Bureau International des Poids et Mesures

Purity Evaluation Guideline: Zearalenone [PEG-01]

1st edition 2019

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des Poids et Mesures

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Purity Evaluation Guideline: Zearalenone [PEG-01]

Contents

1. Scope

This document has been prepared to provide guidance for the value assignment of the mass fraction of zearalenone (ZEN) present in a purified solid ZEN material intended for use as Primary Reference Material.

The information summarized in the document was obtained as part of the BIPM Metrology for Safe Food and Feed Programme for Capacity Building and Knowledge Transfer on the production and characterization of reference materials for mycotoxin analysis.

2. Introduction

In collaboration with the National Institute of Metrology, China (NIM) and the National Metrology Institute of South Africa (NMISA), the BIPM initiated in 2016 a Capacity Building and Knowledge Transfer program for Metrology for Safe Food and Feed in Developing Economies. [1] This project is designed to allow NMIs to work together to strengthen the worldwide mycotoxin metrology infrastructure; provide knowledge transfer to scientists developing capabilities in this area and to enable NMIs in developing regions to provide calibrants, matrix reference materials and proficiency test samples that support testing activities and laboratory services for mycotoxin analysis within their countries.

As for all other areas of organic analysis primary reference materials consisting of well characterized, high purity compounds are required for each analyte subject to investigation. These materials are the source of higher-order metrological traceability for the assigned values of derived calibration solutions, reference materials, proficiency test samples and ultimately the results of routine analysis. Access to pure organic compounds and calibration solutions prepared from these materials is an essential element in the measurement infrastructure supporting the delivery of reliable, comparable results. In the case of mycotoxins purity analysis of source materials involves additional challenges linked to the limited amount of available material and its potential toxicity.

Zearalenone, a fungal secondary metabolite produced by *Fusarium spp*, is a frequent contaminant of food and animal feed, in particular of maize but is also found in barley, oats and wheat. [2], [3] It is a non-steroid estrogenic compound which can cause changes in reproductive organs and fertility loss and has been shown to have several other toxic effects. [4] The ability to undertake robust and reliable analysis for zearalenone and related compounds is required for health and food safety and for trade in primary produce for countries which produce or consume large quantities of corn grains and wheat. [5]

An essential requirement of this project was to obtain and characterize a primary reference material for zearalenone that could be used subsequently to establish a calibration heirarchy to underpin the metrological traceability of results linked through calibration to this material. [6] This guideline summarizes characterization and purity assignment studies to assess identity and purity of a Primary Reference Material ISO 17511:2003 for zearalenone used to deliver the BIPM MMCBKT program and is intended to be of use to other metrology institutes and reference measurement service providers needing to characterize their own source material for zearalenone analysis. Particular reliance was placed on nuclear magnetic resonance spectroscopy (NMR) studies both to confirm the qualitative identity of the main component of the material and to assign the mass fraction content of zearalenone it contained.

Due to the relatively complex structure of zearalenone, the assignment by qNMR only provides in the first instance an estimate of the total zearalenone and related structure impurity content. This initial value needed to be corrected for the relevant related structure impurity content as assigned separately by LC-MS/MS and LC-DAD methods to give the final value for the absolute zearalenone content of the material. Additional analyses for the assessment of other potential impurities were undertaken to support and confirm the value assigned through combination of the qNMR and LC data.

3. Nomenclature and Ring numbering

Throughout this report the nomenclature and abbreviations proposed by Metzler [8] for the specification of zearalenone and related compounds are used. Zearalenone is abbreviated as ZEN, while noting that in other literature ZON or ZEA are also used for the same structure. The abbreviations for the related zearalenols which retain the alkene bond in the macrocyclic ring are based on ZEL and those for the zearalanols, in which the ring alkene bond is no longer present, are based on ZAL.

For the numbering of the carbon skeleton the IUPAC system which assigns the C-atoms of ZEN from 1–18 is used. This supercedes systems based on use of the numbers 1–6 for the aromatic ring and 1'–12' for the aliphatic carbons of the macrocyclic ring. The structure of ZEN with the current and historical ring numbering schemes are shown respectively in Figure 1 and Figure 2.

The full structures and abbreviations for ZEN, its related metabolite family and impurities identified in the course of this work program are shown in Annex A1.1.

4. Properties of Zearalenone

4.1. Hazard Identification

The substance poses high potential risks for human health if handled inappropriately. It is toxic by inhalation, in contact with skin and if swallowed. Exposure to ZEN may cause cancer.

DISCLAIMER: The safety recommendations given in this section are based on literature reported best practice and have not been verified by the BIPM.

4.1.1. Protective measures

Avoid breathing of dust, vapours, mist or gas. Wear full-face particulate filtering respirator type N100 (US) or type P3 (EN 143) respirator cartridges when working with the solid material. Wear protective gloves, goggles and clothing. Take special care to avoid skin exposure if handling solutions and work in adequately ventilated areas. Wash hands thoroughly after handling.

4.1.2. Emergency procedures

General advice	Immediately call a POISON CENTER or doctor/physician. Show this safety data sheet to the doctor in attendance. Move out of dangerous area.
If inhaled	Move person into fresh air. If not breathing give artificial respiration. Consult a physician.
In case of skin contact	Wash off with soap and plenty of water. Consult a physician.
In case of eye contact	Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
If swallowed	Immediately call a POISON CENTRE or doctor/physician. Never give anything by mouth to an unconscious person. Rinse mouth with water.

4.1.3. Spillage / Projections

Contain spillage and then collect by wet-brushing and place in container for disposal. Keep in suitable, closed containers for disposal according to local regulations.

4.2. Physical and Chemical Properties

Common Name:	Zearalenone
IUPAC Name:	(3S,11E)-14,16-Dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione
Synonyms:	(S)-Zearalenone, *trans*-Zearalenone, ZEN, ZON, ZEA

CAS Registry Number:	17924-92-4
Molecular Formula:	$C_{18}H_{22}O_5$
Molar Mass:	318.364 $gmol^{-1}$
Monoisotopic mass:	318.147
Melting point:	187−189 °C (D/L) ; 164−165 °C (L) [9]
Appearance:	White crystalline powder
Solubility:	Insoluble in water; soluble in aqueous alkali. Slightly soluble in hexane; progressively more soluble in benzene, acetonitrile, methylene chloride, chloroform, methanol, ethanol, acetone and DMSO.
UV maxima	CH_3OH: 236 nm ($\varepsilon = 29,700$), 274 nm (13,909), 316 nm (6,020) [9] CH_3CN: 274 nm ($\varepsilon = 12,623 \pm 111$) [10]

4.3. Structure

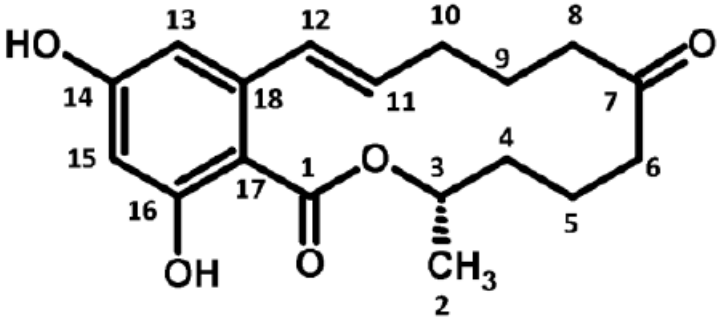


Figure 1 — Structure of ZEN with IUPAC-recommended ring numbering

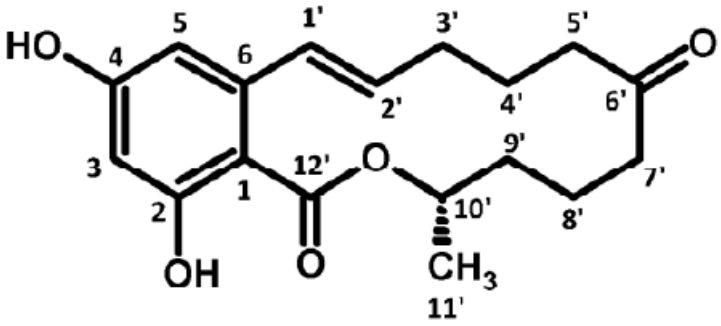


Figure 2 — Structure of ZEN with alternative ring numbering scheme

4.4. Qualitative NMR

4.4.1. Materials and methods

Chemicals:

- Zearalenone (ZEN); BIPM Reference OGO.178a
  Supplier: First Standard, Product No. 1ST7204, Lot ALT601341

NMR Solvents:

- Dimethylsulfoxide-d_6 (DMSO-d_6); BIPM Reference OGS.027c
- Deuterated chloroform ($CDCl_3$); BIPM Reference OGS.026b
- Acetone-d_6; BIPM Reference OGS.029

Solvents were purchased from a commercial supplier and used without further treatment.

4.4.2. Sample preparation

For qualitative NMR analyses an individual sample size of approximately 10 mg of ZEN was made up in 1 mL of deuterated solvent in a glass vial. The sample solution was mixed in a vortex shaker and transferred into NMR tubes (HG-Type: high grade class, 8 in, 5 mm o.d., with PE caps) using disposable glass pasteur pipettes.

4.4.3. NMR acquisition parameters

A JEOL ECS-400 spectrometer operating at 9.4 T (400 MHz for proton) equipped with a direct type automatic tuning (Royal) probe was used for all data acquisition. For qualitative analyses, ^{1}H spectra were acquired for both solvent blank and the ZEN sample using a simple pulse-acquire sequence with the parameters presented in Table 1.

Table 1. Acquisition parameters for exploratory ^{1}H analyses.

Parameter	Value
Number of Transients	512
Receiver gain	34
Acquisition time (s)	3.27
Relaxation delay (s)	1.0
Pulse offset (ppm)	7.0
Spectral width (ppm)	20.0
Data points	32768
Temperature (K)	298
Spinning	Off

^{13}C experiments were conducted using an ordinary power gated sequence (pulse-acquire in ^{13}C channel with proton decoupling both during acquisition and the relaxation delay) using the parameters shown in Table 2.

Table 2. Acquisition parameters used for ^{13}C analyses.

Parameter	Value
Number of Transients	1024
Receiver gain	50
Acquisition time (s)	1.04
Relaxation delay (s)	2.0
Pulse offset (ppm)	100
Spectral width (ppm)	250
Data points	32768
Temperature (K)	298
Spinning	Off

4.4.4. 1D ^{1}H and ^{13}C spectra

The simple ^{1}H and ^{13}C NMR spectra of the ZEN material are shown in Figure 3 and Figure 4. The results obtained were consistent with literature assignments. [10], [11] Figure 5 shows the attached proton test (APT) ^{13}C spectrum of ZEN. Inverted signals correspond to methylene or quaternary carbons and normal signals to methine or methyl carbons.

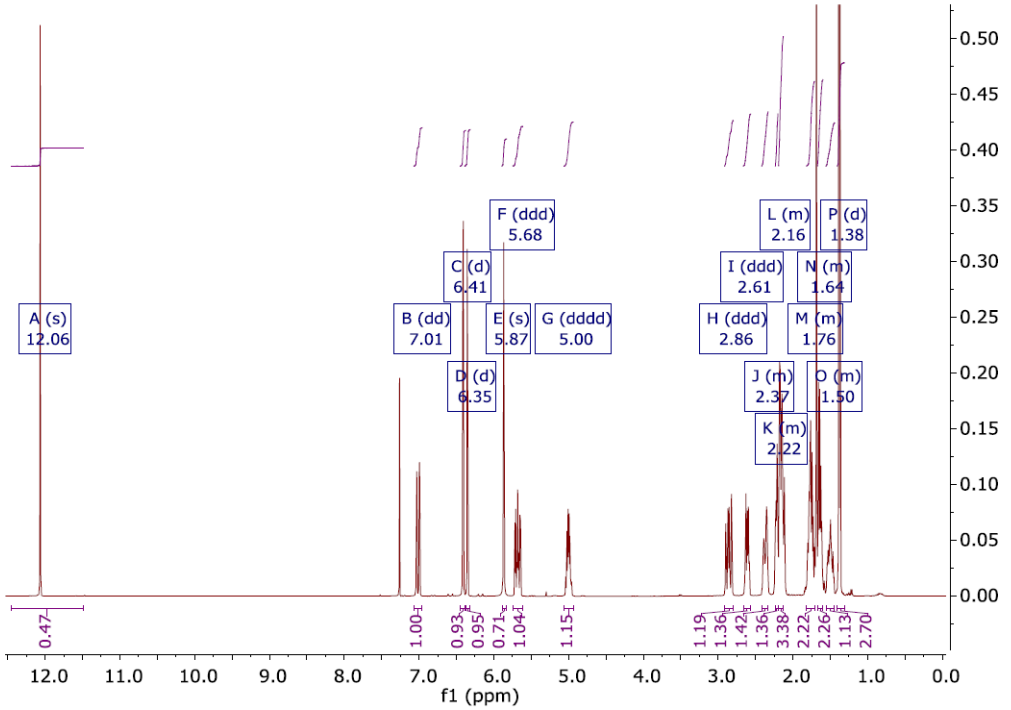


Figure 3 — ^{1}H NMR spectrum of the ZEN in $CDCl_3$.

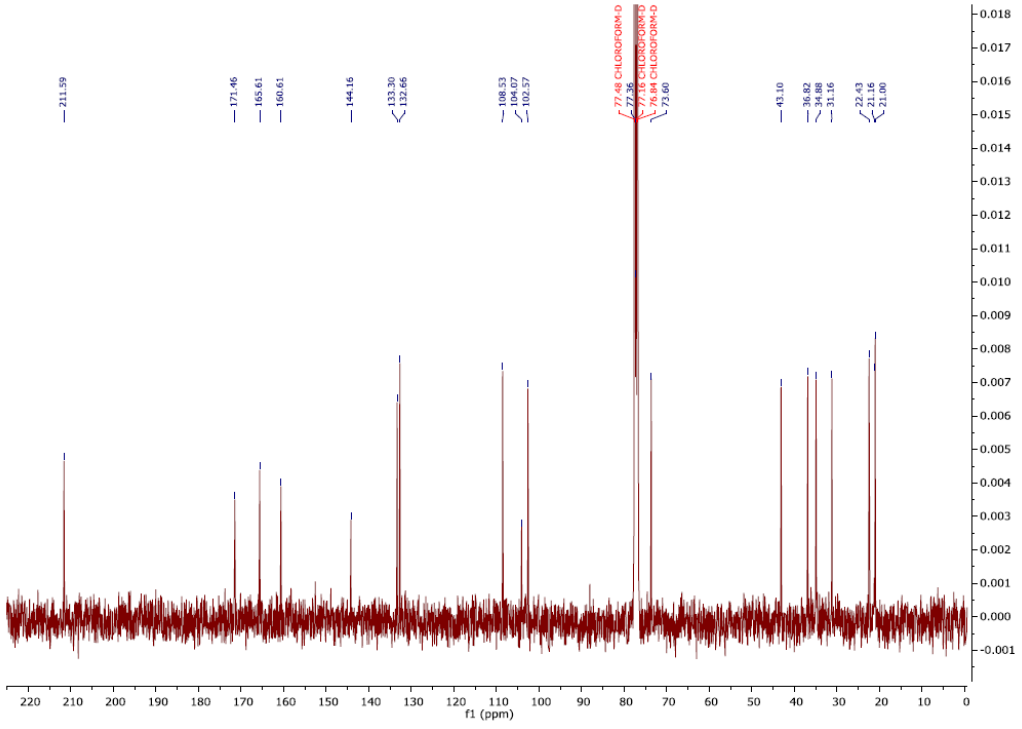


Figure 4 — ^{13}C spectrum of ZEN in $CDCl_3$.

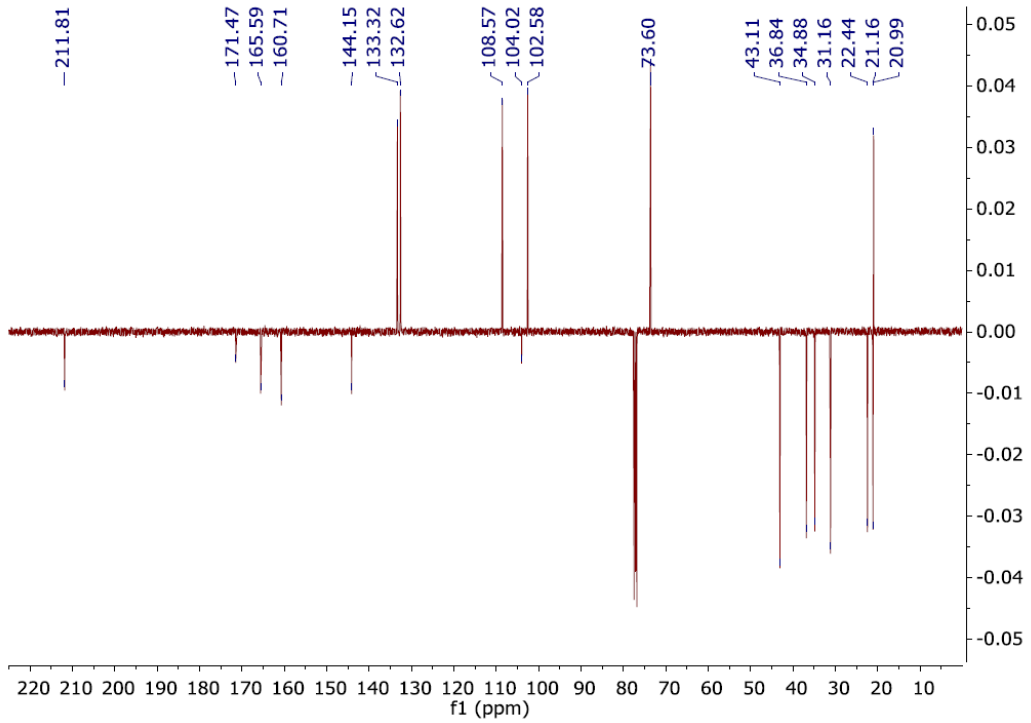


Figure 5 — APT spectrum of ZEN. Down = CH_2/C_q; Up = CH/CH_3.

4.4.5. 2D NMR spectra

To confirm the identification and assignment of the signals, two-dimensional homonuclear correlated spectroscopy (COSY), heteronuclear single-quantum correlation spectroscopy (HSQC) and total correlation spectroscopy (TOCSY) spectra [11], [12] were acquired. The individual spectra are reproduced in Annex A1.2. From the combined data the peak assignments are summarized in Table 3 below. The results are fully consistent with the literature assignments and established the identity of the main component in the material as ZEN.

Table 3. ^{1}H and ^{13}C peak assignments for ZEN in OGO.178.a.

ZEN	^{1}H-NMR (ppm) [a]	^{13}C-NMR (ppm)	COSY	HSQC (ppm)
1	-	171.5	-	-
2	1.38 (3H)	21.0	Couples with 5.00 only	Couples with 20.9
3	5.00	73.6	Couples with 1.38 and 1.64	Couples with 73.5
4	1.64 (2H)	34.9	Couples with 1.38 and 1.76	Couples with 34.8
5	1.76 (2H)	22.4	Couples with 1.64, 2.16 and 2.61	Couples with 22.4
6	2.16 and 2.61	43.1	2.16 with 1.76 and 2.61 2.61 with 1.76 and 2.16	Both with 43.0
7	-	211.8	-	-
8	2.22 and 2.86	36.8	2.22 with 1.50 and 2.86 2.86 with 1.50, 2.16 and 2.86	Both with 36.8

ZEN	^{1}H-NMR (ppm) [a]	^{13}C-NMR (ppm)	COSY	HSQC (ppm)
9	1.50 and 2.16	21.2	1.50 with 2.16, 2.22 and 2.86 2.16 with 1.50, 2.37,2.86 and 5.68	Both with 21.1
10	2.37 and 2.16	31.2	2.16 with 1.76, 2.37, 5.68 2.37 with 1.76, 2.16, 5.68, 7.01	Both with 31.1
11	5.68	132.6	Couples with 2.16, 2.37 and 7.01	Couples with 132.6
12	7.01	133.3	Couples with 2.37 and 5.68	Couples with 133.1
13	6.41	108.6	Couples with 6.35 only	Couples with 108.5
14	-	165.6	-	-
15	6.35	102.6	Couples with 6.41 only	Couples with 102.5
16	-	160.7	-	-
17	-	104.0	-	-
18	-	144.2	-	-
14-OH	5.87	-	-	-
16-OH	12.06	-	-	-

(*a*) All reported ^{1}H signals correspond to one proton except where noted in brackets

4.4.6. Residual solvent content by NMR

In the ^{1}H NMR spectrum of the BIPM material it was possible to detect impurity peaks not present in the solvent blank originating from residual solvents: a singlet at 5.3 ppm from dichloromethane, a singlet at 3.33 ppm from methanol and a quartet at 3.5 ppm which could be either ethanol or diethyl ether. The latter is the more likely according to previously reported chemical shifts. [13]

To obtain an accurate quantification of these small signals a spectrum was acquired using 512 transients, a relaxation delay of 60 s between scans and applying the parameters optimized for quantitative analysis of ZEN (see Table 5 in Section 5.2, see p. 18). From this spectrum, the mass fractions of the residual solvents were calculated from the ratio of the signal integral to that of reference peaks in the ZEN spectrum. Two possible scenarios for the origin of the quartet at 3.5 ppm (either ethanol or diethyl ether) were considered. Two different ZEN peaks were used as reference values and the average values were considered as fit-for-purpose estimates of the mass fractions of the residual solvent content. To investigate the possibility that the quantification of these residual solvent peaks was influenced by contributions from ^{13}C satellite peaks of adjacent ZEN peaks an acquisition using ^{13}C-decoupling and otherwise the same parameters was also performed and the results compared.

The data from the two experiments (with and without ^{13}C-decoupling) with calculation relative to two different ZEN peaks provided a combined result derived from the four calculated values for the levels of each residual solvent. The measurement uncertainty for this result includes contributions from the uncertainty in the molar masses of both ZEN and the

solvents in addition to the pooled variation between the different measurement procedures (2 peaks, 2 acquisitions). The difference in the combined residual solvent content due uncertainty in whether ethanol or diethyl ether is present is negligible, but in order to maintain metrological traceability, an additional uncertainty component was added to take this into account. On the basis of the observed chemical shift the more likely identity is diethyl ether. If desired the identities of the residual solvents could be independently established and quantified using headspace GC-MS based technique. The results for residual solvent content assigned by the relative NMR response and their associated measurement uncertainties are summarized in Table 4. Representative spectra showing each solvent signal relative to the adjacent ZEN peaks are given in Annex A1.4.

Table 4. Estimated mass fraction content of the residual solvents detected in the OGO.178a material.

Solvent	Mass fraction (mgg^{-1})	U_{95} (mgg^{-1}) ($k = 2$)
CH_2Cl_2	1.28	0.023
MeOH	0.18	0.004
Et_2O	0.47	0.053
Total	1.93	0.06

4.5. UV-Vis spectrophotometry

Methods were developed for wavelength scan and fixed wavelength measurements:

Scan wavelength:

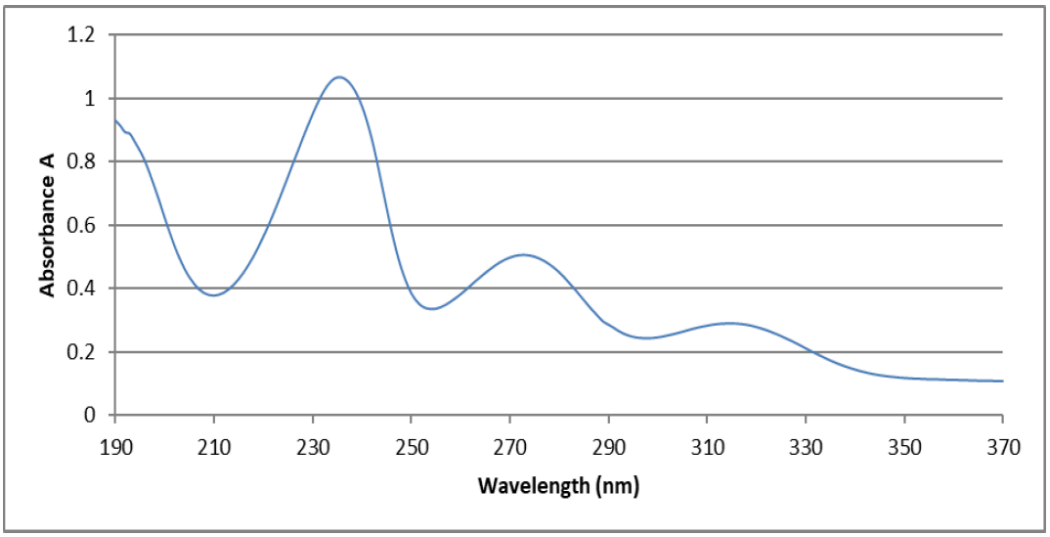
- Deuterium lamp: on
- Tungsten lamp: on
- Scan from 370.00 nm to 190.00 nm
- Data interval: 1.00 nm, scan speed: 266.75 nm min^{-1}
- Slit: 2 nm

Fixed wavelength:

- Deuterium lamp: on
- Tungsten lamp: on
- Wavelengths: 235 nm, 274 nm and 314 nm (for OGP.025, only 274 nm and 235 nm)
- Cycle: 3
- Slit: 1 nm
- Gain: Auto
- Response 0.2 s

- No cell changer
  Reference cell contains pure acetonitrile.
  Temperature was controlled and fixed at 20 °C.

Minimum sample intake: 50 µL



Equivalent UV data for ZEN is reported in reference [10]

4.6. Mass spectrometry

Reference MS and MS/MS data for ZEN under a variety of ionization conditions are available under the entry for "zearalenone" from various open access online databases including the European Mass Bank, the Mass Bank of North America and PubChem.

5. Purity assignment of Zearalenone

5.1. Introduction

This section of the Guideline describes the approach developed during the BIPM MMCBKT program for the purity assignment of the ZEN source material. It is based on a quantitative NMR (qNMR) measurement [14], [15] to quantify the total ZEN and related structure impurity content with correction of the raw qNMR result for the ZEN-related impurity content quantified by LC methods.

The qualitative identity of the ZEN material was established and an estimate of residual solvent impurity content in the material was obtained using the combination of 1D- and 2D-NMR techniques described in Section 4.4 above. This identification was supported by determination of the mass spectrometric and UV-Vis spectrophotometric properties of the material. The assignment of the ZEN content by qNMR through the selection of quantification peak(s), the identification of an appropriate internal standard and the choice of a deuterated solvent is described in Section 4.2.

The development and application of methods for the identification and quantification of the ZEN-related impurity content of the material by LC-MS/MS and LC-DAD is described in Section 4.3. These results were used to correct the “raw” qNMR value for ZEN and ZEN-related impurity content in the source material to give the final assignment of the actual ZEN content of the material.

Supporting analyses undertaken to detect other impurity classes are summarized in Section 4.4 and the selection and combination of the data to give the final purity assignment of the material is described in Section 4.5.

Another approach for the purity assignment of ZEN has been reported through a combination of data obtained by NMR, LC-MS, UV spectrophotometry and DSC. [10]

DISCLAIMER: Commercial NMR and LC instruments, software and materials are identified in this document in order to describe some procedures. This does not imply a recommendation or endorsement by the BIPM nor does it imply than any of the instruments, equipment and materials identified are necessarily the best available for the purpose.

5.2. qNMR

5.2.1. Materials

Chemicals

- Zearalenone (ZEN); BIPM Reference OGO.178a
  Supplier: First Standard, Product No. 1ST7204, Lot ALT601341
- Zearalanone (ZAN); BIPM Reference OGO.182a
  Supplier: First Standard, Product No. 1ST7203, Lot LZ106742
- Dimethylterephthalate (DMTP); BIPM Reference OGE.022b was used as the qNMR internal standard BIPM-2019/1. The mass fraction content of DMTP in the material was assigned as 999.3 ± 0.8 mgg^{-1} $(k = 2)$ by qNMR at the BIPM.

NMR Solvents:

- Acetone-d_6; BIPM Reference OGS.029
- Dimethylsulfoxide-d_6 (DMSO-d_6); BIPM Reference OGS.027c
- Deuterated chloroform ($CDCl_3$); BIPM Reference OGS.026b

Deuterated solvents were purchased from a commercial supplier and used without further treatment. NMR tubes were HG-Type: high grade class, 8 in, 5 mm diameter rated for use with 600 MHz spectrometers fitted with PE caps.

5.2.2. Sample preparation

Gravimetric operations were performed using a Mettler Toledo XP2U ultramicrobalance. Prior to all weighing operations the repeatability of the balance was assessed for suitability to the preparation of qNMR samples by repeat mass determinations of an empty weigh boat. The general recommendations of Yamazaki et al [17] for qNMR sample preparation were used.

Four separate samples were prepared. The individual sample sizes were in the range 4 mg—10 mg for the ZEN material and 2 mg to 4.5 mg for the internal standard DMTP. Each sample was separately weighed into an aluminium weighing boat and then to avoid contact of the solvent with the metal boat, the contents of both were carefully transferred into a common glass vial and each emptied boat was reweighed. The amount of ZEN and DMTP transferred into the glass vial was determined by difference and this value was used for subsequent qNMR calculations. 1 mL of deuterated solvent was added to the vial and the sample solution was mixed in a vortex shaker and checked visually for completeness of dissolution. Approximately 800 µL of this solution was transferred into an NMR tube (HG-Type: high grade class, 8 in, 5 mm o.d., with PE cap) using a glass pasteur pipette.

5.2.3. Choice of solvent and quantification signals

Because of the complexity of the ZEN proton spectrum in the upfield section of the spectrum ($\delta < 5$ ppm) the potential quantification peaks are limited to those occurring at chemical shift between 5 ppm and 7 ppm, corresponding to the aromatic (H-13 and H-15), olefinic (H-11 and H-12) and lactone bridge (H-3) protons.

$CDCl_3$, DMSO-d_6 and acetone-d_6 were investigated as possible solvents. The hydrogen peak from the phenol at position 14 overlays the signal for H-11 at 5.7 ppm, rendering this peak unsuitable for quantification. The signals due to the two aromatic hydrogens centered at 6.4 ppm were associated in this material with small impurities at the baseline of the peak which were considered too close to be subtracted. The most attractive signals for quantification purposes were that at 7.0 ppm corresponding to the H-12 proton and that at 5.0 ppm due to H-3. The peak at 5.0 ppm is a complex multiplet with lower intensity compared to the peak at 7.0 ppm resulting in a lower relative signal to noise ratio. In addition all impurities in the material from either the ZEN or ZAN family will have a signal at a similar chemical shift. It was known from the LC characterization of the material (see Section 4.3, see p. 10) that ZAN was one of the major impurities in the material.

The peak at 7.0 ppm was judged as more suitable for quantification as it would not include any contribution from the ZAN impurity or ZAN-related impurities. However in $CDCl_3$ this peak is overlaid by the residual chloroform ^{13}C satellite. In DMSO-d_6 the chemical shift of the peak moves to 6.6 ppm. It is now in too close proximity to the signals for the aromatic hydrogen

to be used for qNMR. However for acetone-d_6 the H-12 signal chemical shift remains at 7.0 ppm and the residual solvent peak is well separated from the quantification region.

An unanticipated advantage was also discovered in the use of acetone-d_6 as solvent. It was observed that a significant curve occurred in the baseline of the spectra of ZEN in solution in $CDCl_3$ or DMSO-d_6 which was not in evidence with spectra in acetone-d_6. This may simply result from a contribution to the baseline from a broad acidic hydrogen signal in the aprotic solvents that is exchanged out in solution in acetone-d_6 due to the unavoidable presence therein of a small amount of water associated with the solvent. Whatever the source of the interference a bias to lower values was observed when qNMR was carried out on ZEN in solution $CDCl_3$ and DMSO-d_6 compared with the value obtained in solution in acetone-d_6. It is strongly advised to ONLY use acetone-d_6 for qNMR studies of ZEN materials.

Spectra illustrating the contrast between the baseline of the NMR spectrum of ZEN in acetone-d_6 and $CDCl_3$ are reproduced in Annex A1.6.

DMTP was selected as the internal standard selected for the qNMR study. BIPM-2019/1 This material is readily soluble and stable in both non-polar and semi-polar solvents such as acetone-d_6. The signal due to the four equivalent aromatic protons in DMTP which occur as a sharp singlet at 8.0 ppm was used for quantification. The integration ratio was calculated against both the multiplet ZEN H-12 signal at 7.0 ppm and the H-3 signal at 5.0 ppm. The initial qNMR result for the quantification against the signal at 5.0 ppm must be corrected for contributions from all three impurities identified by LC-methods (see Section 4.3, see p. 10) whereas the result using the signal at 7.0 ppm need only be corrected for contributions from 6-dehydro ZEN and *cis*-ZEN impurities.

5.2.4. NMR acquisition parameters

A JEOL ECS-400 spectrometer operating at 9.4 T (400 MHz for proton) equipped with a direct type automatic tuning (Royal) probe operating using the Delta software was used for all NMR data acquisition.

The general recommendations for optimizing spectrometer performance, determining the relevant NMR experiment parameters and undertaking a qNMR experiment as described in the BIPM Internal Standard Reference Data report for the use of DMTP for qNMR measurements BIPM-2019/1 were followed. The final qNMR acquisition parameters are summarized in Table 5.

Table 5. Acquisition parameters for qNMR.

Parameter	Value
ZEN Sample size (mg)	4 – 10
DMTP Sample size (mg)	1.8 – 4.3
Number of Transients	64
Receiver gain	36

Acquisition time (s)	4
Relaxation delay (s)	56
Pulse offset (ppm)	7.3
Spectral width (ppm)	400
Data points	639652
Temperature (K)	298
^{13}C-Decoupling	On
Spinning	Off
Integral ratio (ZEN:DMTP)	0.25 – 0.48

5.2.5. qNMR signal integration

A baseline correction window of eighty times the FWHM was applied to each integrated signal. The integration range start and end points were placed fifty Hz beyond the visible edge of each signal. Results from four independent sample mixtures each measured four times were obtained.

5.2.6. Value assignment and measurement uncertainty

Results from four independent sample mixtures each measured four times on the day of preparation were obtained with quantification using the one proton signal due for H-12 in ZEN at $\delta = 7.0$. The measurement uncertainty budget is reproduced below in Table 6. The integral ratio is the overall mean of the four replicate values obtained for each of the four samples, normalized for the different sample sizes used in their preparation. The standard uncertainty of the normalized ratio is the standard deviation of the mean based on the use of four independent samples. The relative contribution of each component to the uncertainty of the result for this material is displayed in Figure 6. The mass fraction content of "ZEN" in the material from this analysis, quantified against the 7.0 ppm NMR peak in "ZEN", was 998.0 ± 1.8 mgg^{-1}, bearing in mind that this estimate includes the contributions from the 6-dehydro ZEN and *cis*-ZEN impurities.

Table 6. **Uncertainty budget for ZEN purity[1] by qNMR using DMTP in acetone-d_6.**

1

ZEN value uncorrected for 6-dehydroZEN and cis-ZEN impurities (1)

[1] ZEN value uncorrected for 6-dehydroZEN and cis-ZEN impurities

Uncertainty sources	Value	Type	Standard Uncertainty	Sensitivity coefficient	Uncertainty Component
I_S/I_A (repeatability)	0.3382	A	0.00011	2950.768839	3.28E-01
Analyte signal ^1H Nuclei	0.9998	B	0.0003	-998.0293134	2.99E-01
IS signal ^1H Nuclei	3.9992	B	0.0004	249.5400266	9.98E-02
Analyte Molar Mass	318.364	B	0.0167	3.134241647	5.22E-02
IS Molar Mass	194.186	B	0.0085	-5.138525474	4.36E-02
Analyte mass (mg)	4.0700	B	0.00124	-245.1670043	3.05E-01
IS mass (mg)	1.8326	B	0.00124	544.488545	6.77E-01
IS purity (mgg^{-1})	999.3	B	0.4	0.998528678	3.99E-01
				Combined Uncertainty	9.08E-04

Purity of ZEN $998.0 \pm 1.8\ mgg^{-1}$

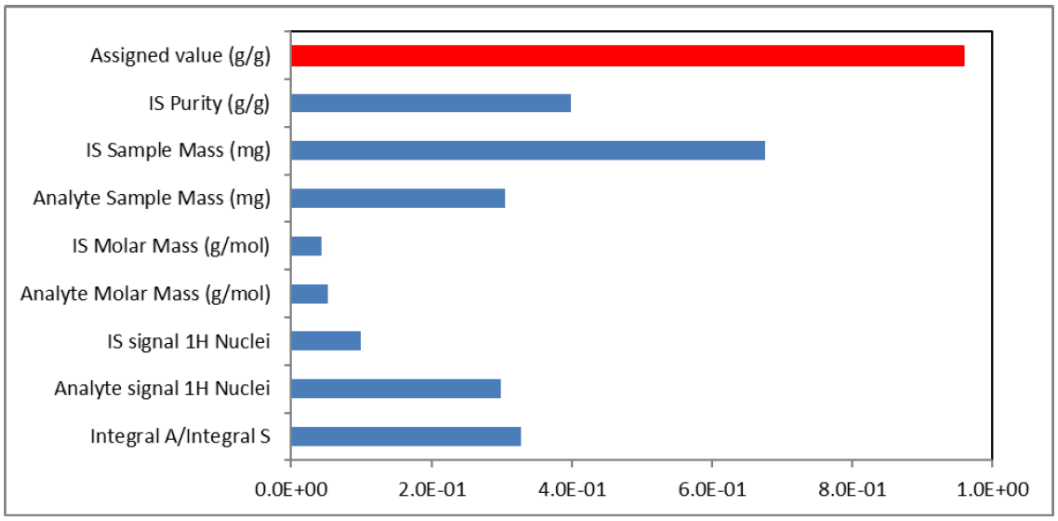


Figure 6 — Relative uncertainty components: ZEN assignment using DMTP in acetone-d_6.

Note in the uncertainty budget that the contribution from the gravimetric operations and the purity of the internal standard are as important to the overall uncertainty of the purity assignment as the contribution due to the repeatability of the integral ratio determination.

The qNMR assignment was repeated using the same set of NMR data obtained on the day of preparation of the sample but with quantification against the one proton signal for H-3 in ZEN at δ 5.0. It was also repeated using the same samples and NMR acquisition and processing parameters three and seven days after the original sample preparation in order to evaluate the

stability of the ZEN in solution. The qNMR assignments were obtained for each data set with quantification against both the δ 7.0 ppm and δ 5.0 ppm signals. The combined assignments are summarized in Table 7

Table 7

Time after prep (days)	ZEN content [(a)] (mg/g, $\delta = 7.0$)	ZEN content [(b)] (mg/g, $\delta = 5.0$)
$T = 0$	998 ± 2	995 ± 2
$T = 3$	996 ± 2	991 ± 2
$T = 7$	997 ± 2	994 ± 2

(*a*) ZEN value uncorrected for 6-dehydroZEN and cis-ZEN impurities

(*b*) ZEN value uncorrected for ZAN, 6-dehydroZEN and cis-ZEN impurities

5.3. Related structure impurities by LC-UV and LC-MS/MS

5.3.1. Materials

Chemicals:

- Zearalenone (ZEN); BIPM Reference OGO.178a
  Supplier: First Standard, Product No. 1ST7204, Lot ALT601341
- Zearalanone (ZAN); BIPM Reference OGO.182a
  Supplier: First Standard, Product No. 1ST7203, Lot LZ106742
- α-Zearalenol (α-ZEL), β-Zearalenol (β-ZEL), α-Zearalanol (α-ZAL), β-Zearalanol (β-ZAL) were all provided as 100 ppm solutions in acetonitrile by First Standard

5.3.2. Standard solutions

Two standard solutions of mass fractions of approximately 200 $mgkg^{-1}$ were prepared using the ZEN (OGO.178) and ZAN (OGO.182) materials. Each stock solution consisted of approximately 4.2 mg of material weighed accurately and made up to the mark in a 25 mL flask with acetonitrile to a final weight of approximately 19.4 g. Gravimetric operations were undertaken using a Mettler AX-504 balance. The ambient temperature during gravimetric operations was 22 °C and relative humidity 54 %. For further calculations the mass fraction content of the ZEN and ZAN was based on the purity assigned for these materials at the BIPM by qNMR. For the solutions of α/β-ZAL and α/β-ZEL the values provided by the supplier were used.

From these six standard solutions a high concentration mixed working solution (HMC) of ZEN, ZAN, α/β-ZAL and α/β-ZEL was gravimetrically prepared by weighing accurately approximately 72 mg of three stock solutions (ZEN, ZAN, β-ZAL) and 110 mg of the other stock solutions (α-ZEL, β-ZEL, α-ZAL) in a 1.5 mL HPLC vial and adding acetonitrile to a final weight of approximately 0.9 g to obtain a mixed working solution. From this high concentration mixed working solution, a middle concentration mixed working solution (MMC) was prepared by accurately weighing approximately 460 mg of the HMC solution in a 1.5 mL HPLC vial and adding water to a final weight of approximately 1 g

A series of five standard dilutions for each of “day 1” and “day 2” measurements were gravimetrically prepared by further dilution of the MMC solution.

5.3.3. LC-DAD method development

A Phenomenex Kinetex EVO C-18 100Å column with dimensions 250 × 4.6 mm and 2.6 μm particle size was used to separate the ZEN, ZAN and related structure compounds in the ZEN material. According to previous research [10] ZEN is of limited stability in solution in methanol and this solvent should be avoided for use in the mobile phase for chromatographic analysis of ZEN. A method using as mobile phase an acetonitrile/water gradient was optimized to achieve adequate resolution of the six components of the test mixture in the smallest possible runtime.

Column:	Phenomenex Kinetex EVO C_{18} 100Å,(250 × 4.6 mm, 2.6 μm) (OGLC.65)	
Column temperature:	25 °C	
Detector:	Qtrap, UV lamp and visible lamp required	
Detection wavelength:	**274** nm **(reference wavelength 360** nm**)**	
Mobile phase:	1. acetonitrile:H_2O = 40:60 (v/v) + 0.1% HCOOH	
	2. acetonitrile (+ 0.1% 0.1% HCOOH)	
Operation mode:	Gradient (inclusive cleaning gradient)	
Solvent gradient:	Time(min)	Mobile phase A
	0.0	100%
	45.0	100%
	46	5.0%
	47	5.0%
	48	100%
	65	100%
Flow rate:	0.6 mL min^{-1}	
Detector:	DAD 274 nm	
	scan range: 190 nm−600 nm; step width: 2.0 nm;	
	slit width: 4 mm;	
Injection Mode:	Standard	
Injection volume:	10 μL	

A chromatogram of the separation of the ZEN, ZAN, α/β-ZAL and α/β-ZEL HMC standard solution obtained using this method with detection at 274 nm is shown in Figure 7.

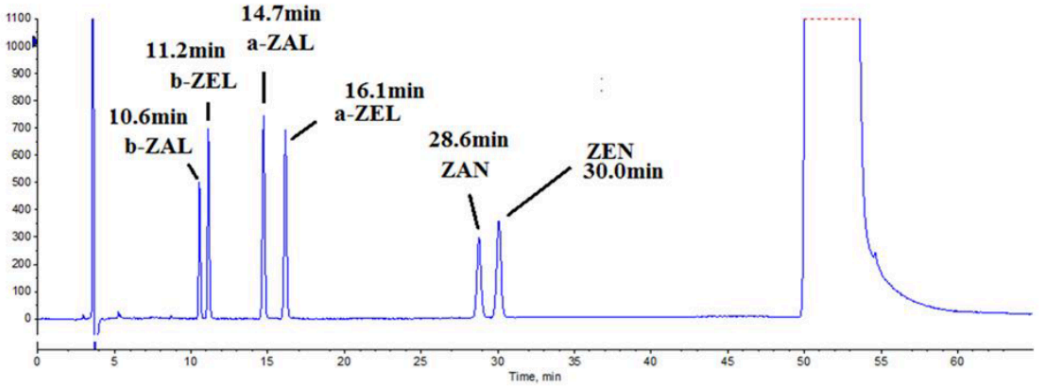


Figure 7 — Chromatographic separation of the test mixture with LC-DAD detection

5.3.4. LC-DAD method performance

Method validation consisted of the evaluation of the method limit of detection (LOD), limit of quantification (LOQ), linearity, sensitivity, repeatability and intermediate precision.

Performance characteristics (limits of detection (LOD) (S/N = 3), limits of quantification (LOQ) (S/N = 6), sensitivity, etc. were calculated corresponding to the 95 %—confidence level (CI) of the y-intercept according to the German standard DIN 32645 for each of ZEN, ZAN, α/β-ZAL and α/β-ZEL. The characteristics were evaluated from the calibration curves obtained from triplicate determinations of standard solutions.

In total, five mass fraction levels were analysed based on the amount of impurity estimated to be present in the ZEN material. The results for ZEN and ZAN are displayed in Table 8.

Table 8. Performance characteristics for ZEN and ZAN. Values in brackets and "CI (95 %)" values were obtained from calculations using the tool "Validata".

Compound	ZEN		ZAN	
Measurement series	Day 1	Day 2	Day 1	Day 2
Mass fraction range (ngg^{-1})	218.9-3428.8	215.9-3436.4	229.1-3588.6	225.9-3596.5
Mass fraction levels	5	5	5	5
Replicates	4	4	4	4
LOD (ngg^{-1})	127.3	109.1	109.9	103.5
LOQ (ngg^{-1})	254.8	218.1	219.9	207.0
Sensitivity (PA*s g/ng)	0.0314	0.03062	0.0313	0.03028
CI (95%) Sensitivity (PA*s g/ng) : -/+	0.0312/ 0.0316	0.03029/ 0.03095	0.0310/0.0315	0.03000/ 0.03056
y-Intercept (PA*s)	0.0616	0.42405	0.1463	0.35645
CI (95%) y-Intercept(PA*s) : -/+	0.33249/ 0.90697	-0.1620/ 1.0101	-0.33646/ 0.62900	-0.18029/ 0.89319
Coefficient of correlation	0.9999	0.9998	0.9998	0.9999
Rel. std error of estimate (%)	0.97	2.00	1.48	1.7

5.3.5. Impurities in ZEN by LC-DAD

Two samples of the ZEN source material OGO.178a were analysed for related impurities using this method. About 4.2 mg of the ZEN material was transferred by aluminium weighing boat into a tared class A 25 mL volumetric, taken up in pure acetonitrile and allowed to stand over night at 4 °C. Aliquots of each solution were transferred into HPLC screw top vials for analysis. The target mass fraction content of ZEN in the solution was aimed to be about 200 $\mu g g^{-1}$ in order to be able to detect low levels of related structure impurities. The mass fraction of each identified impurity was derived by external calibration over an appropriate linear mass fraction range.

For a ZEN sample at a mass fraction of 200 $\mu g g^{-1}$ for the major component, 0.1 % (or 0.2 $\mu g g^{-1}$) of an impurity corresponds to an absolute mass fraction of 200 $n g g^{-1}$. The absolute LODs corresponding to 3*S/N approach for the absorbance at 274 nm range from 53 $n g g^{-1}$ for α-ZEL to 127 $n g g^{-1}$ for ZAN, giving confidence that this method would be able to detect resolved ZEN-related impurities present at relative levels above 0.1 %.

The chromatogram obtained for the ZEN material in solution in acetonitrile at 200 $\mu g g^{-1}$ is shown in Figure 8. The upper pane shows the 274 nm response and the lower pane the TIC obtained by a parallel LC-MS/MS analysis of the same solution. Two impurities were detected using the LC-DAD method. The impurity eluting at 28.5 min was identified as ZAN based on its retention time and this assignment was subsequently confirmed by LC-MS/MS. The other impurity corresponded closely but not exactly to the retention time α-ZEL. Subsequent LC-MS/MS analysis identified this impurity as 6-dehydroZEN.

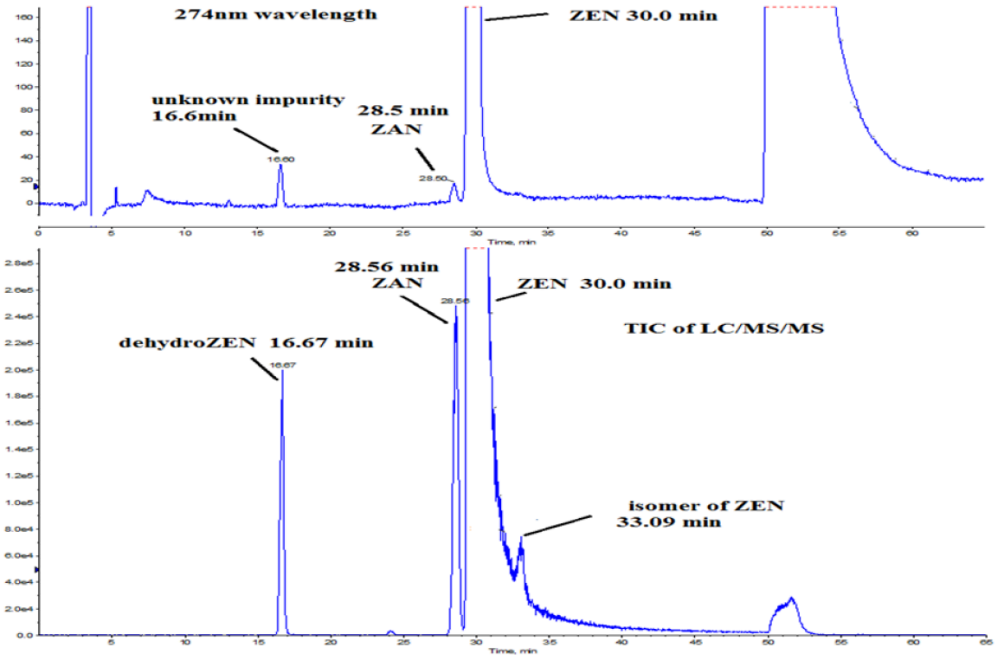


Figure 8 — Chromatogram of LC-DAD (top) and LC-MS/MS (bottom) analysis of a solution of the ZEN OGO.178a source material

No significant amounts of α/β-ZAL or α/β-ZEL were observed in the ZEN material by LC-DAD and this conclusion was subsequently confirmed by LC-MS/MS analysis.

The quantification of the ZAN content in the ZEN material was undertaken using an external calibration curve based on the available ZAN standard. For the 6-dehydro ZEN a calibration curve based on α-ZEL was used since they had similar retention time and no authentic standard

was available for the impurity. The impurity mass fraction values and expanded measurement uncertainties as determined by the LC-DAD method are shown in Table 9.

Table 9. Related structure impurity content of OGO.178a by LC-DAD

Impurity	Mass fraction (mgg^{-1})	U(mgg^{-1})
6-DehydroZEN	1.033	0.052
ZAN	1.325	0.008

5.3.6. LC-MS/MS method development

A method of liquid chromatography with tandem mass spectrometry (LC-MS/MS) was also developed for the simultaneous determination of ZEN, ZAN, α/β-ZAL and α/β-ZEL in the ZEN material. The same column and gradient method described for the LC-DAD analysis were used as the DAD and MS/MS measurements were undertaken in tandem on column eluant from the same LC system.

The MS parameters in negative electrospray ionization (ESI neg.) mode were optimised by direct infusion into the ionization source of the QTrap of single LC standards of ZEN, ZAN, α/β-ZAL and α/β-ZEL and the MRM parameters of each compound were optimized. Each measurement using the optimized parameters was undertaken in triplicate. Optimized intensity was obtained at capillary voltage of −4500 V and source temperature of 550 °C. Nitrogen was used as the ion source gas, curtain gas and collision gas. The Gas 1 and Gas 2 of ion source were at 55 psi and 50 psi; the curtain gas (CUR) was set at 15 psi. The Collision Gas (CAD) was set at Mid. Table 10 lists the results for each compound's optimization in MRM mode.

The transitions of impurities in the material for which no authentic sample was available (6-dehydroZEN, dihydroxy-6-dehydronsZEN, *cis*-ZEN) were determined by direct observation.

Table 10. Ions transitions and MS/MS parameters of ZEN and its impurities in MRM mode

Compounds	Q1 m/z	Q3 m/z	Time (ms)	DP(V)	CE(V)	EP(V)	CXP(V)
Zearalenone (ZEN)	317.2	131.1*	50	-95	-40	-11	-10
		175.1	50	-95	-30	-11	-10
		187.0	50	-95	-27	-11	-10
Zearalanone (ZAN)	319.3	275.0*	50	-110	-30	-11	-10
		205.1	50	-110	-33	-11	-10
Zearalenol (α/β-ZEL)	319.3	275.0*	50	-110	-30	-11	-10
		160.1	50	-110	-41	-11	-10
Zearalanol (α/β-ZEL)	321.3	277.1*	50	-110	-33	-11	-10
		303.2	50	-110	-31	-11	-10
Dehydrozearalenone (dehydroZEN)	315.3	175.1*	50	-90	-30	-11	-10
		271.1	50	-90	-30	-11	-10
dehydroZEN +O2	347.2	315.3*	50	-90	-30	-11	-10
		271.1	50	-90	-30	-11	-10
HYD	335.1	187.0	50	-90	-30	-11	-10

Compounds	Q1 m/z	Q3 m/z	Time (ms)	DP(V)	CE(V)	EP(V)	CXP(V)
HYD-CO2	290.1	187.0	50	-90	-30	-11	-10
HYD-H2O	316.0	187.0	50	-90	-30	-11	-10
HYD-C9H18O3	160.0	149.0	50	-90	-30	-11	-10

The TIC chromatogram of the ZEN, ZAN, α/β-ZAL and α/β-ZEL standard solution using this method is shown in Figure 9.

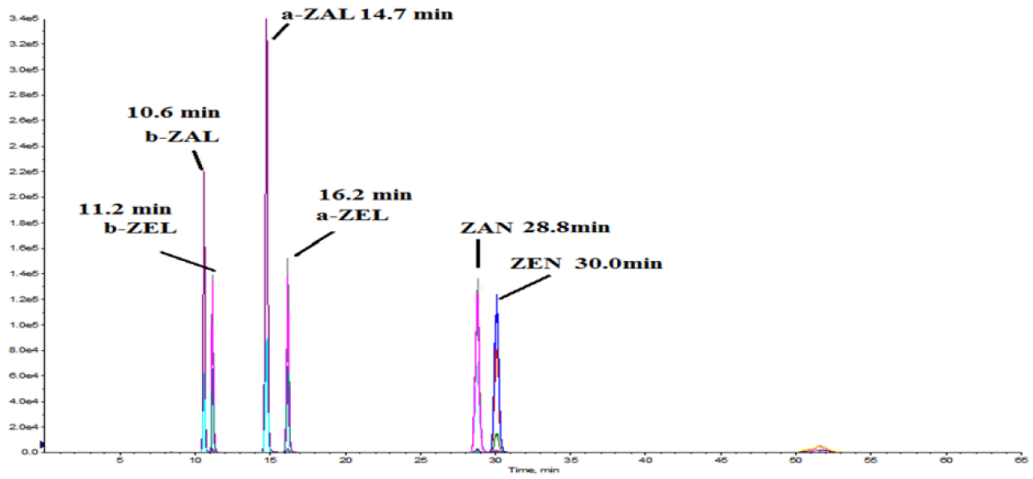


Figure 9 — TIC chromatogram of the standard mixture.

5.3.7. LC-MS/MS method performance

Method validation consisted of the evaluation of the limit of detection (LOD), limit of quantification (LOQ), linearity, sensitivity, repeatability and intermediate precision.

Performance characteristics (limits of detection (LOD) (S/N = 3), limits of quantification (LOQ) (S/N = 6), sensitivity, etc. of the method were calculated corresponding to the 95 % —confidence level (CI) of the y-intercept according to the German standard DIN 32645 for ZEN, ZAN, α/β-ZAL and α/β-ZEL. The characteristics were evaluated from the calibration curves obtained from triplicate determinations of standard solutions.

In total, five mass fraction levels were analysed based on the amount of impurity estimated to be potentially present in the ZEN material. The method performance for ZEN and ZAN is displayed in Table 11.

Table 11. Performance characteristics for ZEN and ZAN. Values in brackets and "CI (95%)" values were obtained from calculations using the tool "Validata"

Compound	ZEN		ZAN	
Measurement series	Day 1	Day 2	Day 1	Day 2
Mass fraction range (ngg^{-1})	28.1-431.8	26.9-432.7	29.4-454.5	28.2-451.9
Mass fraction levels	5	5	5	5
Replicates	4	4	4	4

LOD (ngg^{-1})	4.67	11.8	5.3	15.3
LOQ (ngg^{-1})	9.34	23.6	10.5	30.6
Sensitivity (PA*s g/ng)	10012.4	8368.8	9744.5	8530.1
CI (95%) Sensitivity (PA*s g/ng) : -/+	9871.0 / 10153.9	8068.4/8669.2	9602.3/ 9886.7	8166.3/8893.9
y-Intercept (PA*s)	5367.6	28353.5	22500.8	6523.4
CI (95%) y-Intercept(PA*s) : -/+	-26551.6/ 37286.6	-38731.1/ 95438.0	-12559.6/ 57561.1	-82242.7/ 95289.4
Coefficient of correlation	1.000	0.9999	1.000	1.000
Rel. std error of estimate (%)	2.61	6.73	2.7	7.9

The linearity of the working range was evaluated by constructing calibration curves at five different concentration levels. In order not to reject the linearity hypothesis, a determination coefficient (r^2) of at least 0.999 was required. Sensitivity was also determined from the calibration curve, being equal to the angular coefficient.

Note that the LOD/LOQ estimates for the LC-MS/MS method shown in Table 11 are an order of magnitude smaller than those for the LC-DAD analysis of the same samples listed in Table 10.

5.3.8. Impurities in ZEN by LC-MS/MS

The MS3-IDA-EPI mode of the Qtrap4000 was applied to identify these impurities with a Valco valve function included in the method to cut off the main component from introduction into the ionization chamber.

The HPLC chromatography conditions were listed in the following:

Column Kinetex C18 (2.6 μm · 150 mm · 3.0 mm) OGLC056

Column temperature 35 °C

Mobile phase Acetonitrile+MeOH+H_2O (with 10 mM NH_4AC) =10+45+45

Operation mode isocratic

Flow rate 0.25 mL min^{-1}

Injection Mode Standard

Injection volume 2.0 μL

Duration 30.0 min

A solution of approximately 1.0 mgg^{-1} of ZEN was prepared by accurately weighing approximately 7.7 mg of material into a 10 mL flask and adding methanol to a final weight of approximately 7.85 g. The calculated mass fraction of the ZEN material in the solution was 977.9 μgg^{-1}. To avoid contamination of the LC-MS/MS instrument by the high content of ZEN component, the LC method was slightly changed. After chromatographic separation the mobile phase was switched to waste during elution of the ZEN component, to cut out this peak from the ionization chamber so as to be able to better detect and analyse the minor impurities present in the material. Figure 10 shows the TICs of the ZEN solution in the MS3-IDA-EPI mode of the Qtrap4000 in the upper pane with the LC-DAD response at 274 nm in the lower pane. Four impurities, marked as impurity 1, 2, 3 and 4, were observed in the TIC. Two of these corresponded to peaks also seen at 274 nm using the LC-DAD. After comparison

with the authentic standard, the impurity 3 was confirmed as ZAN. Impurity 4 has the same precursor ion and fragment ions as ZEN and was identified as *cis*-ZEN. The precursor ions and fragmental ions of the main component (ZEN) and the four impurities are listed in Table 12.

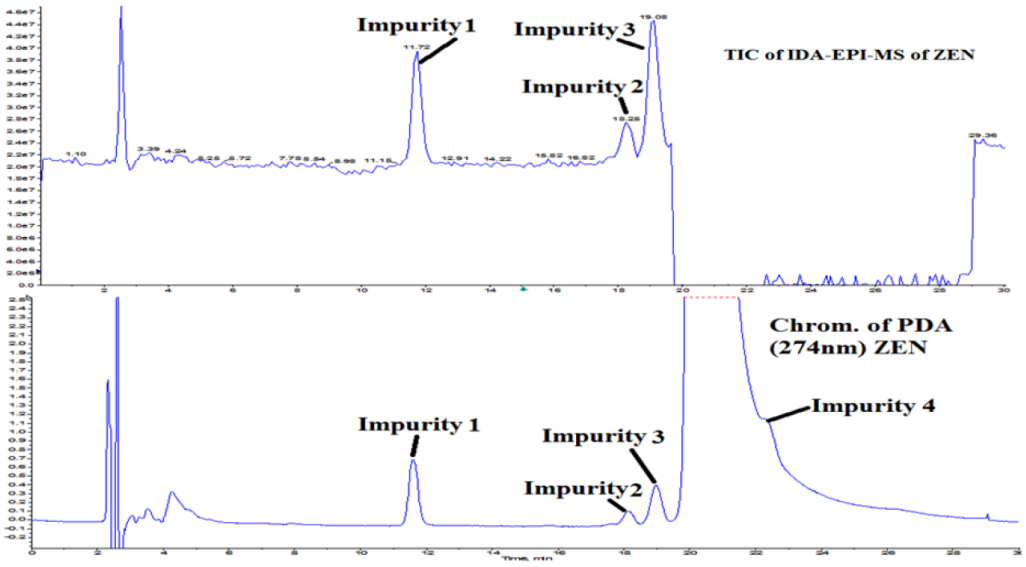


Figure 10 — TIC chromatogram (upper pane) and LC-DAD (lower pane) of OGO.178.a

Table 12. Identification of impurities in ZEN material with QTRAP-MS

Compound	Impurity 1	Impurity 2	Impurity 3	Main component	Impurity 4
RT (min)	11.73	18.31	19.13	20.37	22.48
Precursor ions	315.1	347.1	319.1	317.2	317.3
Fragment ions	/	315.1	/	/	/
	297.1	297.1	301.2	299.3	299.2
	271.2	271.0	275.2	273.2	273.2
	255.1	255.1	257.3	255.1	255.2
	227.2	227.2	231.1	231.4	231.4
	201.0	200.8	205.1	203.0	203.0
	187.1	187.1	187.0	187.1	187.1
	175.0	175.0	174.8	175.0	175.0
	161.0	161.0	161.0	161.0	160.9
	149.0	149.0	148.9	148.9	148.9
				131.0	131.0

Prediction of probable compounds according to the parent ions and series fragment ions

Molecular formula	$C_{18}H_{20}O_5$	$C_{18}H_{20}O_7$	$C_{18}H_{24}O_5$	$C_{18}H_{22}O_5$	$C_{18}H_{22}O_5$
Molecular weight (Da)	316.3484	348.3510	320.38016	318.364	318.364

Prediction* Compound	7'-dehydrozearalenone (7'-dehyZEN)	7-dehyZEN+O2 (15-O-desmethyl-(5Z)-7-oxozeaenol)	Zearalanone (ZAN)	S-zearalenone (S)-ZEN	isomer-zearalenone ®-ZEN

Quantitative estimates of the three major impurities present in the ZEN material identified by LC-MS/MS were undertaken. The quantification of the ZAN content was undertaken using an external calibration curve prepared with the ZAN standard. As no authentic standards were available for the other impurities the quantification of 6-DehydroZEN was undertaken using the α-ZEL calibration curve and for *cis*- ZEN using the ZEN calibration curve. The impurity mass fraction values and corresponding expanded measurement uncertainties are summarised in Table 13.

Table 13. Related structure impurity content of ZEN material by LC-MS/MS

Impurity	Mass fraction (mg/g)	U (mg/g)
6-DehydroZEN [(a)]	1.035	0.053
ZAN	1.138	0.014
Cis- ZEN [(b)]	0.289	0.004

(*a*) a-ZEL calibration curve and uncertainty used;

(*b*) ZEN calibration curve and uncertainty used;

5.4. Water content by Karl Fischer titration

5.5. Final ZEN Purity assignment

In the case of the OGO.178.a material the initial value of the purity of the uncorrected total "ZEN" content was 998.0 ± 1.8 mgg^{-1}. This was the mean of sixteen qNMR assignments (four samples each analysed in quadruplicate) using freshly prepared sample quantified against the signal at 7.0 ppm.

The estimates of the impurity components in the material form the LC methods were:

Impurity	Content (mgg^{-1})	u (mgg^{-1})	Assignment
7-dehydro ZEN	1.03	0.027	LC-MS/MS and LC-DAD
ZAN	1.23	0.007	LC-MS/MS and LC-DAD
Iso-ZEN	0.29	0.002	LC-MS/MS
Total Residual solvent	1.93	0.115	NMR

For the final assignment of true ZEN content the initial qNMR value was corrected for 7-dehydroZEN and *cis*-ZEN content only as ZAN does not contribute to the signal at 7.0 ppm. This gave an assigned value of the ZEN content of OGO.178a as 996.7 ± 1.9 mgg^{-1}, or for reporting purposes 997 ± 2 mgg^{-1}

6. Acknowledgements

All NMR and LC studies were carried out by the co-authors of this document in the course of secondments at the BIPM. The support of the parent institution of each scientist in making them available for secondment to the BIPM is gratefully acknowledged.

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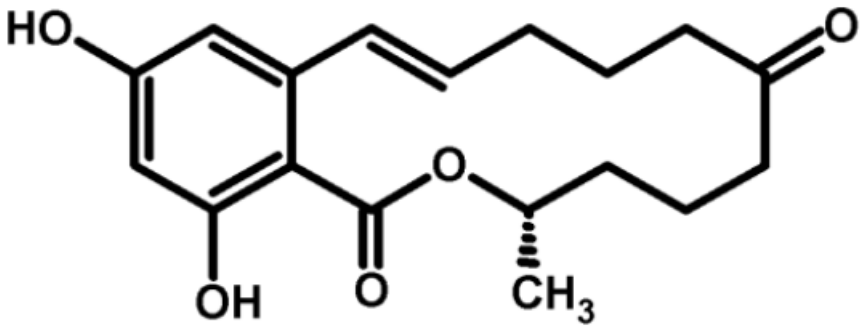
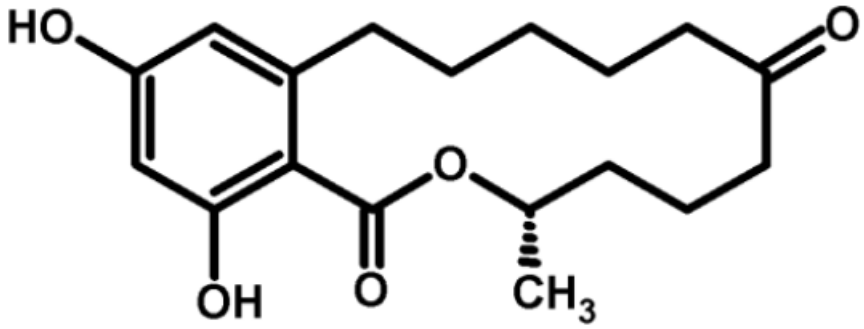
Annex 1. Annexes

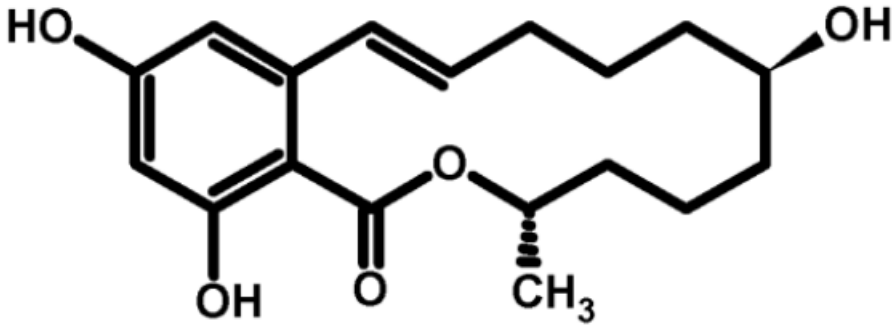
A1.1. Chemical structures of ZEN and related substances



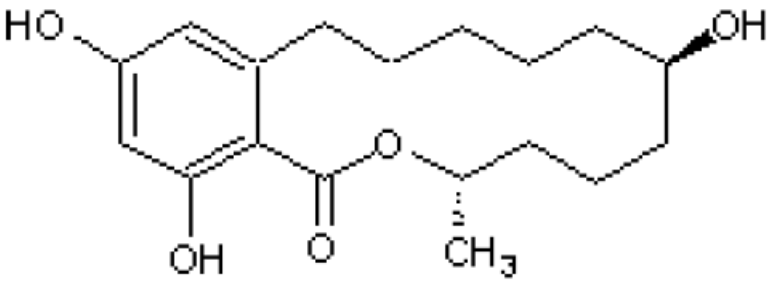
Zearalenone (ZEN)



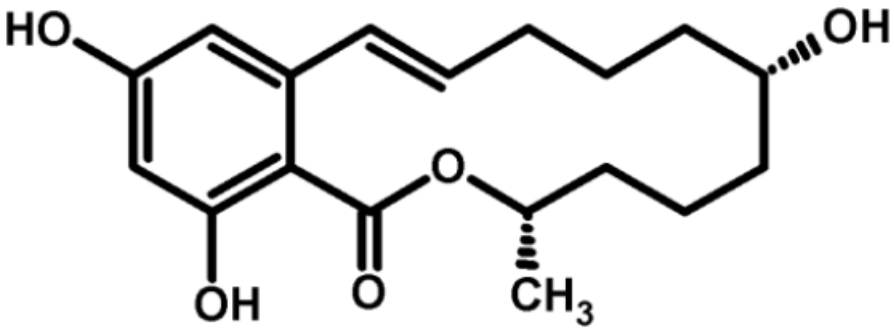
Zearalanone (ZAN)



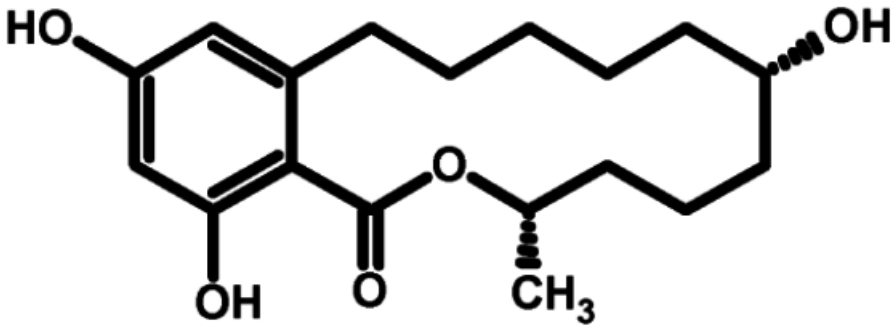
α - Zearalenol (α-ZEL)



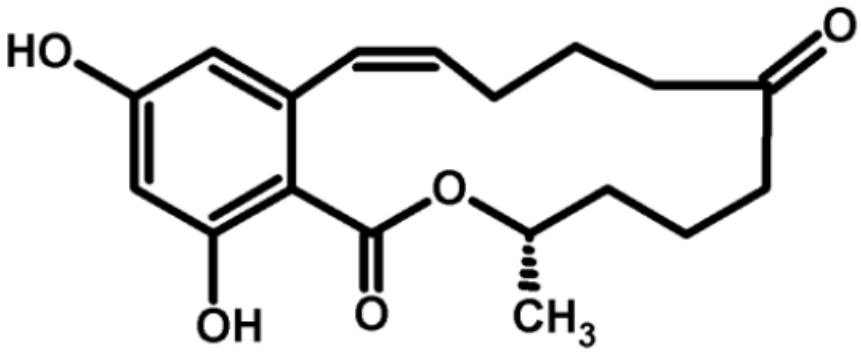
α - Zearalanol (α-ZAL)



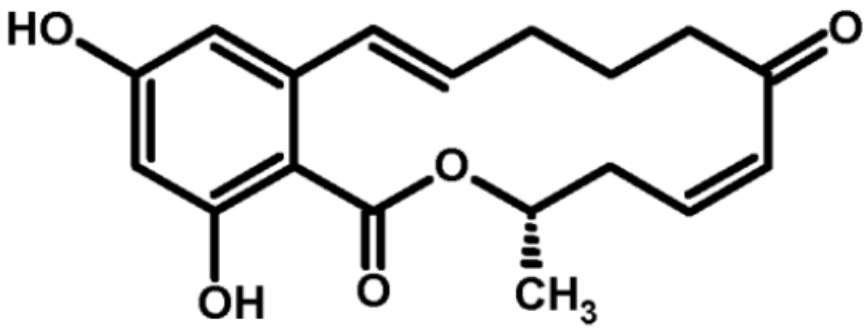
β - Zearalenol (β-ZEL)



β - Zearalanol (β-ZAL)



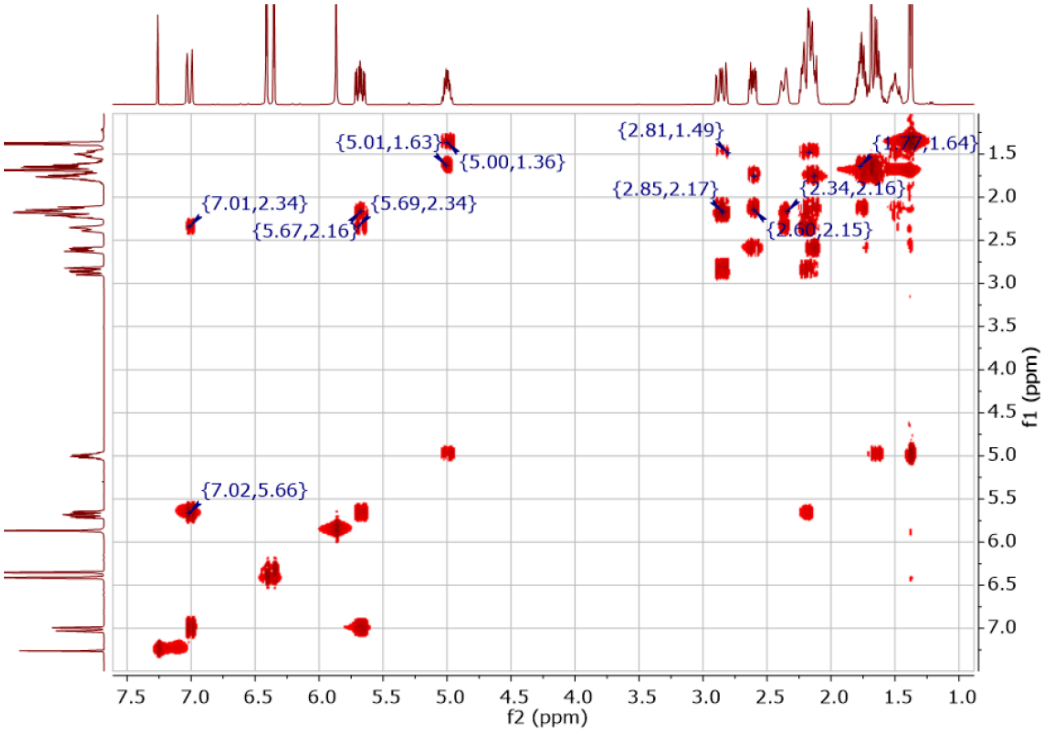
cis-Zearalenone (cis-ZEN)



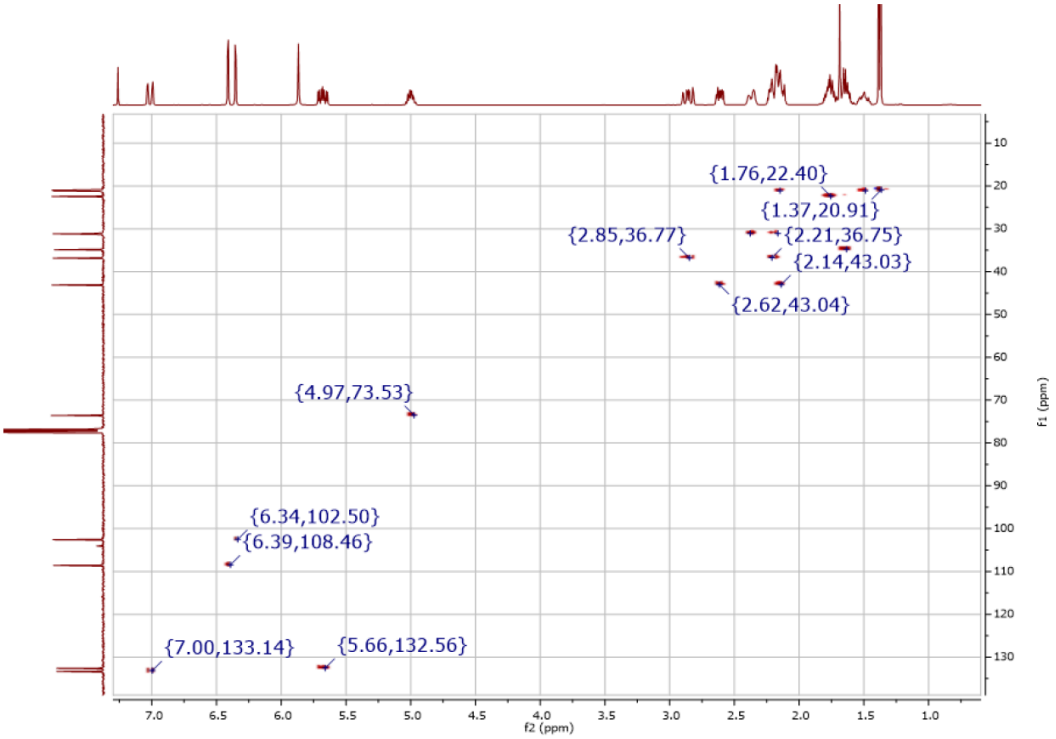
6-Dehydrozearalenone (6-dehydroZEN)

A1.2. 2D-NMR of ZEN

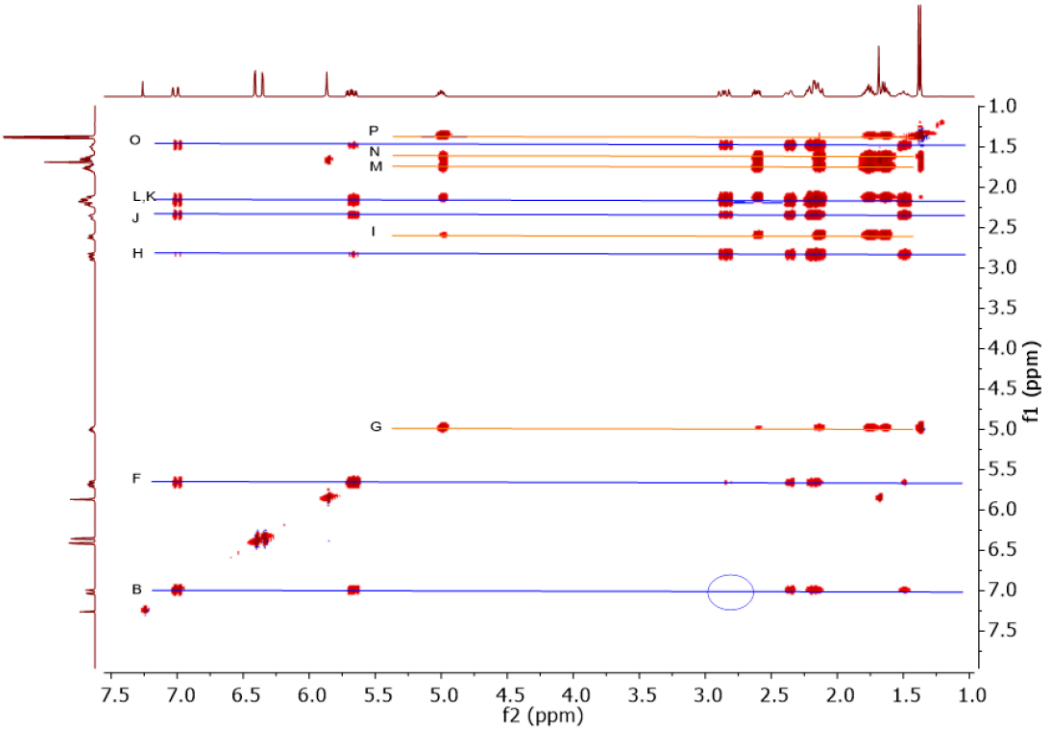
A1.2.1. COSY



A1.2.2. HSQC

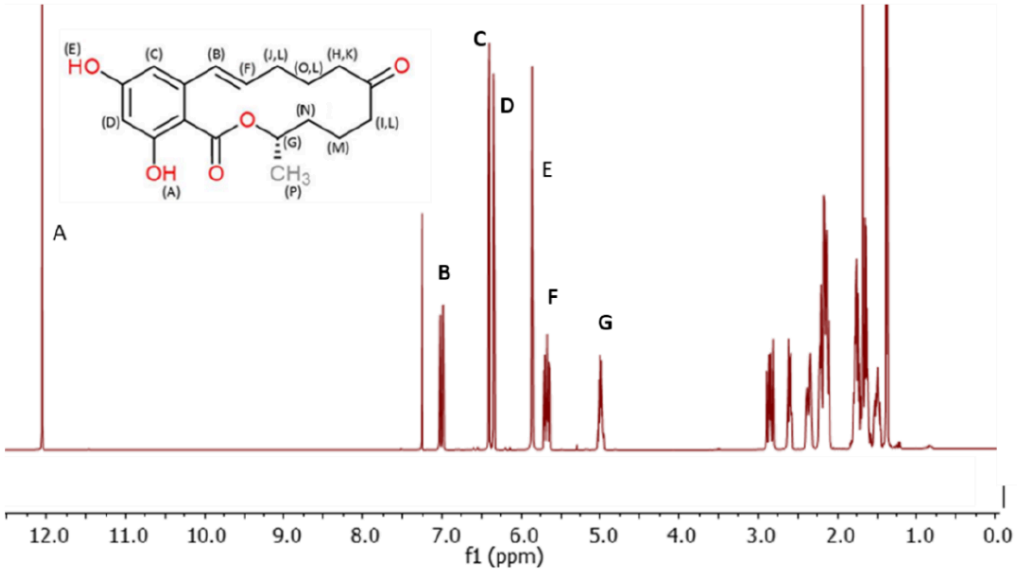


A1.2.3. TOCSY

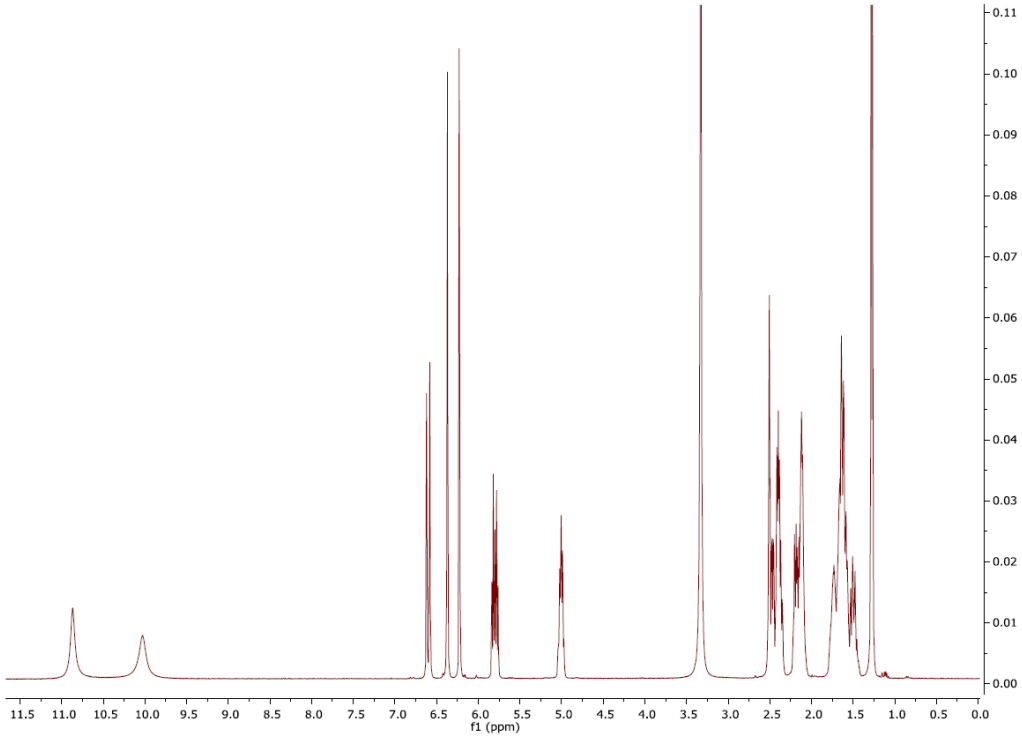


A1.3. ^{1}H NMR of ZEN

A1.3.1. ZEN in $CDCl_3$



A1.3.2. ZEN in DMSO-d_6



A1.3.3. ZEN in acetone-d_6

A1.4. Residual solvent content by NMR

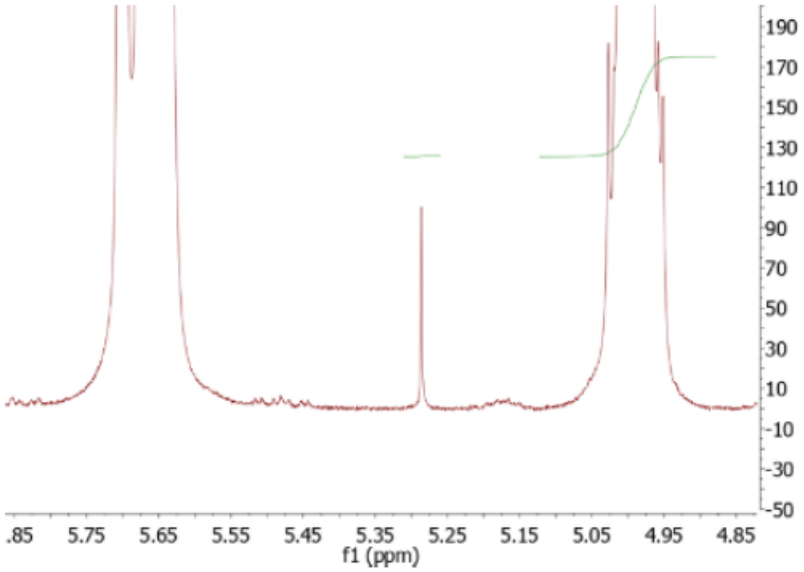


Figure A1.1 — CH_2Cl_2 residue in ZEN

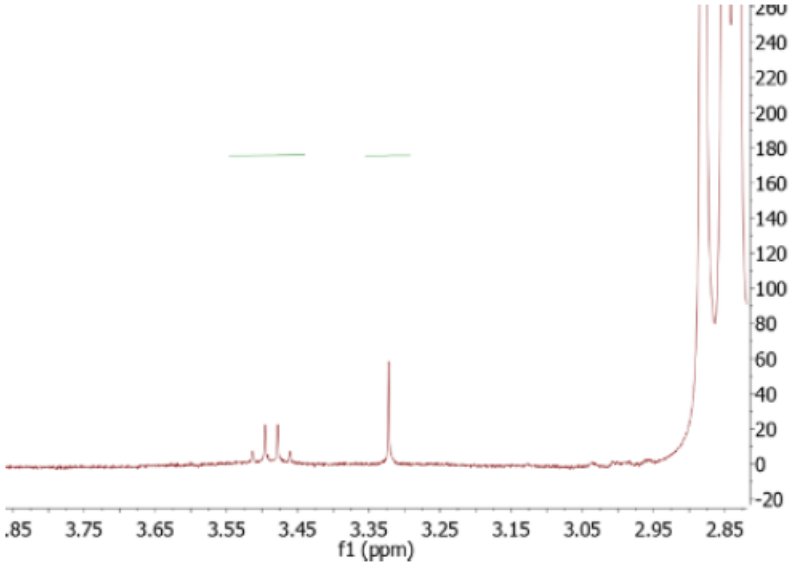


Figure A1.2 — CH_3OH and Et_2O (?) residue in ZEN

A1.5. qNMR of ZEN

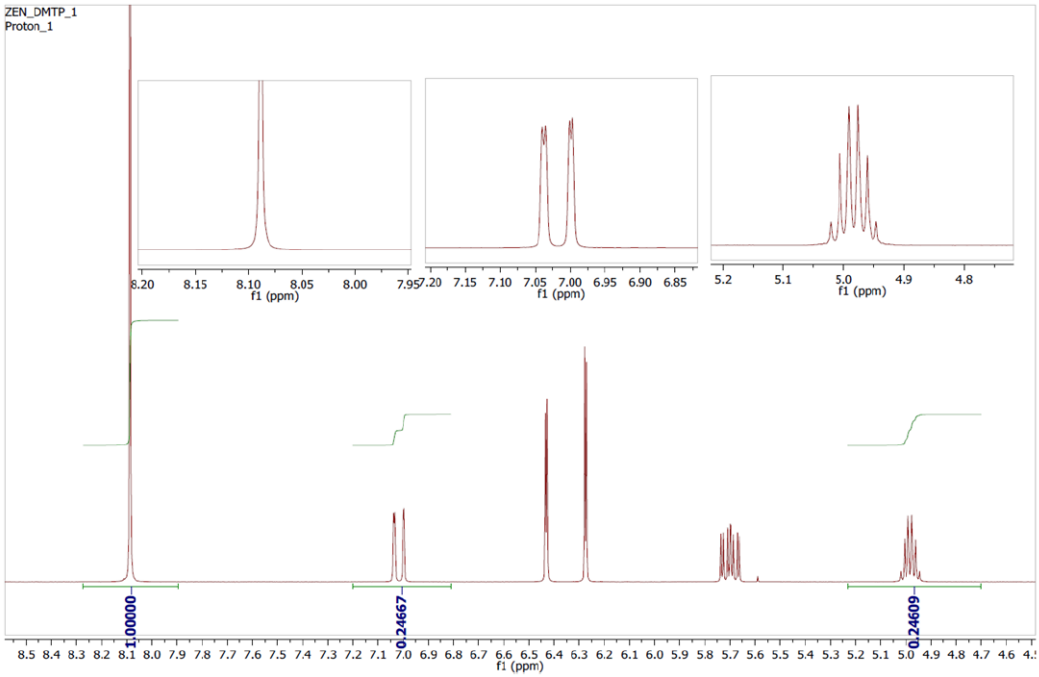


Figure A1.3 — ^{1}H qNMR spectrum of ZEN and DMTP in $CDCl_3$.

A1.6. Baseline contrast $CDCl_3$ v. Acetone-d_6

Figure A1.4 and Figure A1.5 display the observed, expanded baseline of the NMR spectrum of ZEN in $CDCl_3$ and acetone-d_6 respectively.

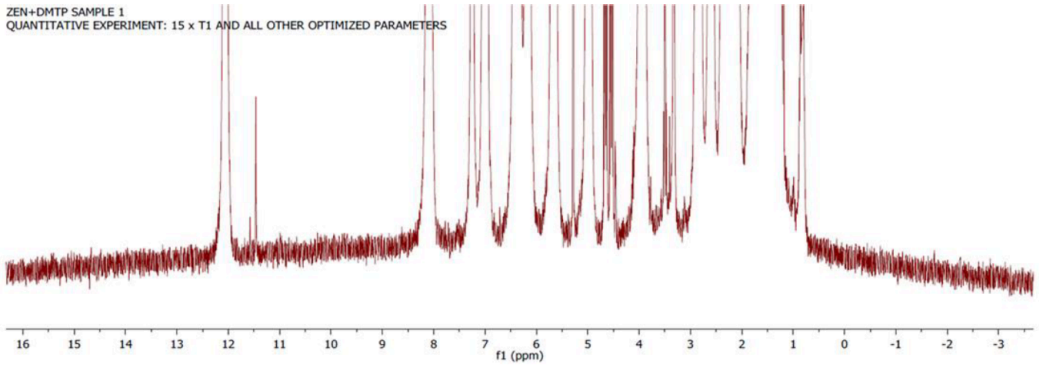


Figure A1.4 — NMR spectrum of ZEN in $CDCl_3$

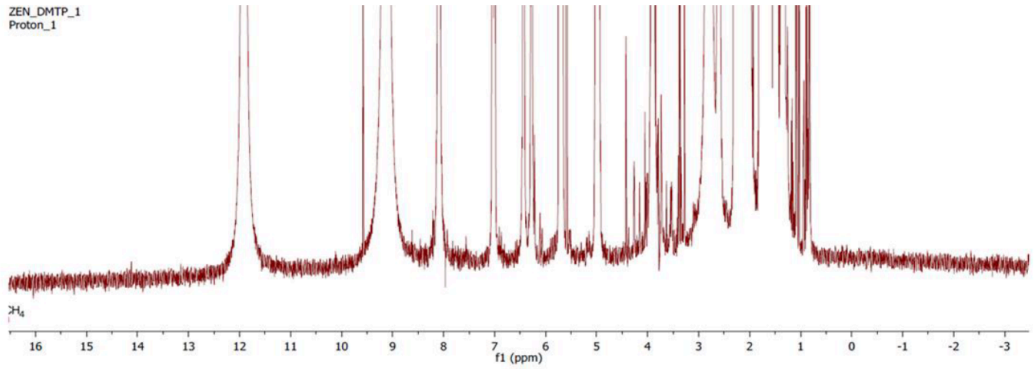


Figure A1.5 — NMR spectrum of ZEN in acetone-d_6

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