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## Production of a calibrant certified reference material for determination of the estrogenic mycotoxin zearalenone

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**Abstract** Several previous interlaboratory studies in the field of mycotoxin analysis have revealed considerable problems, apparent as high between-laboratory standard deviations, or rather non-comparable and non-traceable results. A major reason is lack of proper calibrants for external calibration. Public awareness of substances that mimic or interfere with the activity of natural hormones (endocrine disrupters) has led to increased interest in mycotoxins with estrogenic potential, e.g. zearalenone (ZON). During a large-scale standard measurement and testing (SMT) project of the European Commission (EC) dealing with the preparation and certification of reference materials for determination of the mycotoxin ZON in maize, a ZON calibrant in acetonitrile was prepared and intensively checked for purity, homogeneity, and stability. Preparation of the material, study of its homogeneity and stability, and characterisation of the calibrant on the basis of its preparation, with discussion of the results obtained, are described in this paper. The certified value of  $9.95 \mu\text{g mL}^{-1}$  for ZON in acetonitrile and its corresponding expanded uncertainty of  $\pm 0.30 \mu\text{g mL}^{-1}$  were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM).

**Keywords** Calibrant · Certified reference material (CRM) · Expanded uncertainty · Mycotoxin · Zearalenone

### Introduction

Mycotoxins are toxic natural secondary metabolites formed by fungi on agricultural commodities in the field or during storage, which cause food- and feed-borne intoxication in man and farm animals. The only class of estrogenic mycotoxins so far identified are the zearalenones. Their precursor, zearalenone (ZON, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)- $\beta$ -resorcylic acid lactone, CAS No.: 17924–92–4), is a secondary fungal metabolite produced by several species of the *Fusarium* fungi, especially *F. graminearum* and *F. culmorum* in both the temperate and the warm regions of the world [1, 2]. In relation to food and feed, ZON is, with aflatoxin B<sub>1</sub> and M<sub>1</sub>, deoxynivalenol, ochratoxin A, and the fumonisins, one of the most important mycotoxins world-wide [3]. Although the reported incidence rates and concentration levels in cereals and mixed feed vary considerably, maize is the most frequently contaminated commodity. Although ZON has been characterised as a compound that is not acutely toxic, because a single oral dose of up to  $20 \text{ g kg}^{-1}$  b.w. does not cause death in mice and rats [4], its marked estrogenic and anabolic properties (endocrine disrupter) can cause severe reproductive and infertility problems in farm animals, especially swine [5].

The potential health risks of ZON contamination to humans and animals have been addressed by several food authorities in different countries [6] and will increasingly become a quality criterion in trade. Hence, accurate and reliable analytical methods are required which are capable of determination of ZON at levels down to  $50 \mu\text{g kg}^{-1}$  in naturally contaminated samples of cereals and cereal-based food and feed [7, 8].

Two international interlaboratory studies on ZON in maize organised by IFA-Tulln in 1996 [9] and 1998 [10] produced different results. Satisfactory relative between-

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laboratory standard deviations ( $RSD_R$ ) of 15.0 to 27.7% for two ZON spiked maize materials and 16.6% for a naturally ZON contaminated maize were obtained in the interlaboratory study in 1996. The reasonable precision and accuracy were mainly because of the use of a common ZON calibrant provided by the IFA-Tulln which had to be used for calibration by the participating laboratories. The interlaboratory study performed in 1998 was designed without providing a common ZON calibrant. High  $RSD_R$  of 27.7% for ZON-spiked maize and 40.5 to 41.2% for two naturally ZON-contaminated maize materials were obtained under realistic routine analytical conditions using in-house ZON calibrants. These results confirmed the findings from the study of 1996 that besides matrix-certified reference materials (CRM) for ZON in maize a certified ZON calibrant is also crucial to improve and ensure the comparability and reliability of measurement results for the determination of ZON.

For this reason, a large-scale project [11] involving 27 laboratories was launched in 1998, in the framework of the Standard Measurement and Testing (SMT) programme, by the European Commission (EC), with the major aim of producing two matrix CRM for ZON in maize. Because  $RSD_R$  in the 1996 and 1998 studies were not acceptable for the purpose of characterisation of CRM, even though the  $RSD_R$  criteria of  $\leq 40\%$  for ZON at concentration levels of  $>100 \mu\text{g kg}^{-1}$  established by CEN [12] were usually fulfilled, a comprehensive feasibility study was performed at the beginning of the project. After the laboratories improved their analytical performance within the feasibility study one material for ZON in maize (BCR-717 "low level ZON") was successfully certified for a value of  $83 \mu\text{g kg}^{-1}$  with a corresponding uncertainty of  $9 \mu\text{g kg}^{-1}$  by means of the characterisation. Because of the very low level of ZON contamination of the other maize reference material (BCR-716 "very low level ZON") it was only possible to certify the ZON content as being less than  $5 \mu\text{g kg}^{-1}$  [13].

Besides production of two CRM for ZON in maize, it was also intended to prepare a ZON calibrant to serve as a common calibrant during the feasibility study and later as a commercially available CRM [14].

In contrast with the certification of the ZON content in the maize CRM, the ZON calibrant has been certified on the basis of preparation. Besides the influence of inhomogeneity and instability, the certified value is mainly based on the purity and weighed amount of crystalline ZON and solvent (acetonitrile) rather than on the mean value of measurement results obtained by several laboratories for this calibrant. However, the concentration thus calculated was also confirmed by UV spectrophotometry in a mini-intercomparison study. Therefore, as a first step, the molar absorptivities of ZON in acetonitrile had to be determined, by a mini-interlaboratory study among the three main project partners, by UV spectrophotometry. Furthermore, the concentrations of the common ZON calibrant and the ZON calibrants individually prepared by the three main partners were determined by UV spectrophotometry in another mini-interlaboratory study. The concentrations

primarily calculated on the basis of the preparation for the common and individual ZON calibrants confirmed the results obtained by UV spectrophotometry [15].

In the following discussion the whole approach for production of the calibrant CRM for ZON in acetonitrile, named BCR-699, in compliance with the GUM [16], ISO Guide 31 and 34 [17, 18] and will be described, as a typical example. Nomenclature according to ISO Guide 34 (1998) is used in this paper which means "production" of a reference material (RM) refers to the process comprising planning, material processing, homogeneity and stability testing, characterisation, and value assignment. Production of a material in a technical sense is referred to as "processing".

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### Purity of the material

First, the purity of one lot (993 mg) of crystalline ZON purchased from Sigma-Aldrich (Dorset, UK) was investigated. The purity of the ZON was examined by UV spectrophotometry (UV), liquid chromatography (LC) with diode-array detection (DAD) and fluorescence detection (FLD), ion chromatography (IC), liquid chromatography with tandem mass spectrometry (LC-MS-MS),  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy, and differential scanning calorimetry (DSC) as described in detail by Krška et al. [19].

DSC analysis and the UV spectrum of ZON showed no detectable impurities. Likewise, no impurities were observed by LC analysis with both DAD and FLD detection. IC showed negligible contamination of the ZON with chloride of  $0.020 \pm 0.005\%$  and nitrate of  $0.016 \pm 0.006\%$ . Zearalanone (ZAN) was identified as one of two minor impurities (0.2%) by LC-MS-MS. The  $^1\text{H}$  NMR measurements showed an additional peak, which has not been previously reported in the literature. It was identified as part of the ZON spectrum – the signal arising from the phenolic proton attached to  $\text{C}_4'$ . The manufacturer declares an impurity of 0.2% dichloromethane, which was confirmed to be 0.1% by  $^1\text{H}$  NMR. Other minor impurities, whose structures remain unknown, were discovered at 3.5 and  $<1$  ppm. On the basis of on these findings, the total amount impurities might thus range from 0.34% on the basis of UV, LC-DAD, DSC, LC-MS-MS and IC measurements to 1% on the basis of NMR measurements. Therefore, a purity of 99.5% with a tolerance of  $\pm 0.5\%$  was finally attributed to the ZON studied in this project. The conservative assumption of a rectangular distribution was made and the tolerance divided by the square root of 3 resulting in an uncertainty of  $u_c(P) = 0.3\%$  for the purity.

The purity of the acetonitrile was determined by  $^1\text{H}$  and  $^{13}\text{C}$  NMR measurements. It was concluded that within the sensitivity of the instrument used the acetonitrile was 100% pure. Thus, the associated uncertainty was assumed to be negligible and was not considered, in accordance with the GUM [16].

## Processing of the material

A stock solution of  $1000 \mu\text{g mL}^{-1}$  was produced gravimetrically by dissolving  $502.41 \pm 0.01$  mg of the crystalline ZON in  $394.74 \pm 0.01$  g acetonitrile ( $502.41$  mL at  $20^\circ\text{C}$ ). Acetonitrile was HPLC Grade S (far-UV) from one batch produced by Rathburns Chemicals (Walkerburn, EH43 65U, UK) as described in detail by Josephs et al. [15].

About 5 L of a solution of approx.  $10 \mu\text{g mL}^{-1}$  ZON in acetonitrile were prepared which served as a common ZON calibrant during the whole study. This calibrant was also subjected to certification on the basis of preparation as described in this paper. Therefore,  $39.16 \pm 0.01$  g of the  $1000 \mu\text{g mL}^{-1}$  stock solution ( $49.84$  mL at  $20^\circ\text{C}$ ) was transferred to a 5-L flask. The flask was diluted to volume with acetonitrile and weighed  $3916.10 \pm 0.01$  g ( $4984.2$  mL at  $20^\circ\text{C}$ ). The final solution was subdivided to ten 500 mL bottles. In the end, a volume of 4 mL of the final solution was dispensed into amber glass ampoules under dry ice conditions using an electronic pipette. One thousand two hundred and fifteen ampoules were sealed with an ampoule sealer (Adelphi Twen Jet) using a supply of natural gas and compressed air.

The ampoules and solutions were stored in a freezer at  $-18^\circ\text{C}$  between use for preparation of dilute solutions. A solution of approx.  $30 \mu\text{g mL}^{-1}$  ZON in acetonitrile, to be used for the determination of the molar absorptivity, was also prepared on the basis of the  $1000 \mu\text{g mL}^{-1}$  stock solution.

## Characterisation

Characterisation refers to the measurements that form the basis of the value that is assumed to be the best estimate of the “true content” of the material. Typically, characterisation is carried out by one or more laboratories performing measurements by one or more analytical methods. For most matrix CRM, the preferred option is to randomise method biases by using several different, validated methods performed by several independent laboratories. Estimation of the uncertainty of the batch characterisation will be performed either by use of a model based on analytical results submitted or an approach based on the full uncertainty budgets submitted by the participants [20].

However, in this characterisation of a ZON calibrant CRM an approach involving assessment of the mass concentration and its corresponding characterisation uncertainty ( $u_{\text{char}}$ ) on the basis of the preparation of the calibrant solution was found to provide the best estimate of the “true content”. In addition, the ZON mass concentration of the gravimetrically prepared calibrant was confirmed by a mini-interlaboratory study as described in detail by Josephs et al. [15].

For calculation of the characterisation uncertainty the main contributions were selected and expressed together with the corresponding uncertainties. The purity of the crystalline ZON (Purity), the weighing procedure ( $m(\text{ZON})$ )

and three gravimetric dilution steps with acetonitrile ( $V_X(\text{ACN})$ ) could be identified as the main contribution to the uncertainty.

As mentioned already, the purity of the crystalline ZON was found to be 99.5% assuming a maximum tolerance of  $\pm 0.5\%$  which results in an uncertainty of  $u_c(\text{Purity})=0.3\%$  considering the conservative assumption of a rectangular distribution.

For the preparation of the first solution of ZON in acetonitrile, the mass of the crystalline ZON was calculated to be  $m(\text{ZON})=502.41$  mg. An uncertainty of  $u_c(m(\text{ZON}))=0.032$  mg was estimated taking into account the repeatability (0.03 mg) and linearity (0.012 mg) of the microbalance.

Apart from the repeatability and the linearity of the weighing process the uncertainties of the three gravimetric dilutions are mainly influenced by the volume expansion of the acetonitrile and as a consequence of the temperature. The laboratory was maintained at  $20 \pm 2^\circ\text{C}$  throughout all the work described. Therefore, all calculations of mass to volume for acetonitrile were made using the density of  $0.7857 \text{ g cm}^{-3}$  at  $T=20^\circ\text{C}$ . For further calculations of the volume expansion of acetonitrile the temperature was considered by assuming a maximum temperature variation of  $\pm 2^\circ\text{C}$ . The respective relative volume expansion coefficient of acetonitrile is  $1370 \times 10^{-6} \text{ }^\circ\text{C}^{-1}$  [21]. In consideration of the rectangular distribution the volume expansion term has to be divided by the square root of 3. Thus, volume expansion uncertainties of  $u_{V_1}(\text{Vol.exp.})=0.795$  mL for  $V_1=502.41$  mL,  $u_{V_2}(\text{Vol.exp.})=0.079$  mL for  $V_2=49.841$  mL and  $u_{V_3}(\text{Vol.exp.})=7.885$  mL for  $V_3=4984.2$  mL were calculated.

According to Eq. (1) combination of the uncertainty contributions of the linearity, repeatability, and volume expansion uncertainties results in combined uncertainties of  $u(V_1)=0.795$  for  $V_1$ ,  $u(V_2)=0.080$  for  $V_2$  and  $u(V_3)=7.885$  for  $V_3$  for the three acetonitrile dilutions:

$$u_c(V_X) = \sqrt{u(\text{Rep})^2 + u(\text{Lin})^2 + u_{VX}(\text{Vol.exp.})^2} \quad (1)$$

As an estimate for the characterisation uncertainty ( $u_{\text{char}}$ ) the expanded uncertainty of the ZON concentration was obtained by quadratic summation of the standard uncertainties determined above and by multiplication by a coverage factor of  $k=2$ . The summation was performed according to Eqs. (2) and (3):

$$U_c(c_{\text{ZON}}) = c_{\text{ZON}} \times \sqrt{\left(\frac{u_c(P)}{P}\right)^2 + \left(\frac{u_c(m)}{m}\right)^2 + \left(\frac{u_c(V_1)}{V_1}\right)^2 + \left(\frac{u_c(V_2)}{V_2}\right)^2 + \left(\frac{u_c(V_3)}{V_3}\right)^2} \quad (2)$$

$$c_{\text{ZON}} = \frac{1000 \times m \times V_2 \times P}{V_1 \times V_3} \quad (3)$$

Utilising a ZON concentration of  $c_{\text{ZON}}=9.950 \mu\text{g mL}^{-1}$ , ZON purity of  $P=0.995$ , ZON mass of  $m=502.41$  mg, dilution volumes of  $V_1=502.41$  mL,  $V_2=49.841$  mL and

$V_2=4984.2$  mL and the corresponding uncertainties of  $u_c(P)=0.003$ ,  $u_c(m)=0.032$  mg,  $u_c(V_1)=0.795$  mL,  $u_c(V_2)=0.080$  mL and  $u_c(V_3)=7.885$  mL a combined standard uncertainty of  $U_c(c_{ZON})=0.0398$   $\mu\text{g mL}^{-1}$  and relative combined standard uncertainty of  $RU_c(c_{ZON})=0.40\%$  was obtained for ZON in acetonitrile.  $U_c(c_{ZON})$  was multiplied by a coverage factor of  $k=2$  to obtain the expanded uncertainty of ZON in acetonitrile of  $U(c_{ZON})=0.0796$   $\mu\text{g mL}^{-1}$  which corresponds to a relative expanded uncertainty of  $RU(c_{ZON})=0.80\%$ .

Finally, a value of  $9.95$   $\mu\text{g mL}^{-1}$  for the ZON mass concentration was attributed to ZON calibrant in acetonitrile. The combined standard uncertainty of  $\pm 0.0398$   $\mu\text{g mL}^{-1}$ , corresponding to  $0.40\%$ , was used as an estimate of the characterisation uncertainty ( $u_{\text{char}}$ ). The total combined uncertainty of the ZON calibrant was then multiplied by a coverage factor of  $k=2$  (cf. certified value and its associated uncertainty).

This ZON mass concentration was also subjected to a comparison with the mean of means of  $9.81\pm 0.20$   $\mu\text{g mL}^{-1}$ ,  $10.11\pm 0.16$   $\mu\text{g mL}^{-1}$  and  $10.07\pm 0.28$   $\mu\text{g mL}^{-1}$  obtained from a mini-intercomparison study on the basis of UV spectrophotometric measurements at 236 nm, 274 nm and 314 nm, respectively. No statistical deviation could be observed by a  $t$ -test as described by Josephs et al. [15].

## Homogeneity testing

Homogeneity testing of the ZON calibrant was performed by selecting approximately every 50th ampoule ( $n=24$ ) of the 1215 ampoules during the filling sequence. The analytical sequence for the ampoules was selected by use of a random-number generator. The homogeneity measurements were made by employing an UV spectrophotometer. Measurements were performed in duplicate ( $n=2$ ) at a wavelength of 274 nm with a bandwidth of 0.5 nm. Quartz cuvettes ( $d=10$  mm) were used under temperature-controlled conditions ( $+25$  °C) and a molar absorptivity of  $\epsilon(274 \text{ nm})=12623\pm 111$  (CI(95%)) was applied, which was determined for ZON in acetonitrile by the three main partners of the project [15]. The UV spectrophotometer was calibrated according to the AOAC Official Method [22] procedure employing potassium dichromate NIST SRM 935. An additional check was performed using the optical density CRM from the Laboratoire National d'Essais, Paris [23].

The results of the final homogeneity testing for ZON in acetonitrile were evaluated by ANOVA [24], which enables separation of the method variation ( $s_{\text{wb}}$ ) from the experimental averages over one unit ( $u_{\text{c,bb}}$ ) to obtain an estimation for the real variation between units ( $s_{\text{bb}}$ ) as shown in Eq. (4):

$$u_{\text{c,bb}}^2 = s_{\text{bb}}^2 + \frac{s_{\text{wb}}^2}{n} \quad (4)$$

The standard deviation between the units was used as the estimator for the between-units variance. The measurement variation sets a lower limit  $u_{\text{bb}}^*$  to this estimator, which is given by the Eq. (5):

$$u_{\text{bb}}^* = \sqrt{\frac{MS_{\text{within}}}{n}} \sqrt{\frac{2}{v_{MS_{\text{within}}}}} \quad (5)$$

where  $MS_{\text{within}}$ ,  $n$ , and  $v_{MS_{\text{within}}}$  are the mean squares within units, the number of measurements per unit ( $n=2$  for duplicate analysis), and the degrees of freedom of  $MS_{\text{within}}$ , respectively. The uncertainty of the homogeneity ( $u_{\text{bb}}$ ) is consequently estimated as  $s_{\text{bb}}$  or  $u_{\text{bb}}^*$ , depending on which of these is larger. Results from homogeneity testing of the ZON calibrant are summarised in Table 1.

Moreover, linear regressions were calculated for the results due to filling and analysis order. The slopes of the lines were tested for significance on a 95% confidence level to check for significant trends. The normalised results due to the filling sequence are presented in Fig. 1. No trends due to the filling and analysis sequences and no significant inhomogeneities were detected for the ZON calibrant. The final  $u_{\text{bb}}$  of  $0.92\%$  was used for evaluation of the total combined uncertainty of the ZON calibrant (cf. certified value and its associated uncertainty).

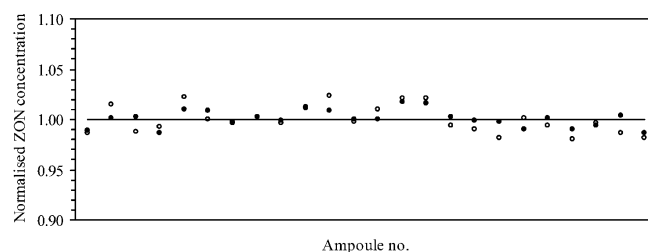
## Stability studies

Stability of (C)RM should also be tested under relevant conditions, which usually requires two stability studies. The short-term stability study elucidates possible degradation during transport whereas the stability of a (C)RM during storage at a particular storage temperature is assessed by a long-term stability study. Because the ISO Guides 31 and 34 [17, 18] require that certifying bodies and producers of (C)RM inform the users about the ex-

**Table 1** Summarised results of the homogeneity testing of the ZON calibrant

|                   | ZON in acetonitrile         |
|-------------------|-----------------------------|
| Mean of means     | 9.926 $\mu\text{g mL}^{-1}$ |
| SD                | 0.100 $\mu\text{g mL}^{-1}$ |
| $N$               | 24                          |
| $s_{\text{wb}}$   | 0.65%                       |
| $s_{\text{bb}}$   | 0.92%                       |
| $u_{\text{bb}}^*$ | 0.25%                       |
| $u_{\text{bb}}^a$ | 0.92%                       |

<sup>a</sup>Higher value ( $u_{\text{bb}}^*$  or  $s_{\text{bb}}$ ) taken as uncertainty estimate for the homogeneity



**Fig. 1** Homogeneity of ZON in acetonitrile – filling sequence (normalised with respect to the mean value)

**Table 2** Sampling scheme for short- and long-term stability studies of the ZON calibrant

| Storage temperature | Number of ampoules |   |   |   |               |   |   |    |    |    |
|---------------------|--------------------|---|---|---|---------------|---|---|----|----|----|
|                     | Time (weeks)       |   |   |   | Time (months) |   |   |    |    |    |
|                     | 0                  | 1 | 2 | 4 | 0             | 3 | 6 | 12 | 24 | 36 |
| +40 °C (UV)         | –                  | 2 | 2 | 2 | –             | – | – | –  | –  | –  |
| +40 °C              | –                  | 2 | 2 | 2 | –             | 2 | 2 | 2  | 2  | 2  |
| +25 °C              | –                  | 2 | 2 | 2 | –             | 2 | 2 | 2  | 2  | 2  |
| +4 °C               | –                  | 2 | 2 | 2 | –             | 2 | 2 | 2  | 2  | 2  |
| –18 °C              | 2                  | – | – | – | 2             | – | – | –  | –  | –  |

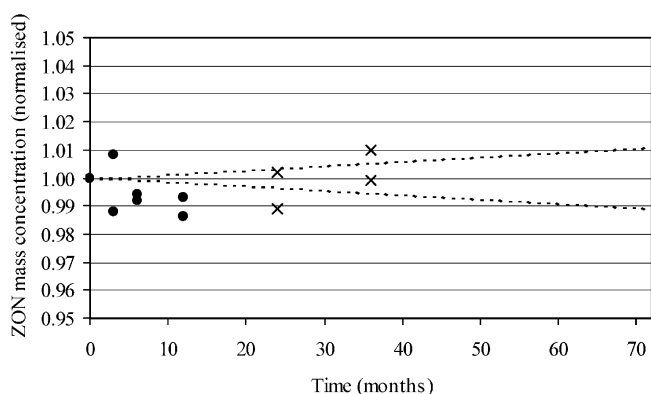
pected lifetime of a (C)RM the shelf life and the corresponding uncertainty is assessed on the basis of the data obtained in the long-term stability study.

First, the short-term stability of the ZON calibrant was measured under five different conditions (+4 °C, +25 °C, +40 °C, and under additional UV exposure at +40 °C) at four storage times of 0, 1, 2, and 4 weeks. The long-term stability study was performed for storage times of 0, 3, 6, 12, 24, and 36 months under three storage conditions (+4 °C, +25 °C, and +40 °C). Both stability studies were carried out as “isochronous measurements” [25] to enable measurements under repeatability conditions utilising both UV spectrophotometry and LC–FLD. According to the “isochronous measurements” design the ampoules were removed after their allocated storage times at the temperatures as mentioned in Table 2 and set to a reference temperature of –18 °C (two ampoules at each time and temperature). The thus collected samples were then analysed together after 4 weeks to determine short-term stability. To determine long-term stability results from two isochronous studies performed after 12 and 36 months were combined as described below.

Determination of the concentration of ZON by UV spectrophotometry was performed as described already for homogeneity testing. The analytical sequence for the ampoules was selected by using a random-number generator. In addition, LC analysis was performed employing a Gilson 231 autosampler and Gilson 307 pump equipped with a Phenomenex Prodigy 5 ODS3 100A column (150 mm × 4.6 mm). The LC was operated isocratically at ambient temperature using methanol–water (60:40, v/v) at 1.0 mL min<sup>-1</sup>. An injection volume of 10 µL was used. A Jasco FPL 520 detector was connected for fluorescence detection; excitation and emission wavelengths were 275 nm and 450 nm, respectively.

Statistical analysis of the results obtained by UV spectrophotometry and LC–FLD was performed to investigate if there were any significant trends in the concentration of ZON in the ampoules as a result of each storage condition. The slopes were therefore tested for significance on a 95% level. However, only results from UV spectrophotometric determination were used for calculation of the stability uncertainty whereas LC–FLD was used as separation method to determine if other degradation products were present that might not be seen by UV spectrophotometry.

Evaluation of the results from UV spectrophotometric determination of the short-term stability of the ZON cali-



**Fig. 2** UV spectrophotometry of ZON in acetonitrile – normalised results of the long-term stability study at +4 °C. The results after 12 and 36 months are separated graphically (dots represent the 12-month study and crosses the 36-month study)

brant showed no significant trend. The normalised results proved no degradation occurred at +4 °C, +25 °C, +40 °C and not even at +40 °C with UV exposure.

For evaluation of the long-term stability study the results from the 12 and 36 months measurements were combined in a manner similar to the approach used to prolong the validity of (C)RM certificates described by Linsinger et al. [26]. The ZON mass concentrations were normalised with respect to the average mass concentration of the individual study and not expressed as concentration in µg mL<sup>-1</sup>, because this would have introduced a bias, because the averages of the combined 12 and 36 months measurements were not the same. Normalisation served only as a means to make the 12 and 36 months measurements comparable. Any degradation would result in higher values at the beginning and lower values towards the end of the study. The uncertainties of the long-term stability study ( $u_{\text{its}}$ ) were estimated by regression analysis of the combined long-term stability data with subsequent testing of the slope of the regression lines for significance at the 95% level. No slope was found to be statistically significant, therefore, a shelf life (e.g. 6 years) was chosen and the uncertainty connected with this time estimated via the standard deviation of the slopes. For a shelf life of 6 years, the  $u_{\text{its}}$  for the ZON calibrant amounts to 4.1%, 2.1% and 1.1% for storage at +40 °C, +25 °C and +4 °C, respectively. The normalised results for the crucial long-term stability study at +4 °C employing UV spectrophotometry

are presented graphically in Fig. 2. Dotted lines in the long-term stability show the uncertainty of the certified value of the reference material because of possible instability.

The outcome of UV spectrophotometric determination of the short- and long-term stability was underpinned by employing LC-FLD. Using LC-FLD the slopes were also found to be not significant, even at the elevated temperatures, and additional UV exposure at +40 °C. The results of the trend analysis showed that there were no significant trends due to any storage condition. However, closer inspection of the LC chromatograms of undiluted ZON calibrants showed very small peaks presumably due to ZON degradation at +25 °C after 36 months.

It can be concluded that both UV spectrophotometric and additional LC-FLD determination showed no significant trend and no degradation, not even at +40 °C with UV exposure, for short-term stability, which describes the stability of the ZON calibrant during transport. Therefore, the material will be shipped under ambient conditions. The uncertainty contribution of the short-term stability ( $u_{\text{sts}}$ ) is assumed to be negligible, because no degradation is expected during this short time.

Moreover, no significant trend was observed in the long-term stability study employing both UV spectrophotometric and LC-FLD determination. No significant degradation of ZON had occurred after 36 months for all storage temperatures. According to this the ZON calibrant is stable at +4 °C, showing no signs of degradation, whereas a very small but insignificant decrease in the concentration of the ZON calibrant stored at +25 °C was measured by LC-FLD. As a result of these findings a storage temperature of +4 °C is recommended for long-term storage. Assuming a shelf life of 6 years the uncertainty of the long-term stability ( $u_{\text{lts}}$ ) of the ZON calibrant amounts to  $u_{\text{lts}}=1.1\%$  under these conditions. This uncertainty ( $u_{\text{lts}}$ ) was included in the combined uncertainty of the ZON calibrant (cf. certified value and its associated uncertainty).

The shelf life and, hence, the validity of the certificate can be prolonged if additional data are obtained from supplementary stability monitoring schemes which will be performed over a long period of several years by the Institute for Reference Materials and Measurements (IRMM) [24].

### Certified value and its associated uncertainty

Estimation of the final uncertainty of the certified value must consider all sources relevant to the user [20]. This includes not only the uncertainty of the batch characterisation ( $u_{\text{char}}$ ), but also uncertainties related to possible between-unit variation ( $u_{\text{bb}}$ ), instability during long-term storage ( $u_{\text{lts}}$ ) and instability during transport to the customer ( $u_{\text{sts}}$ ). The combined standard uncertainty for a CRM can, consequently, be expressed by Eq. (6):

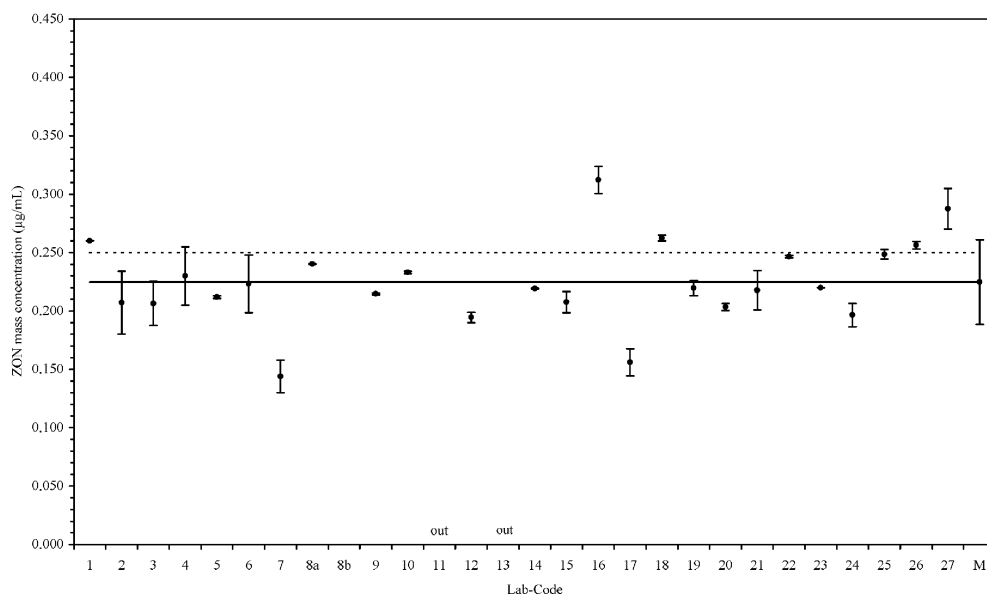
$$U_{\text{CRM}} = \sqrt{u_{\text{char}}^2 + u_{\text{bb}}^2 + u_{\text{sts}}^2 + u_{\text{lts}}^2} \quad (6)$$

Furthermore, ISO Guides 31 and 34 [17, 18] require that certifying bodies and producers of CRM not only include contributions connected to homogeneity and stability in the combined standard uncertainty but also state shelf lives for their CRM.

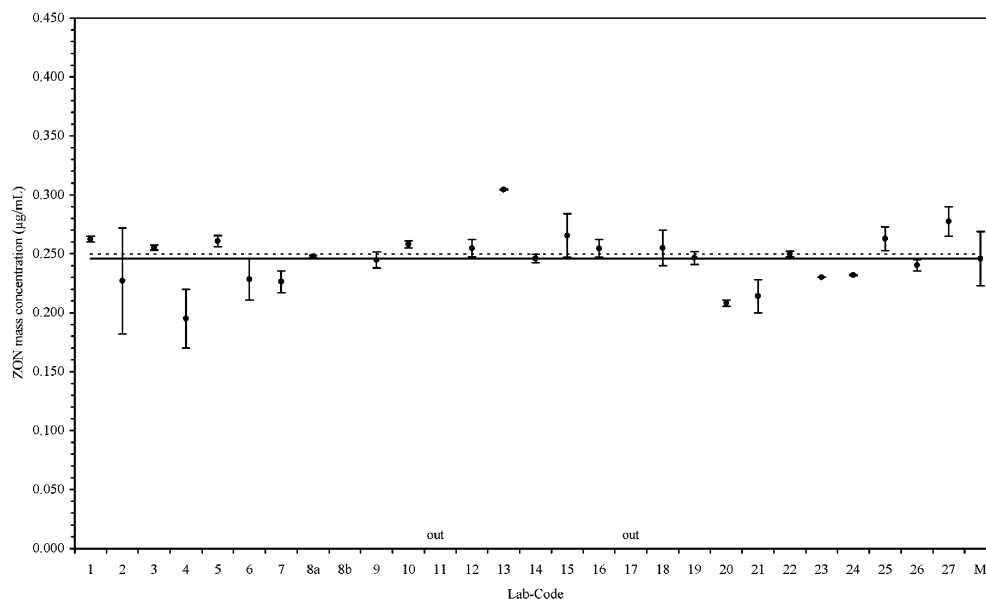
For the ZON calibrant a total expanded uncertainty ( $U_{\text{CRM}}$  multiplied by a coverage factor of  $k=2$ ) of  $\pm 0.30 \mu\text{g mL}^{-1}$  for the certified value of  $9.95 \mu\text{g mL}^{-1}$  was calculated by combination of 0.40%, 0.92% and 1.1% for  $u_{\text{char}}$ ,  $u_{\text{bb}}$ , and  $u_{\text{lts}}$ , respectively, obtained as described above. The relative total expanded uncertainty of the ZON calibrant was consequently estimated to be 3.0%. The validity of certified value and its total expanded uncertainty was certified assuming a shelf life of 6 years and storage at +4 °C.

Without including  $u_{\text{lts}}$ , the relative total expanded uncertainty would only be 2.0%, which is only two-thirds of

**Fig. 3** Laboratories' mean results for ZON in "unknown" solution using their own ZON calibrants: *full line*, assigned mean of means of  $0.225 \mu\text{g mL}^{-1}$ ; *dotted line*, target concentration of  $0.250 \mu\text{g mL}^{-1}$ ; *out*, outlying results; *error bars* denote standard deviations



**Fig. 4** Laboratories' mean results for ZON in "unknown" solution using the common ZON calibrant: *full line*, assigned mean of means of  $0.246 \mu\text{g mL}^{-1}$ ; *dotted line*, target concentration of  $0.250 \mu\text{g mL}^{-1}$ ; *out*, outlying results; *error bars* denote standard deviations



the value including  $u_{\text{ts}}$ . This demonstrates the strong influence of the long-term stability study on the total expanded uncertainty of the ZON calibrant.

#### Interlaboratory study

An interlaboratory study carried out to certify the ZON mass concentration in maize clearly demonstrated the improved comparability of measurement results for the determination of ZON as a result of the availability of this well characterised ZON calibrant [13].

In this interlaboratory study a solution of "unknown" ZON concentration was analysed (in duplicate on two days) by 28 laboratories using their LC methods. The participants were asked to perform external calibrations using both the common ZON calibrant, which was subjected to certification, and their own in-house ZON calibrants.

After rejection of two outlying results (laboratories 11 and 13) a mean of means of  $0.225 \mu\text{g mL}^{-1}$  with a relative between-laboratory standard deviation ( $RSD_R$ ) of 16% was obtained by use of their own ZON calibrants. The deviation of the mean of means from the target concentration ( $0.250 \mu\text{g mL}^{-1}$ ) of the "unknown" ZON solution was 10% as depicted in Fig. 3.

However, using the common ZON calibrant for external calibration a mean of means of  $0.246 \mu\text{g mL}^{-1}$  with a smaller  $RSD_R$  of 9% was achieved subsequent to the rejection of two outlying results (laboratories 11 and 17). In addition, a smaller deviation of only 1.6% from the target concentration ( $0.250 \mu\text{g mL}^{-1}$ ) of the "unknown" ZON solution was obtained, as shown in Fig. 4.

These findings clearly emphasize the major influence of a properly produced calibrant, here a CRM for ZON in acetonitrile, on the quality of analysis in the field of mycotoxins, in which external calibration is still mainly used for quantification.

#### Conclusions

Certification is an integrated process consisting of batch characterisation and homogeneity and stability testing. The approach outlined was used to determine a certified value and corresponding uncertainty for a calibrant CRM for ZON in acetonitrile in compliance with the GUM [16]. It resulted in satisfactory overall uncertainty of  $0.30 \mu\text{g mL}^{-1}$  for a certified mass concentration of  $9.95 \mu\text{g mL}^{-1}$  of ZON in acetonitrile. The relative overall uncertainty of 3.0% is still sufficient to make the CRM useful and can even guarantee a shelf life of 6 years on storage at  $+4^\circ\text{C}$ . The uncertainty about the long-term stability of the ZON calibrant was identified by this study as the main contribution to the overall uncertainty.

Anyway, the major impact of the new CRM for ZON in acetonitrile (named BCR-699) on the quality of analysis, as a result of improving comparability and traceability, was demonstrated in an interlaboratory study performed on an "unknown" ZON solution. In addition, the common ZON calibrant was employed in a preliminary interlaboratory study involving analysis of ZON in naturally contaminated maize [27] and for the characterisation of a matrix CRM for ZON in maize (named BCR-716 and BCR-717) [13]; this resulted in  $RSD_R$  of 8% and 4.3%. Compared with  $RSD_R$  of approximately 40% obtained in an interlaboratory study on naturally ZON-contaminated maize performed in 1998 [10] the  $RSD_R$  improved dramatically, the common ZON calibrant being a major reason for this improvement.

Independent confirmation of the usefulness of characterising uncertainty, calculated on the basis of preparation for the first time, came from a mini-interlaboratory study performed by three laboratories, by comparing results obtained by UV spectrophotometry with the calculated values. However, a thorough evaluation of the purity of the compound and its solvent and detailed planning of the

gravimetric preparation is necessary to enable assessment of the characterisation uncertainty on the basis of the preparation [15].

All these findings underline the importance of an appropriate and well characterised calibrant CRM in the field of mycotoxin analysis and encouraged us perform an EC project (funded under contract no. G6RD-2002-00853) dealing with a feasibility study to produce calibrant CRM for mycotoxins belonging to the B-trichothecenes group, which are highly desired by laboratories working in the field of mycotoxin analysis [28].

The Institute for Reference Materials and Measurements (IRMM) of the EC Directorate General Joint Research Centre (JRC) will presumably make the certified ZON calibrant, under the name BCR-699, available for sale by end of 2003. It will probably be released together with the two matrix CRM for ZON in maize BCR-717 "low level ZON" successfully certified for a ZON content of  $83 \pm 9 \mu\text{g kg}^{-1}$  and BCR-716 "very low level ZON" certified for a ZON content less than  $5 \mu\text{g kg}^{-1}$  [13].

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## References

- Betina V (1989) (ed) CRC handbook of naturally occurring food toxicants, CRC Press, Boca Raton
- Gareis M, Bauer J, Enders C, Gedek B (1989) In: Chelkowski J (ed) *Fusarium* mycotoxins, taxonomy, pathogenicity. Elsevier, Amsterdam, pp 441-473
- Task force report No. 139 (2003) Mycotoxins: risks in plant, animal, and human systems. Council for Agricultural Science and Technology, Ames
- Kuiper-Goodman T, Scott PM, Watanabe H (1987) Regul Toxicol Pharmacol 7:253-306
- Scientific Committee on Food (1999) Opinion on *fusarium* toxins, part 2: zearalenone. European Commission, Brussels
- Food and Agriculture Organization of the United Nations (1997) World-wide regulations for mycotoxins – a compendium. FAO Food and Nutrition Paper 64, Rome
- Krska R (1998) J Chromatogr A 815:49-57
- Krska R, Josephs R (1997) Fresenius J Anal Chem 369:469-476
- Schuhmacher R, Krska R, Grasserbauer M (1997) Fresenius J Anal Chem 359:510-515
- Josephs RD, Schuhmacher R, Krska R (2001) Food Addit Contam 18:417-430
- Standard Measurement and Testing project "ZONMAIZE" (1998) Contract number SMT4-CT98-2228. European Commission, DG12, Brussels, Belgium
- CEN Report (1999) Food analysis – biotoxins – criteria of analytical methods of mycotoxins CR 13505. European Committee for Standardization, Brussels
- Krska R, Josephs RD, MacDonald S, Pettersson H (2003) The certification of the mass concentration of zearalenone in acetonitrile (BCR-699) and mass fraction of zearalenone in maize - very low level (BCR-716) and zearalenone in maize - low level (BCR-717), BCR-Information, Report EUR-Report, Luxembourg (2003) EUR20782EN, ISBN 92-984-6094-6
- Krska R, Josephs RD, Parich A, Schuhmacher R, Pettersson H, MacDonald S, Brereton P (2001) In: de Koe WJ, Samson RA, van Egmond HP, Gilbert J, Sabino M (eds) Mycotoxins and phycotoxins in perspective at the turn of the millennium. Wageningen, pp 77-86
- Josephs RD, Krska R, MacDonald S, Wilson P, Pettersson H (2003) J AOAC Int 86:50-60
- International Organization for Standardization (1995) Guide to the expression of uncertainty in measurement. GUM, Geneva
- International Organization for Standardization (1998) Reference materials – contents of certificates and labels. ISO Guide 31, Geneva
- International Organization for Standardization (1999), General requirements for competence of reference material producers. ISO Guide 34, Geneva
- Krska R, Welzig E, Josephs RD, Kandler W, Pettersson H, MacDonald S, Charlton A, Brereton P, Hametner C, Berner D, Zoellner P (2003) J AOAC Int 86(4):77-728
- Josephs RD, Koeber R, Bernreuther A, Linsinger TPJ, Schimmel H (2003) Development of certified reference materials for mycotoxins, in: Barug D, van Egmond HP, Lopez-Garcia R, van Osenbruggen T, Visconti A (eds) Meeting the mycotoxin menace, Wagening Academic Publishers, Wageningen, The Netherlands (2003) 235-252 (ISBN 90-769-9828-0)
- Flick EW (1996) Industrial solvents handbook, 3rd edn. Noyes, Westwood
- AOAC International (1990) Official methods of analysis, 15th edn. AOAC International, Arlington, section 970.44
- LNE (1987) Note de Laboratoire, Matériaux de Référence de Densité Optique (solutions), Bilan de l'étude de faisabilité, S.M.R./D/207/87, Paris
- Linsinger TPJ, Pauwels J, van der Veen AMH, Schimmel H, Lamberty A (2001) Accred Qual Assur 6:20-25
- Lamberty A, Schimmel H, Pauwels J (1998) Fresenius J Anal Chem 360:359-461
- Linsinger TPJ, Pauwels J, Lamberty A, Schimmel H, van der Veen AMH, Siekmann L (2001) Fresenius J Anal Chem 370: 183-188
- Josephs RD, Krska R (2000) Mycotoxin Res 16:217-220
- Krska R, Baumgartner S, Josephs RD (2001) Fresenius J Anal Chem 371:285-299