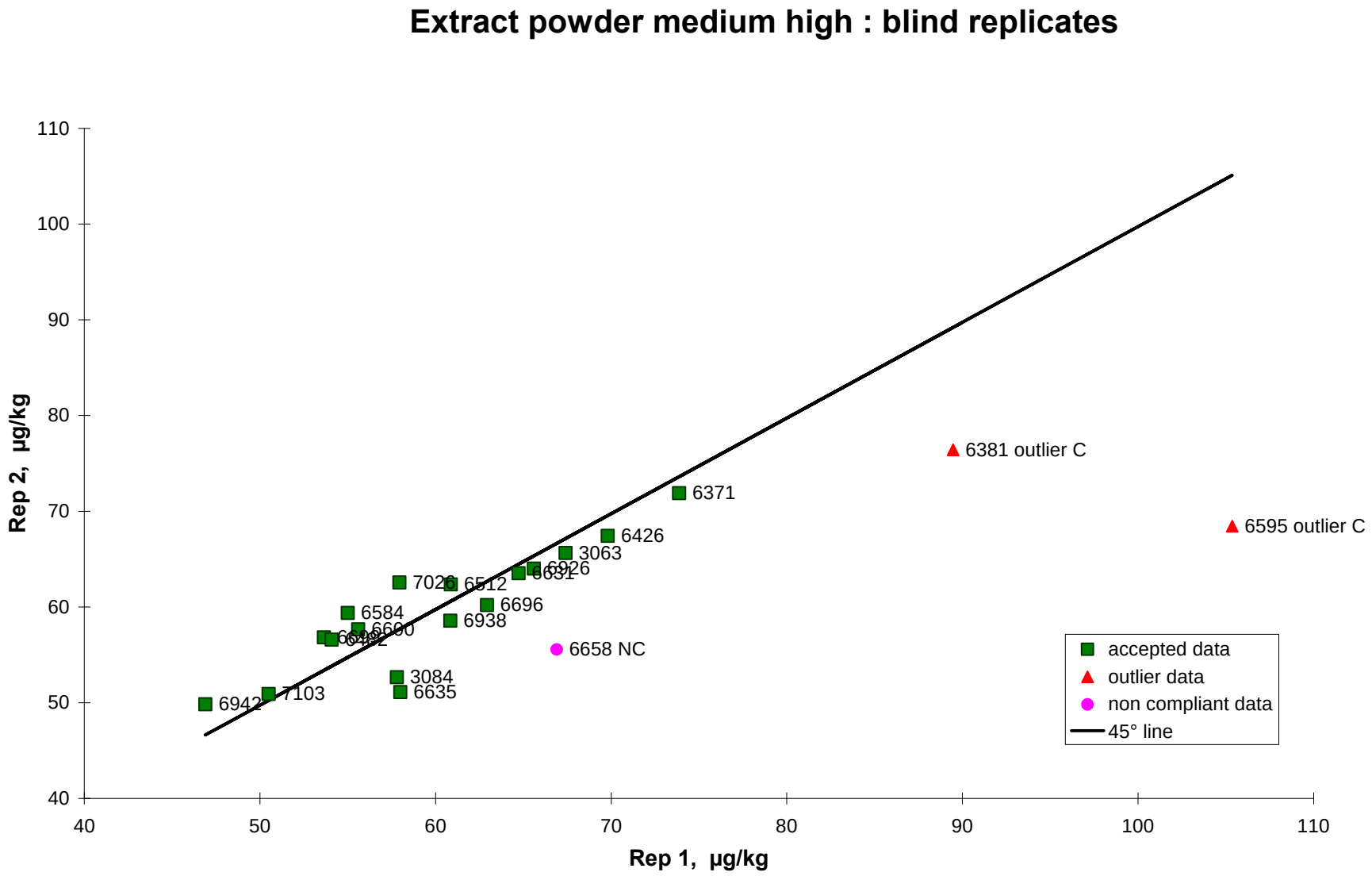


Table 18: Extract powder high

Participant	Result 1	Result 2
3063	103.41	105.42
3084	92.48	95.96
6371	112.74	108.71
6381	107.46	111.6
6426	110.15	107.64
6482	88.88	87.37
6512	102.66	99.57
6584	86.05	92.08
6595	131.68	114.21
6600	99.17	93.59
6631	93.95	94.89
6635	85.93	79.36
6658	90.93	98.7
6696	94.24	100.36
6699	103.66	96.32
6926	100.8	99.6
6938	78.3	97.2
6942	73.72	82.15
7026	93.38	98.06
7103	69.19	84.73

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Figure 23: Distribution of individual results of replicate measurements.

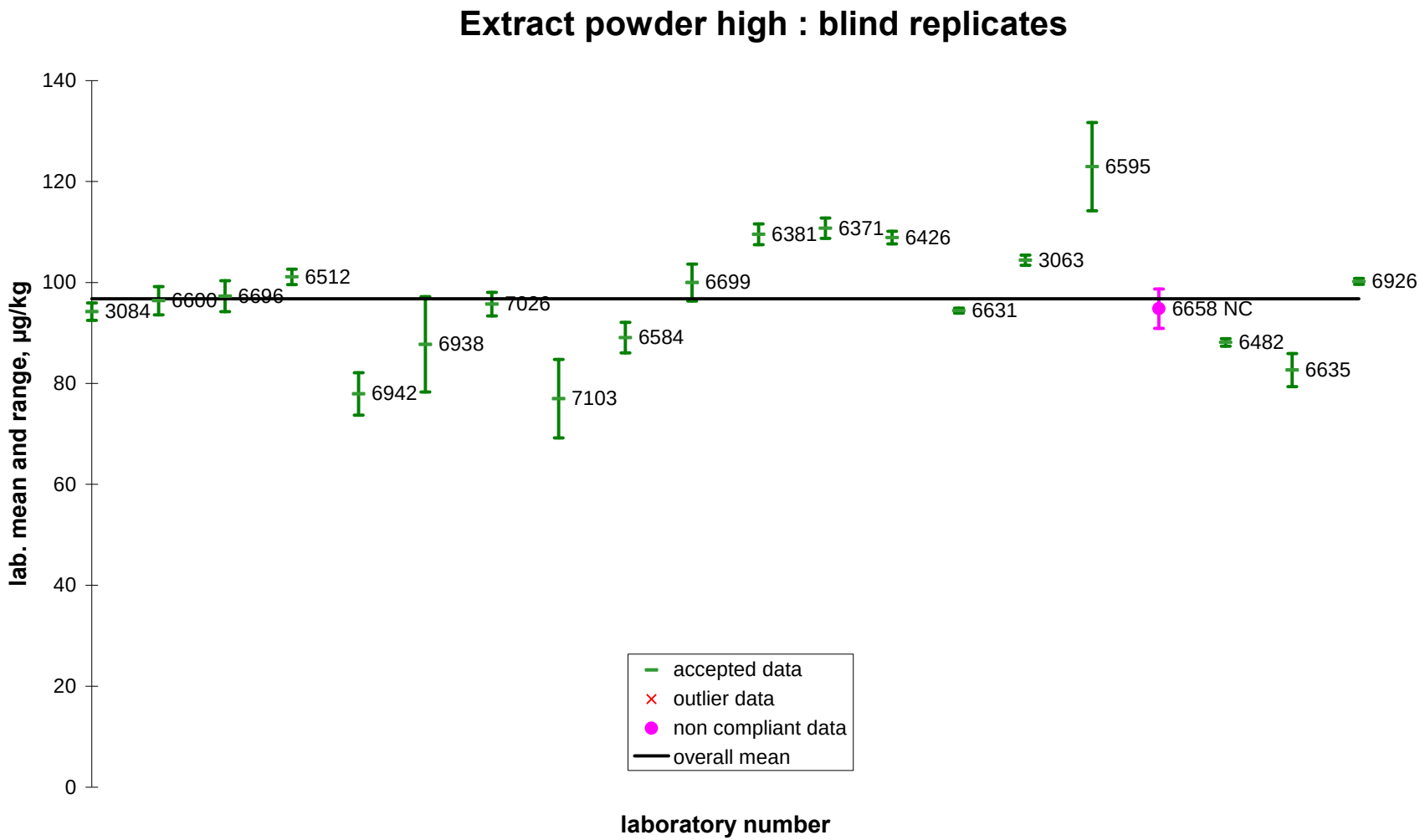


Figure 24: Youden Plot

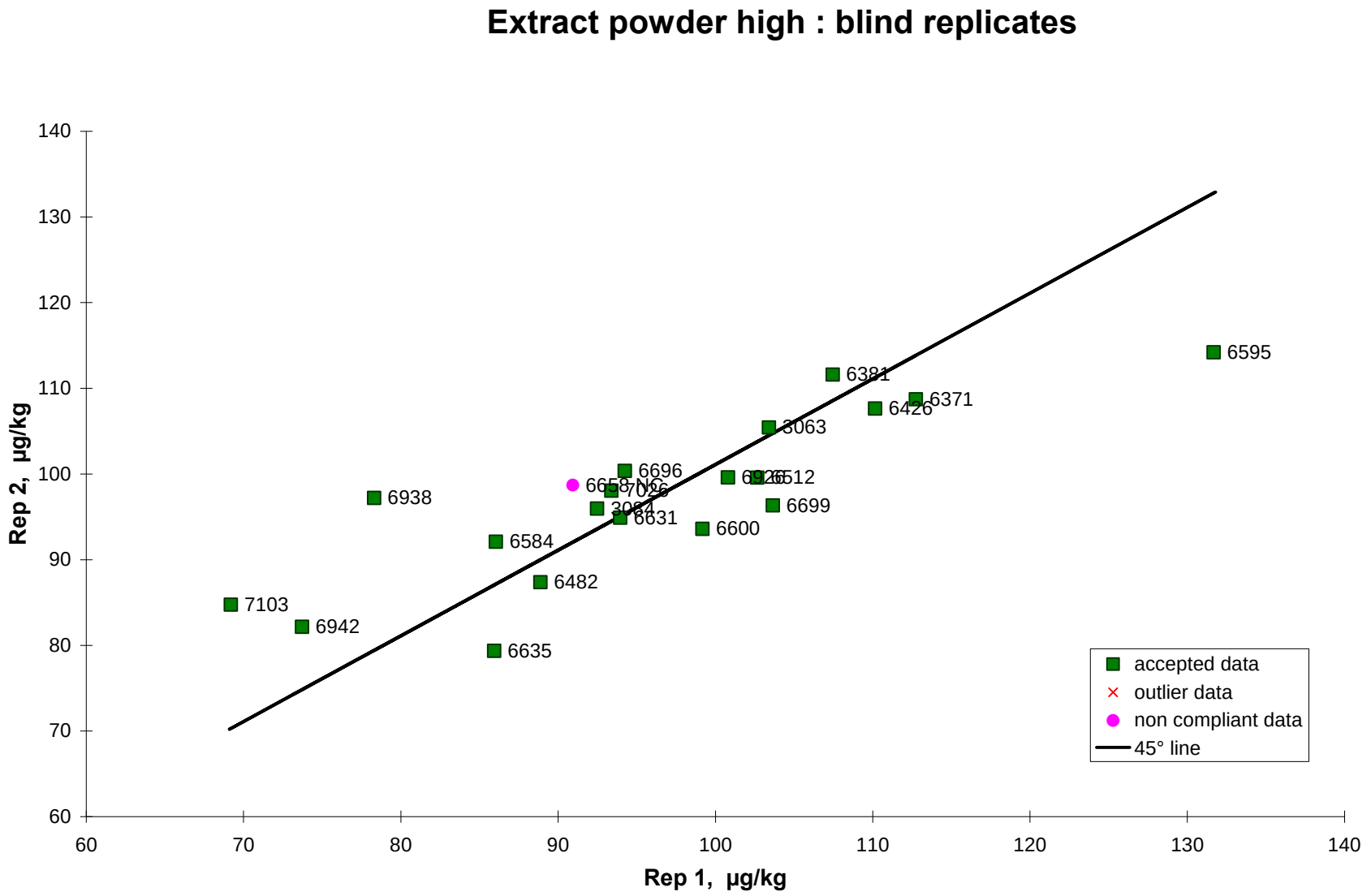


Table 19: Root high

Participant	Result 1	Result 2
3063	23.65	25.71
3084	24.24	23.86
6371	26.79	23
6381	24.25	23.24
6426	67.21	20.78
6482	20.06	21.01
6512	22.39	24.25
6584	20.84	22.15
6595	19.16	17.44
6600	20.26	23.89
6631	49.43	54.35
6635	19.64	20.41
6658	45.3	29.95
6696	23.54	21.87
6699	19.8	19.45
6926	30.8	26.4
6938	22.07	20.95
6942	20.82	22.49
7026	19.29	24.22
7103	18.96	11.94

Lab 6658 was considered as a non compliant due to incorrect resolution and integration of the OTA peak.

Lab 6426 was considered as an outlier applying the Cochran test.
Lab 6631 was considered as an outlier applying the Grubb's single outlier test

Figure 25: Distribution of individual results of replicate measurements.

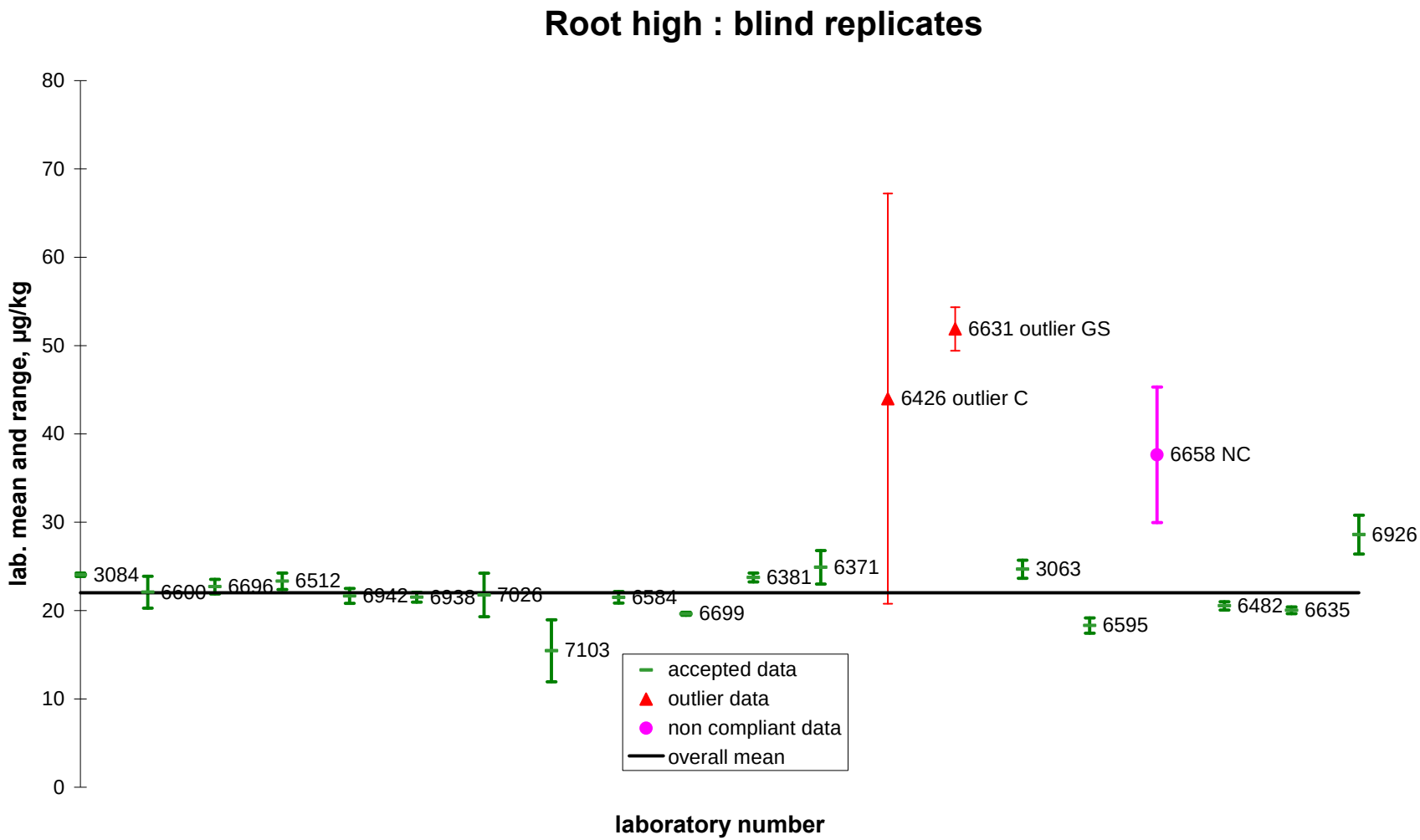
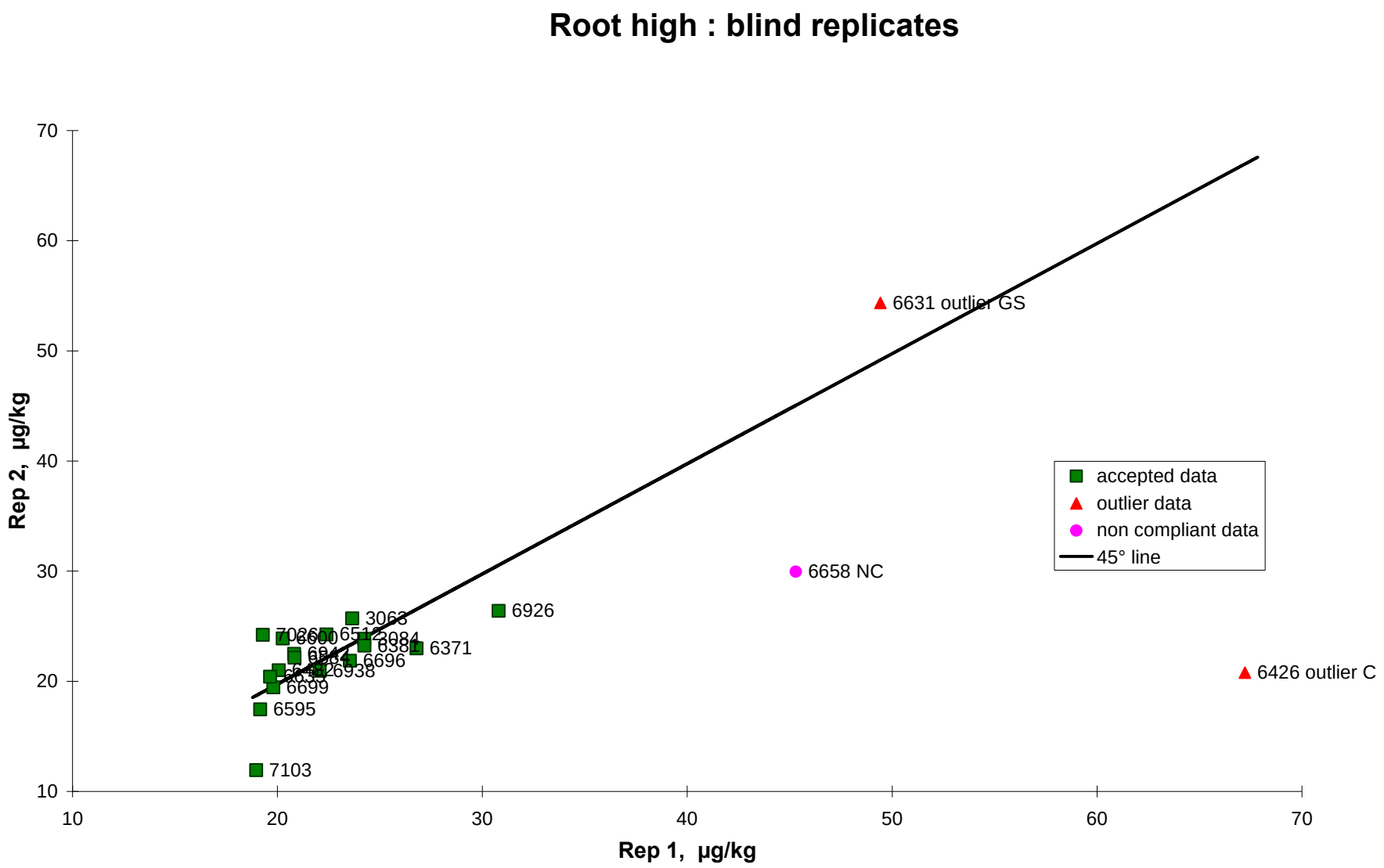


Figure 26: Youden Plot



ANNEX 4 - Questionnaire output: general

Participant background-1

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?

LabID	Years of experience	Accredited	Accredited matrices
3063	More than 25 years	NO	
3084	for more the 10 years	YES	Wheat and wheat products, coffee. Sometimes we do ring trials for other product with success
6371	15	YES	Unprocessed cereals, products derived from cereals, unprocessed and processed, raw coffee, roasted coffee, soluble coffee, spices, dried fruits, grape juice
6381	> 12 years	YES	All Foodstuffs
6426	10	YES	Coffee, cereals and cereal products, raisins
6482	More than 20 years	YES	Baked beans, beer, cereals & cereal products, cocoa, chocolate & chocolate products, coconut, dried pulses, dried fruit, duplicate diets, coffee (green, roasted & instant), nuts & nut butters, pork products, wine, spices, special dietary infant foods, baby food including dry mixes & rusks.
6512	19	NO	N/A
6584	about 13 years	YES	
6595	6	YES	Animal Feed and Food from Plant Origin
6600	11	YES	Cereals, raisins, wine, fruit juices, coffee, fish, kidneys, animal feed
6631	10	YES	Coffee, Cereals, Wine, Beer, Chocolate, Paprika, Chilli, Baby food, Dried Vine Fruit, Liquorice.
6635	11 years	YES	ISO 17025 Accreditation with Ochratoxin A analysis by HPLC included in scope (no specific matrix)
6658	four	YES	dried fruits
6696	10	YES	Cereals [grains and flour], beers (validation for kidneys and lever; expected accreditation for February 2011)
6699	15	YES	cereals, cereals products, dried fruits, coffee, feed
6926	Since 1996 (using TLC). Since 2007 (using HPLC)	YES	Wine, Animal feeds, Grains an by products, Grapes an by products
6938	More than 30 years	YES	Cereals, coffee, paprika, beans, lenses, peas, rice, raisins, dried fruit, nuts.
6942	16 years	YES	Cereals
7026	since 1999	YES	Feeding stuff
7103	2 years	NO	

Participant background-2

How many samples does your laboratory analyse per year?
Please report the most frequent matrices
Which of the following matrices does your laboratory analyse for the determination of Ochratoxin A on a routine basis?

LabID	Samples/ Year	Most frequent matrices	OTA routine
3063	More_500	Cereals (wheat, barley, coffee), wine, grape and beer.	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Roasted coffee, Wine, Grape juice
3084	More_500	Wheat and wheatproducts Coffee Cocoa and chocolate	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Spices
6371	More_500	unprocessed cereals, products derived from cereals, unprocessed and processed	Liquorice, Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Grape juice, Babyfood, Spices
6381	50_149	It depends on the Year´s Programme (Paprika, wine, cereal-based babyfoods, meat products, beer, etc.)	Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Babyfood, Spices, Beer, Meat products, Cocoa products
6426	150_500	coffee, cereals and cereal products	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee
6482	150_500	Cereals and cereal based infant foods	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Babyfood, Wine 1 batch of 20 samples + QA per year, Spices 2 or 3 batches of 20 samples + QA per year, Feed 1 batch of 20 samples + QA per year
6512	50_149	Infant and breakfast cereals, infant formula (milk and soy), human milk, coffee, dried fruit, organ meats.	Cereals for direct human consumption, Babyfood
6584	More_500		Liquorice, Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Spices, Feed
6595	150_500	Cereals, feed, raisins, coffee	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Grape juice, Feed
6600	More_500	cereals, raisins, wine, kidneys, animal feed, spices (estimated most frequent)	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Grape juice, Babyfood, Spices, Feed, Other, thee with liquorice
6631	150_500	We analyse approximately equal numbers of all 10 matrices listed above and also a range of proficiency tests from proficiency test providers.	Liquorice, Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Grape juice, Babyfood, Spices, Chocolate and beer
6635	More_500	soy protein, oats, spices, raisins	Liquorice, Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Babyfood, Spices, Feed
6658	More_500	cereals, spices, dried fruits (e.g. raisins), cacao and coffee beans	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Spices, Feed, Other, cacao beans

LabID	Samples/ Year	Most frequent matrices	OTA routine
6696	50_149	grains and flour	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Roasted coffee, Spices, Feed, (if feed then only feed based on Cereals for direct human consumption or cereal derivatives)
6699	5_49	cereals, cereals products, feed	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Feed
6926	More_500	Wine, Animal feeds, Grains an by products, Dried Grapes	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Wine, Grape juice, Babyfood, Feed
6938	50_149	Cereals, raisins. (In 2010 dried fruit)	Unprocessed Cereals for direct human consumption, Vine fruit
6942	50_149	Cereals, coffee, raisins	Unprocessed Cereals for direct human consumption, Cereals for direct human consumption, Vine fruit, Roasted coffee, Wine, Babyfood, Spices
7026	5_49	individual feed (cereals, etc.), compound/mixed feed, supplementary feed, premix, mineral feed, additive	Unprocessed Cereals for direct human consumption, Feed
7103	5_49	Dried Fruit, Cereals and Spices.	Cereals for direct human consumption, Vine fruit, Spices

ILC-MVS Organisation-1
Did you find the instructions distributed for this MVS adequate?
If NO, which parts do you think could be improved?
What do you think about the reporting by electronic forms?

LabID	Instructions	Proposed improvements	Electronic forms
3063	YES		It is simple, rapid and clear.
3084	YES		If it's works, good.
6371	YES		Very practical
6381	YES		Convenient
6426	YES		easy to use
6482	YES		No problems encountered so far.
6512	YES	N/A	It is fine, however I still need to send you a printed version so this does not save me any time.
6584	YES		very comfortable
6595	YES		OK.
6600	YES		
6631	YES		Very user friendly.
6635	YES		No Problem
6658	YES		fastest way to submit data
6696	YES		easy and fast
6699	YES		OK
6926	YES		It´s OK
6938	YES		Good (We do not have a fax any longer in our departement)
6942	YES		Easy to understand and use it.
7026	YES		ok
7103	YES		Satisfactory

ILC-MVS Organisation-2

Did you have any problems in using the forms?
If YES, which were these problems?
Any other comments you wish to address?

LabID	Problems	Problems description	Comments
3063	NO		
3084	NO		
6371	NO		
6381	NO		
6426	NO		
6482	NO		
6512	YES	I had a problem when using this form and adding the resolution of OTA and two contiguous peaks. For each sample ID number there are two possible resolutions. This form did not give enough spaces to add all of the samples that I had <1.5 and didn't distinguish between the two values.	
6584	YES	In the table of calibration it is not possible to write digits >999 (e.g. 1000µl is displayed as 1,000µl)	
6595	NO		
6600	NO		
6631	NO		
6635	NO		
6658	NO		Our estimated LoQ was 20 ppb, which is not sufficient regarding the lowest MRL for liquorice (20 ppb in herbal infusions). To lower the LoQ substantially, there should be no fixed ration between V3 and injection volume. To increase the separation, it should be possible to change the particle size of the HPLC column from 4.6 to 3.5 micrometers. Thus, a lower ration between V3 and injection volume might be justified.
6696	YES	I received a message: "this operation is not allowed". Typing OK it seems not to do any trouble.	
6699	NO		we forgot to write the number of standard solution
6926	NO		
6938	NO		
6942	NO		
7026	NO		no
7103	NO		None

Method

Did you find the method description adequate?
If NO, in which part(s) could it be improved?

LabID	Method description	Proposed improvements
3063	YES	
3084	YES	
6371	YES	
6381	YES	
6426	YES	
6482	YES	1. 4.23 - A note about accurately determining the concentration of the stock solution will need to be included when the method is written for further distribution (we understand this instruction was limited for the MVS). 2. 4.24 Could include an option to pipette ""150µl or a volume of stock solution exactly equivalent to 2µg ochratoxin A"" to produce a solution of exactly 0.10µg/ml. this would avoid the need to adjust all the values of the calibration graph at a later stage. 3. The NOTE 3 in section 6.4. On first reading it was not clear that this note was explaining how the injection volume could be increased to accommodate larger volume injections if this was needed due to HPLC equipment. It might be clearer if some text was added to add a statement after (V3) something like 'This will ensure the correct mass equivalent of test sample is injected and the calibration series in Table 2 is still correct. It may perhaps help avoid confusion because at first we though it was an instruction on how to vary the sensitivity the method, or to dilute samples that were outside the calibration range.
6512	YES	N/A
6584	YES	
6595	YES	
6600	YES	
6631	YES	
6635	YES	
6658	YES	
6696	YES	
6699	YES	
6926	YES	
6938	YES	
6942	YES	
7026	YES	
7103	YES	

ANNEX 5 - Questionnaire output: deviations from SOP and the analytical process

Specific deviations-1
Did you follow the method in all details?
5 - Apparatus
6.1 – Spiking

LabID	Deviations	Apparatus	Spiking
3063	NO	Analytical column: XTerra ® RP18 Column, 5 µm, 4.6 x 250 mm (Waters)	
3084	NO	Waters HPLC with fluorescence detection. Lichrospher C18 endcapped 250*4mm	
6371	NO		
6381	NO	Analytical Column: Spherisorb ODS2 C18, 250 x 4.6 mm, 5 um	
6426	NO	we used the analytical column ZORBAX eclipse XDB-C18, 4.6x150mm 5 micron	
6482	NO	We purchased a guard column but could not use it as it did not fit in the guard column holders that we had in the laboratory. Therefore the analysis was carried out using an in-line HPLC filter before the analytical column and no guard column.	As SOP
6512	NO		
6584	NO	analytical column	
6595	NO	Zorbax SB-C18 3.0x150mm 5-Micron	
6600	NO	Column: LichroCART 250-4, Lichrospher 100, RP-18 (5 µm)	
6631	NO	Waters Spherisorb 5 um ODS2 4.6 x 250 mm.	1 hour.
6635	YES	Originally tried 2 other C18 HPLC columns that were used in the lab but the resolution was not adequate. We had to purchase and use the Zorabax column as listed in the protocol to perform the evaluation	
6658	NO	As analytical column, ACE 5 C18 (250 mm x 4,6 micrometers particle size) was used.	none
6696	NO	Instead of conical flask: Polypro High density bottle with scew cap (500ml). shaker: over head shaker (type: REAX II) NO filtration but our routine method: a first centrifugation at 5000 rpm (extraction flask) and a second one on 20ml aliquot (30 ml centrifuge tube) at 10000rpm. We obtained solutions ready for the immunocolumn (OchraTest). HPLC column: Alltech Alltima 5 µmC18, 250 x 4.6 mmID	
6699	YES		
6926	NO	Cromatographic column: Phenomenex Gemini C18, 250 x 4.60 mm, 5 um, 110 A	
6938	YES		
6942	NO	Analytical reverse-phase HPLC separating column SphereClone ODS (2) with dimensions of 150 x 4.6 mm I.D. and particles of size 5 um.	
7026	YES		
7103	YES		

Specific deviations-2
6.2 - Extraction
6.3 – IAC clean-up

LabID	Extraction	IAC clean-up
3063		
3084	The only problem we had here is sample 5130 and 2431. The sampling itself was very difficult due to the viscosity, even with heating is was not liquefied.	no deviation
6371		
6381		
6426		
6482	As SOP	As SOP
6512	For samples 5435 and 5691 weighed samples to one decimal place.	I took the reservoirs off to add the 1mL of Tween 20 2% solution and the methanol. Collected eluent in 13 x 100 mm culture tubes instead of 2 mL vials.
6584	extraction in 250ml brown glass bottles	
6595		
6600		Tween 80 in stead of Tween 20
6631	Samples were extracted in 500 ml Schott Duran bottles.	No deviation.
6635		
6658	none	none
6696	All extraction were done on 10gr sample (for both liquorice root and extract) because the lack of material to reach 20 gr for some root samples.	
6699		
6926		
6938	The sample+extraction solvent must be shaken by hand more than just "a few seconds" to obtain a homogenous suspension.	
6942		
7026		4.17: We did use PBS solution prepared from commercially available PBS material <Phosphate Buffered Saline / PBS (Dulbecco A), BR00536; OXOID Limited, Basingstoke, Hampshire, England>. Alternatively to 4.7, 4.8, 4.9, 4.10.
7103		

Specific deviations-3
6.4 - Concentration
7.1 – HPLC operating conditions

LabID	Concentration	HPLC	Different HPLC column
3063			YES
3084	For the samples we re-dissolved in 800µl instead of 200µl For the injection we used 50 µl (standards) and 200µl sample solutions so that the procedure was respected in the dilutions.	flow: 1ml/min isocratic solvent: water/acetonitrile/acetic acid (50/50/1) runtime: 30 min	YES
6371		washing step in gradient with 90% methanol was 5 min, total run time for one run was 30 min No column oven was used, the temperature in the laboratory was 21-22 C	NO
6381			YES
6426	we used reconstitution volume=0.5ml and injection volume=0.050ml		YES
6482	As SOP	No guard column, but otherwise as SOP.	NO
6512		Column oven temperature (including the guard column) was set to 25C as 22C ± 1C was impossible to maintain in the lab. A temperature of minimum 30C is recommended.	NO
6584	injection volume 40µl - reconstitution volume 400µl		YES
6595		flow 0.6 ml/min - Gradient table: 0 min B 100%-10 min B 30%-15 min B30%-15.01 min B100%-20 min 100% Mobile phase A - MeOH; B - MeOH/H2O (1:1)	YES
6600		gradient: 10.1 in stead of 10.01 and 20.1 in stead of 20.01	YES
6631	Reconstitution volume was 200 ul Injection volume was 20 ul	Bandwidth was 10 nm.	YES
6635			NO
6658	injection volume = 50 microliters reconstitution volume = 500 microliters	none	YES
6696	Use of centrifuge vacuum evaporation (SpeedVac).	We used the gradient and flow from the procedure	YES
6699		we eluated the standards for 10 minutes	NO
6926	Reconstitution volume: 400uL-Injection volume: 40uL		YES
6938			NO
6942	Reconstitution volume 500 ul and injection volume 50 ul		YES
7026			NO
7103			NO

Specific deviations-4
7.2 – 7.1 - Calibration
7.4 – Determination

LabID	Calibration	Determination
3063		
3084	no deviation, except exact concentration is not known (+/-13.5µg/ml?)	no deviation
6371		
6381	Part 4.24: The OTA standard solution was prepared by pippeting 190 ul OTA stock solution into a 25 ml volumetric flask and following the procedure consequently.	
6426		
6482	As SOP	As SOP
6512		
6584		
6595		
6600		
6631	All standards were prepared using a Hamilton Diluter. We never use volumetric flasks for this operation.	
6635		
6658	none	none
6696	We used the true concentration values instead the nominal concentration	
6699		
6926	Standard solutions injected on duplicate Y axis=area X axis=ng injected	
6938		
6942		
7026		
7103		

Specific deviations-5
Others

LabID	Any other deviation
3063	
3084	Calibration: injection sequence Calibration standard: exact concentration? al this is based on a concentration of 13.5 µg/ml like mentioned in the procedure
6371	
6381	
6426	
6482	1. There may be a transcription error for two samples. On the sample receipt form they were recorded as 4275 (spiked sample) and 1380. However the analyst has labelled them as 4875 and 1330. We no longer have the vials to check which is correct. 2. The ampoule number for the calibration standard was not recorded and the empty ampoule has been discarded. 3. There are no results for samples 1369 and 3090 - they were not missed out from the analytical batch.
6512	
6584	
6595	
6600	
6631	
6635	
6658	none
6696	
6699	the sample 1585 weight was 9.19 g and was very viscous
6926	
6938	
6942	
7026	
7103	

Analytical process at the participants' laboratory – general - 1
Did you encounter any problem during the analysis?
If YES, what were the specific problems and to which samples do they apply?

LabID	Problem	Description	Sample
3063	NO		
3084	YES	The gradient didn't work well and destabilized our system, no stable baseline. For this reason we applied a isocratic run with sufficient elution time.	For all samples
6371	NO		
6381	NO		
6426	NO		
6482	NO		
6512	NO	N/A	
6584	YES	it is difficult to evaporate all the cleaned samples to dryness because of a low water content after IAC	
6595	NO		
6600	NO		
6631	YES	Samples 3831 and 3546 were difficult to weigh out. It proved necessary to take <10 g sample but this was extracted with a 20:1 ratio of extracting solution to sample. We tried heating the samples prior to weighing but this did not help.	Liquorice extract (paste)
6635	NO		
6658	NO		
6696	YES	At the first beginning, for the preparation of the std curve. We got some problem with the STD ampoule that was very difficult to get out of his plastic container (diameter to close of which of the ampoule) and difficult to break (ampoule not prepared to be broken and no tool to break it).	STD solution
6699	NO		
6926	NO		
6938	YES	The two viscous extracts impossible to dissolve before shaking.	Liquorice extract (Paste)
6942	NO		
7026	NO		
7103	NO		

Analytical process at the participants' laboratory – general - 2
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.

LabID	Abnormality	Description	Sample
3063	NO		
3084	NO		
6371	NO		
6381	NO		
6426	NO		
6482	NO		
6512	YES	Samples 5742 and 4809 were a paste and harder to weigh (even when warmed in a hot water bath). I had to scrape the samples along the inside of the Erlenmeyer flask in order to get them into the flask.	Liquorice extract (paste)
6584	NO		
6595	NO		
6600	NO		
6631	YES	Sample no. 3982 needed an extra free air push.	Liquorice root
6635	NO		
6658	NO		
6696	NO		
6699	NO		
6926	NO		
6938	NO		
6942	NO		
7026	NO		
7103	YES	Slow IACs: 1006; 1042; 2163. One sample (paste) 2450 was very viscous making it quite difficult to mix with solvent	Liquorice extract (powder) and liquorice root. Liquorice extract (paste)

Analytical process at the participants' laboratory – general - 3

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 SOP)*

How long did it take the whole processing of the MVS samples (from the preparation to the reporting of the results)? (hours)

Any other information you wish to add

LabID	Familiarity	Paragraph #	Analysis time (hours)	Additional information
3063	YES		50	
3084	YES		16 hours	
6371	YES		Difficult to say, samples were analyzed in 5 different days	In SOP point 6.3: We'd prefer to use a larger volume V2 for transferring the filtered extract than 0.5 ml. The extract is viscous and now we used reversed pipetting with displacement pipette. If the volume is larger, an additional diluting is needed.
6381	YES		20 hours (Extraction+ Purification) + 24 hours (HPLC+processing) = 44 hours	We spent one additional week trying to find the most suitable column (among the available columns in our lab), which could be able to separate conveniently the OTA peak from the interferences peaks present in the final extracts. Apart of the finally selected column, we also checked the following ones: Tracel Extrasil C18 ODS, Atlantis C18 and Luna C18. We obtained bad resolutions and very high pressure values (3200 psi) with these 3 columns.
6426	YES		40	
6482	NO	Para. 6.3 We found the approach to dilute the filtrate by adding it to the PBS in the reservoir of the IAC unusual - as some PBS had already started to go through the IAC before the filtrate was added. In our laboratory we would allow PBS to pass through the IAC, dilute the filtrate with a known volume of PBS & add this to the IAC reservoir. I guess this is a measure to save some time, but limits the possibility to automate this step if this is critical?	12 - 15 hours	We would like to see a second calibration series injected at the end of the sequence OR for different standards to be injected through the sequence to provide more confidence in the stability of the system and the calibration graph. At the moment there are only single values for most of the calibration points, only Std. 3 has replicate injections. We also thought the calibration series was oddly spaced. There was very little difference in response between the two lowest concentration standards, but much larger gaps between higher concentration calibration points. The range of the series seems very large - it could be reduced and this would allow the lower points to be spaced differently.
6512	YES	N/A	70	

LabID	Familiarity	Paragraph #	Analysis time (hours)	Additional information
6584	YES		14	it is not practicable to handle low sample volumes of 200µl. Filtration for HPLC analysis is not possible.
6595	YES		8	
6600	YES		27 (estimated)	We would prefer to inject higher volumes of the sample. The peaks of the standards 0,5 and 1 ng/ml were very small (estimated S/N 3-6). The viscosity of some samples was rather high, even after heating in a water bath.
6631	YES		Approximately 30-32 hours including the HPLC analysis.	Additional samples for which a resolution <1.5 was obtained are as follows: 3759 (1.33), 3868 (1.41), 4386 (1.41), 3544 (1.27), 3627 (1.26), 3780 (1.24), 3982 (1.27), 3669 (1.36)
6635	YES		approximately 25 hours	
6658	YES		six hours for extraction and IAC by 4 persons; HPLC runs over night	none
6696	YES		3 x (14 hours - total lab work) = 42 hours ;	Because of the disponibility of the lab technician, we had to split the liquorice series on three days. Labo work was done during the day. The HPLC analysis begun so far the samples were prepared for the analytical process. Usually that begun at about 4.00h pm and ran over night. N.B.: at point 6, night hours not taken into account. Next day morning, overview and compilation of the results while a second serial of liquorice samples was treated in the lab. Doing so, we didn't need an overnight stop of the global procedure.
6699	YES		30 hours	our samples stated over night after ICA column cleanup before evaporation in refrigerator at 4 degrees of C
6926	YES		40 hours	
6938	YES			Preparation of the samples were done during four days, 10, 13, 15, 16 December. The eluats from the IACs were frozen the same day. Concentration and HPLC-analysis another day, 20 December. One long run, new samples were put into the autoinjector the day after.
6942	YES		10 h	
7026	YES		For 10 spiking samples about 21 hours, for 18 samples about 28 hours.	
7103	YES		52 hours	

Analytical process at the participants' laboratory – Overnight stops

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.

LabID	Stops	Samples and stage
3063	NO	
3084	NO	
6371	YES	1st calibration was made 30.11.2010 and was used to calculate results for samples analyzed 30.11, 1.12 and 2.12. The validity of the calibration was checked every day with STD-3. 2nd calibration was made 7.12.2010 and was used to calculate results for samples analyzed 7.12 and 8.12. The validity of the calibration was checked every day with STD-3. All samples were prepared during the daytime and HPLC-run was during the following night. This is our normal procedure in routine work
6381	NO	Although samples were extracted and purified along different days, their corresponding final extracts were stored under frozen conditions and injected in the HPLC in a single sequence.
6426	NO	
6482	NO	
6512	NO	N/A
6584	NO	
6595	NO	
6600	NO	
6631	YES	Samples were weighed out on Day 1 and on Day 2 the remainder of the procedure was performed.
6635	NO	
6658	NO	
6696	NO	
6699	NO	
6926	NO	
6938	YES	See point 7.
6942	NO	
7026	NO	
7103	NO	

Analytical process at the participants' laboratory – Integration
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? (Numeric value)
Which global settings did you use for automatic integration (e.g. valley-to-valley or horizontal baseline or tangential, etc.)?

LabID	Integration	Visual check	Chromatograms #	Integration mode
3063	NO			
3084	NO			
6371	NO			
6381	YES	YES	0	valley to valley
6426	NO			
6482	YES	YES	10	Used HPChem station. Integration events - Detector Default Integration Event Table "Event FLD" Slope sensitivity 1.000, Peak Width 0.040, Area Reject 1.000, Height Reject 1.700, Shoulders OFF Not sure how these relate to the parameters listed. Most integration was good, samples with unsatisfactory integration (all under integrated) were re-integrated manually.
6512	YES	YES	0	Traditional integration, baseline to baseline
6584	NO			
6595	NO			
6600	YES	YES	25	not specified in the software
6631	YES	YES	20	Valley-to-valley.
6635	YES	YES	2	valley to valley
6658	NO			
6696	YES	YES	9	valley to valley where applicable
6699	NO			
6926	YES	YES	12	valley to valley, retention time, peak width, threshold
6938	YES	YES	9	Valley-to-valley
6942	NO	NO		
7026	NO			Annotation: We could observe that for all of the 10 spiking samples automatically integration (e.g. valley-to-valley) of the signals was appropriate. For 16 of the 18 samples (with higher amounts of Ochratoxin A) manually integration of signals was explicitly more reliable than automatically integration.
7103	YES	YES	5	Valley-to-valley

Analytical process at the participants' laboratory – Resolution

Did you measure a < 1.5 resolution between the OTA peak and the two contiguous interferences for any of the samples?

LabID	Insufficient resolution	Samples	R_S
3063	YES	3826, 3598, 4498, 3695, 4309, 4011, 3853, 3587, 3098, 3422	0.8, 0.85, 0.9, 0.9, 1.44, 1.3, 0.92, 0.91, 0.9, 0.89
3084	NO		
6371	NO		
6381	YES	1842	0.495
6426	NO		
6482	NO		
6512	YES	Attachments of chromatograms	
6584			
6595	NO		
6600	NO		
6631			
6635	NO		
6658	YES	3755, 3914, 3959, 4134, 4137, 4289, 4328, 4521, 4531, 4705, 5038	1.16, 0.97, 0.93, 1.12, 1.1, 0.97, 1.02, 1.09, 1.13, 0.96, 1.1
6696	NO		
6699	YES	1020, 1020, 2835, 1435, 1435, 2059, 2059, 2517, 2725, 2725	2.64, 3.69, 4.47, 1.53, 1.99, 2.86, 5.2, 7.9, 6.12, 2.2
6926	YES	2186, 3355, 2998, 1799, 2112, 2009, 2799, 1798	1.3, 1, 1.1, 1, 1, 1.4, 1.3, 0.4
6938	NO		
6942	YES	4940, 5446	1.03, 1.05
7026	NO		
7103	NO		

ANNEX 6 - Supporting documents: Method and spiking protocols

SOP of the Method

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

EURL
European Union Reference Laboratory
Mycotoxins

Determination of Ochratoxin A in liquorice root and liquorice extracts by immunoaffinity column clean-up and High Performance Liquid Chromatography with fluorescence detection.

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

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 liquorice root and liquorice extracts (in the form of both powder and paste) using liquid-chromatography with fluorescence detection. This candidate method will be validated for the determination of OTA via the analysis of naturally contaminated and spiked 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 dried, reconstituted and quantified by reverse-phase high performance liquid chromatography (RP-HPLC) coupled with fluorescence detection.

4 Reagents

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

4.5 Toluene, UV grade

4.6 Sodium hydrogen carbonate, ($NaHCO_3$)

4.7 Sodium chloride, (NaCl)

4.8 Disodium hydrogen orthophosphate, ($Na_2HPO_4 \cdot 12\ H_2O$)

4.9 Potassium dihydrogen phosphate, (KH_2PO_4)

4.10 Potassium chloride, (KCl)

4.11 Sodium hydroxide, (NaOH)

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

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

Dilute 8,28 ml of hydrochloric acid solution (4.12) to 1 l with water.

4.14 Tween® 20

4.15 Tween® 20 solution, *c*(Tween®20) = 20 g/l (2%)

Add 20 g of Tween® 20 (4.14) to 1000 ml of grade 3 water.

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

Dissolve 8 g NaOH (4.11) in 1 l of water

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

Dissolve 8 g NaCl (4.7), 2.9 g Na_2HPO_4 x 12H2O (4.8), 0.2 g KH_2PO_4 (4.9) and 0.2 g KCl (4.10) in 900ml of water. After dissolution, adjust the pH to 7,4 with hydrochloric acid solution (4.13) or sodium hydroxide solution (4.16) as appropriate, then dilute to 1 l with water.

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

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

Add 30 g of sodium hydrogen carbonate (4.6) to 1000 ml of grade 3 water.

4.19 Extraction solvent

Mix 50 parts per volume of methanol (4.2) to 50 parts per volume of sodium hydrogen carbonate solution (4.18). Mix well.

4.20 HPLC mobile phase A

Mix 70 part per volume of methanol (4.3) with 30 parts per volume of grade 3 water and with 1 part per volume of glacial acetic acid (4.4).

4.21 HPLC mobile phase B

100% methanol (4.3).

Degas mobile phase solvents A and B (4.20 and 4.21) with for example helium or equivalent methods.

4.22 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 (4.17) 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.

4.23 OTA stock solution

A solution in toluene/acetic acid 99:1(≈ 13,5 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.

4.24 OTA standard solution, *c*(Ochratoxin A) ≈ 0.10 µg/ml

Pipette 150 µl OTA stock solution (4.23) into a 20 ml volumetric flask (5.18), bring it to dryness by applying a gentle flow of nitrogen (4.1), add about 5 ml of mobile phase A (4.20) and shake vigorously. Then dilute to 20 ml (up to the mark) with the mobile phase A (4.20) and shake again. This gives a standard solution containing ≈ 100 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.

4.25 OTA spiking standard solutions

The solutions are provided with the samples and contain OTA in acetonitrile/acetic acid 99:1 at different concentration levels.

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 1 000 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 20 ml and 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 Solvent vacuum filtration system, suitable for 47 mm filter

5.13 Automated SPE Vacuum System

5.14 Vortex mixer

5.15 Sample concentrator, with temperature control and gas supply (either vacuum centrifugation – e.g. Genevac or equivalent - or evaporation under nitrogen stream)

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

5.17 Glass insert vials, ~ 250 µL capacity and crimp caps or equivalent

5.18 Volumetric flasks, of various capacities (e.g. 10 ml, 20 ml, 25 ml)

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

5.20 HPLC apparatus, comprising the following:

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

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

5.20.3 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.

5.20.4 Recorder, integrator or computer based data processing system

5.20.5 Analytical reverse-phase HPLC separating column Zorbax® SB-C18 with column dimensions of 250 mm x 4.6 mm I.D. stationary phase with particles of size 5 µm. This column ensures a baseline resolved resolution of the Ochratoxin peak from interferences. If another column should be used, a separation factor (α) of at least 1,5 between OTA peak and the contiguous ones is required.

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

5.20.7 Degasser, optional, for degassing HPLC mobile phases (4.20) and (4.21)

---

[1] In alternative a filter of the same typology with 47mm diameter can be used. See 6.2.

5.20.8 **Column oven,** capable to operate at 22 ºC ± 1 ºC

6 Procedure

6.1 Spiking procedure

Please follow the spiking instructions given in the attached **SPIKING PROTOCOL.**

6.2 Extraction

Weigh 10 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 (4.19). 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 (5.19) for further analysis[2]. Proceed immediately with the immunoaffinity column clean-up procedure (6.3)

6.3 Immunoaffinity column cleanup

Connect the immunoaffinity column (4.22) to the vacuum manifold (5.13), and attach a reservoir of 20 ml or 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) 15 ml of PBS (4.17), transfer 0.5 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 5.13). 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 with1 ml Tween® 20 2% (4.15) followed by 10 ml of water at a rate not exceeding 3 ml/min. Repeat these washing steps and dry the column by passing nitrogen or air through it for about 1 - 2 seconds. Then discard all the eluent from this stage of the clean-up procedure.

---

[2] In alternative a vacuum filtration can be applied, by using a vacuum system (5.12) and a 47 mm glass fibre filter (see note 1). Add approximately 10 ml of the centrifuged extract (**Error! Reference source not found.**) onto the glass fibre filter and apply a slight vacuum. Discard this volume and filter again at least 20 ml of the remaining extract in another 250 ml screw cap flask for further analysis. Proceed immediately with the immunoaffinity column clean-up procedure (6.3)

Finally, place a 2 ml vial (5.16) under the column and pass 0.5 ml of methanol (4.3) through the column, collecting the eluate. After the last drops of methanol have passed through the column, wait approximately 1 minute. Then add a further 0.5 ml of methanol (4.3) applying the same procedure and collecting the eluate. Finally add 0.50 ml of methanol (4.3) and continue to collect the eluate. Carefully pass air through the column in order to collect any final drops.

NOTE 2: Alternatively to a manual procedure the immunoaffinity clean-up and elution can be performed with an automatic sample preparation unit, provided that volumes and aliquots remain unchanged.

Make note of columns with exceptionally fast or slow flow rates

6.4 Sample purified extract concentration

Evaporate the methanolic eluate to dryness applying either gentle stream of nitrogen at about 30-35°C (5.15) or vacuum centrifugation (5.15).

Re-dissolve the purified sample residues in 200 µL [V_3] mobile phase A (4.20), cap vial and shake on vortex mixer for at least 15 sec, making sure the lower part of the vial is thoroughly rinsed by the solvent. Transfer the **test solution** so obtained in insert vials (5.17) and analyse.

NOTE 3: If necessary due to the HPLC apparatus characteristics, the injected volume of the purified sample could differ from 20 µl but, due to sensitivity reasons, it is recommended to maintain a ratio of 10 between V_3 and the injection volume. For instance, the injection volume could be increased to 40 µl but then the sample is to be re-dissolved in 400 µl of mobile phase A (V_3). The same injection volume should be consistently applied for the calibration solutions.

7 HPLC Analysis

7.1 HPLC operating conditions

When the column specified in (5.20.5) and the mobile phases A and B, specified in 4.20 and 4.21 respectively, were used, the following settings were found to be appropriate:

Table 1: gradient conditions

Time min	Flow rate ml/min	Mobile Phase A %	Mobile Phase B %
0.00	1.0	100	0
10.00	1.0	100	0
10.01	1.0	10	90
20.00	1.0	10	90
20.01	1.0	100	0
30.00	1.0	100	0

- Column oven temperature (including the guard column) is 22 °C ± 1 °C;
- injection volume is 20 µl;
- autosampler (optional) temperature is 15 °C to 20 °C;
- Detector wavelength: excitation 332 nm, emission 476 nm

The recommended flow rate is 1 ml per minute for the chosen column (5.20.5). Ochratoxin A elutes with retention of approximately 10 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 3 (see Table 2) and one chromatogram for each test sample where the separation of OTA peak from the matrix peaks is evidenced.

7.2 Preparation of calibration solutions for HPLC

Prepare six HPLC calibration solutions from the standard solutions prepared (4.24).

With appropriate calibrated pipettes or microsyringes (5.8) the volumes of the ochratoxin A standard solution (4.24) listed in Table 2 are to be distributed separately into a set of volumetric flasks (5.18). After having added the standard solution (4.24), add the mobile phase A (4.20) up to the mark, close and mix manually. This will result in 6 OTA solutions with approximately the concentrations listed in Table 2.

These six solutions cover a range from 4 µg/kg to 160 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 °C. Peak areas corresponding to the same calibration solution injected at regular intervals should be within ± 3 %.Ochratoxin A calibration standard solutions

These solutions shall be used directly for injection into the HPLC system, after transferring them in the vials (5.16).

Table 2: Preparation of HPLC calibration solutions

STD	µL Std (4.24)	Final Volume (ml)	Nominal concentration (ng/ml)	Corresponding contamination level (µg/kg)
STD 1	125	25	0.5	4
STD 2	100	10	1	8
STD 3	500	10	5	40
STD 4	1000	10	10	80
STD 5	1500	10	15	120
STD 6	2000	10	20	160

NOTE 4: The exact concentrations of OTA in the OTA standard solution (4.24) and in the calibration standard solutions (7.2) have to be derived from the concentration of the reference standard material (4.23) and the volumes used.

7.3 Calibration curve

Prepare a calibration curve by injecting 20 µl of the six ochratoxin A calibration solutions (7.2) 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.

7.4 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 A (4.20)
- The six calibration standards obtained from the standard solution (4.24) at the concentrations included in Table 2 from the lowest to the highest level
- Mobile phase A (4.20)
- Test solutions (from samples and from spiking experiments)

Every 10 test solutions, one injection of STD 3 (Table 2) has to be performed (control standard). If the area of the standard deviates for more than ±20% 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.

7.5 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 3 (Table 2) 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.

8 Calculation

8.1 Preparation of the calibration graph

Plot the peak signals as area or height (y-axis) against the concentration of the OTA calibration standard solutions (7.2) [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.

8.2 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

$$w_{OTA} = \frac{C_{OTA} \times V_1 \times V_3}{W \times V_2}; \qquad \text{(i.e. } w_{OTA} = C_{OTA} \times 8\text{)}$$

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.

9 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

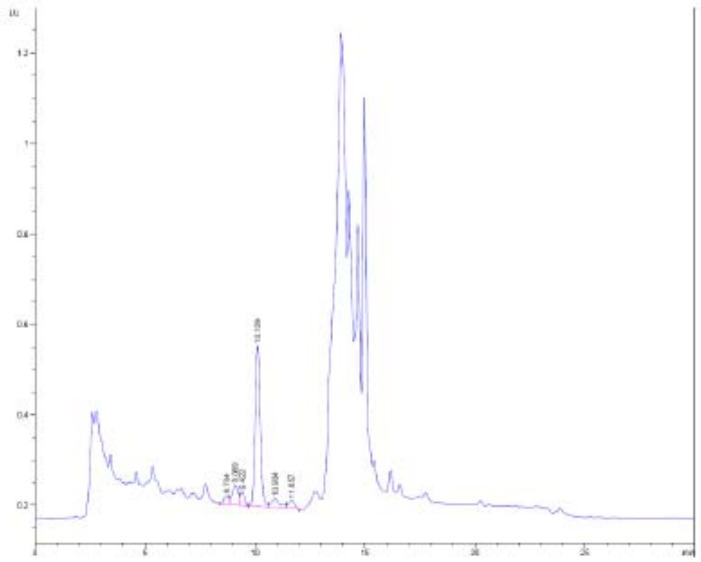
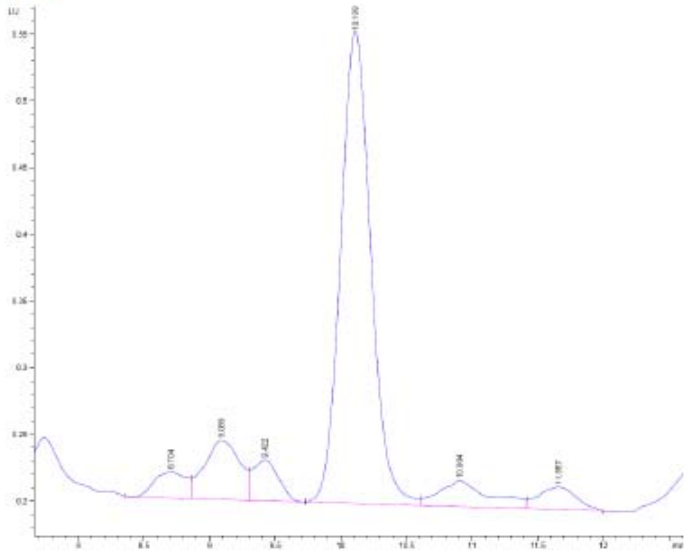


Figure 2



Key:
LU: fluorescence
Ochratoxin A: peak at 10.042 min (resolution: > 3 with both preceding and following peaks)

Figure 1 and 2 — Liquorice powder (Ochratoxin A concentration ~ 80 µg/kg)

Operating conditions for Figure 1 and 2:

Column:	Zorbax® SB-C18 with column dimensions of 250 mm x 4.6 mm I.D. stationary phase with particles of size 5 µm.
Flow rate:	1 ml/min
Mobile phase:	HPLC gradient (see 7.1)
Column	22 ° C controlled
Injection volume:	20 µL
Detection:	Fluorescence, 332 nm excitation, 476 nm emission, bandwidth ≤16 nm

Spiking protocol

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
Community reference laboratory for mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

SPIKING PROTOCOL FOR THE METHOD VALIDATION STUDY ON THE DETERMINATION OF OCHRATOXIN A IN LIQUORICE

This box contains ten samples labelled "SAMPLE for SPIKING nnnn", where "nnnn" indicates a unique numerical code. These are blank and naturally contaminated materials to be spiked and the results reported will be used by the organiser for recovery determinations.

There are also ten ampoules labelled as "Spiking solution nnnn" where "nnnn" indicates a unique numerical code corresponding to the sample which shall be spiked with that solution. The solvent for the Ochratoxin A (OTA) spiking solutions is acetonitrile/acetic acid (99/1, v/v).

The spiking samples and the respective spiking solutions are coded with the same unique 4 digits number. **Each spiking solution has to be used exclusively to spike the sample with the same code**.

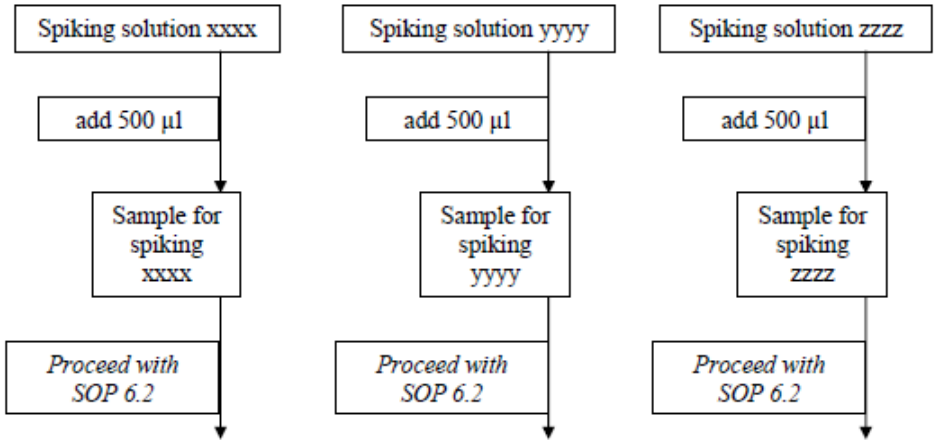
For the recovery determination, proceed as follows for each of the samples for spiking:

- Weigh **10.0 g**, to the nearest 0.1 g, of the test sample into a. 500 ml conical flask or similar recipient. Please follow the procedure as described in the standard operating procedure (SOP) in **paragraph 6.2**.
- Add **exactly** 500 µL of "Spiking solution" to the respective (with the same code as the spiking solution) "SAMPLE for SPIKING" in the recipient and repeat it for all the spiking solutions and samples for spiking. You will end up with ten spiked samples, each spiked with one spiking solution. For reporting, please use the code on the ampoules (which coincides with the code on the sample spiked with that 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 content of OTA according to the method protocol. For this, proceed with the addition of the required amount extraction solvent (see **paragraph 4.19 and 6.2** of the SOP for the solvent composition and volumes required) as described in **paragraph 6.2** in the method protocol.

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

Diagram for the spiking procedure (example):



ANNEX 7 - Supporting documents: communication

Announcement of the study

Method validation study on a HPLC-FLD based method for determination of Ochratoxin A in liquorice based food

Privacy statement | Legal notice

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Search IRMM Internet

EUROPA > European Commission > JRC > IRMM > EU Reference Laboratories > EURL mycotoxins > Interlaboratory comparisons

Font Size: A A A A

News | Links | Press corner | Site map | Contact

Main Menu

- About IRMM
- Activities
- Reference materials
- EU Reference Laboratories
- Interlaboratory comparisons
- Job opportunities
- Events
- Training
- Calls
- Publications

EURL Mycotoxins

About us | Legislation | Network laboratories | Interlaboratory comparisons | What's new? | Contacts | Network pages

News archive

- Environmental analysis
- Nuclear research
- Reference materials and measurements
- Food, biotechnology and health

catalogue

ERM

EURL

CRL Feed Additives

EUFRAT

Method validation study on a HPLC-FLD based method for determination of Ochratoxin A in liquorice based food

The European Union Reference Laboratory for Mycotoxins (EU-RL Mycotoxins) will organise in the second half of 2010 an inter-laboratory comparison for the validation of an analytical method (MVS) to determine ochratoxin A (OTA) in liquorice root and liquorice extracts, along the requirements of the Commission Regulation No 105/2010.

The method is based on solvent extraction, immunoaffinity column purification (IAC), and detection / quantitation by high performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD).

Design of the study

The MVS will be conducted and evaluated according to ISO 5725-2:1994.

The participants to the study will be asked to analyse a total of about 20 samples, including liquorice extract (as paste and as powder), liquorice root and candies containing liquorice extract.

The concentration levels will be between 3 and 200 µg/kg.

The starting date of the comparison will be decided upon the amount of laboratories expressing their interest in participating in the study. Dispatch will be pre-announced at least two weeks in advance.

The participants will have 6 weeks from dispatch date to report back the results.

The MVS will be open to all interested laboratories, e.g. NRLs, official food control laboratories, private food control laboratories, academia, and laboratories from industry.

If you are interested in participating to this MVS, please contact:

Donata Lerda

Institute for Reference Materials and Measurements (IRMM)

EU-RL Mycotoxins

Retieseweg 111

B-2440 Geel, Belgium

Tel: +32-14-571 826

Fax: +32-14-571 783

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

Latest update 14 June, 2010

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL MYCO
Sent: Wednesday, July 28, 2010 14:49
To:
Subject: Method validation study for Ochratoxin A in liquorice
Importance: Normal

Dear Madam/ Dear Sir,

The EU-RL Mycotoxins is organising a Method Validation Study on Ochratoxin A in Liquorice.
If you should be interested in participating, please read the attached invitation letter.

You can also go to the link:
http://irmm.jrc.ec.europa.eu/html/CRLs/crl_mycotoxins/interlaboratory_comparisons/Ochratoxin_A_in_Liquorice_index.htm

Please let us know, by writing to this mailbox as soon as possible, whether you would like to participate.

Thank you and best regards,

Donata

Invitation letter for OTA in l...

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

Invitation letter for the method validation study by Inter-laboratory comparison (ILC-MVS) for the determination of

Ochratoxin A (OTA) in liquorice

Dear Colleague,

The European Union Reference Laboratory (EU-RL) for Mycotoxins would like to invite you to participate in a method validation study for the determination of ochratoxin A in liquorice root and liquorice extracts, along the requirements of the Commission Regulation No 105/2010.

The method is based on solvent extraction, immunoaffinity column purification, and detection / quantitation by high performance liquid chromatography coupled with fluorescence detection.

This study is open to all laboratories experienced in this subject.

The participants will have to analyse a total of 20 samples, including liquorice extract (as paste and as powder), liquorice root and candies. The target levels will be between 3 and 200 µg/kg.

The study will start within October 2010. Dispatch will be pre-announced at least two weeks in advance. The participants will have 6 weeks from dispatch date to report back the results.

For questions and additional information please feel free to contact or by phone (+32 014 571826).

If you wish to participate please send an e-mail with your details (phone, e-mail, postal address and contact name) to Donata Lerda (jrc-irmm-crl-mycotox@ec.europa.eu) as soon as possible. There is no deadline at this moment, but once the number of possible participants has been reached, the registration procedure will be closed.

For questions and additional information please feel free to contact Donata by e-mail or by phone (+32 014 571826).

Subscription

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL MYCO
Sent: Wednesday, September 15, 2010 12:12 AM
To:
Subject: Method validation study for Ochratoxin A in liquorice
Importance: Normal

Dear Madame / Sir, **JRC D08/DL/hn/ARES 592899(2010)**

The European Union Reference Laboratory for Mycotoxins announced on the 5th of August 2010 that an interlaboratory comparison for the validation of a method to determine Ochratoxin A (OTA) in liquorice and liquorice extracts was planned to be organised this year. The invitation for expression of interest in participating in the study was also forwarded by CEN TC 275/WG 5 secretariat.
As a first step of the study we would like you to send back to us your comments about the standard operating procedure (SOP) and the outline of the study which are herein attached.

You are also asked to confirm, by filling in and signing the attached FORM, your interest in participating in the study (please note that some of the fields are required to be filled in before being able to send back the FORM.

We would be grateful if you could reply, by sending back the FORM both via e-mail and via FAX (or e-mail if the signed form is saved as PDF), before **1 October 2010**.

FORM_subscription to MVS on OT...

Draft_SOP_MVS for determinatio...

MVS OTA in iquorice_Outline o...

Thank you in advance for the co-operation and best regards,
Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

European Union Reference Laboratory
Mycotoxins

Geel,

Subscription questionnaire for inter-laboratory study

Determination of **ochratoxin A in liquorice root and liquorice extracts** 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

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

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

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 must be rigidly met;

c. The method must be strictly followed;

d. Samples must be handled according to instructions;

e. A qualified operator must perform the measurements

2. Comments you wish to address before participation

About this form:

About the outline:

About the method:

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, Donata Lerda, Retieseweg 111, B-2440 Geel, Belgium; **Fax: +32 14 571 783**

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

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

EURL
European Union Reference Laboratory
Mycotoxins

Method validation study on the determination of ochratoxin A in liquorice root and liquorice extracts 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 liquorice root and extracts. The study is foreseen to take place in **autumn 2010**.

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 send the **day before dispatch** of samples and participants will receive a dispatch note containing all data for tracking the shipment.

After dispatch of samples, participants will have **five weeks** for reporting the data back to the EU-RL with 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. 18 samples for single analysis with different content levels of OTA(the final number of samples could slightly deviate from this number)
   b. 10 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:

Donata Lerda
Institute for Reference Materials and Measurements (IRMM)
EU-RL Mycotoxins
Retieseweg 111
B-2440 Geel, Belgium
Tel: +32-14-571 826
FAX: +32-14-571 783
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: Donata Lerda, Frans Verstraete, Franz Ulberth, Anne-Mette Jensen

Dispatch of materials

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL MYCOTOX
Sent: Tuesday, October 19, 2010 5:12 PM
To:
Subject: MVS OTA in liquorice: preannouncement of the sample dispatch
Importance: Normal

Dear Madam, dear Sir,

We are planning to dispatch the samples on week 45-46.
Dispatching will be done via DHL and according the international rules the content will be classified as DANGEROUS GOODS in EXCEPTED QUANTITIES.
The samples will be described as samples for laboratory use.

Please let us know if you should need further description and/or a proforma invoice for an efficient and quick custom clearance of the parcel.

Thanks for the co-operation and best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL MYCOTOX
Sent: Monday, November 15, 2010 3:17 PM
To:
Subject: MVS OTA in liquorice: samples dispatch

Dear .....,

We sent out the samples today afternoon.
To view your shipment tracking details, please click on the following link:
http://www.dhl.com/cgi-bin/tracking.pl?AWB=xxxxxxx

The following lines report the description of the parcel.
WEIGHT: 2,4 kg
PIECES: 1
CONTENTS: Dangerous Goods in excepted quantities

Please, send back to us the **sample receipt** included in the parcel as soon as you receive it.

Please, remember to store the samples, IAC, and solutions at **-20 ºC (in the freezer)** till the use.

Best regards,

Donata

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium

Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements
Community Reference Laboratory for Mycotoxins

EURL
European Union Reference Laboratory
Mycotoxins

RECEIPT FORM

Surname of Participant	
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) One inter-laboratory comparison sample receipt form (= this form)
b) A printout of the PT outline
c) A printout of the instructions
d) The SOP of the method
e) The spiking protocol
f) Your participation code (LAB ID)
g) 18 coded test materials for direct analysis
h) 10 coded test materials for spiking before analysis
i) A standard solution
j) 10 spiking standard solutions
k) 35 immunoaffinity columns
l) Safety sheets for solvents and OTA

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	
Serial number of the samples to be spiked/spiking solutions you received	

SIGNATURE: _____

Please fax or email the completed form to:

Donata Lerda
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

Instructions

From: LERDA Donata (JRC-GEEL) **On Behalf Of** JRC IRMM CRL MYCOTOX
Sent: Thursday, November 18, 2010 5:30 PM
To:
Subject: ARES (2010) 803738: MVS on OTA in Liquorice: Reporting of results
Importance: High

Ares(2010)_803738

Dear Madam, dear Sir,

Parcels containing the samples to be analysed in the course of this collaborative trial were dispatched on Monday.
Starting **from now till the 10/01/2011** you can report the results and the questionnaire.

Please, use the three PDF FORMs attached to this mail to send back your results, calibration curve and notes to us, following carefully the procedure reported at the beginning and end of each FORM.
WARNING: when filling in the FORM for calibration you might receive twice the error message "This operation is not permitted". Please click on OK and proceed. The filling in and sending out will work anyhow.
In case you should have any doubt or question, please do not hesitate contacting us.

Thanks again for joining the study.

Best regards,

Donata

ARES 52_Instructions_MVS

MVS OTA in liquorice_Outline o..

SOP_MVS for determination of O..

Spiking protocol for OTA in li...

Results MVS OTA in liquorice_F...

Questionnaire MVS OTA in liquo...

Calibration MVS OTA in liquori...

Donata Lerda
Food Safety and Quality Unit
Institute for Reference Materials and Measurements
(EC – JRC – IRMM)
Postal address: Retieseweg 111, B-2440 Geel, Belgium
Phone: +32 14 571 826
Fax: +32 14 571 783
e-mail: donata.lerda@ec.europa.eu

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for reference materials and measurements
EU reference laboratory for mycotoxins

European Union Reference Laboratory
Mycotoxins

Geel, 29/10/2010
JRC.DG.D.6/DL/iv /ARES (2010) 759462

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 liquorice and liquorice extracts.

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 calibrants 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 SHOULD BE FOLLOWED AS CLOSELY AS PRACTICABLE** and 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 6.2 of the method protocol). This is of crucial importance due to the material homogeneity requirements
   - Some samples, in particular the extracts in form of **paste**, should be, if necessary **heated** in a water bath to get a workable consistence. Before sampling the amount required for the analysis (10 g) from the recipient, the sample should be **mixed**, e.g. with a spatula, to obtain a homogeneous sample. As a matter of fact, for this materials aliquots were positively assessed for homogeneity before dispatch, but a phase separation could occur before the sample is analysed at the laboratory participating to the study

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

- 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 **four digits code preceded by the specification of the matrix (Root or Extract).** 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 shall be used for reporting of all results.
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 **10/01/2011** which gives a time period of **five 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

Donata LERDA (deputy in case of absence Zoltan KUNSAGI)

Fax: 0032-14-571783

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: Donata Lerda, Frans Verstraete, Franz Ulberth, Anne-Mette Jensen, Zoltan Kunsagi

ANNEX 8 - Supporting documents: FORMS

Form for reporting results

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 liquorice and Liquorice extracts

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

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.

Please remember also to send a printout (or its PDF) of one chromatogram obtained by injecting STD 3 (see Table 2) and one chromatogram for each test sample where the separation of OTA peak from the matrix peaks is evidenced.

>> 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. Your Name (First name + SURNAME), you should write your name in normal letters and the surname in capital letters).
3. Please always report in the first column of the table the four digits code of the sample, also when you do not report any results for it
4. The results to be reported have to be calculated applying the Equations 1 and 2 in paragraph 8.1 and 8.2 respectively of the SOP.
5. Whenever the result obtained is above the LOQ, you should just fill the fields in the first column (column head "Sample code") and in the second column (column head: "OTA content")
6. For all samples where the result obtained should be below the LOQ, please digit YES in the field corresponding to the sample in the third column (column head "Below LOQ") and the LOQ value, expressed in µg/kg and with one decimal in the fourth column (column head "If YES, please report the LOQ you estimated")
7. All the fields in the second and fourth column of the Table of results, are numeric fields: **do not try to enter other formats**.
8. If you could not analyse a particular sample, simply leave the result field empty (but remember to report the corresponding sample code)
9. 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.
10. The Table of results should be filled in for both non spiked (18) and spiked (10) samples

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

Participant details

1. Your Laboratory ID (4 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 (4 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				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				

ATTENTION

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

Please, send back this FORM before the 10/01/2011

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, Donata Lerda, 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

Form for the 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 liquorice and Liquorice extracts

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 3 (see Table 2) and one chromatogram for each test sample where the separation of OTA peak from the matrix peaks is evidenced.

>> 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. Your Name (First name + SURNAME), you should write your name in normal letters and the surname in capital letters).
3. When numeric values are required, please **do not try to enter other formats**.

Participant details

1. Your Laboratory ID (4 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 per year?*

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

Please report the most frequent matrices

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

☐	Liquorice and liquorice extracts
☐	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
☐	Spices
☐	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. Did you follow the method in all details?*

◯	◯
YES	NO

If NO, in which part(s) did you deviate from the protocol?*

Method paragraph	Description of the deviation applied
5 - Apparatus	(e.g. the analytical column)

Method paragraph	Description of the deviation applied
6.1 - Spiking	(e.g. evaporation time after spiking of the sample)
6.2 - Extraction	
6.3 – IAC clean-up	
6.4 - Concentration	(e.g. injection volume and reconstitution volume)

Method paragraph	Description of the deviation applied
7.1 – HPLC operating conditions	(e.g. different gradient/flow due to analytical column different from the one mentioned in the protocol)
7.2 – 7.1 - Calibration	
7.4 - Determination	
Others	

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. How long did it take the whole processing of the MVS samples (from the preparation to the reporting of the results)? (hours)

7. 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.)?

4. Did you measure a < 1.5 resolution between the OTA peak and the two contiguous interferences for any of the samples?*

$$\text{Resolution} = R_S = \frac{2X[t_{R,2} - t_{R,1}]}{W_{b,1} + W_{b,2}}$$

Where:

$t_{r,1}$ and $t_{r,2}$ = retention times of peak 1 and peak 2 respectively (e.g. OTA peak and a matrix peak)s

$W_{b,1}$ and $W_{b,2}$ = peak widths at the base of peak 1 and peak 2 respectively

◯	◯
YES	NO

If YES, please report the corresponding sample code(s) and resolution (numeric value)*

Sample code	Resolution

ATTENTION

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

Please, send back this FORM before the 10/01/2011

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, Donata Lerda, 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

Form for reporting the calibration

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 calibration for the participants to the Inter-laboratory comparison for the validation of a method to determine Ochratoxin A in liquorice and Liquorice extracts

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

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.

>> 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. All the fields in the second and fourth column of the Table of results, are numeric fields: **do not try to enter other formats**.
3. Results obtained shall be reported:
   - as numbers and with the format obtained from the instrument for the areas
   - in the unit required on top of the respective column and with **two decimals** (e.g. 12.13) for the volumes and the solutions contents. Please enter ONLY ONE datum for each field.
4. Insert as many areas per level as many are the calibrations performed for the analysis of all the samples (e.g. two sets in case the analysis was performed in two different days)

Participant details

1. Your Laboratory ID (4 digits number)*:

2. The code of the calibrant (4 digits number on the ampoule labelled as "Ochratoxin A in Toluene - HAc 99:1 / EU-RL - MYC - 08)

Table of Calibration

Level	Volume Std (4.24) (µl)	Final Volume (ml)	Concentration (ng/ml)	Area 1st calibration	Area 2nd calibration	Area 3rd calibration	Area 4th calibration	Area 5th calibration
STD 1								
STD 2								
STD 3								
STD 4								
STD 5								
STD 6								

ATTENTION

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

Please, send back this FORM before the 10/01/2011

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, Donata Lerda, 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

European Commission

EUR 24778 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Report on the inter-laboratory comparison organised by the European Union Reference Laboratory for Mycotoxins for the determination of Ochratoxin A in liquorice and liquorice extracts - Method based on immunoaffinity column clean-up with high performance liquid chromatography and fluorimetric detection method
Authors: Donata Lerda, Zoltan Kunsagi, Helena Ernst, Massimo Ambrosio, and Joerg Stroka
Luxembourg: Publications Office of the European Union
2011 – 125 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-19831-1
doi:10.2787/39928

Abstract
The European Union Reference Laboratory for Mycotoxins (EU-RL Mycotoxins), operated by the Institute for Reference Materials and Methods (IRMM) of the Joint Research Centre (JRC), organised a method validation study (MVS) for evaluating the effectiveness of a method for the determination of Ochratoxin A (OTA) in liquorice root and liquorice extracts.

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 dried, reconstituted and quantified by high performance liquid chromatography-flourimetric detection.

Twenty laboratories from 13 EU Member States, a laboratory in Uruguay, one in Turkey, one in Canada, and one in US participated in this study. Contents of OTA ranged from 26 to 141 µg/kg and from 8 to 52 µg/kg for liquorice extracts and root material respectively.

Mean recoveries were calculated as 87 % for liquorice root, and 84 to 88 % for liquorice extracts.

Based on results for the spiked and naturally contaminated samples the relative standard deviations for reproducibility (RSD_R) ranged from 10 to 17 % and from 11 to 22 % in liquorice extracts and liquorice root respectively. Standard deviations for repeatability (RSD_r) ranged from 4 to 9 % and from 6 to 9 % in liquorice extracts and liquorice root respectively.

The Commission Regulation (EC) No 401/2006 lays down performance criteria that must be met by a method to determine OTA in food when used for official control purposes. These criteria have been met by this method for both the liquorice root and the liquorice extracts.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LA- NA- 24778- EN- C

Publications Office

ISBN 978-92-79-19831-1