Federal Institute for Materials Research and Testing

European Reference Materials

# CERTIFICATION REPORT

## The certification of the mass fractions of deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZON) in wheat flour

## Certified Reference Material

## ERM®-BC600

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## SUMMARY

This report describes the certification of a wheat flour material intended for the determination of the contained *Fusarium* mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZON). Detailed information are given regarding the preparation of the material, the homogeneity and stability studies, the used analytical methods and the results of the certification study. The certified values and the uncertainties are:

| ***Fusarium* mycotoxins in wheat flour** | | | |
|---|---|---|---|
| Compound [1)] | No. of accepted data sets | Certified value [2)] | Uncertainty [3)] |
| | | Mass fraction in µg kg$^{-1}$ | |
| Deoxynivalenol (DON) | 17 | 102 | 11 |
| Nivalenol (NIV) | 10 | 1000 | 130 |
| Zearalenone (ZON) | 20 | 90 | 8 |

[1)] DON, NIV and ZON as measured by using appropriate sample preparation techniques (e.g. solvent extraction, clean-up, derivatisation), instrumental separation (HPLC, GC) and detection techniques as specified on page 13 of this report, corrected for extraction efficiency/recovery.

[2)] Unweighted mean of accepted mean values, independently obtained in different laboratories using various methods. The certified values are traceable to the SI.

[3)] Estimated expanded uncertainty $U$ with a coverage factor of $k = 2$, corresponding to a level of confidence of about 95 %, as defined in the Guide to the expression of uncertainty in measurement (GUM), ISO, 1995. Uncertainty contributions arising from characterisation as well as from homogeneity and stability testing were taken into account.

## LIST OF ABBREVIATIONS

(if not explained elsewhere)

| | |
|---|---|
| ANOVA | Analysis of Variance |
| ESI | Electro-Spray-Ionisation |
| FLD | Fluorescence detection |
| GC | Gas chromatography |
| GUM | Guide to the Expression of Uncertainty in Measurement |
| HPLC | High performance liquid chromatography |
| IAC | Immunoaffinity column (clean-up) |
| ILC | Interlaboratory comparison study |
| ISO | International Organization of Standardization |
| ISTD | Internal Standard |
| LC | Liquid chromatography |
| MRM | Multiple Reaction Monitoring |
| MS | Mass spectrometry |
| NIV | Nivalenol |
| ZON | Zearalenone |

**CONTENTS**

# 1 Introduction

Food safety and consumer protection have gained an increased importance in the last decade. Due to serious toxic effects caused by mycotoxins, the surveillance, determination and reduction of these compounds in food and feed is subject to the work of legislative bodies, industry and chemical laboratories. In the frame ERM® a new certified reference materials for *fusarium* mycotoxins in wheat flour (ERM®-BC600) was developed at BAM Federal Institute for Materials Research and Testing.

Mycotoxins are secondary fungal metabolites partially associated with human and animal diseases. Several *fusarium* species are able to produce a large number of structurally different mycotoxins, e.g. fumonisins, zearalenone (ZON) and trichothecenes. The latter group mainly contain type A toxins (e.g. T-2, HT-2) and type B toxins like nivalenol (NIV) and deoxynivalenol (DON). *Fusarium* toxins occur worldwide in a large variety of food and feed commodities, especially in cereals and cereal based products.
Legal limits, already existing for DON and ZON [*Commission Regulation EC 1881/2006 and EC 1126/2007*], are currently under discussion for other *fusarium* toxins (T-2 / HT-2). It is therefore essential to develop and validate analytical methods for the determination of *fusarium* mycotoxins in different foodstuffs that are reliable and capable to detect the toxins within their legal limits.

Food and feed reference materials and especially certified reference materials (CRM) are versatile tools in the verification of the accuracy of analytical measurements. They can be used for the measurement uncertainty estimation, to assess the traceability of the analytical results, or the calibration of analytical instruments.
The reference material ERM®-BC600 was produced for the purpose of quality assurance and quality control for the determination of DON, NIV and ZON in wheat flour. The ERM®-BC600 material is a wheat flour sample from a non-commercial source, not intended for human consumption and naturally contaminated with DON, NIV and ZON.
A total number of 21 laboratories were selected based on documented experience and proficiency and invited to participate in the interlaboratory comparison study (ILC) for certification of the candidate material prepared at BAM.

This report describes the preparation, characterisation and certification of the wheat material including homogeneity and stability studies. The certified mass fractions for DON, NIV and ZON, their uncertainties and the shelf lives were evaluated according to internationally accepted procedures.

# 2 Production of the candidate material

## 2.1 Preparation of the candidate material

Wheat was infected with *fusarium culmorum* during its growth on a test field in the surroundings of Berlin, Germany. After harvest about 21 kg of *fusarium* contaminated wheat kernel were taken as starting material for ERM candidate preparation.

The kernels were ground by a centrifugal mill (ZM 1000, Retsch) to obtain a final particle size smaller than 1 mm. This material was homogenised by means of a drum hoop mixer for 12 hours. Further homogenisation and bottling was done using a version of the so-called "cross riffling" procedure [*van der Veen* et al.]. A total of 256 units were bottled in 250 mL amber glass bottles containing (81 ± 1) g sealed with screw caps containing PTFE-inlays, and numbered in the order of leaving the bottling process. Immediately after bottling the whole batch was stored at -20 °C in a freezer. **Table 1** summarizes the secondary matrix characterisation.

**Table 1**: Matrix characterisation of ERM-BC600

| Measurand | Value | Method |
|---|---|---|
| Particle size range | < 1000 µm | Dry sieving |
| Water content | (10.6 ± 0.2) % | Coulometric Karl-Fischer-Titration |
| C,H,N-Analysis | w(C) = (47.4 ± 0.4) %<br>w(H) = (7.2 ± 0.1) %<br>w(N) = (2.4 ± 0.1) % | Catalytic combustion |

## 2.2 Analytical method

Analyses for homogeneity- and stability studies as well as for certification purposes were carried out at BAM by using the following HPLC-MS/MS method based on a stable isotope dilution analysis (SIDA):

*Sample preparation*

About 5 g of the wheat flour sample were weighed into a 60 mL glass centrifugation tube sealed with a screw cap with PTFE-inlay. An internal standard (ISTD) solution containing $^{13}C_{15}$DON and $^{13}C_{18}$ZON was added to the sample. The sample was suspended in 40 mL acetonitrile : water (84:16; v:v) and extracted by shaking using a horizontal mixer (400 $min^{-1}$) for one hour. The suspension was centrifuged at 3.000 g to separate the solid particles.

For ZON analysis 8 mL of the extract were taken for further steps. For acidification a volume of 80 µL of glacial acetic acid was added to this extract followed by a purification procedure by using a MultiSep 226 AflaZON+ column (RomerLabs, Austria). The purified extract was analysed by HPLC-MS/MS using the LC- and MS- parameter as described below.

For DON/NIV analysis 8 mL of the raw extract were purified by using a MultiSep 225 Trich column (RomerLabs, Austria). The purified extract was analysed by HPLC-MS/MS using the HPLC- and MS- parameters as described below.

**Table 2**: Parameters of the HPLC-MS/MS system

| | Instrument / Measurement conditions |
|---|---|
| **HPLC** | |
| Instrument | Agilent 1100 |
| Column | Eurosphere 100, RP-18, 250 x 4 mm, Particle size 5 µm (Knauer) |
| Mobile phase (Eluent) | A = water, containing 0.1 % acetic acid<br>B = methanol containing 0.1 % acetic acid) |
| Gradient program | **Time (min) / Eluent A / Eluent B**<br>0.00 / 85.0 / 15.0<br>10.00 / 85.0 / 15.0<br>19.00 / 0.0 / 100.0<br>25.00 / 0.0 / 100.0<br>28.00 / 85.0 / 15.0<br>35.00 / 85.0 / 15.0 |
| Flow | 0.5 mL $min^{-1}$ |
| Oven temperature | 30 °C |
| Injection volume | 50 µL |
| **MS/MS detection** | |
| Mass spectrometer | Applied Biosystems API 4000 |
| Ionisation | ESI negative (-) |
| Ion source temperature | 450 °C |
| Modus | Multiple reaction monitoring (MRM) |

The following mass transitions were monitored and used for quantification:

**Table 3**: MRM transitions for native and isotopic mycotoxins

| **Compound** | **MRM transition (m/z)** | **Dwell time (ms)** | **DP (V)** | **CE (eV)** | **CXP (V)** |
|---|---|---|---|---|---|
| DON | 295 $[M-H]^-$ → 265 | 200 | -40 | -22 | -13 |
| $^{13}$C-DON | 310 $[M-H]^-$ → 279 | 200 | -40 | -22 | -13 |
| NIV | 311 $[M-H]^-$ → 281 | 200 | -45 | -22 | -15 |
| ZON | 317 $[M-H]^-$ → 131 | 200 | -80 | -42 | -8 |
| $^{13}$C-ZON | 335 $[M-H]^-$ → 290 | 200 | -80 | -34 | -5 |

**DP**: Declustering potential; **CE**: Collision energy; **CXP**: Cell exit potential

Six-point calibrations were used for quantification of the measured area ratios. Each calibration solution was freshly prepared by weighing. The calibration functions for DON, NIV and ZON (**figures 1a-c**) were assumed to be linear and obtained by regression analysis.

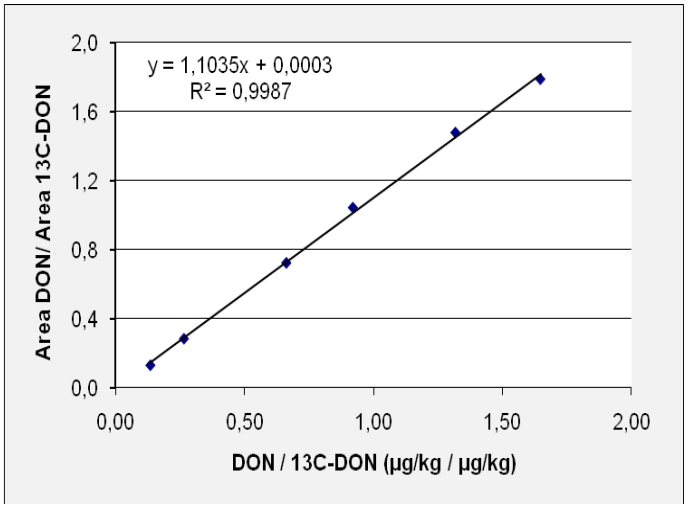


**Figure 1a**: Linear calibration function for deoxynivalenol (DON)

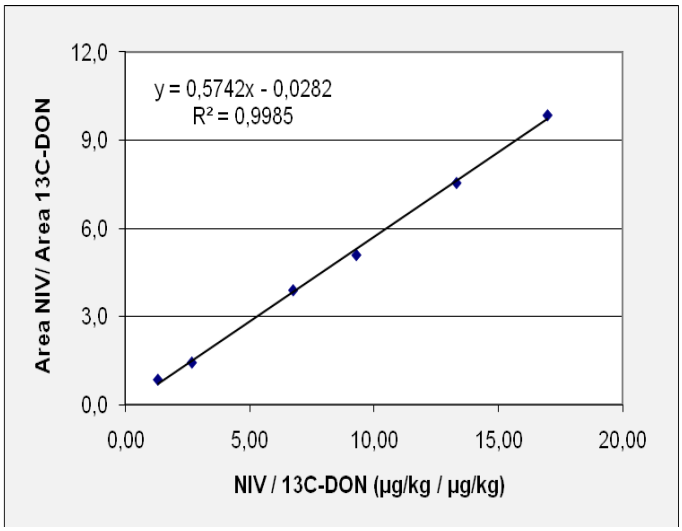


**Figure 1b**: Linear calibration function for nivalenol (NIV)

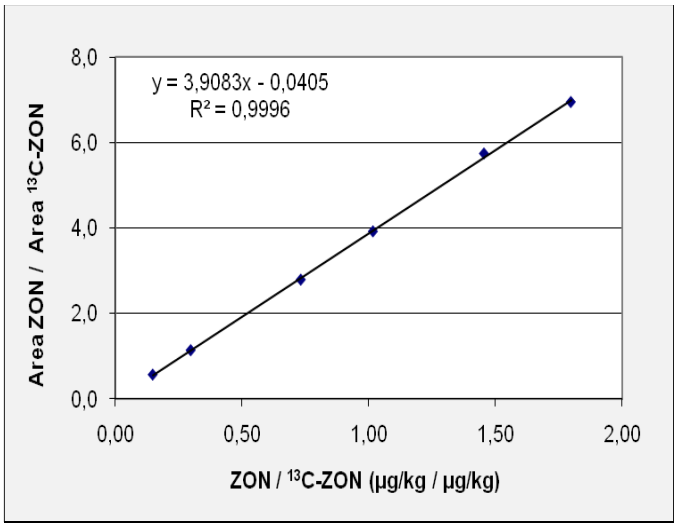


**Figure 1c**: Linear calibration function for zearalenone (ZON)

The isotopic labelled internal standards $^{13}$C-DON and $^{13}$C-ZON were used for quantification of the respective native compounds. $^{13}$C-DON was furthermore used as ISTD for the quantification of NIV. The native mycotoxin calibration standards (DON: 99.4 %, NIV: 98.4 %, ZON: 99.4 %) were purchased from Biopure, Tulln, Austria.

### 2.3 Minimum sample size

The minimum sample intake for one determination should be chosen in a way that no significant heterogeneity within the bottle is to be expected. Homogeneity measurements were successfully evaluated for 5 g sample intake for a single determination. Therefore, a minimum sample intake of 5 g is recommended on the certificate.

## 3 Homogeneity study

Based upon thorough batch homogenisation, and the results of preliminary studies, a satisfactory level of sample homogeneity was expected. For further quantitative demonstration, 10 units were selected randomly from the whole set of 256 bottles and analysed four times each according to the analytical method described before (section 2.2). All 10 units were extracted and processed once under repeatability conditions followed by the second set of extractions and processing in a randomised manner again under repeatability conditions and so on.

Processed extracts were analysed by HPLC-MS/MS (MRM mode) under repeatability conditions guaranteeing that all 40 extracts were quantified versus one calibration after randomisation. The ANOVA results are displayed in **table 4** together with the estimations of the contributions due to the between bottle inhomogeneity ($u_{bb}$) according to [*ISO Guide 35*]. For raw data see Annex A.

**Table 4:** Analysis of Variance (ANOVA) and estimates for uncertainty contribution according to ISO Guide 35

| Compound | $MS_{between}$ ($\mu g^2\ kg^{-2}$) | $MS_{within}$ ($\mu g^2\ kg^{-2}$) | Test criterion $MS_{between} / MS_{within}$ | Critical value $F(f1, f2, 5\%)$ | $u_{bb}$ ($\mu g\ kg^{-1}$) | $u_{bb\_rel}$ (%) |
|---|---|---|---|---|---|---|
| DON | 97.838 | 95.164 | 1.0281 | 2.2107 | 2.478 | 2.47 |
| NIV | 26088.683 | 17194.326 | 1.5173 | 2.2107 | 47.155 | 4.10 |
| ZON | 132.423 | 98.890 | 1.3391 | 2.2107 | 2.895 | 3.03 |

For calculation of $u_{bb}$ the following equations were applied:

$$u_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \qquad \textbf{(1)}$$

$$u_{bb} = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{df_{within}}} \qquad \textbf{(2)}$$

| | |
|---|---|
| $u_{bb}$: | uncertainty between bottles |
| $MS_{between}$: | mean of squared deviations between bottles |
| $MS_{within:}$ | mean of squared deviations within bottles |
| n*:* | Number of replicate analysis |
| $df_{within}$: | Degrees of freedom (within groups) |

Because the test criterion is lower than the critical value, no significant inhomogeneity of the batch was detected. A contribution $u_{bb}$ to the overall uncertainty of the certified reference material was nevertheless derived from the ANOVA results and included in the uncertainty budget of the certified value. For that purpose the higher value for $u_{bb}$ was taken calculated on the basis of **equation 1** and **equation 2**, respectively.

# 4 Stability study

## 4.1 Initial stability study

From experience a temperature-driven deterioration of the mycotoxin contents was to be expected also for this material. Selected units of the candidate material were submitted to accelerated ageing at temperatures between 4 °C and 60 °C over periods of 0.25 months to 12 months as shown in **table 5** to perform a so-called isochronous stability study [*Lamberty et al. 1998*]. Annex B shows the raw data of the initial stability study.

**Table 5**: Accelerated ageing of exposed samples to perform an isochronous stability study

| Ageing [months] | Storage temperature | | | | Remark |
|---|---|---|---|---|---|
| | 4 °C | 23 °C | 40 °C | 60 °C | |
| 0.25 | x | x | x | x | Initial study |
| 0.50 | x | x | x | x | |
| 0.75 | x | x | x | x | |
| 1 | x | x | x | x | |
| 3 | x | x | x | | |
| 6 | x | x | x | | |
| 12 | x | x | x | | |
| 24 | x | x | | | Post-certification monitoring |
| 36 | x | x | | | |
| 48 | x | x | | | |
| 60 | x | x | | | |

After the respective periods of time the exposed units were transferred into a freezer at -20 °C. All units were analysed for DON, NIV and ZON content using the method described in section 2.2 under repeatability conditions together with 4 reference samples which had been kept at -20 °C over the whole period of the initial stability study. Two independent extracts were obtained for each exposed sample and each reference sample. The extracts of the reference samples were evenly distributed over the whole measurement sequence and measured together with the exposed samples.

Data processing and result assessment was carried out in accordance with [*Bremser et al*.] assuming an *Arrhenius* model for the dependence of the reaction rate k(T) on temperature. The plots of the logarithm of the reaction rate ln(k_eff) over the inverse temperature for DON, NIV and ZON are given in **figures 2a-c**.

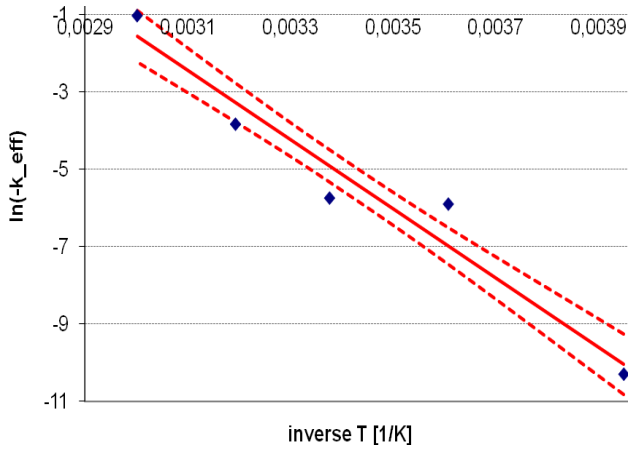


**Figure 2a**: Effective reaction rate for DON in dependence on the inverse temperature (semi-logarithmic plot)

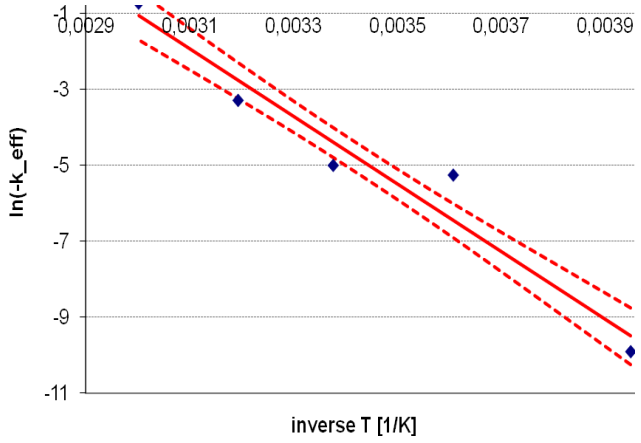


**Figure 2b**: Effective reaction rate for NIV in dependence on the inverse temperature (semi-logarithmic plot)

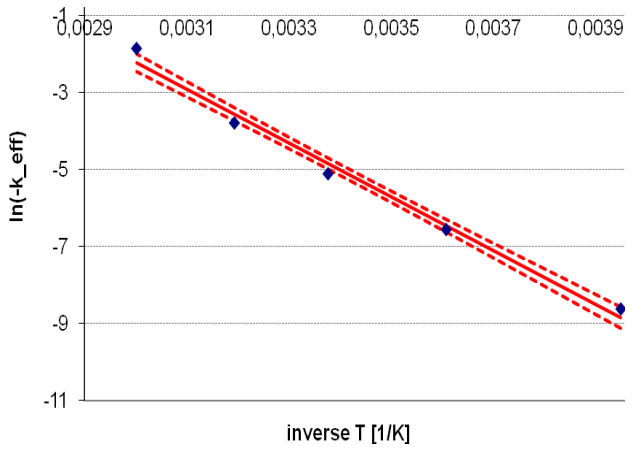


**Figure 2c**: Effective reaction rate for ZON in dependence on the inverse temperature (semi-logarithmic plot)

As obvious from the graph, the temperature dependence can indeed be approximated by a straight line. The corresponding confidence interval for the line is also given in the figure. The estimated activation energies ΔE are: DON: 74.3 kJ/mol; NIV: 73.9 kJ/mol; ZON: 58.0 kJ/mol. These values are in acceptable agreement with activation energies determined for a large variety of organic compounds. By using these data and the assumed model, an estimate can be obtained when degradation will presumably force the mycotoxin content to fall short of the certified lower expanded uncertainty limit. In the sense of a worst-case estimation, these calculations are carried out for the reaction rates at the upper confidence limit of the line as shown in **figure 2**. The results are given in **table 6**.

**Table 6**: Estimation of shelf life

| Temperature °C | Expiry (months) | | |
|---|---|---|---|
| | DON | NIV | ZON |
| -20 | 1209 | 877 | 489 |
| 4 | 77 | 56 | 50 |
| 23 | 10 | 8 | 10 |
| 40 | 2 | 1 | 3 |
| 60 | 0 | 0 | 1 |

The data table will be updated during post-certification monitoring. Shelf life at a storage temperature of 4 °C is considerable but not quite enough for a desirable minimum shelf life of 5 years. This shelf life can reliably be assumed at a storage temperature of -20 °C for all three mycotoxins. However, exposure to temperatures higher than room temperature may reduce the time of validity of ERM-BC600 drastically. Therefore, a common user-end expiry date of **one year after delivery from storage** is established provided the sample is stored at -20 °C at the user's site. Transportation/delivery time should be kept at the possible minimum and any exposure to heat should be avoided.

### 4.2 Post-certification stability monitoring

The first rough estimation of stability will be updated by annual measurements of units stored at -20 °C (reference), 4 °C and 23 °C over the period of availability of the material.

# 5 Certification study

## 5.1 Design of the study

Two units of the candidate reference material (sample_1 and sample_2) were to be analysed by each laboratory in duplicate. In addition, each participant received one unit of the candidate reference material for determination of the overall method recovery. Results ought to be reported based on total mass intake (no dry mass determinations) indicating whether the determined method recovery had already be taken into account or not. Results returned to BAM were scrutinised for consistency.

For measurement control purposes, two HPLC vials containing DON, NIV and ZON in acetonitrile were dispatched for direct HPLC analysis.

## 5.2 Participants of the ILC

A total number of 21 laboratories (**table 7**) were selected to participate in the interlaboratory comparison study (ILC) based on their approved expertise in the field of mycotoxin analysis.

**Table 7**: Participants of the interlaboratory comparison study for certification of ERM-BC600

| Laboratory | City / Country | Compound | | |
|---|---|---|---|---|
| | | DON | NIV | ZON |
| Agentur für Gesundheit und Ernährungssicherheit | Linz, Austria | x | x | x |
| Bundesanstalt für Materialforschung und -prüfung | Berlin, Germany | x | x | x |
| Chemisches und Veterinäruntersuchungsamt | Sigmaringen, Germany | x | x | x |
| Chemisches Untersuchungsinstitut der Stadt Leverkusen | Leverkusen, Germany | x | x | x |
| Eurofins / Wiertz-Eggert-Jörissen | Hamburg, Germany | x | x | x |
| Food GmbH | Jena, Germany | x | x | x |
| Friedrich-Löffler-Institut | Braunschweig, Germany | x | | x |
| General Chemical State Laboratory | Athen, Greece | x | | x |
| Kantonales Labor | Zurich, Switzerland | x | x | |
| Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen | Dresden, Germany | x | x | x |
| Landwirtschaftliche Untersuchungs- und Forschungsanstalt Nordrhein-Westfalen | Münster, Germany | x | | x |
| Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer | Speyer, Germany | x | | x |
| Max-Rubner Institut | Detmold, Germany | x | | x |
| Niedersächisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit | Stade, Germany | x | | x |
| Peri Medizinische Analytik | Sindelfingen, Germany | x | x | x |
| Public Analyst's Laboratory | Dublin, Ireland | x | | x |
| R-Biopharm AG | Darmstadt, Germany | x | | x |
| SGS Germany GmbH | Hamburg, Germany | x | x | x |
| Staatliches Veterinäruntersuchungsamt | Arnsberg, Germany | | | x |
| UIS Umweltinstitut synlab GmbH | Stuttgart, Germany | x | x | x |
| Zentralinstitut für Ernährungs- und Lebensmittelforschung | München, Germany | x | | x |

### 5.3 Methods used by ILC-participants

The participants of the ILC applied methods of their own choice with own calibration standards of known purities.

Extraction was mostly performed by shaking or ultrasonication using acetonitrile : water mixtures for all three compounds (e.g. acetonitrile : water = 84:16, v:v) or pure water for DON and NIV, respectively. Sample preparation, generally including dilution of extract, clean-up and derivatisation steps, was handled in different ways. When a clean-up was applied various kinds of solid phase extraction columns (e.g. MultiSep®, MycoSep®, Bond Elute®) or immunoaffinity columns (IAC) were applied to purify the extracts for DON, NIV and ZON. In some cases (HPLC-MS/MS using internal standards) a clean-up step was omitted. A derivatisation of DON and NIV was necessary either to achieve sufficient sensitivity by using fluorescence detection (FLD) or to increase the volatility to apply GC analysis. For the latter a silylation of DON / NIV was preferably done. The application of a HPLC-FLD method for DON and NIV involves a post-column derivatisation by thermal treatment with sodium hydroxide followed by a chemical reaction to analyse the derived formaldehyde.

For separation of the purified extract mostly liquid chromatography (HPLC) but also gas chromatography (GC) were applied. Different types of detectors (HPLC: UV, FLD, MS // GC: ECD, MS) were used for DON, NIV and ZON depending on sample preparation and separation technique. The tandem MS detection (HPLC-MS/MS) is suited for a sensitive and selective measurement of all three compounds. The HPLC-FLD was an alternative method frequently used for ZON analysis by the participants.

### 5.4 Evaluation of ILC results

The submitted results of the certification study were technically and statistically evaluated in accordance with ISO Guide 35 and the specific requirements of the ERM agreement (for detailed information see: www.erm-crm.org/ermcrm).

**DON**: Deletion of the results of two laboratories (Q and R) for low recovery at the control solutions and high results for the wheat flour sample (**figure 3**). After deletion of Q and R, the result of lab N was detected as outlier (Nalimov test at 0.05 and 0.01 level; Grubbs at 0.05 level) and therefore deleted.

**NIV**: One outlying value (lab G) was detected for NIV (Nalimov test at 0.05 and 0.01 level; Grubbs at 0.05 level) and not taken into account for further processing.

**ZON**: No outlier detected.

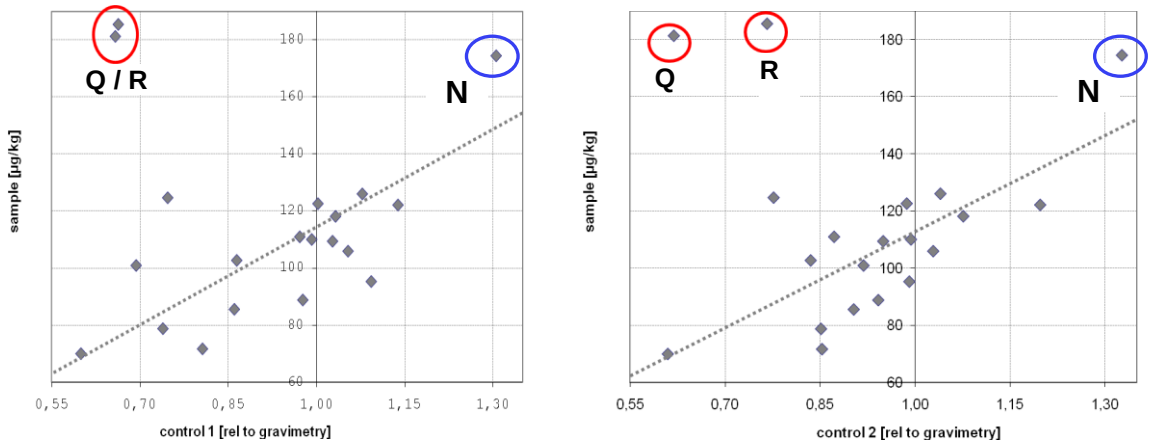


**Figure 3**: Youden plot of the lab mean values for the wheat flour sample (DON) against normalized value of control solution_1 (left) and control solution_2 (right). The suspected labs Q and R are marked in red. Lab N (deleted based on Nalimov and Grubbs test) is marked in blue.

After removal of the indicated laboratories, the accepted data sets for DON, NIV and ZON as shown in **tables 8, 9** and **10** were used for further processing.

**Table 8**: Accepted laboratory data sets for DON

| Lab | ERM-BC600 (sample A) in µg kg$^{-1}$ | | ERM-BC600 (sample B) in µg kg$^{-1}$ | | Lab mean in µg kg$^{-1}$ |
|---|---|---|---|---|---|
| A | 86.3 | 84.2 | 84.2 | 87.4 | 85.5 |
| B | 86.7 | 90.3 | 65.9 | 72.0 | 78.7 |
| C | 62.7 | 74.4 | 68.6 | 74.2 | 70.0 |
| D | 68.9 | 68.9 | 71.9 | 77.0 | 71.6 |
| E | 87.3 | 89.2 | 121.3 | 105.7 | 100.9 |
| F | 145.2 | 101.7 | 96.1 | 97.1 | 110.0 |
| G | 115.4 | 112.1 | 123.2 | 137.6 | 122.1 |
| H | 110.0 | 133.0 | 115.0 | 146.0 | 126.0 |
| I | 97.5 | 106.3 | 121.3 | 118.8 | 110.9 |
| J | 106.6 | 102.5 | 121.8 | 106.6 | 109.4 |
| K | 123.6 | 121.4 | 115.9 | 111.6 | 118.1 |
| L | 93.7 | 112.6 | 91.6 | 83.2 | 95.3 |
| M | 143.8 | 120.8 | 132.3 | 101.7 | 124.6 |
| O | 113.1 | 108.4 | 96.0 | 106.2 | 105.9 |
| P | 116.2 | 122.5 | 131.0 | 120.5 | 122.6 |
| S | 92.5 | 89.7 | 85.6 | 87.3 | 88.8 |
| T | 98.3 | 95.5 | 107.0 | 109.9 | 102.7 |

**Table 9**: Accepted laboratory data sets for NIV

| Lab | ERM-BC600 (sample A) in µg kg$^{-1}$ | | ERM-BC600 (sample B) in µg kg$^{-1}$ | | Lab mean in µg kg$^{-1}$ |
|---|---|---|---|---|---|
| A | 805.1 | 773.5 | 802.0 | 810.2 | 797.7 |
| B | 875.9 | 913.4 | 1088.6 | 963.5 | 960.3 |
| D | 887.3 | 962.0 | 1046.8 | 945.0 | 960.3 |
| E | 802.8 | 769.5 | 1091.8 | 878.3 | 885.6 |
| H | 1259.8 | 1186.6 | 1219.5 | 1251.2 | 1229.3 |
| I | 1140.0 | 1180.0 | 1360.0 | 1400.0 | 1270.0 |
| M | 1245.5 | 993.9 | 1230.3 | 916.7 | 1096.6 |
| O | 947.7 | 1006.7 | 1126.9 | 1023.0 | 1026.1 |
| S | 866.3 | 848.0 | 857.6 | 822.3 | 848.5 |
| T | 933.0 | 940.0 | 937.0 | 901.0 | 927.8 |

**Table 10**: Accepted laboratory data sets for ZON

| Lab | ERM-BC600 (sample A) in µg kg$^{-1}$ | | ERM-BC600 (sample B) in µg kg$^{-1}$ | | Lab mean in µg kg$^{-1}$ |
|---|---|---|---|---|---|
| A | 95.9 | 90.4 | 106.8 | 89.0 | 95.5 |
| B | 93.4 | 84.5 | 89.0 | 86.8 | 88.4 |
| C | 92.1 | 85.9 | 77.8 | 75.0 | 82.7 |
| E | 67.6 | 85.8 | 71.0 | 76.2 | 75.2 |
| F | 97.3 | 124.3 | 91.5 | 104.9 | 104.5 |
| G | 95.1 | 92.8 | 90.4 | 88.1 | 91.6 |
| H | 85.8 | 89.7 | 89.6 | 103.0 | 92.0 |
| I | 120.0 | 98.0 | 98.0 | 92.0 | 102.0 |
| J | 104.0 | 108.1 | 93.6 | 93.6 | 99.8 |
| K | 79.7 | 72.9 | 85.4 | 84.3 | 80.6 |
| L | 79.1 | 73.6 | 82.4 | 81.3 | 79.1 |
| M | 124.0 | 110.4 | 104.2 | 100.0 | 109.6 |
| N | 75.3 | 69.1 | 77.3 | 80.4 | 75.5 |
| O | 91.8 | 88.0 | 86.7 | 88.7 | 88.8 |
| P | 105.5 | 90.6 | 115.0 | 94.1 | 101.3 |
| Q | 92.5 | 87.4 | 93.9 | 109.1 | 95.7 |
| R | 62.5 | 80.8 | 83.7 | 63.5 | 72.6 |
| S | 106.0 | 105.1 | 107.9 | 107.7 | 106.7 |
| T | 95.1 | 104.0 | 101.1 | 101.4 | 100.4 |
| U | 72.0 | 78.0 | 69.0 | 76.0 | 73.8 |

Values given in **tables 8, 9** and **10** are corrected for method recovery determined by each laboratory but not corrected for the purity of the individual calibration standards. The means of laboratory means, displayed in **table 11**, were taken as the uncorrected (for purity) estimates for the values to be certified ($w_{char}$).

**Table 11**: Statistical parameters of the accepted data sets of ILC

| Compound | Mean of means $w_{char}$ (µg kg$^{-1}$) | SD (µg kg$^{-1}$) | u(x) (µg kg$^{-1}$) | CI (µg kg$^{-1}$) | TI (µg kg$^{-1}$) | Pooling | Data sets |
|---|---|---|---|---|---|---|---|
| DON | 102.59 | 18.31 | 4.44 | 9.42 | 52.34 | no | 17 |
| NIV | 1000.21 | 156.6 | 49.53 | 112.05 | 529.25 | no | 10 |
| ZON | 90.79 | 11.85 | 2.65 | 5.55 | 32.61 | no | 20 |

CI Confidence interval of the mean of means at a 0.05 significance level
TI Tolerance interval of the mean of means at a 95 % confidence level

Participants of the ILC used different methods or implementations for extraction, clean-up, HPLC/GC and detection. Obviously there was no good reason for assuming that the single values measured by the different laboratories would belong to a common population. Single measurement results cannot be pooled, and therefore the means of laboratory means were considered to be appropriate estimates for the mycotoxin mass fractions of the reference material.

### 5.5 Certified values and uncertainty budget

The estimate $w_{char}$ (mean of lab means according to results given in **table 11** for DON, NIV and ZON) must be corrected for the purity of the calibration standard ($f_{pur}$) used at coordinator's site according to **equation 3** to give the certified value ($w_{cert}$) listed in **table 12**.

$$w_{cert} = w_{char} \cdot f_{pur} \qquad \textbf{(3)}$$

**Table 12**: Estimates for the certified values for DON, NIV and ZON

| Compound | $w_{char}$ in µg kg$^{-1}$ | $f_{pur}$ | $w_{cert}$ in µg kg$^{-1}$ |
|---|---|---|---|
| Deoxynivalenol (DON) | 102.59 | 0.997 | 102.28 |
| Nivalenol (NIV) | 1000.21 | 0.992 | 992.21 |
| Zearalenone (ZON) | 90.79 | 0.997 | 90.52 |

The values of $f_{pur}$ given in **table 12** were calculated based on a rectangular distribution of the certified purities of the calibration standards. The combined uncertainty ($u_{com}$) is composed from the contributions in **equation 4**, which are summarized in **table 13**.

$$u_{com}^2 = u_{char}^2 + u_{bb}^2 + u_{lts}^2 + u_{pur}^2 + u_{trc}^2 \qquad \textbf{(4)}$$

**Table 13**: Contributions to the uncertainty of the mycotoxin mass fractions of ERM-BC600

| Uncertainty contribution | | DON | | NIV | | ZON | |
|---|---|---|---|---|---|---|---|
| | | rel. (%) | µg kg$^{-1}$ | rel. (%) | µg kg$^{-1}$ | rel. (%) | µg kg$^{-1}$ |
| Uncertainty of characterisation (standard deviation of the mean of lab means) | $u_{char}$ | 4.3 | 4.43 | 5.0 | 49.38 | 2.9 | 2.64 |
| Contribution from a possibly undetected inhomogeneity | $u_{bb}$ | 2.47 | 2.52 | 4.10 | 40.65 | 3.03 | 2.75 |
| Contribution from long term stability (sufficiently stable for shelf lives up to 5 years) | $u_{lts}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| Uncertainty of the purity of used calibration standard | $u_{pur}$ | 0.173 | 0.177 | 0.461 | 4.583 | 0.173 | 0.157 |
| Traceability adjustment (half of the difference between BAM value and mean of the ILC) | $u_{trc}$ | | 1.66 | | 12.93 | | 0.989 |
| Combined uncertainty of ERM-BC600 | $u_{com}$ | | 5.36 | | 65.42 | | 3.94 |
| Expanded uncertainty of ERM-BC600, coverage factor $k$ = 2 | $U_{ERM}$ | | 10.73 | | 130.9 | | 7.88 |

The certified values for ERM-BC600 are summarized in **table 14** together with the expanded uncertainty $U_{ERM}$ calculated based on a coverage factor $k$ = 2. The values and the expanded uncertainties are rounded according to the recommendations of the Guide to the Expression

of Uncertainty in Measurement [*ISO Guide 98, Part 3*] and are given with respect to raw sample mass.

The water content was seen to remain stable if the material is handled according to the instructions in the certificate (see also clause 6).

**Table 14**: Certified mass fractions of ERM-BC600

| Compound | Mass fraction in µg kg$^{-1}$ | | |
|---|---|---|---|
| | **Certified value** | **Uncertainty** | **Expanded uncertainty** |
| DON | 102 | 5 | 11 |
| NIV | 1000 | 65 | 130 |
| ZON | 90 | 4 | 8 |

### 5.6 Traceability

Beside the fact that all laboratories providing accepted data used validated and calibrated methods, traceability of the certified values was directly established to stated references of the pure mycotoxins using the BAM certification method – stable isotope dilution analysis (SIDA) using $^{13}$C-isotopic labelled internal standard for HPLC-MS/MS measurement. These measurements took traceability from pure reference substances (DON: 99.4 %, NIV: 98.4 %, ZON: 99.4 %; Biopure, Tulln, Austria) with purities independently confirmed by UV absorption measurements.

## 6 Information on the proper use of ERM-BC600

### 6.1 Shelf life

From the initial stability study, a considerably large shelf life well above a period of 5 years at a storage temperature of -20 °C was estimated. Since the dispatch to the end user may occur at any time during this period the certified properties will be valid for 12 months beginning with the dispatch of the material from BAM. The validity of this information will be maintained by the post-certification monitoring.

### 6.2 Transport, storage and use

Due to the proved stability of the reference material a cooled dispatch is not necessary during transport. On receiving, the bottle is to be stored at a temperature equal to or lower than -20 °C. Before withdrawing a sub-sample the bottle should be allowed to reach room temperature and be mixed thoroughly. Thereafter, the bottle must be closed tightly and stored at a temperature equal to or lower than -20 °C. The water content remains stable when the material is treated as described.

### 6.3 Safety instructions

No hazardous effects are to be expected when the material is used under conditions usually adopted for the analysis of foodstuff matrices low / moderately contaminated with DON, NIV and ZON. It is strongly recommended to handle and dispose of the reference material in accordance with the guidelines for hazardous materials legally in force at the site of end use and disposal.

### 6.4 Legal notice

Neither the BAM Federal Institute for Materials Research and Testing nor any person acting on their behalf make any warranty or representation, express or implied, that the use of any information, material, apparatus, method or process disclosed in this document may not infringe privately owned rights, or assume any liability with respect to the use of, or damages resulting from the use of any information, material, apparatus, method or process disclosed in this document.

## 7 References

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# 8 Annexes

**Annex A**: Raw data of homogeneity testing for DON, NIV and ZON in ERM-BC600

| | DON content (µg kg$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bottle-No.** | **1** | **2** | **3** | **4** | **Mean** | **SD** | **RSD (%)** |
| 9 | 102.52 | 105.09 | 92.85 | 100.82 | 100.32 | 5.28 | 5.26 |
| 52 | 85.55 | 89.87 | 79.45 | 105.12 | 90.00 | 10.95 | 12.17 |
| 79 | 107.53 | 90.08 | 116.70 | 96.13 | 102.61 | 11.85 | 11.55 |
| 95 | 114.58 | 110.32 | 101.24 | 101.30 | 106.86 | 6.68 | 6.25 |
| 110 | 113.82 | 102.30 | 96.41 | 115.55 | 107.02 | 9.20 | 8.59 |
| 150 | 101.79 | 106.25 | 104.60 | 89.84 | 100.62 | 7.42 | 7.38 |
| 177 | 110.46 | 89.72 | 86.86 | 97.26 | 96.08 | 10.55 | 10.98 |
| 206 | 108.73 | 93.71 | 106.18 | 97.49 | 101.53 | 7.09 | 6.99 |
| 227 | 115.02 | 90.71 | 103.75 | 87.45 | 99.23 | 12.66 | 12.76 |
| 256 | 114.76 | 101.73 | 102.71 | 84.31 | 100.88 | 12.53 | 12.42 |
| **Mean** | | | | | **100.51** | | |

| | NIV content (µg kg$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bottle-No.** | **1** | **2** | **3** | **4** | **Mean** | **SD** | **RSD (%)** |
| 9 | 1121.83 | 1177.19 | 1495.09 | 1434.61 | 1307.18 | 185.11 | 14.16 |
| 52 | 1106.11 | 1114.78 | 973.78 | 1240.28 | 1108.74 | 108.87 | 9.82 |
| 79 | 1148.48 | 1204.43 | 1176.29 | 1069.60 | 1149.70 | 58.08 | 5.05 |
| 95 | 1156.37 | 1317.80 | 1194.69 | 996.01 | 1166.22 | 132.73 | 11.38 |
| 110 | 1210.02 | 1008.60 | 1072.28 | 1029.19 | 1080.02 | 90.63 | 8.39 |
| 150 | 1253.74 | 998.17 | 912.81 | 913.35 | 1019.52 | 161.22 | 15.81 |
| 177 | 1258.07 | 1150.12 | 981.93 | 1060.10 | 1112.56 | 118.88 | 10.69 |
| 206 | 1303.52 | 1047.76 | 999.93 | 1249.84 | 1150.26 | 148.90 | 12.94 |
| 227 | 1355.95 | 1152.49 | 1119.79 | 1065.87 | 1173.52 | 126.75 | 10.80 |
| 256 | 1389.42 | 1219.44 | 1285.46 | 1066.93 | 1240.31 | 135.11 | 10.89 |
| **Mean** | | | | | **1150.80** | | |

| | ZON content (µg kg$^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Bottle-No.** | **1** | **2** | **3** | **4** | **Mean** | **SD** | **RSD (%)** |
| 9 | 100.18 | 99.70 | 106.96 | 89.51 | 99.09 | 7.19 | 7.26 |
| 52 | 94.32 | 95.33 | 74.71 | 86.75 | 87.78 | 9.52 | 10.84 |
| 79 | 82.61 | 86.70 | 104.74 | 110.20 | 96.06 | 13.46 | 14.01 |
| 95 | 95.11 | 87.70 | 100.17 | 104.73 | 96.93 | 7.30 | 7.53 |
| 110 | 91.73 | 85.60 | 93.67 | 84.89 | 88.97 | 4.38 | 4.93 |
| 150 | 86.50 | 108.46 | 77.65 | 90.87 | 90.87 | 12.95 | 14.25 |
| 177 | 85.13 | 109.15 | 105.32 | 86.34 | 96.48 | 12.52 | 12.98 |
| 206 | 95.92 | 88.65 | 105.20 | 96.87 | 96.66 | 6.77 | 7.01 |
| 227 | 91.11 | 112.69 | 114.08 | 113.81 | 107.92 | 11.23 | 10.40 |
| 256 | 86.54 | 86.30 | 107.09 | 95.84 | 93.94 | 9.82 | 10.46 |
| **Mean** | | | | | **95.47** | | |

**Annex B**: Raw data of stability testing for ERM-BC600. The mass fractions of DON, NIV and ZON are given in µg $kg^{-1}$.

| DON | Storage temperature (°C) | | | |
|---|---|---|---|---|
| **Time (months)** | **4** | **23** | **40** | **60** |
| 0.25 | 129.6420684 | 118.6655278 | 102.5521 | 84.65902 |
| 0.25 | 129.1912076 | 94.80983493 | 111.3507 | 75.50005 |
| 0.5 | 120.3241713 | 98.42057098 | 121.4215 | 68.86757 |
| 0.5 | 93.88053998 | 117.0520657 | 118.0445 | 65.66632 |
| 0.75 | 121.9435731 | 121.0645179 | 113.7293 | 66.35728 |
| 0.75 | 118.4515361 | 109.7369607 | 128.7232 | 84.15299 |
| 1 | 120.1404701 | 130.50264 | 128.0524 | 54.39192 |
| 1 | 118.8603776 | 113.5631736 | 100.503 | 86.89142 |
| 3 | 126.7283413 | 121.24904 | 109.586 | |
| 3 | 120.8294967 | 110.8416584 | 100.2709 | |
| 6 | 107.8279679 | 127.9618219 | 77.94277 | |
| 6 | 95.54425059 | 129.3112054 | 102.3402 | |
| 9 | 99.52132262 | 115.5554556 | 73.46481 | |
| 9 | 103.4633354 | 121.4473492 | 96.16578 | |
| 12 | 120.320775 | 123.7393023 | 110.8923 | |
| 12 | 126.0855581 | 110.2355228 | 90.00545 | |

| NIV | Storage temperature (°C) | | | |
|---|---|---|---|---|
| **Time (months)** | **4** | **23** | **40** | **60** |
| 0.25 | 900.7491207 | 988.6197689 | 1051.415 | 759.2306 |
| 0.25 | 952.0026473 | 812.6403733 | 985.9687 | 711.7057 |
| 0.5 | 1004.148787 | 1017.759996 | 941.8066 | 734.2201 |
| 0.5 | 998.0012292 | 846.9520954 | 1013.257 | 753.0591 |
| 0.75 | 1051.352243 | 987.9806045 | 861.8703 | 680.1644 |
| 0.75 | 1030.825182 | 961.7486251 | 942.7972 | 716.5724 |
| 1 | 912.7621299 | 1020.694581 | 898.6353 | 537.9358 |
| 1 | 1044.838936 | 1071.828749 | 886.7833 | 552.8058 |
| 3 | 933.3289718 | 958.6593439 | 782.7585 | |
| 3 | 980.7888873 | 910.2159079 | 906.7836 | |
| 6 | 950.1508652 | 1077.954206 | 688.14 | |
| 6 | 950.5558723 | 1014.431105 | 735.4884 | |
| 9 | 875.5502353 | 918.1010101 | 632.5341 | |
| 9 | 991.2323743 | 952.1406053 | 699.864 | |
| 12 | 1019.869492 | 1092.254536 | 739.8909 | |
| 12 | 942.6209423 | 929.394517 | 675.6395 | |

| ZON | Storage temperature (°C) | | | |
|---|---|---|---|---|
| **Time (months)** | **4** | **23** | **40** | **60** |
| 0.25 | 98.16955741 | 109.2881607 | 86.88635 | 78.95129 |
| 0.25 | 112.3427414 | 90.8154189 | 89.35201 | 88.49337 |
| 0.5 | 96.37206785 | 87.48536447 | 87.27933 | 94.5344 |
| 0.5 | 97.44528412 | 101.9478253 | 100.5834 | 75.34041 |
| 0.75 | 100.5864887 | 105.6556967 | 93.81981 | 93.19071 |
| 0.75 | 95.27651754 | 92.82480945 | 111.4209 | 83.18278 |
| 1 | 109.6532918 | 110.1818378 | 94.95568 | 68.91159 |
| 1 | 99.77675953 | 103.6568992 | 96.70716 | 82.96029 |
| 3 | 96.13590121 | 92.75390291 | 88.52773 | |
| 3 | 94.53551682 | 88.479492 | 94.93456 | |
| 6 | 93.21133599 | 103.3991904 | 65.98704 | |
| 6 | 114.5990171 | 105.6591489 | 85.37849 | |
| 9 | 115.461442 | 81.40661513 | 79.22527 | |
| 9 | 87.61750358 | 98.06478711 | 71.56406 | |
| 12 | 93.08486908 | 84.21245749 | 83.70388 | |
| 12 | 119.2943068 | 106.247443 | 69.87231 | |