Preparation and Characterization of Zearalenone Standard Solution for Mycotoxin Analysis

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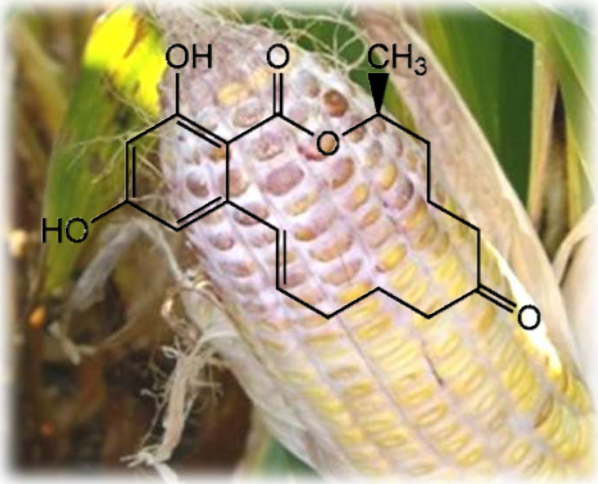
21st June 2019

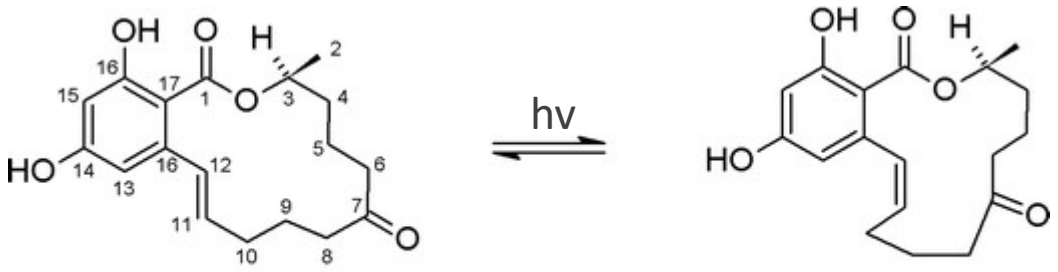
Zearalenone (ZEN)

- Zearalenone (ZEN) is a fungal mycotoxin produced by *Fusarium spp*. and present in several types of food but especially in maize and wheat
- It is a non-steroid estrogenic compound which can cause changes in reproductive organs and fertility loss and plus several other toxic effects
- JECFA- provisional maximum tolerable daily intake for ZEN and its metabolites (including α-zearalanol) - 0.5 µg/kg BW/day (CX/FAC 00/19-2000)

EC 1126/2007: setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products (20- 400µg/kg for ZEN)



Zearalenone (ZEN)



trans-zearalenone *cis*-zearalenone

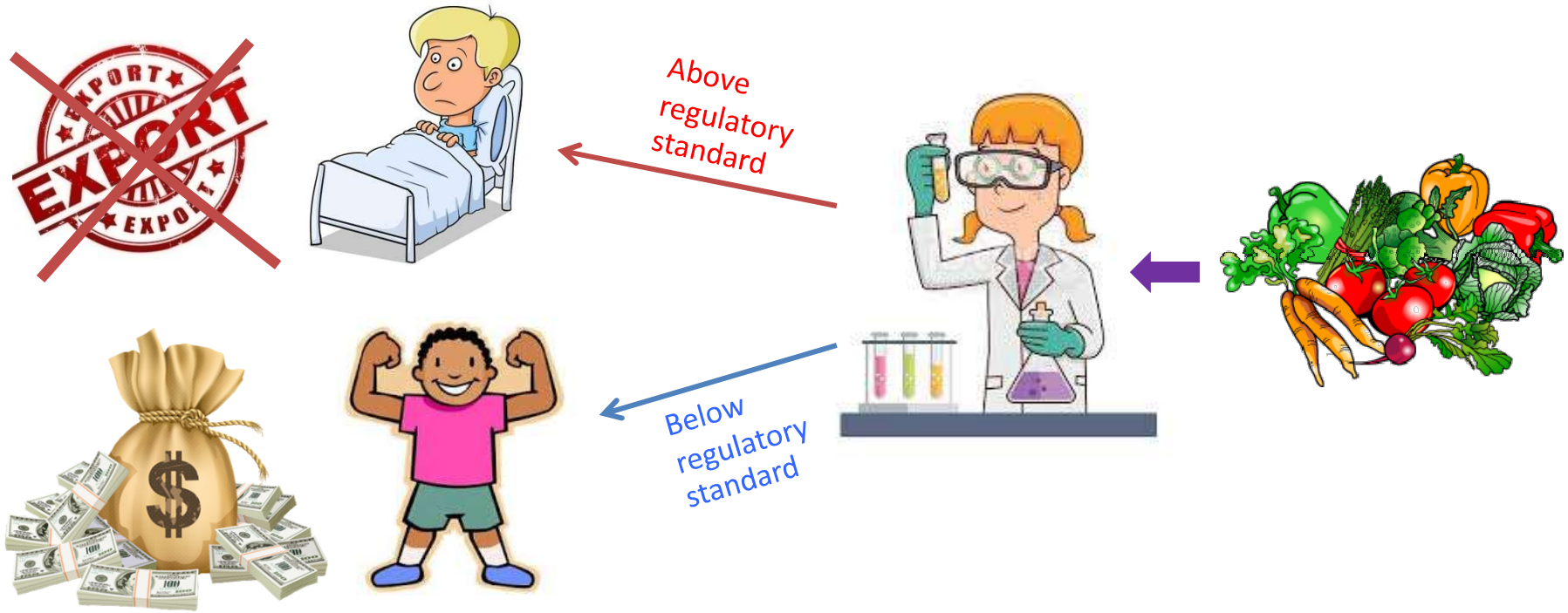
Figure 1. Stereoisomerization of *trans*-zearalenone and *cis*-zearalenone at the double bond C11-C12

- ZEN is biosynthesized in the *trans*-configuration
- ZEN has been shown to readily isomerize to the cis-configuration upon the influence of light

For Food safety: Ensuring Analytical reliability

- Food producers need to ensure that their products are toxicologically safe and testing compliance with all legal requirements through the use of analytical.

- An analytical reliability in the testing and monitoring should be ensure



Gravimetric preparation and value assignment of zearalenone (ZEN) in acetonitrile (ACN)

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Bureau International des Poids et Mesures

International interlaboratory comparison: CCQM-K154.a

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Gravimetric Preparation of ZEN solution

Homogeneity study

Stability study

Verification

ZEN stock solution

Bureau International des Poids et Mesures

ZEN calibration solution

- Determine and Compare ZEN mass fraction
- UV spectrophotometry
- LC-UV

Gravimetric value

Analytical results

Gravimetric preparation of Zearalenone calibration solution

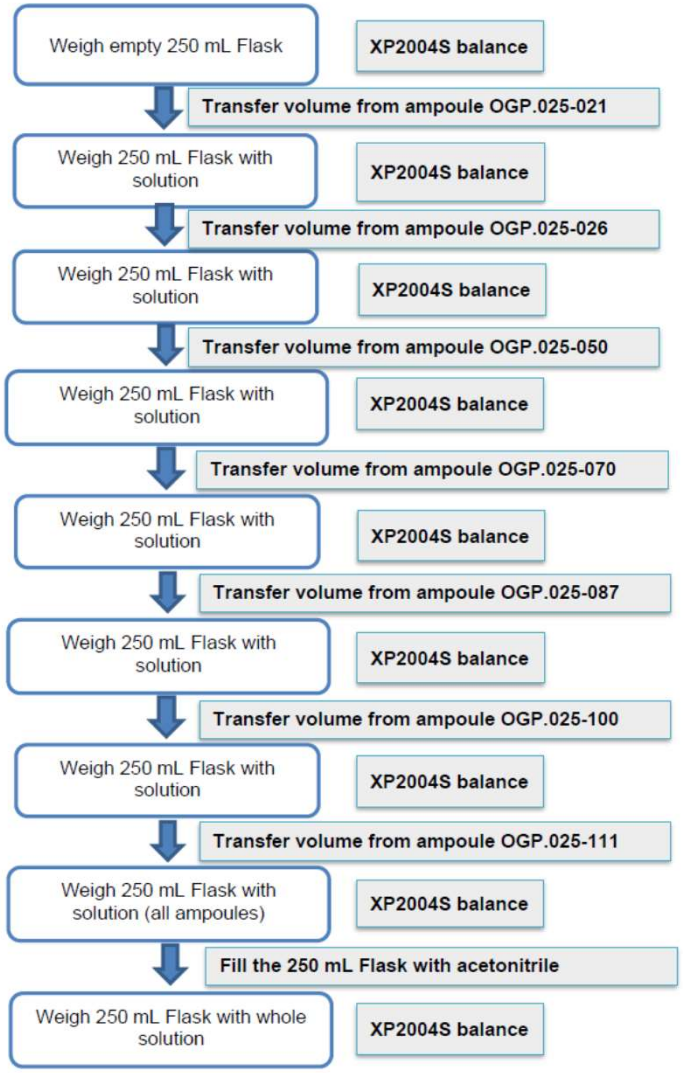


Figure 2. ZEN stock solution OGP.025

Concentration 130.1± 2.2 µg/g (k=2)

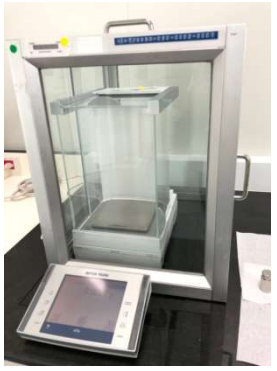


Figure 3. Preparation of ZEN calibration solution

(Volume: 250 mL,Stored at -20 °C (dark))

Gravimetric preparation of Zearalenone calibration solution

Measurement equation:

$$w(xi) = \frac{w_z * m_z}{m_{total}} \qquad (1)$$

where;

$w(xi)$	mass fraction of the prepared solution, µg/g
w_z	mass fraction of the stock solution prepared from (OGP.025), µg/g
m_z	mass of the stock solution (OGP.025) added (g)
m_{total}	mass of the total solution (g)

Measurand	Mass Fraction (µg/g)
trans-zearalenone	14.73

Homogeneity study of Zearalenone calibration solution

- ✓ Ten samples were taken in randomly stratified interval
- ✓ The main component ZEN (*trans*-zearalenone) was measured directly without further dilution
- ✓ Aliquots from ampoules were analyzed by HPLC-PDA
- ✓ Three independent replicates in randomly stratified.
- ✓ Trend tests for filling/analysis sequence (linear regressions)
- ✓ Uncertainty of homogeneity was evaluated by one-way ANOVA (u_{bb})

Homogeneity study of Zearalenone calibration solution

LC parameters
Column: Luna C18 100Å, 150 x 4.6 mm Column temperature: 30 °C Mobile phase: ACN:H_2O = 50:50 (v/v) Flow rate: 1.0 mL/min Detector: DAD 254nm (Scan range:190nm-700nm; Slit width: 1.2mm) Injection volume: 10 µL Duration: 15 min

Homogeneity study of Zearalenone calibration solution

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{\nu_{MSwithin}}} \qquad (2)$$

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \qquad (3)$$

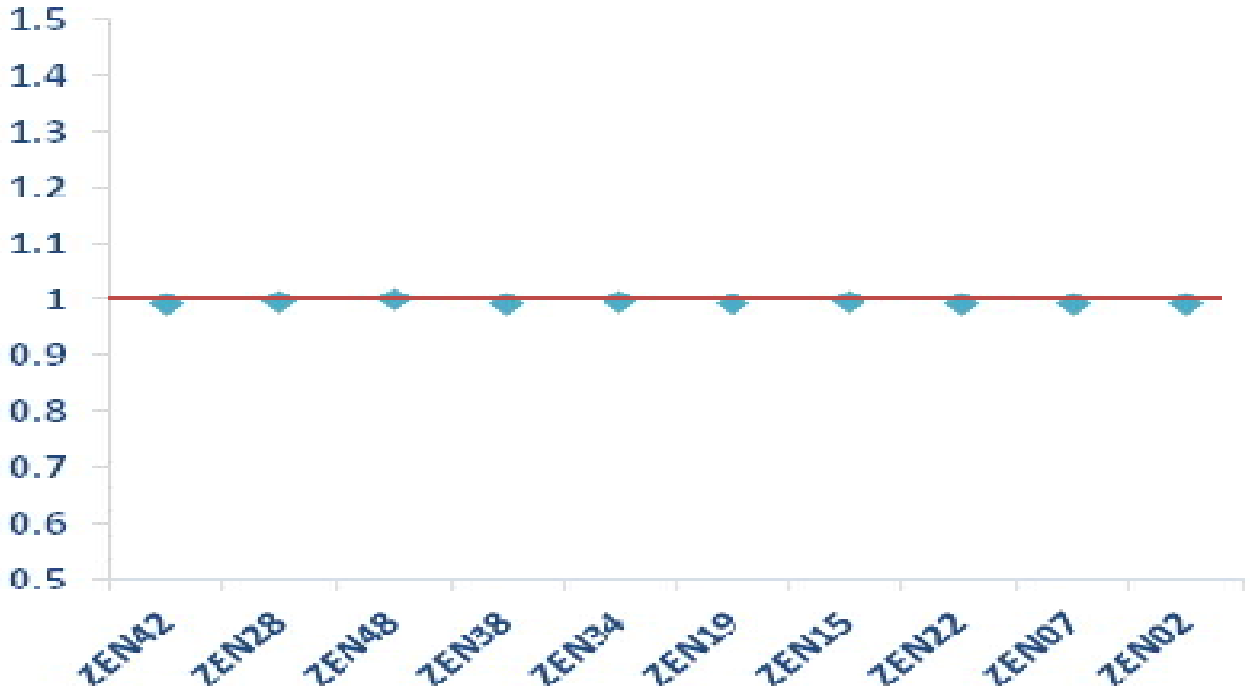
N	s_{wb} (%)	F	F_{crit}	s_{bb} (%)	u^*_{bb} (%)
29	0.75	0.5573	2.3928	-(1)	0.243

(1) Not calculable because $MS_{between} < MS_{within}$

- No differences in the within- and between-sample variances could be detected by the F-tests at the 95 % confidence level
- The % u_{bb} of 0.243% was calculated from equation (2) that was the uncertainty contribution due to homogeneity

d) confirmation, for example by use of an F test, that the between-unit term is not statistically significant at the 95 % level of confidence.

Homogeneity study of Zearalenone calibration solution



- No significant trend was observed for the injection sequence of ZEN
- No evidence of statistically significant inhomogeneity was observed for main component based either on fill order or analysis order
- ZEN calibration solution is therefore considered homogeneous

Stability study of Zearalenone (ZEN) calibration solution

- ✓ The main component of ZEN was measured directly without further dilution
- ✓ Aliquots from ampoules were analyzed by HPLC-PDA
- ✓ Three independent replicates in randomly stratified for the main compound (ZEN) with calibration curves

Linear regressions- Trend tests for stability/analysis sequence

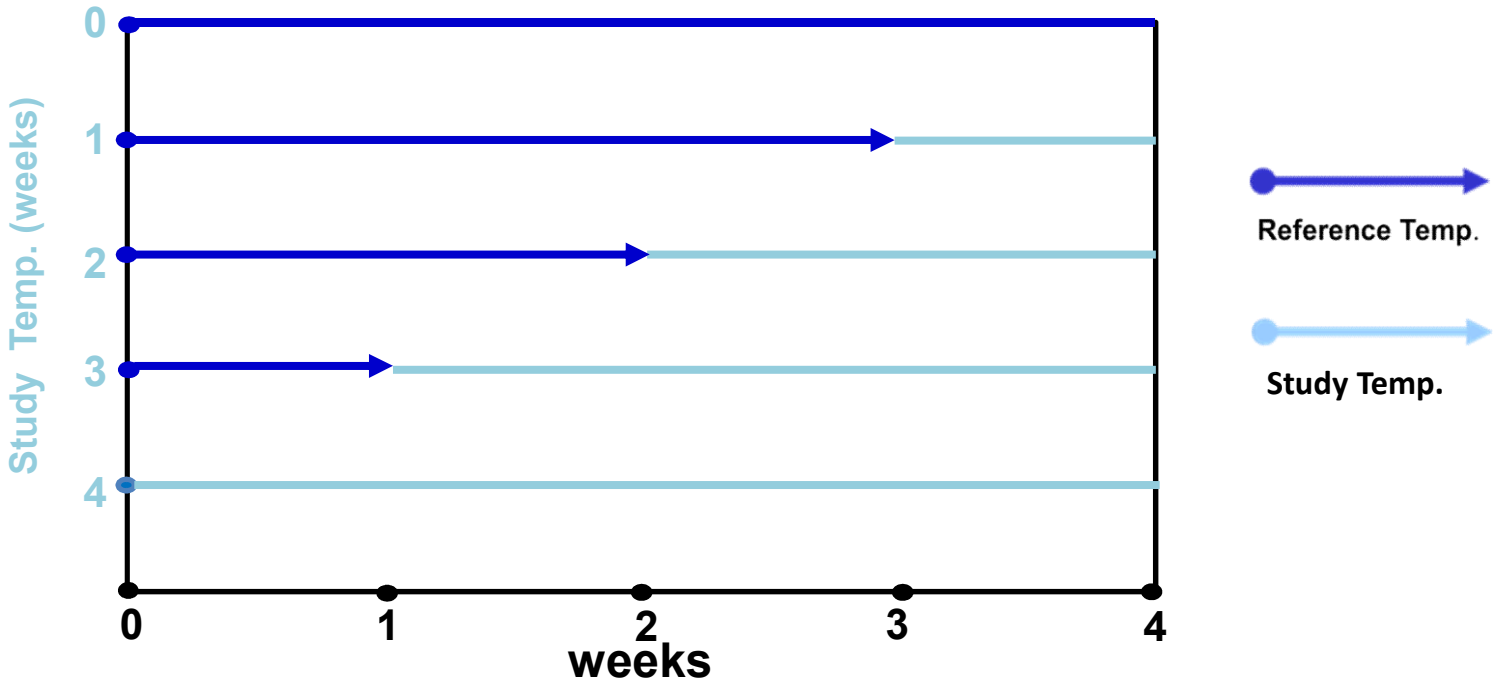
Uncertainty of stability (u_{its})

Stability studies are planned in the form of isochronous measurements

Stability study of Zearalenone (ZEN) calibration solution

Isochronous experiment design

- Reference temp: -20 °C (dark)
- Study temp: 4 °C & 40 °C
- Study duration: 0, 1, 2, 3, 4 weeks (amber ampoule)
- 2 ampoules for each condition, two aliquots for analysis



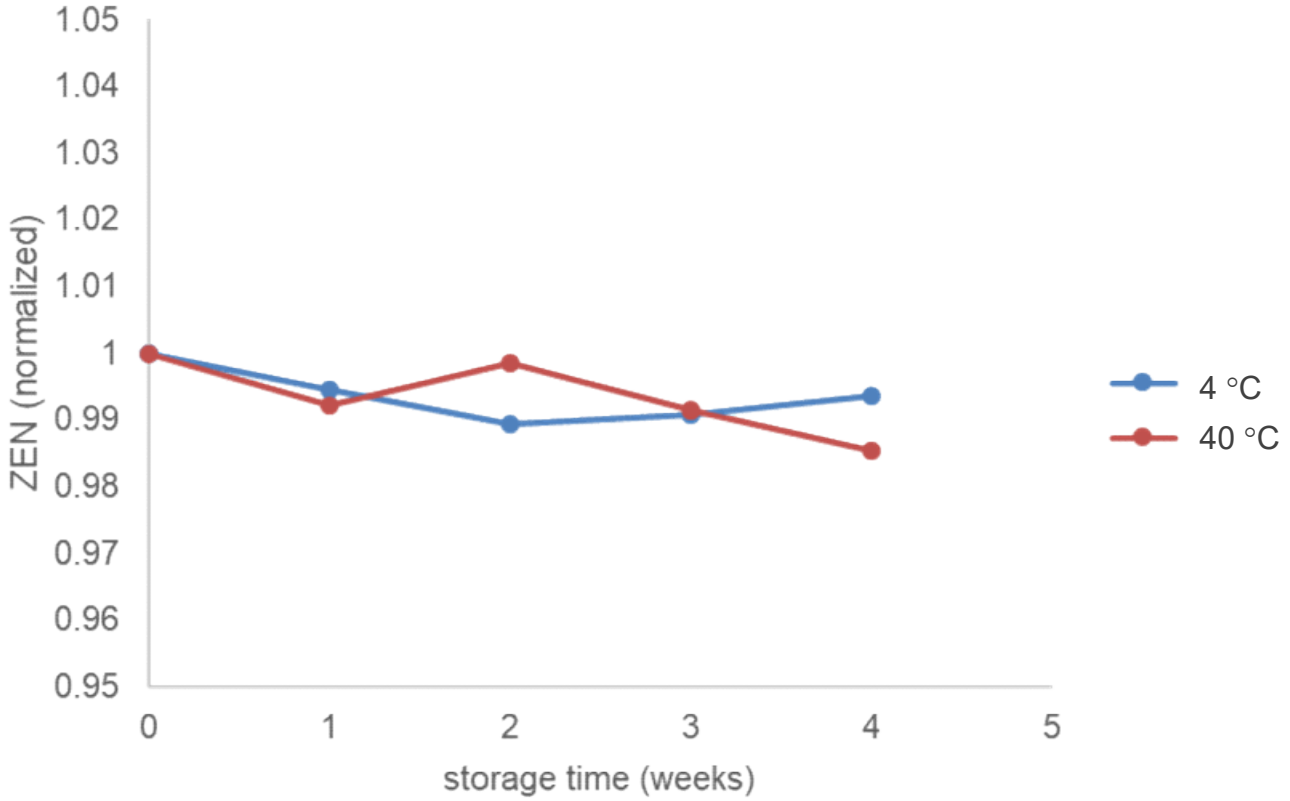
Stability study of Zearalenone (ZEN) calibration solution

Statistical parameters

	4 °C	40 °C
Slope (b), degree	-0.02189	-0.03596
Standard error of slope (s_b), degree/week	0.015907	0.017068
Degree of freedom	7	7
t_{cal} following equation (4)	1.376045	2.107102
t_{crit} = t(0.05, 7)	2.364624	2.364624
Statistical significance at 95% CI	NO	NO
t*s(b1) $u_{(its)}$	0.0048%	0.0051%

- A linear regression functions were calculated for the results according to the conditions during the stability study.
- The slopes were found to be not significant at a 95% confidence level.
- The relative uncertainty due to stability was 0.0048%

Stability study of Zearalenone (ZEN) calibration solution



No drop trends existed for 4°C and 40 °C storage conditions.

✓ ZEN calibration solution is stable at 4°C and 40 °C

Verification for measurement of ZEN calibration solution

- ✓ Two samples were analysed directly without dilution: ZEN 13 and ZEN 33
- ✓ One ampoule of OGP.025 (ZEN stock solution) was used to prepare standards for LC measurements
- ✓ Aliquots from ampoules were taken for HPLC-PDA measurements.
- ✓ Three independent replicates were analyzed for major compound (ZEN) using one point bracketing quantification
- ✓ The analytical results were compared with *t*-test and recovery test

Verification for measurement of ZEN calibration solution

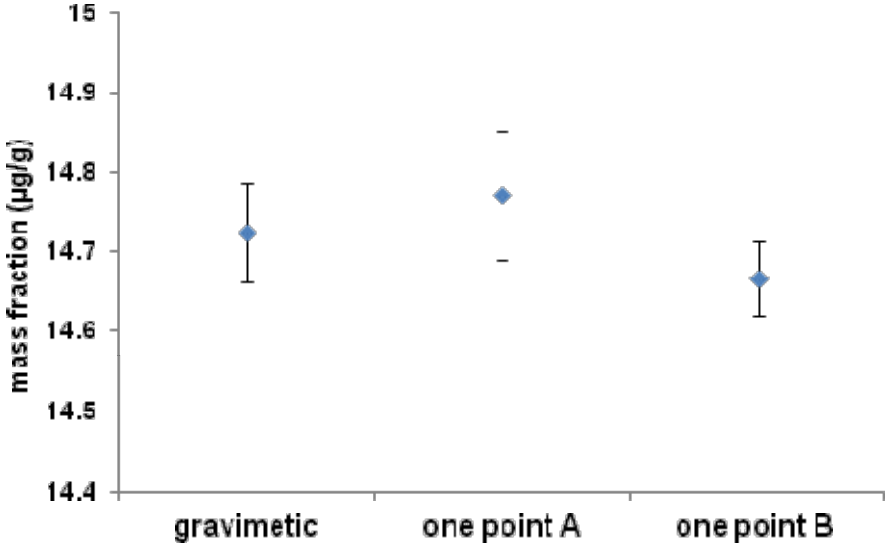


Figure 4. Comparison of mass fraction result

$$t=(|\bar{X}-\mu|\times\sqrt{n})/S \quad (4)$$

		A	B
t-test	t_{cal}	0.6789	1.5324
	t_{cri}	2.5706	2.5706
	$t_{cal} < t_{cri}$	YES	YES
Recovery		100.3%	99.6%

one point bracketing analytical results agreed with the gravimetric value

Measurement uncertainty

Expanded measurement equation:

$$w(xi) = \frac{w_z * m_z}{m_{total}} . F_{stb} . F_{\text{homo}} \qquad (5)$$

where;

w(*xi*)	: mass fraction of the prepared solution, µg/g
w_z	: mass fraction of the stock solution prepared from (OGP.025), µg/g
m_z	: mass of the stock solution (OGP.025) added (g)
m_{total}	: mass of the total solution (g)
F_{stb}	: Stability factor, given a value of 1
F_{homo}	: Homogeneity factor, given a value of 1

Measurement uncertainty

Combined measurement uncertainty:

$$\frac{u(w_{xi})}{w_{xi}} = \sqrt{\left(\frac{u(w_z)}{w_z}\right)^2 + \left(\frac{u(m_z)}{m_z}\right)^2 + \left(\frac{u(m_{total})}{m_{total}}\right)^2 + \left(\frac{u(F_{homo})}{F_{homo}}\right)^2 + \left(\frac{u(F_{stb})}{F_{stb}}\right)^2} \quad (6)$$

where;

$u(w_{xi})$	: standard uncertainty of the prepared standard solution
$u(w_z)$	: standard uncertainty of the stock standard solution obtained from the certificate (OGP.025)
$u(m_z)$,$u(m_{total})$	: standard uncertainties of masses estimated from the bias of balance and the precision of balance
$u(F_{homo})$	: standard uncertainty due to homogeneity factor, estimated from ANOVA
$u(F_{stb})$	: standard uncertainty due to stability testing at 4 °C, estimated from trend analysis

EURACHEM/CITAC Guide CG 4 Quantifying Uncertainty in Analytical Measurement

Measurement uncertainty Budget

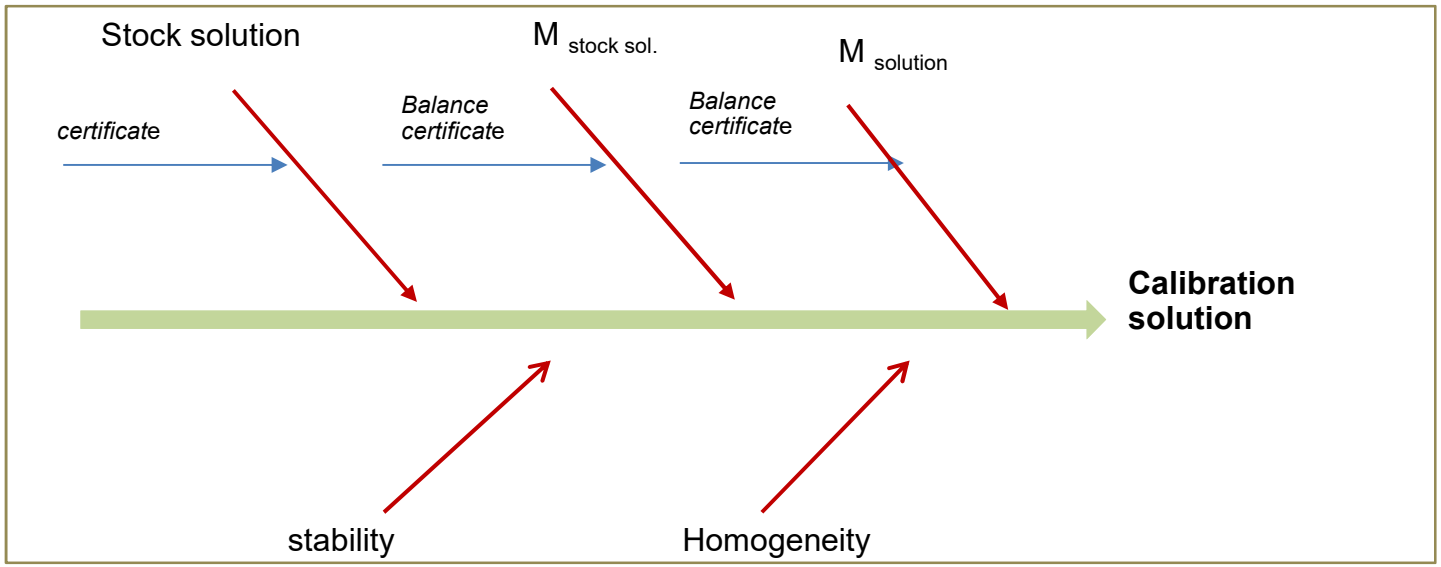


Figure 5 Diagram indicating the input quantities and their related uncertainty sources

Result

Measurand	Mass Fraction (µg/g)	Combined Standard Uncertainty (µg/g)	Coverage Factor (k)	Expanded Uncertainty (µg/g)
trans-Zearalenone	14.73	0.148	2.0	0.30

International interlaboratory comparison: CCQM-K154.a

ZEN solution sent to BIPM

4 ampoules

Determine and Compare ZEN mass fraction by

- UV spectrophotometry
- LC- UV

International interlaboratory comparison: CCQM-K154.a

	ZEN							
			U	U_{rel} %	U	Urel %	DoE	U(DoE)
NIMT	Gravimetric	14.73	0.15	1.00	0.30	2.01		
BIPM	Measured A	14.45	0.13	0.92	0.27	1.84	0.28	0.40
	Measured B	14.51	0.10	0.66	0.19	1.32	0.22	0.35

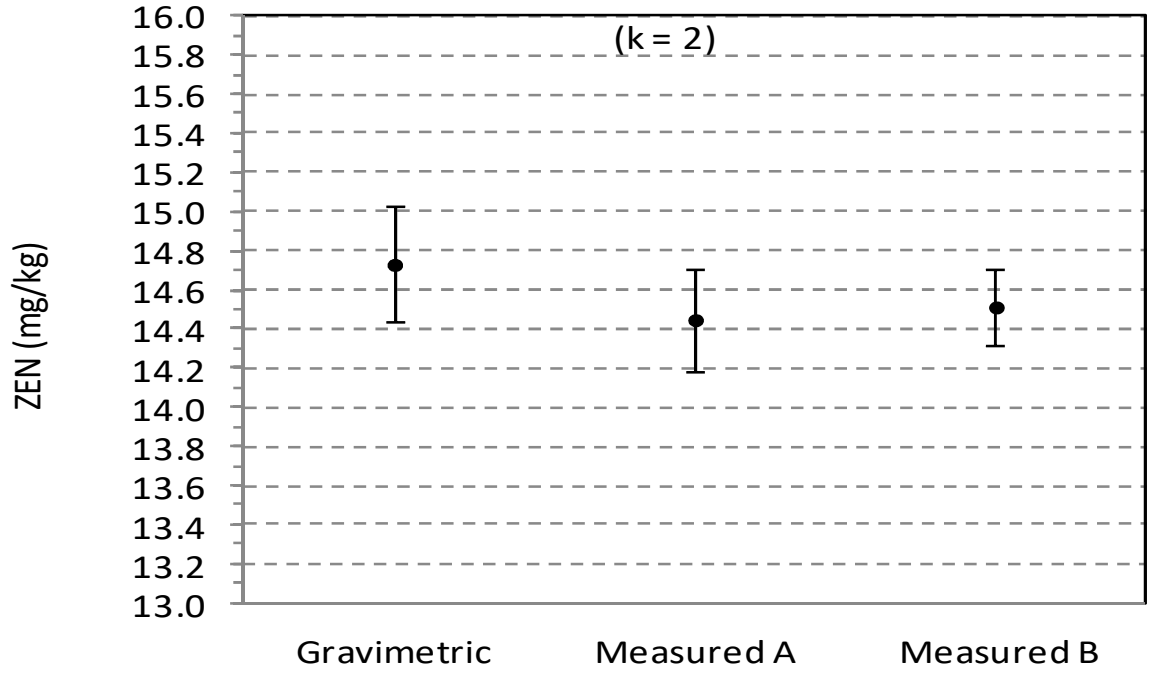
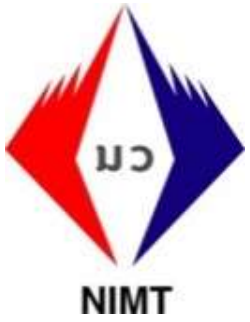


Figure 6. CCQM-K154.a result

Summary

- ✓ ZEN calibration solution in acetonitrile was prepared by gravimetric method
- ✓ Homogeneity studies for ZEN solution showed satisfactory results
- ✓ *trans*-zearalenone (major component) was stable at 4°C and 40 °C conditions during the 4 weeks of study
- ✓ BIPM determined and compared the ZEN mass fractions by UV spectrophotometry and HPLC-UV
- ✓ Successfully, gravimetric value prepared by NIMT agreed with the analytical values obtained from BIPM

Acknowledgement



NIMT

BIPM

PTB

Organic Analysis staffs

Thank you