Metrology for monitoring endocrine disrupting compounds under the Water Framework Directive

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TUBITAK UME

ISO 17025: Metrological Traceability

6.5 Metrological traceability

6.5.1 The laboratory shall establish and maintain metrological traceability of its measurement results by means of a documented unbroken chain of calibrations, each contributing to the measurement uncertainty, linking them to an appropriate reference.

NOTE 1 In ISO/IEC Guide 99, metrological traceability is defined as the "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty".

NOTE 2 See Annex A for additional information on metrological traceability.

6.5.2 The laboratory shall ensure that measurement results are traceable to the International System of Units (SI) through:

a) calibration provided by a competent laboratory; or

NOTE 1 Laboratories fulfilling the requirements of this document are considered to be competent.

b) certified values of certified reference materials provided by a competent producer with stated metrological traceability to the SI; or

NOTE 2 Reference material producers fulfilling the requirements of ISO 17034 are considered to be competent.

c) direct realization of the SI units ensured by comparison, directly or indirectly, with national or international standards.

NOTE 3 Details of practical realization of the definitions of some important units are given in the SI brochure.

ISO 17025: Metrological Traceability

A.2 Establishing metrological traceability

A.2.1 Metrological traceability is established by considering, and then ensuring, the following:

a) the specification of the measurand (quantity to be measured);

b) a documented unbroken chain of calibrations going back to stated and appropriate references (appropriate references include national or international standards, and intrinsic standards);

c) that measurement uncertainty for each step in the traceability chain is evaluated according to agreed methods;

d) that each step of the chain is performed in accordance with appropriate methods, with the measurement results and with associated, recorded measurement uncertainties;

e) that the laboratories performing one or more steps in the chain supply evidence for their technical competence.

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Definitions

Reference Material (RM)

Material, sufficiently **homogeneous** and **stable** with respect to one or more specified **properties**, which has been established to be fit for its intended use in a measurement process.

Properties can be quantitative or qualitative, e.g. identity of substances or species.

Certified Reference Material (CRM)

Reference material characterized by a **metrologically valid procedure** for one or more specified properties, accompanied by a RM **certificate** that provides the **value** of the **specified property**, its associated **uncertainty**, and a statement of **metrological traceability**.

ISO GUIDE 33:2015: Ref. Materials: Good Practice in using RMs

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Key characteristics of a RM material according to common applications

	Precision control	Bias control	Calibration/ conventional scales	Assigning values to other materials
	RM	CRM		
Specification of the property of interest	Required	Required	Required	Required
Property value		Required	Required	Required
Stated uncertainty		Required	Required	Required
Specified level of homogeneity	Required	a	a	a
Specified level of stability	Required	a	a	a
Statement of metrological trace-ability		Required	Required	Required
Instructions for use	Required	Required	Required	Required
Expiry date of the certificate		Required	Required	Required

a Uncertainty contribution included in the stated uncertainty associated with the property value.

ISO GUIDE 33:2015:
Ref. Materials:
Good Practice in using RMs

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ISO 17034: RM Production

General requirements for the competence of reference material producers

1 Scope

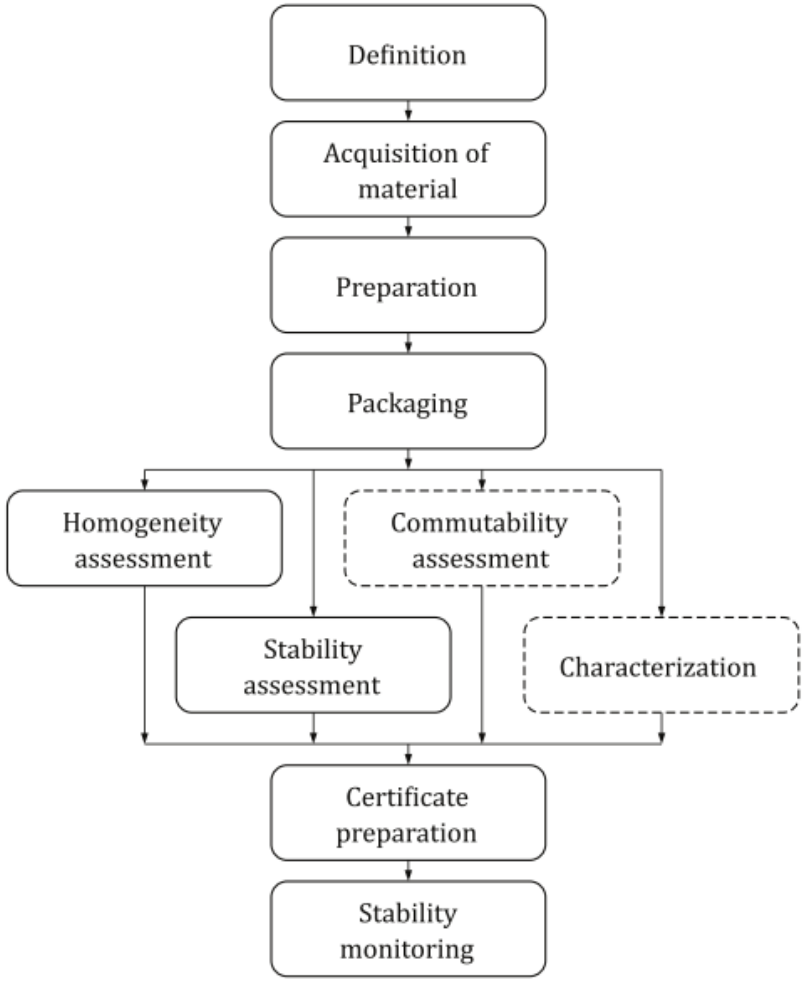
This International Standard specifies general requirements for the competence and consistent operation of reference material producers.

This International Standard sets out the requirements in accordance with which reference materials are produced. It is intended to be used as part of the general quality assurance procedures of the reference material producer.

This International Standard covers the production of all reference materials, including certified reference materials.

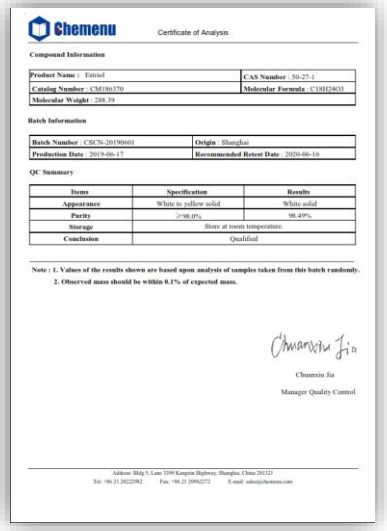
NOTE Reference material producers, regulatory authorities, organizations and schemes using peer assessment, accreditation bodies and others can also use this International Standard in confirming or recognizing the competence of reference material producers.

ISO 17034: Schematic outline of RM Material Project



ISO 17034: Acquisition of material

- Raw material supplied from Chemenu Inc. Shangai China
- Reported purities by producer with HPLC assay ranging 98.49-99.46 %
- Identity confirmation performed by NMR and preliminary investigation for water content done by TGA which is in the range 0.7-1.8 %
- Raw material stored in -20 °C freezer till bottling day


Chemenu Certificate of Analysis

Compound Information

Product Name : Estriol	CAS Number : 50-27-1
Catalog Number : CM186370	Molecular Formula : C18H24O3
Molecular Weight : 288.39	

Batch Information

Batch Number : CSCN-20190601	Origin : Shanghai
Production Date : 2019-06-17	Recommended Retest Date : 2020-06-16

QC Summary

Items	Specification	Results
Appearance	White to yellow solid	White solid
Purity	≥98.0%	98.49%
Storage	Store at room temperature	
Conclusion	Qualified	

Note : 1. Values of the results shown are based upon analysis of samples taken from this batch randomly.
2. Observed mass should be within 0.1% of expected mass.

Chuanxiu Jia
Manager Quality Control

Address: Bldg 5, Lane 3399 Kangyun Highway, Shanghai, China 201321
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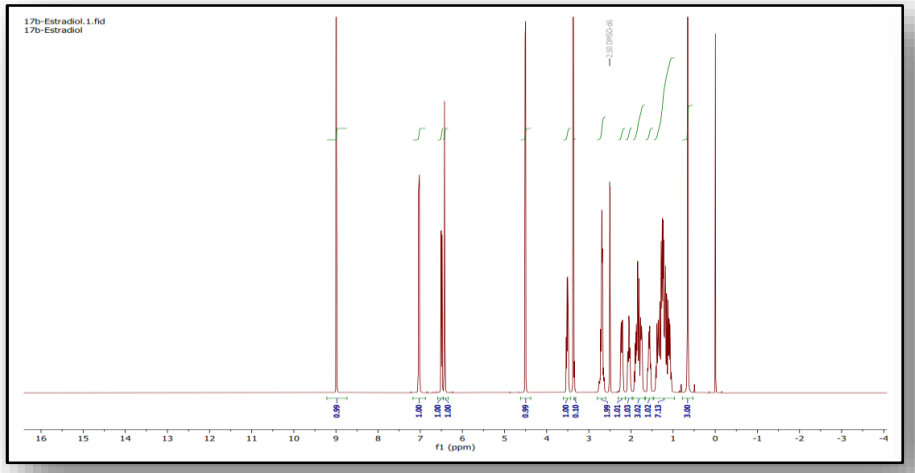

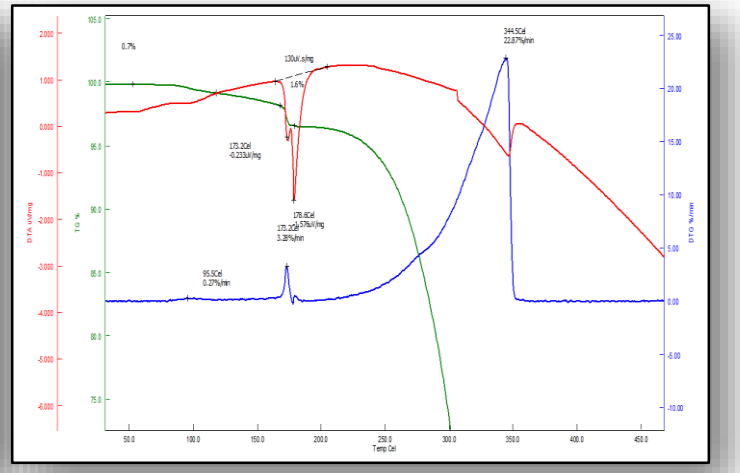

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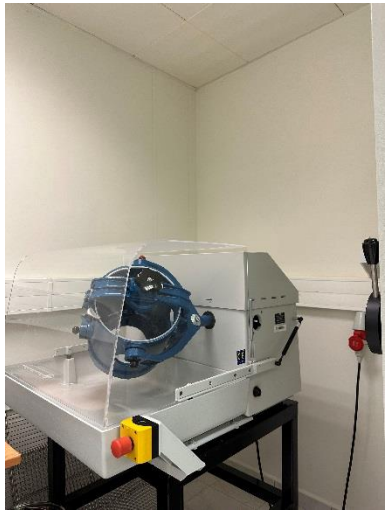
ISO 17034: Packaging

- Before filling raw material equilibrated to room temperature and homogeneized by 3D mixer
- Using by MCPI FD SPA 4A fine dosing machine, powder filled to 8 ml amber bottles under argon atmosphere
- Bottles were capped and labelled according to filling sequence
- Bottles lined up in plastic crates and stored at +4 or -20 °C rooms



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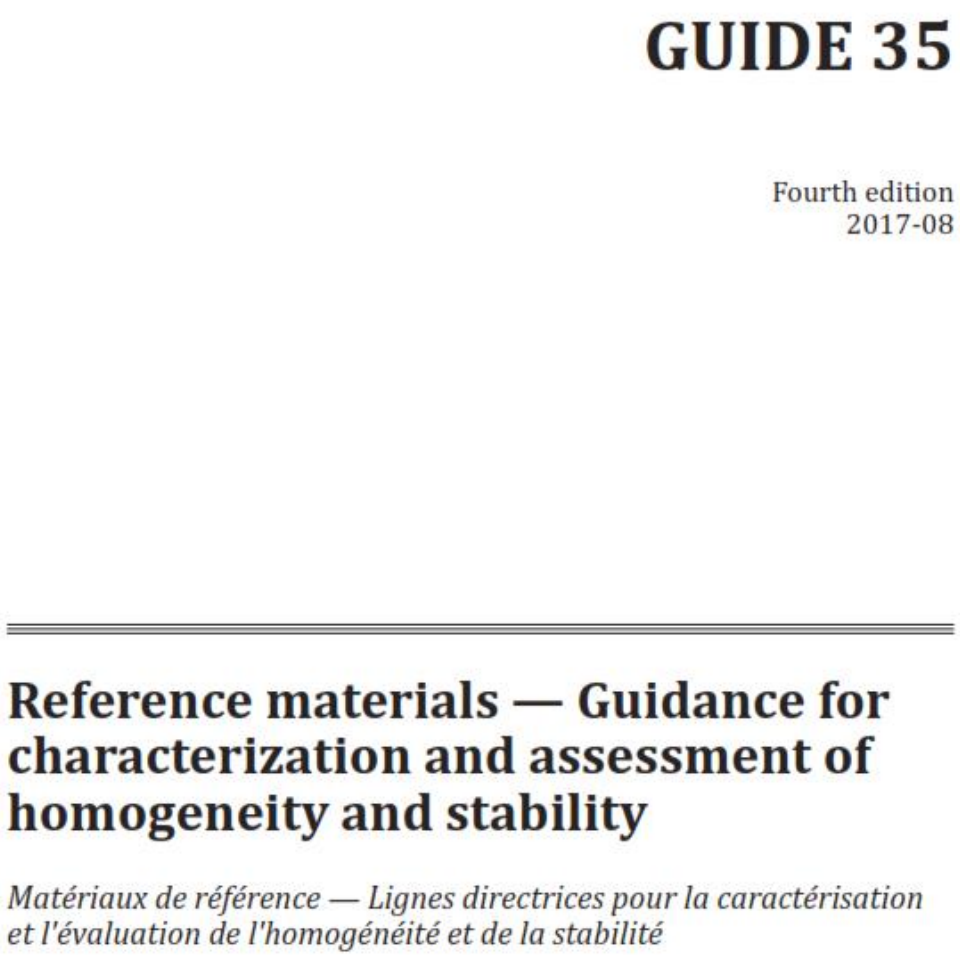
ISO Guide 35: Homogeneity and Stability



GUIDE 35

Fourth edition
2017-08

Reference materials — Guidance for characterization and assessment of homogeneity and stability

Matériaux de référence — Lignes directrices pour la caractérisation et l'évaluation de l'homogénéité et de la stabilité

ISO Guide 35: Assessment of Homogeneity

7 Assessment of homogeneity

7.1 Preamble

Most RMs are prepared as batches of 'units' (e.g. bottles, vials or test pieces). It is important that all distributed units are the same within the stated uncertainty for each property value and, unless sold as single-use units, that the material within each unit is uniform. ISO 17034 accordingly requires the assessment of the homogeneity of a reference material (RM).

Homogeneity can refer either to variation of a property value between separate units of the material, or to variation within each unit. It is always necessary to assess the between-unit variation. Where the intended use permits the use of part of a unit – for example, a small portion of a solid or liquid material, or a small region of the surface – it is also usually necessary either to assess the within-unit variability of the material (within-unit heterogeneity) or to provide instructions for use that control the impact of within-unit heterogeneity. These instructions can include, for example, remixing of the sample and, for granular materials, a minimum sample size, because the within-unit heterogeneity is directly reflected in the minimum size of subsample that is representative for the whole unit.

The assessment of homogeneity may include the use of prior evidence (including prior experimental evidence) of the homogeneity of the material, performing an experimental homogeneity study on the candidate reference material, or both. In most cases, an experimental study is necessary. Exceptions include, for example, batches of a highly homogeneous material, such as a solution for which previous experimental studies have demonstrated that packaging and storage do not affect the homogeneity; or

ISO Guide 35: Minimum Number of Units for Homogeneity Study

$$N_{min} = \max\left(10, \sqrt[3]{N_{prod}}\right) \quad (1)$$

where max(.., ..) indicates the maximum of the terms within the parentheses.

NOTE 1 It is not normally useful to examine more than 30 units of a reference material characterized for a quantitative property.

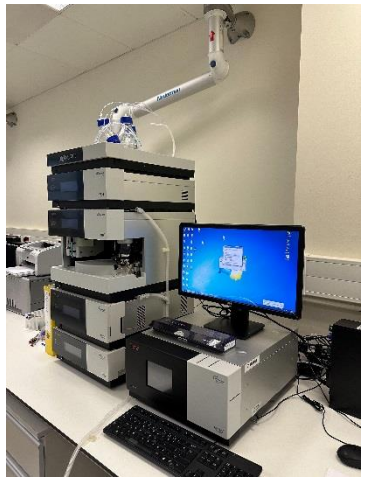
NOTE 2 7.4.1.3 gives further guidance on the minimum number of units for production batches of 100 or fewer units

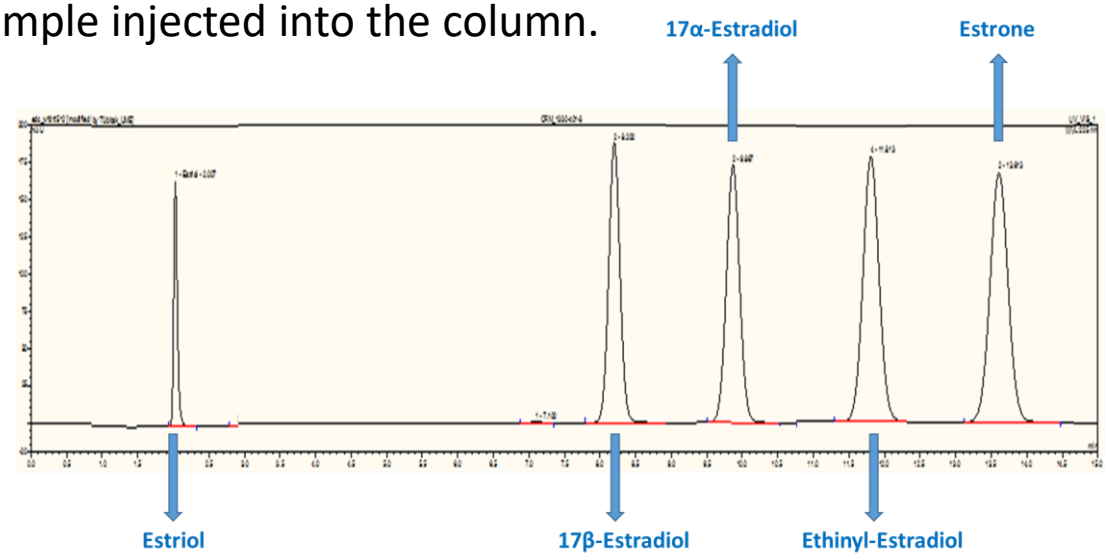
7.4.1.3 Small production batches

Some reference materials are produced in small batches of 50 or fewer units, for example, secondary gas calibration standards. For such small production batches, the minimum number of units specified in 7.4.1.1 usually represents a very large fraction of the available units. Where the batch size is below 100 units, homogeneity should be assessed on the larger of three units or 10 % of the batch size, randomly selected from the batch. Replication should be as high as practically feasible to provide the best available test power for the number of units used. Power analysis (7.4.2) may be used to assist in considering desirable replication levels. For example, with three units, four observations per unit gives

ISO Guide 35: Homogeneity

- Number of units produced ranged from 500 to 780 units
- Each bottle contains minimum of 250 mg of sample
- For homogeneity assessment 10 units are selected
- Units numbers are identified by random stratified sampling with a program
- 3 subsamples are prepared gravimetrically on Mettler Toledo XP205 balance (readability: 0.01 mg) by weighing around 30 mg sample which is dissolved in 30 mL methanol
- Each subsample are transferred to 3 HPLC vials and analyzed by HPLC-UV
- Homogeneity tests were performed by Thermo Dionex Ultimate 3000 HPLC-DAD at 225 nm.
- Troyasil C18 150x 4.6 mm 5 µm analytical column used at 25 °C
- Isocratic mobile phase program used as A:62,5% and B: 37,5% (A: 95:5 % H2O:ACN and B: 100% ACN) for 15 minutes at a flow of 1.250 mL/min, 2 µL sample injected into the column.





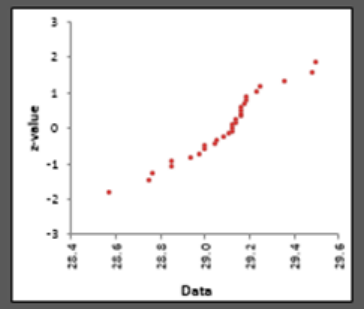
ISO Guide 35: Homogeneity

- The data were evaluated statistically by regression analysis for the presence of any trend in analytical and filling sequence at 95% and 99% confidence level
- Analytical sequence trend was found only for 17β-estradiol, and the data were reprocessed to correct for trend
- Grubbs test (one sided and two sided) was applied to all data for the presence of outlier at 95% and 99% confidence level. No outlier was detected
- Data were visually checked whether all individual data follow a unimodal distribution using histograms and normal probability plots. It was found that the distributions was normal.

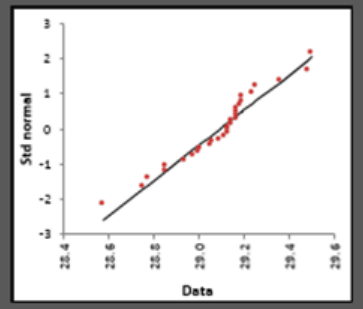
Homogeneity Assessment: Estrone

Normality Test

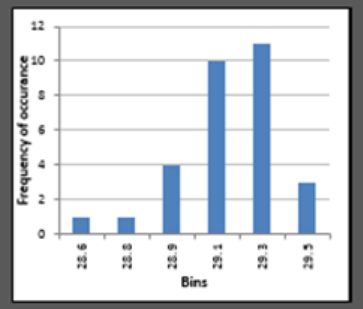
Normal Probability Plot



Quantile-Quantile Plot



Histogram Bin # : 6

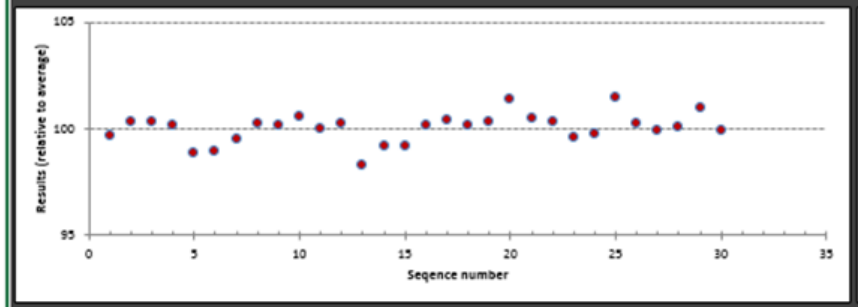


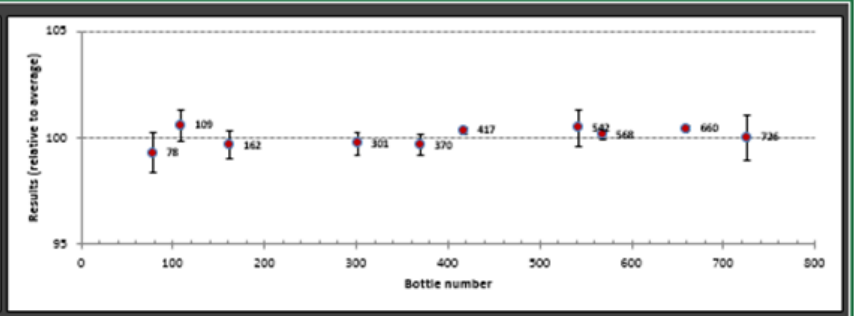
Shapiro-Wilk Test

Alpha:	0.05
W:	9.49E-01
p-value:	1.58E-01
reject normality?	NO

NO: normal distribution
YES: not a normal distribution

GRAPHS FOR THE RELATIVE DATA





Trend Analysis

Analytical Sequence	
95%	NO TREND
99%	NO TREND

Filling Sequence	
95%	NO TREND
99%	NO TREND

Outlier Test

Within Individual Results

Two Sided Grubbs Test

Conf. Int.	Level	Outlier?	Data	Bottle No	Run Order
95%	Higher	NO	-	-	-
	Lower	NO	-	-	-
99%	Higher	NO	-	-	-
	Lower	NO	-	-	-

One Sided Grubbs Test

Conf. Int.	Outlier?	Outlier?	Data	Bottle No	Run Order
95%	Higher	NO	-	-	-
	Lower	NO	-	-	-
99%	Higher	NO	-	-	-
	Lower	NO	-	-	-

Within Bottle Averages

Two Sided Grubbs Test

Conf. Int.	Level	Outlier?	Data	Bottle No
95%	Higher	NO	-	-
	Lower	NO	-	-
99%	Higher	NO	-	-
	Lower	NO	-	-

One Sided Grubbs Test

Conf. Int.	Outlier?	Outlier?	Data	Bottle No
95%	Higher	NO	-	-
	Lower	NO	-	-
99%	Higher	NO	-	-
	Lower	NO	-	-

ANOVA Analysis

Uncertainty

0.21%

ISO Guide 35: Homogeneity

The ANOVA allowed the calculation of the within- (s_{wb}) and between-unit homogeneity (s_{bb}), estimated as standard deviations, according to the following equations:

$$s_{wb} = \sqrt{MS_{within}}$$

MS_{within} : Mean squares within-unit

s_{wb} is equivalent to the s of the method, provided that subsamples are representative for the whole unit

$$s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

When $MS_{between}$ is smaller than MS_{within}, s_{bb} cannot be calculated. Instead, u^*_{bb}, the heterogeneity that can be hidden by the method repeatability, is calculated, according to the following equation

$$u^*_{bb} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{\nu_{MSwithin}}}$$

Homogeneity Assessment: Results Summary

Analyte	$s_{wb,rel}$, %	$s_{bb,rel}$, %	$u^*_{bb,rel}$, %	$u_{bb,rel}$, %
17β-estradiol	0.615	0.16	0.20	0.20
17α-ethinylestradiol	0.845	0.11	0.27	0.27
Estrone	0.658	0.20	0.21	0.21
17α-estradiol	0.499	$MS_{between} < MS_{within}$	0.16	0.16
Estriol	0.824	0.10	0.27	0.27

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ISO Guide 35: Assessment of Stability

6.7 Stability assessment

RMs should be sufficiently stable for their intended use, so that the end user can rely on the assigned value at any point within the period of validity of the certificate. Typically, it is important to consider stability under long-term storage conditions, under transport conditions and, where applicable, the storage conditions at the RM user's laboratory. This can include consideration of stability after opening, if re-use is permitted. Clause 8 provides detailed guidance on stability assessment.

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ISO Guide 35: Assessment of Stability

- Short-term stability → 4 weeks, long-term stability → 6 months
- For stability assessment 2 units are selected for each time point
- Units numbers are identified by random stratified sampling with a program
- 3 subsamples are prepared gravimetrically on Mettler Toledo XP205 balance (readability: 0.01 mg) by weighing around 30 mg sample which is dissolved in 30 mL methanol
- Each subsample are transferred to 3 HPLC vials and analyzed by HPLC-UV
- Stability tests were performed by Thermo Dionex Ultimate 3000 HPLC-DAD at 225 nm.
- Troyasil C18 150x 4.6 mm 5 µm analytical column used at 25 °C
- Isocratic mobile phase program used as A:62,5% and B: 37,5% (A: 95:5 % H2O:ACN and B: 100% ACN) for 15 minutes at a flow of 1.250 mL/min, 2 µL sample injected into the column.

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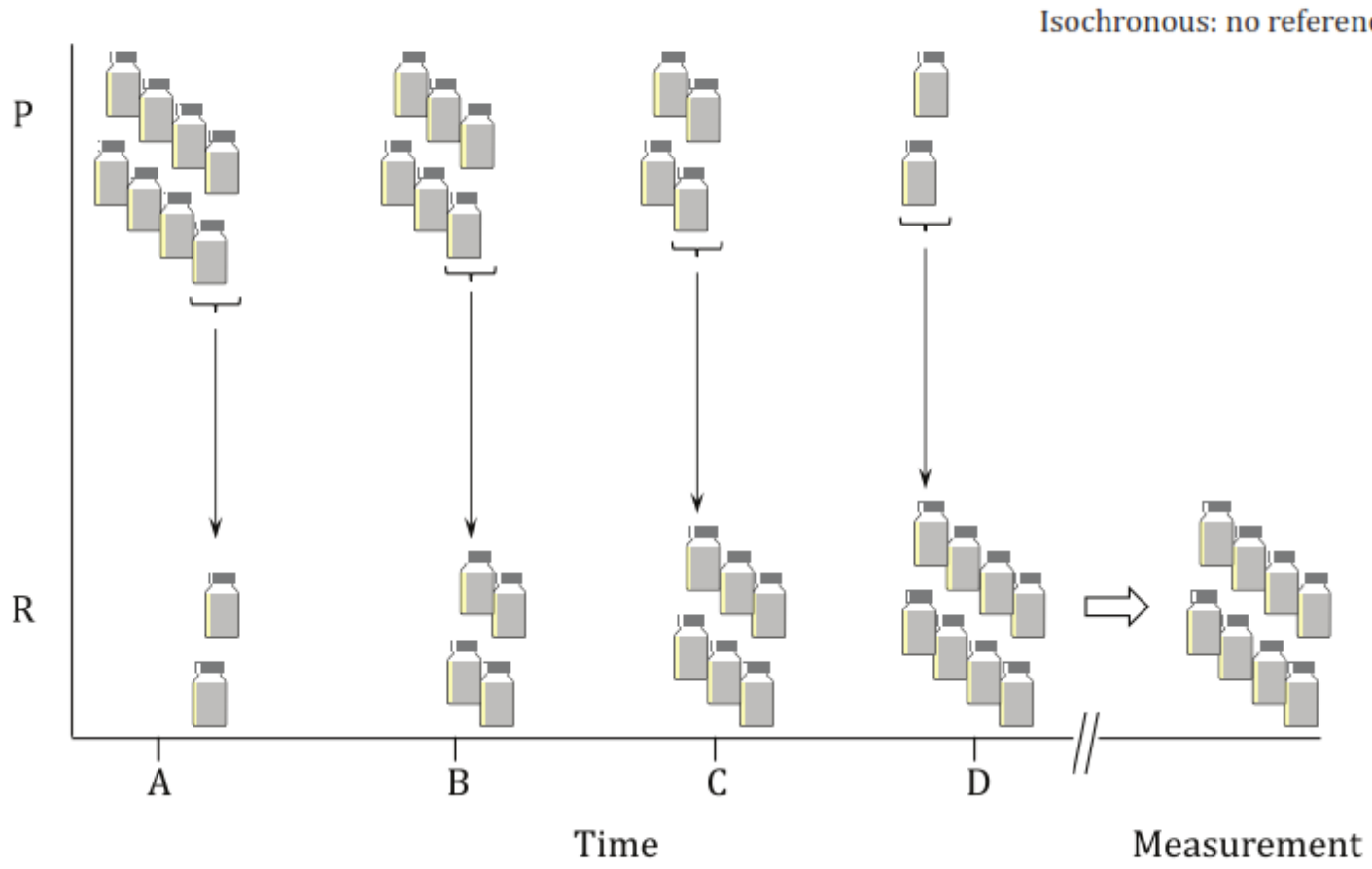

Key

P planned storage conditions

R reference conditions

ISO GUIDE 35:2017(E)

ISO Guide 35: Assessment of Stability

	STS (4 weeks)		LTS (6 months)	
	Ref. (°C)	Test (°C)	Ref. (°C)	Test (°C)
17β-Estradiol	-20	+4; +25	-20	+4
17α-Ethinylestradiol	+4	+25; +45	+4	+20
Estrone	+4	+25; +45	+4	+20
17α-Estradiol	+4	+25; +45	+4	+20
Estriol	+4	+25; +45	+4	+20

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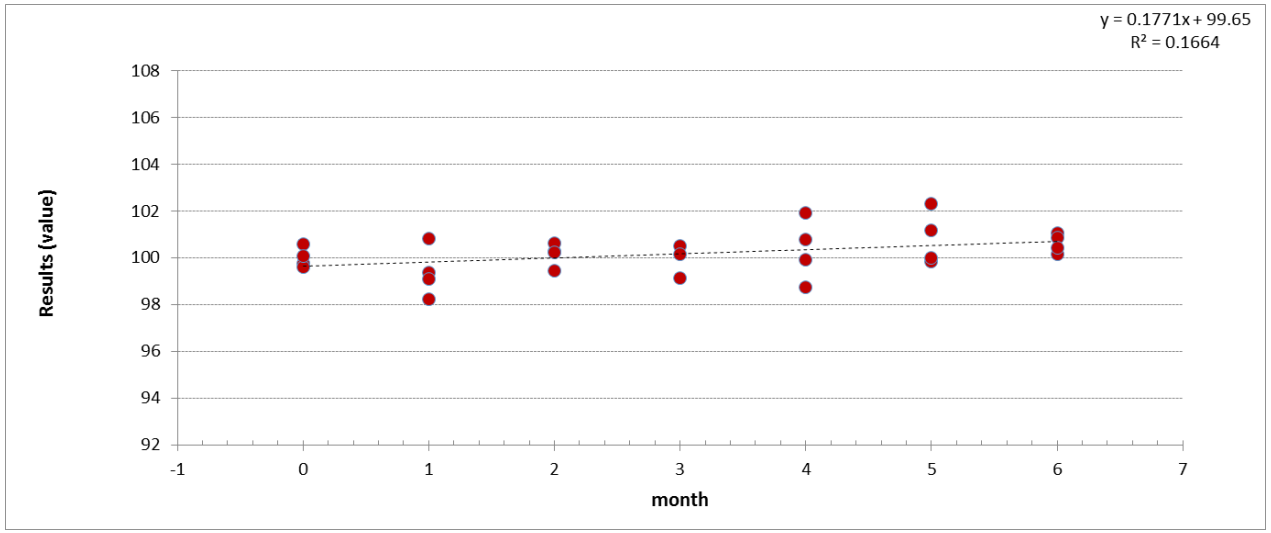
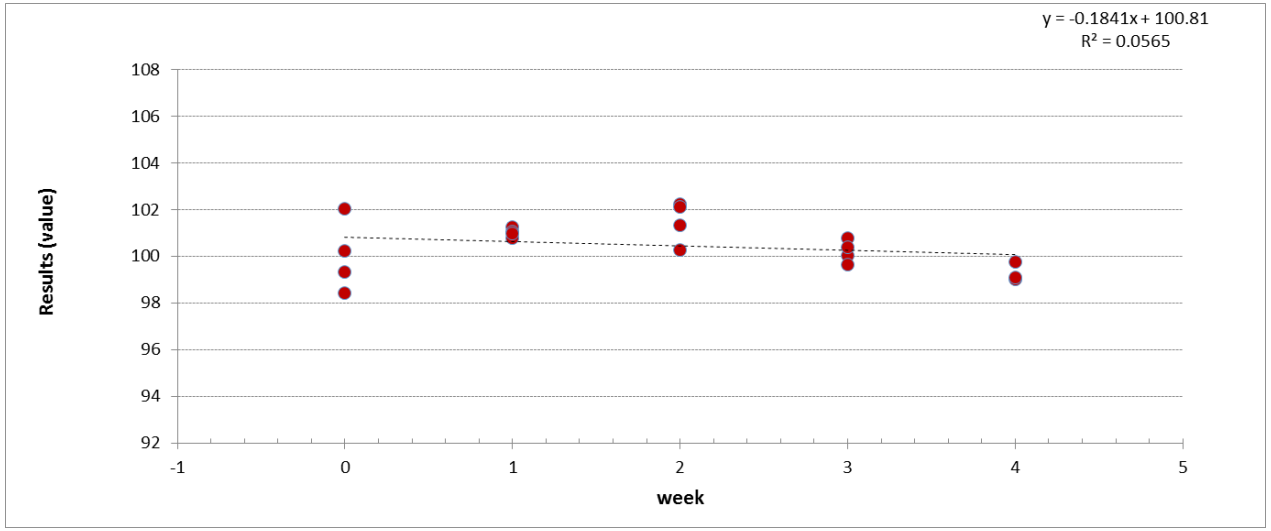
Assessment of Stability: 17α-Estradiol



$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum (t_i - \bar{t})^2}} \times t$$

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum (t_i - \bar{t})^2}} \times t$$



ISO Guide 35: Characterization of the material

Characterization can be achieved by using one or several methods in one or several laboratories[27]. ISO 17034 lists several basic approaches to characterization:

— using a single reference measurement procedure (as defined in ISO/IEC Guide 99) in a single laboratory;

— characterization of a non-operationally defined measurand using two or more methods of demonstrable accuracy in one or more competent laboratories;

— characterization of an operationally-defined measurand using a network of competent laboratories;

— value transfer from a reference material to a closely matched candidate reference material performed using a single measurement procedure performed by one laboratory;

— characterization based on mass or volume of ingredients used in the preparation of the reference material.

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Characterization: Purity Determination of 17α-Estradiol by qNMR

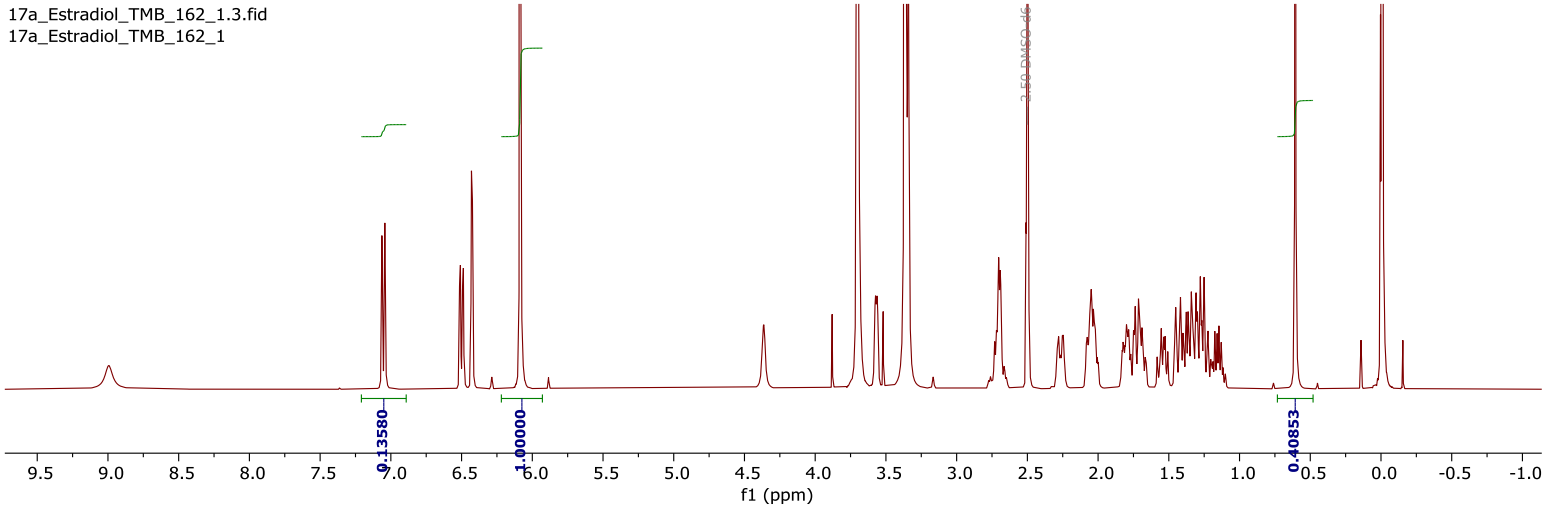
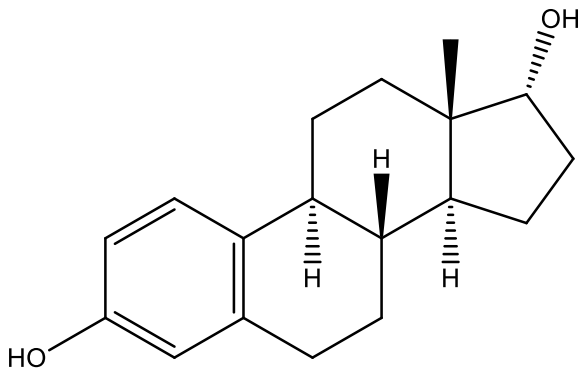


Figure. 1H qNMR spectrum of 17α-Estradiol with 1,3,5-trimethoxybenzene standard in DMSO-d6

Purity: %99.24 ± 0.28



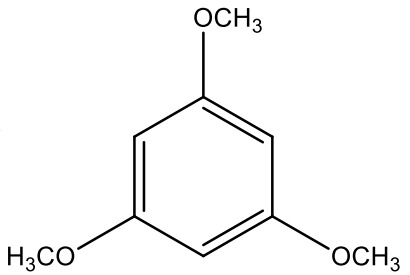
17α-Estradiol

Table 1. Uncertainty Budget of 17α-Estradiol

Uncertainty Budget			
	Value (X)	u(x)	u(x)/X
Purity of Analyte (%)	99.239	0.109189925	0.001100268
Reference Purity (%)	99.798	0.087	0.000871761
Mw Analyte	272.38196	0.008377398	3.07561E-05
Mw Referennce	168.18978	0.00421725	2.50744E-05
m Analyte	10.4067	0.001000001	9.60921E-05
m Reference	12.597	0.001000001	7.93841E-05
			0.001409846
Purity, %	99.239		
upurity	0.140		
Upurity	0.280		

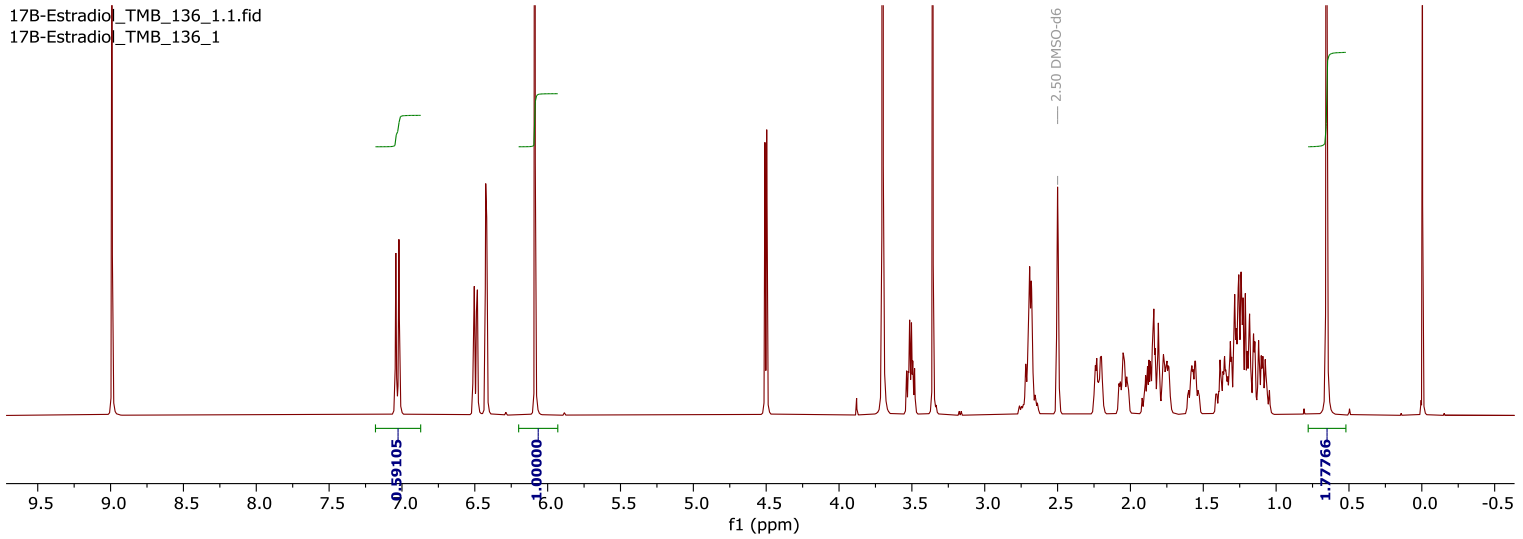
Traceability

UME CRM 1301 →

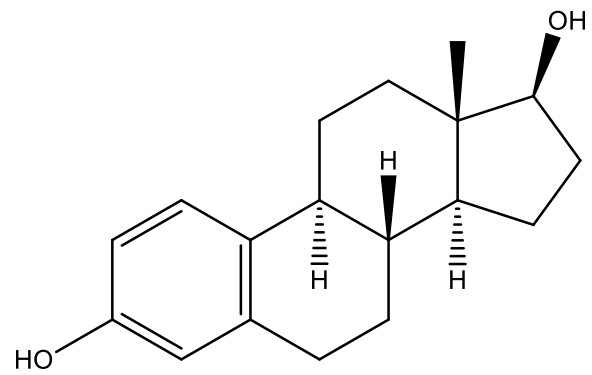


1,3,5-trimethoxybenzene

Characterization: Purity Determination of 17β-Estradiol by qNMR



Purity: %99.26 ± 0.18



17β-Estradiol

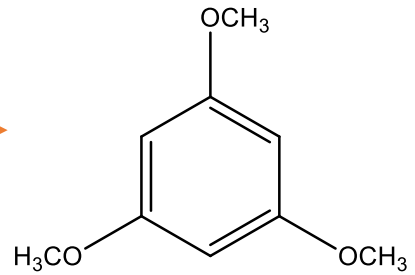
Figure. 1H qNMR spectrum of 17β-Estradiol with 1,3,5-trimethoxybenzene standard in DMSO-d6

Table 2. Uncertainty Budget of 17β-Estradiol

Uncertainty Budget			
	Value (X)	u(x)	u(x)/X
Purity of Analyte (%)	96.263	0.03414642	0.000354721
Reference Purity (%)	99.798	0.087	0.000871761
Mw Analyte	272.38196	0.008377398	3.07561E-05
Mw Referennce	168.18978	0.00421725	2.50744E-05
m Analyte	21.1922	0.000400003	1.8875E-05
m Reference	7.0918	0.000400003	5.64036E-05
			0.000943878
Purity, %	96.263		
upurity	0.091		
Upurity	0.182		

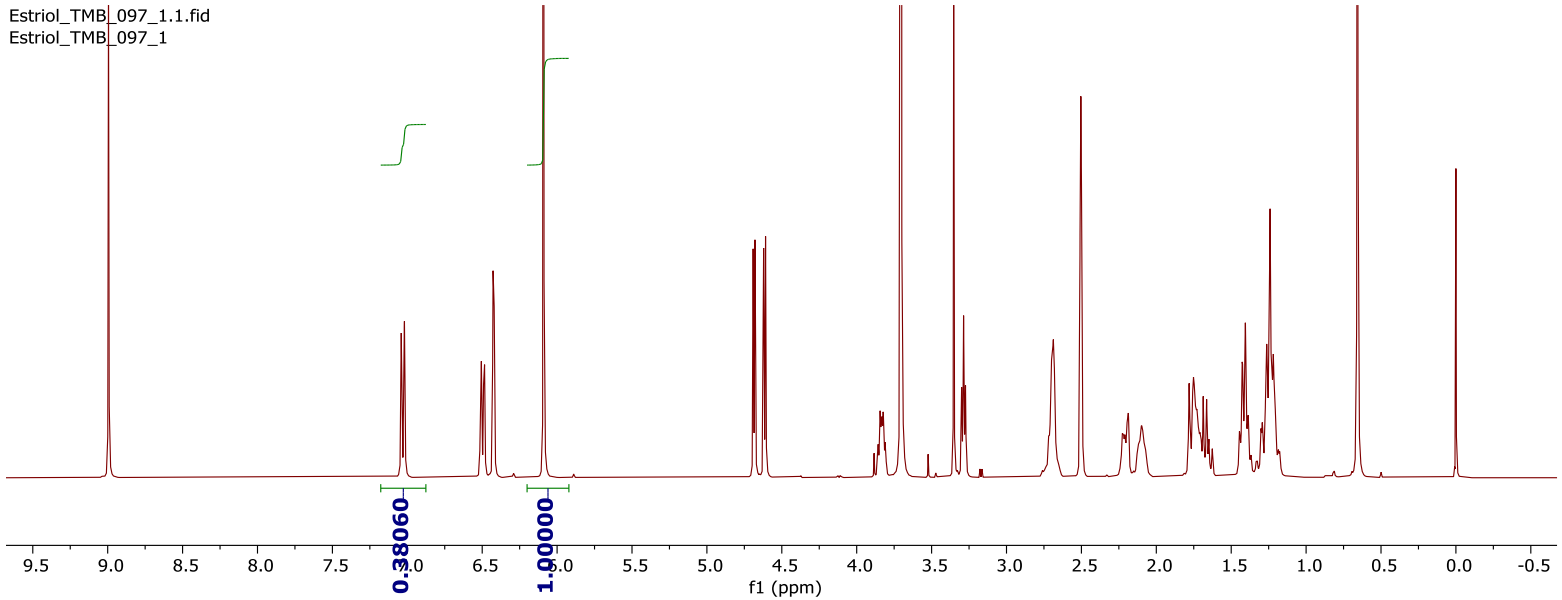
Traceability

UME CRM 1301 →

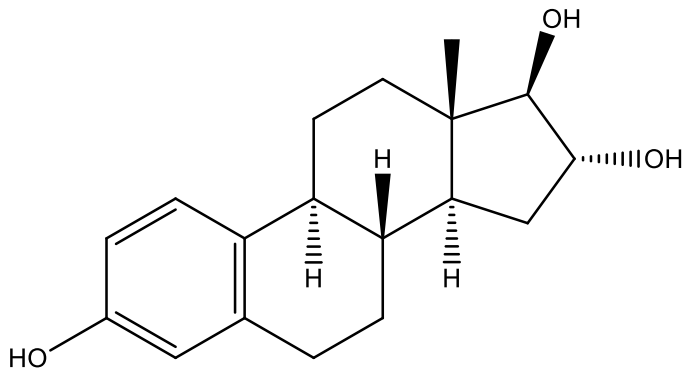


1,3,5-trimethoxybenzene

Characterization: Purity Determination of Estriol by qNMR



Purity: %98.61 ± 0.26



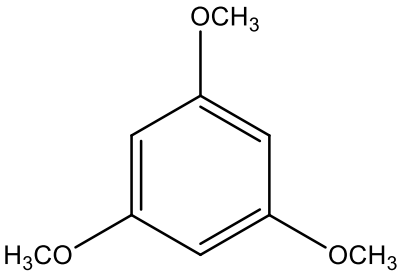
Estriol

Figure. 1H qNMR spectrum of Estriol with 1,3,5-trimethoxybenzene standard in DMSO-d6

Uncertainty Budget			
	Value (X)	u(x)	u(x)/X
Purity of Analyte (%)	98.609	0.099260845	0.001006615
Reference Purity (%)	99.798	0.087	0.000871761
Mw Analyte	288.38136	0.008386346	2.90807E-05
Mw Referennce	168.18978	0.00421725	2.50744E-05
m Analyte	11.2174	0.000400003	3.56591E-05
m Reference	8.1124	0.000400003	4.93076E-05
			0.001333574
Purity, %	98.609		
upurity	0.132		
Upurity	0.263		

Traceability

UME CRM 1301 →



1,3,5-trimethoxybenzene

Characterization: Purity Determination of 17α-ethynylestradiol by qNMR

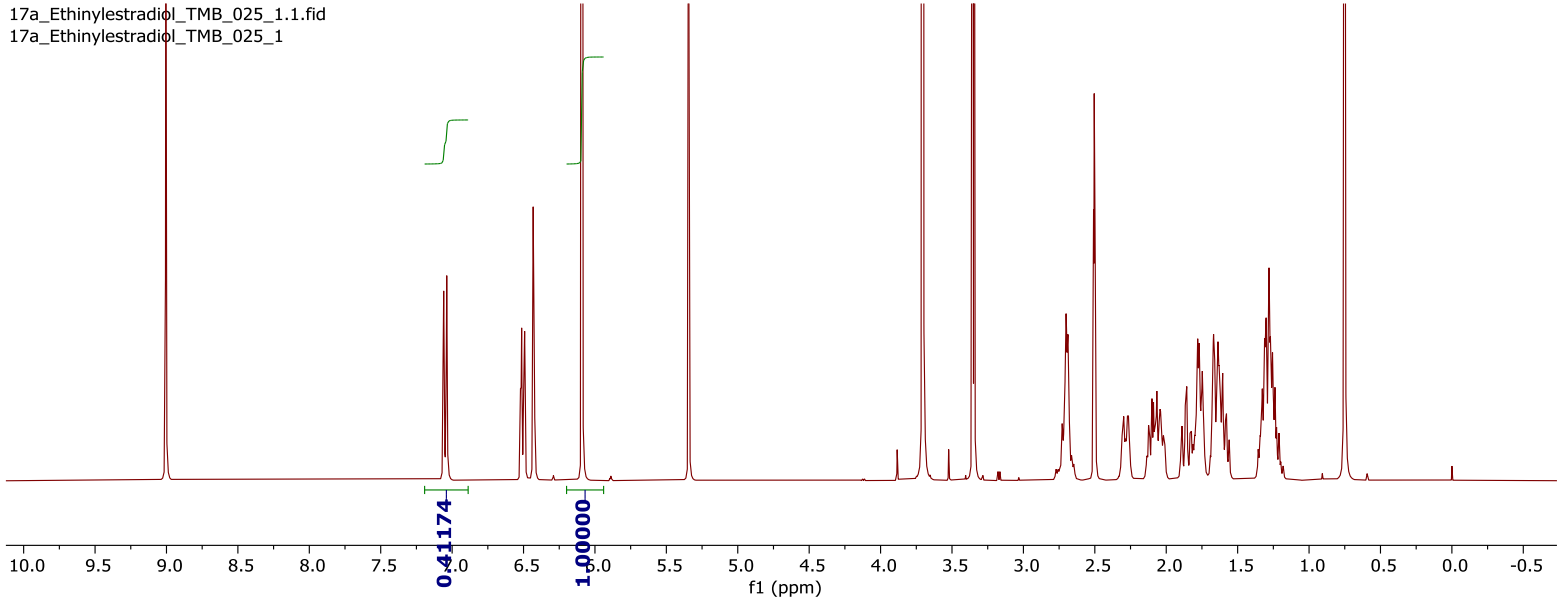
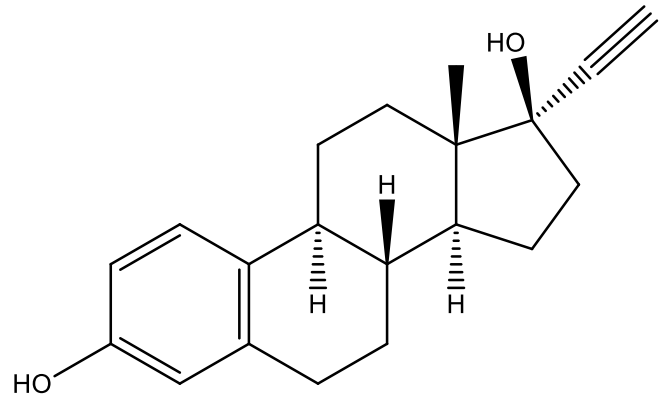


Figure. 1H qNMR spectrum of 17α-ethynylestradiol with 1,3,5-trimethoxybenzene standard in DMSO-d6

Purity: %97.36 ± 0.23

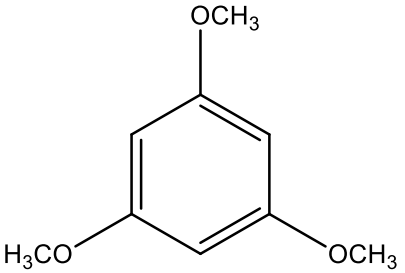


17a-ethynylestradiol

Uncertainty Budget			
	Value (X)	u(x)	u(x)/X
Purity of Analyte (%)	97.355	0.074406029	0.000764272
Reference Purity (%)	99.798	0.087	0.000871761
Mw Analyte	296.40336	0.009294844	3.13588E-05
Mw Referennce	168.18978	0.00421725	2.50744E-05
m Analyte	11.2835	0.000500002	4.43127E-05
m Reference	8.0401	0.000400003	4.9751E-05
			0.001161951
Purity, %	97.355		
upurity	0.113		
Upurity	0.226		

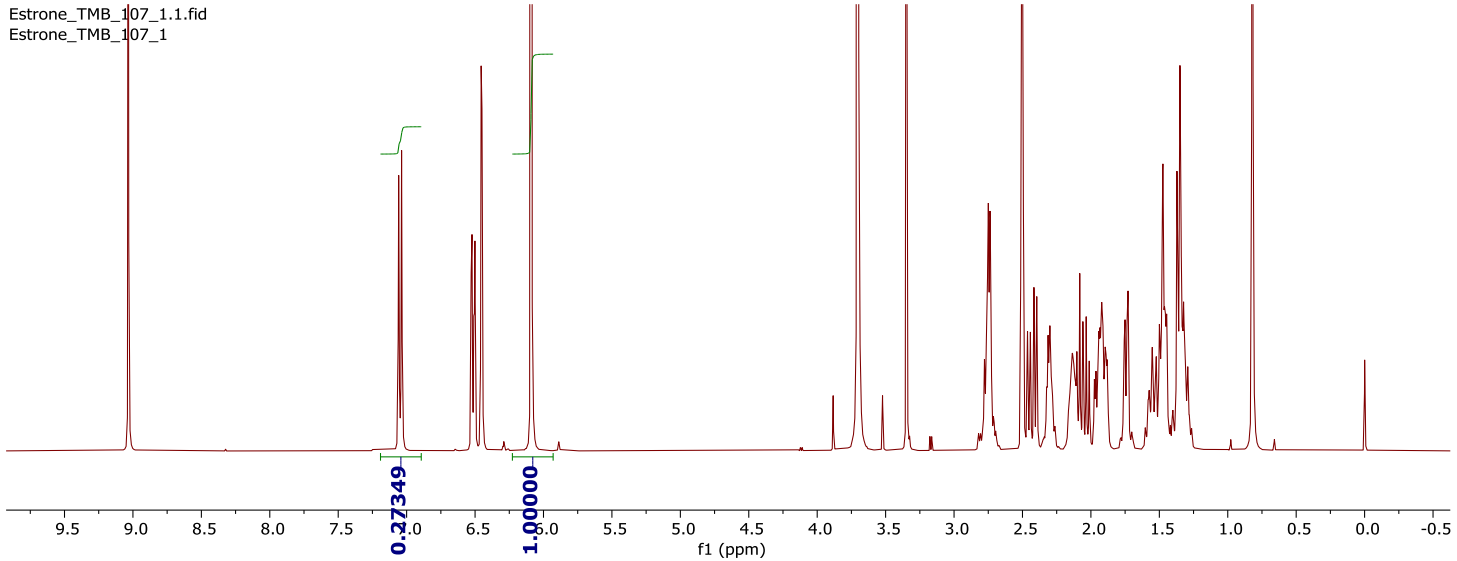
Traceability

UME CRM 1301 →

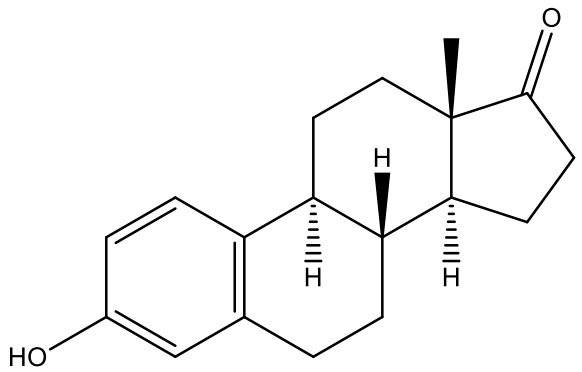


1,3,5-trimethoxybenzene

Characterization: Purity Determination of Estrone by qNMR



Purity: %99.39 ± 0.19



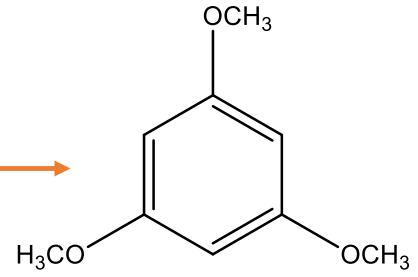
Estrone

Figure. 1H qNMR spectrum of Estrone with 1,3,5-trimethoxybenzene standard in DMSO-d6

Uncertainty Budget			
	Value (X)	u(x)	u(x)/X
Purity of Analyte (%)	99.385	0.039008114	0.000392496
Reference Purity (%)	99.798	0.087	0.000871761
Mw Analyte	270.36608	0.008368425	3.09522E-05
Mw Referennce	168.18978	0.00421725	2.50744E-05
m Analyte	10.4067	0.001000001	9.60921E-05
m Reference	12.597	0.001000001	7.93841E-05
			0.000964957
Purity, %	99.385		
upurity	0.096		
Upurity	0.192		

Traceability

UME CRM 1301 →



1,3,5-trimethoxybenzene

qNMR analyzes for each sample were performed with 9 different samples and 5 instrument replicates by 400 MHz Bruker NMR.

ISO Guide 35: Evaluating measurement uncertainty

10.2 Basic model for a batch characterization

The value of a certified property in a single unit of an RM when delivered to the user can, in principle, be affected by the characterization process, by real variation between individual units (heterogeneity), change over time and changes during transportation and subsequent storage. The model used for evaluating the uncertainty associated with a certified value should allow for all of these effects where they are significant. A convenient simple model for this purpose is as follows:

$$x_{CRM} = y_{char} + \delta_{hom} + \delta_{lts} \quad (16)$$

Usually, any homogeneity and stability studies are designed in such a way that the values of these error terms can be assumed to be zero, but their uncertainties might not be zero.

Formula (16) provides a simple additive 'measurement model' to which the GUM principles can be readily applied. Assuming independence of the variables, the uncertainty associated with a property value of a CRM can be expressed as

$$u_{CRM} = \sqrt{u_{char}^2 + u_{hom}^2 + u_{lts}^2} \quad (17)$$

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UME

ISO Guide 35: Evaluating measurement uncertainty

		u_{char} %	u_{bb} %	u_{STS} %	u_{LTS} %	u_{LTS} % 6 month	u %	Characterization (mg/g)	U (mg/g)
UME CRM 1330	17beta-estradiol	0.091	0.20	0.17	0.12	0.72	0.772	992.63	15.435
UME CRM 1331	Ethinyl-estradiol	0.113	0.27	0.17	0.17	1.02	1.075	973.55	21.494
UME CRM 1332	Estrone	0.096	0.21	0.42	0.17	1.02	1.127	993.85	22.540
UME CRM 1333	17alpha-estradiol	0.140	0.16	0.18	0.13	0.78	0.828	992.39	16.565
UME CRM 1334	Estriol	0.132	0.27	0.16	0.08	0.48	0.588	986.09	11.770

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TÜBİTAK

UME

TUBITAK UME REFERENCE MATERIALS

TÜRKAK

Ref. Material Production
TS EN ISO 17034
AB-0001-RM

Accredited RM Producer since 2016

CRM RESULT EVALUATION APPLICATION

CRM Certified Reference Material RE Result Evaluation

Turkish Accreditation Agency (TURKAK) is a signatory to the European co-operation for Accreditation (EA) Multilateral Agreement (MLA) in the scope of ISO 17034.

https://rm.ume.tubitak.gov.tr/en

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THANKS FOR YOUR ATTENTION!

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