EURAMET-QM-S17
Estrogens in water

Supplementary Comparison
Track C

Study Protocol
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INTRODUCTION

Natural and synthetic estrogens are widely used worldwide, e.g. for contraception. Through application or improper disposal, these estrogens can enter the water cycle unchanged or transformed. They can therefore be detected in surface and groundwater, as well as in treated wastewater. It is known that estrogens may end up in surface waters via wastewater, and due to their physicochemical properties, they can partition in the different compartments (water and suspended particulate matter (SPM)) of water systems. They are of rising concern, due to their high estrogenic activity even at the measured ultra-trace levels (far below ng/l). Therefore, appropriate measurement methods are necessary which allow estrogen levels below their ecotoxicological level e.g. or predicted no effect concentration (PNEC) or environmental quality standard (EQS) to be monitored and to demonstrate if a water body is at risk.

Water is a crucial resource and to satisfy the demand for water quality, an ambitious set of European directives has been put in place under the umbrella of the Water Framework Directive (WFD). One important aspect is, to monitor and control the occurrence and concentrations of potentially polluting substances in the aquatic environment. According to WFD and Directive 2013/39/EU, the monitoring programs should generate high-quality data on the concentrations of priority substances and other pollutants in the aquatic environment.

Decision EU 2015/495 specifies a “Watch List” of substances pursuant to the WFD that must be monitored across Europe. The Watch List (WL) is a mechanism for obtaining high-quality Union-wide monitoring data for potential water pollutants for which only insufficient monitoring data (or data of insufficient quality) are available. Its final goal is to determine the risk they pose at EU level and to decide if EQS should be set for them. Three estrogenic substances: 17-beta-estradiol (17βE2), 17-alpha-ethinylestradiol (17αEE2) and estrone (E1) have been included in the first WL. Required Detection limits were 0.035 ng/L for 17αEE2 and 0.4 ng/L for 17βE2 and E1. The monitoring results for the 1st WL (established by Commission Decision 2015/495) showed that some countries have found it very difficult to reach the necessary LOQs for some substances, among those 17βE2 and 17αEE2. As a consequence, both estrogens were kept on the second Watch list of substances to be monitored in EU surface waters (Commission Implementation Decision (EU) 2018/840).

There are no EN or ISO standards currently available to address the measurement of estrogens by MS-based methods. Consequently, testing laboratories develop their own in-house methods and validate according to internal criteria, as there is a lack of reference materials and proficiency tests. Materials for validation of in-house methods and for the establishment of a reliable quality assurance and control measures are not available as well.

The aim of this interlaboratory comparison (ILC) is being undertaken to allow NMIs and DIs to demonstrate the fitness for purpose of their validated methods for estrogens measurements by MS-based.

Successful participation in this comparison will demonstrate participants' capabilities in determining low-polarity analytes (pKow < -2) with molecular mass range from 170 to 500 g/mol at mass fraction levels of 0.01 ng/kg to 10 ng/kg in water matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) clean-up and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC-MS, GC-MS/MS, GC-HRMS, HPLC-FLD, LC-MS, LC-MS/MS or LC-HRMS.

As a Track C comparison, it is expected that all NMIs or DIs who have or expect to have services related to the capabilities associated to the "How far does the light shines" statement for this key comparison will participate.

Evidence of successful participation in formal, relevant international comparisons is needed to document measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

TIMELINE

Approximately, the ILC takes place according to the following timetable:

Table 1:Timetable of the comparison

Date	Action
February 2024	Proposed to Euramet
April 2024	Proposed to OAWG
February 2025	EURAMET authorized EURAMET_QM.KCXXX
June 2025	Call for participation to EURAMET and OAWG members, beginning of the comparison
Mid-September 2025	Sample Distribution
Mid-December 2025	Deadline for Submission of Results
February 2026	Preliminary Discussion of Results at Euramet meeting
April 2026	Final report, end of the comparison

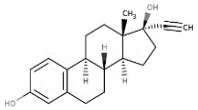
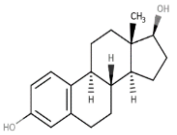
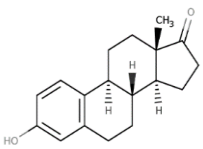
MEASURANDS

Minimum reporting requirements for participants to EURAMET-QM-S17 are the mass fractions of:

- 17-beta-estradiol (17βE2),
- 17-alpha-ethinylestradiol (17αEE2),
- estrone (E1).

Table 1 below displays information of these compounds.

Table 1 — The table summarizes names, abbreviations, structures, CAS numbers, formulas, molecular weights, log Kow of the 3 selected oestrogens

Names	Structure	CAS-RN [a]	Formula	Molecular weight (g/mol)	Log K_{ow}
17alpha- ethinyloestradiol (17αEE2) IUPAC name: (13R,17S)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol		57-63-6	$C_{20}H_{24}O_2$	296.40	4.52
17beta-oestradiol (17βE2) IUPAC name: (8R,9S,13S,14S,17S)-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol		50-28-2	$C_{18}H_{24}O_2$	272.38	4.13
Estrone (E1) IUPAC name: (8R,9S,13S,14S)-3-hydroxy-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthren-17-one		53-16-7	$C_{18}H_{22}O_2$	270.37	3.69

[a] CAS-RN: Chemical Abstracts System Registration Number

STUDY MATERIALS

Each participant will receive separate kits for chemical measurements. In particular, each kit included:

- Ampoules of SPM (suspended particulate matter, estrogen-free, and heat sterilized) and of a simulated dissolved organic carbon (DOC) solution (commercially available humic acid);
- 2 Vials (EURAMET_QM_S17_C1 and EURAMET_QM_S17_C2, for chemical measurements; containing, at different concentration, 3 mL of the standard of the following targeted substances in methanol:
    - 17-beta-estradiol (17βE2),
    - 17 alpha ethinylestradiol (17αEE2),
    - estrone (E1).

The RMs reconstituted were tested by BAM for stability (stable at +4°C for 14 days).

Each unit shall be reconstituted by the laboratory participating in the ILC, following the specific protocol provided by the organizers.

Before the reconstitution, the kit components must be stored in the dark at + 4°C.

Reference material composition

The following **Table 2** shows the composition of the reference materials (RM) reconstituted by the participating laboratories, for the chemical measurements.

Table 2 – RMs composition - Chemical methods

RM 1 EURAMET_QM-S17_C1	Low Concentration Range ng/L	SPM mg/L	DOC mg/L	RM 2 EURAMET_QM-S17_C2	Medium Concentration Range ng/L	SPM mg/L	DOC mg/L
17βE2	0.1-0.5	10	2	17βE2	0.5-2.5	50	5
17αEE2	0.02-0.1			17αEE2	0.1-0.5		
E1	0.2-1.0			E1	0.5-2.5		

ILC Procedure

Test Methods description

Chemical measurements

For chemical measurements are **mandatory**:

- Measurements of the three WFD priority substances: 17-beta-estradiol (17βE2), 17-alpha-ethinylestradiol (17αEE2), and estrone (E1).

The RMs reconstituted shall be analyzed in repeatability conditions in **duplicate** (independent measurements). The overall number of samples for each kit to be analyzed will be four (4) (**EURAMET_QM_S17_C1_1/2** and **EURAMET_QM_S17_C2_1/2**).

Detailed Reference Material (RM) Reconstitution Procedure

For setting up the RM for the ILC it is necessary to mix all the constituents together to the desired whole water sample with a distinct DOC, estrogen content and SPM load. In the following paragraphs the procedure to be used for the RM reconstitution for chemical measurements is reported.

Chemical measurements

The kit provided for the interlaboratory comparison consists of one DOC spiking solution, two EDC spiking solutions (two different concentration levels C1 and C2), and a vial containing the suspended particulate matter (SPM).

Five individual samples must be prepared for the entire interlaboratory comparison. These are summarized in the following table.

Sample	EDC spiking solution	EDC spiking solution volume [µL]	DOC spiking solution volume [µL]	Final DOC [mg L^{-1}]	SPM [mg]	Sample volume [L]
1	EURAMET_ QM-S17_C1	100	1810	2.0	10	1
2	EURAMET_ QM-S17 _C1	100	1810	2.0	10	1
3	EURAMET_ QM-S17 _C2	100	4540	5.0	50	1
4	EURAMET_ QM-S17_C2	100	4540	5.0	50	1
5 (blank)	-	-	-		-	1

The samples must be prepared in amber glass bottles (1000 mL are recommended). The fixed volume of the prepared DOC spiking solution with known DOC concentration (actual 1100 $mg \cdot L^{-1}$) using a 0.45 µm syringe filter (PTFE syringe filters are recommended) giving the final DOC listed in the table must be added to the mineral water. Additionally, an aliquot of 100 µL of the estrogen spiking solution (EURAMET_QM-S17_C1 or EURAMET_QM-S17_C2, 3mL each), containing E1, 17-beta E2, and 17-alpha EE2, in methanol, must be added to the DOC-containing mineral water. Finally, the desired amount of suspended particulate matter (SPM) is added accordingly to the given value in the table. All steps must be controlled gravimetrically. The resulting reference material sample solutions are homogenized on a horizontal shaker or equivalent for at least 10 minutes. Subsequently, an appropriate internal standard mix is used with the needed concentrations. After spiking the samples with the internal standards, they are homogenized again on a horizontal shaker or equivalent for at least 5 to 10 minutes. Store the samples at least for 12 h at +4°C to ensure the equilibrium time. This final RM can be stored for a maximum of two weeks at +4 °C. When analyzing the stored whole water samples allow them to stand at room temperature (+20 °C) for at least one hour.

The procedure can be modified by setting up a batch of DOC-containing mineral water and preparing individual subsamples which can be spiked with the estrogen spiking solution and the desired amount of SPM.

Stability Assessment of solutions

Reference material candidate

A representative water matrix is necessary to be used during the whole period of the comparison with a defined and constant composition. Here, the “synthetic real water matrix” was the candidate

of choice. A defined composition which consists of commercially available mineral water with known ingredients (inorganics, pH, and "one source water") and a simulated dissolved organic carbon (DOC): commercially available humic acid (Sigma-Aldrich) at 7 $mg \cdot L^{-1}$ level was used. The pH value is given by the mineral water itself at 7.3. Additional model suspended particulate matter (SPM, estrogen-free, and heat sterilized) can be added e.g., at a common level of e.g., 50 $mg \cdot L^{-1}$. In comparison to naturally contaminated water this synthetic whole water matrix can be spiked by using an estrogen solution containing all three estrogens at desired concentration level (e.g., EQS).

Processing of humic acid solution:

A HA solution was prepared following an existing method, using technical grade HAs (CAS-No. 68131-04-4, Sigma Aldrich, Taufkirchen, Germany as sodium salt, H16752, LOT #STBJ1504). 7.5 g of solid was dissolved in 1 L of mineral water in an ultrasonic bath and was centrifuged and pooled in a precleaned plastic drum which was left standing overnight to allow sedimentation of particles. Subsequently, the solution was filtered through a 0.2 µm capsule membrane filter with the aid of a vacuum pump. Despite the relatively large surface area, only about 1 L of HA solution could be filtered before the filter had to be replaced. Finally, the HA solution was filled in amber glass bottles. The dissolved organic carbon (DOC) concentration of the HA stock solution was measured in triplicate using a TOC analyzer. Blank measurements with respect to the three estrogens were also performed. In order to avoid biological activity, the HA stock solution (1100 mg DOC L^{-1}) was irradiated by gamma irradiation.

Preparation and selection of the water matrix:

The most suitable water matrix for the test material preparation was selected according to its similarity to natural waters while taking into account several practical aspects.

Demineralized water (>18 $(M\Omega \cdot cm)^{-1}$) was ruled out because of its low ionic strength, as it would certainly not be representative of any environmental water sample.

Use of tap water was also ruled out because possible chlorinated residues in some tap waters can degrade certain analytes.

The use of natural waters directly from a lake or a river would require pre-treatment of the water such as filtration, an exhaustive analysis of blanks and, in case of needing extra water, the repetition of the whole process.

Bottled mineral water offered a good compromise considering all the aspects mentioned above. It is easily obtained and constitutes a natural matrix free of significant amounts of contaminants. The laboratory must used mineral water with defined characteristics presented in the next table.

Concentration (mg/L)	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	Cl^{-}	NO_3^{-}	SO_4^{-}	HCO_3^{-}
minimum	60.0	3.0	3.0	0.5	5.0	<5	5.0	200.0
maximum	120.0	30.0	35.0	5.0	40.0	<5	60.0	400.0

No evaluation of intrabottle inhomogeneity had been carried out but only between bottles with regards to inhomogeneity and stability.

Storage and sample preparation for the stability study

For preparing the individual samples for this study the subsequent workflow had been applied:

1. On day 0 twelve individual whole water samples with a volume of mineral water which is suitable for the individual extraction method containing 7 $mg \cdot L^{-1}$ DOC, 50 mg SPM at pH 7.3 were produced.
2. These samples were spiked with the estrogens at 1 $ng \cdot L^{-1}$ level for each constituent except 17-alphaEE2 which has a level of 0.1 ng L^{-1}. The resulting samples were homogenized e.g., on a horizontal shaker for an appropriate time (e.g., 10 min).
3. Three samples were also spiked with the internal standards.
4. All the twelve sample were stored at +4°C.
5. Respect the equilibrium time of at least 12h.
6. After respecting the equilibrium time, the three samples containing the unlabelled estrogens and internal standards were extracted by the individual appropriate extraction method (SPE disk, SPE) subsequently followed by a further purification step. These extracts were stored at -20°C.
7. The samples for day 1, 5 and 7 were prepared in the same way. All the taken samples were directly extracted and purified at each individual day and were stored at -20°C.
8. LC-MS/MS measurements of all extracts and analysis at least in duplicates after defreezing.

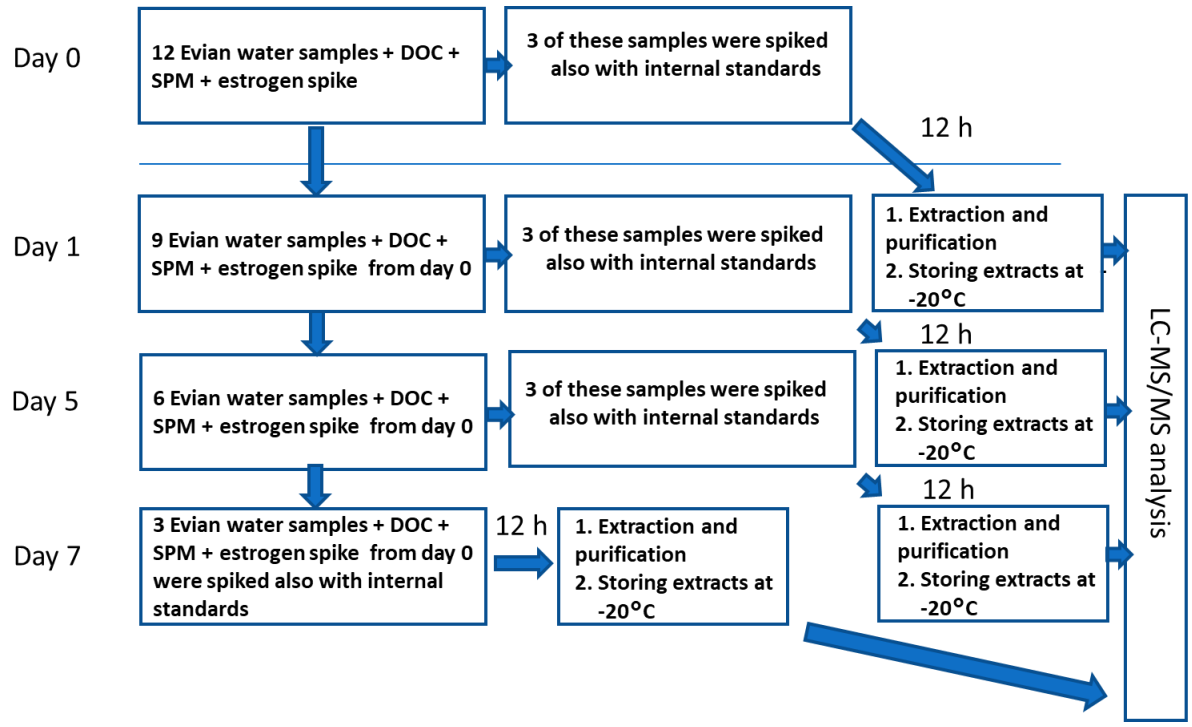


Figure 1: Sample preparation and extraction scheme for the stability study.

Data analysis

The individual recovery rates of each estrogen species in the samples were calculated by using an instrumental calibration.

Time schedule

Eight days for sample preparation, spiking and storage of the samples at +4°C plus one additional week for defreezing, measurements, and data analysis.

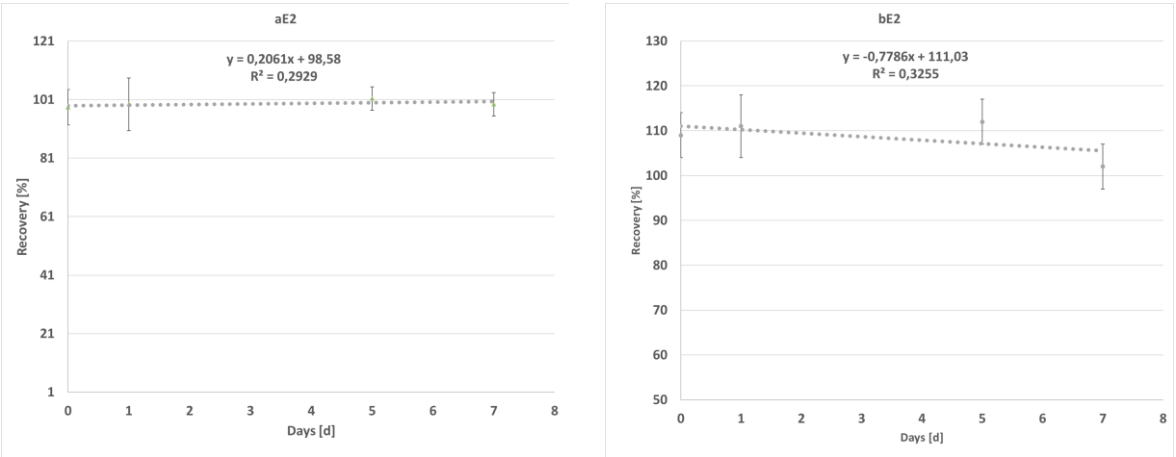


Figure 5: Left: Stability of aE2 extracts. Right: Stability of bE2 extracts.

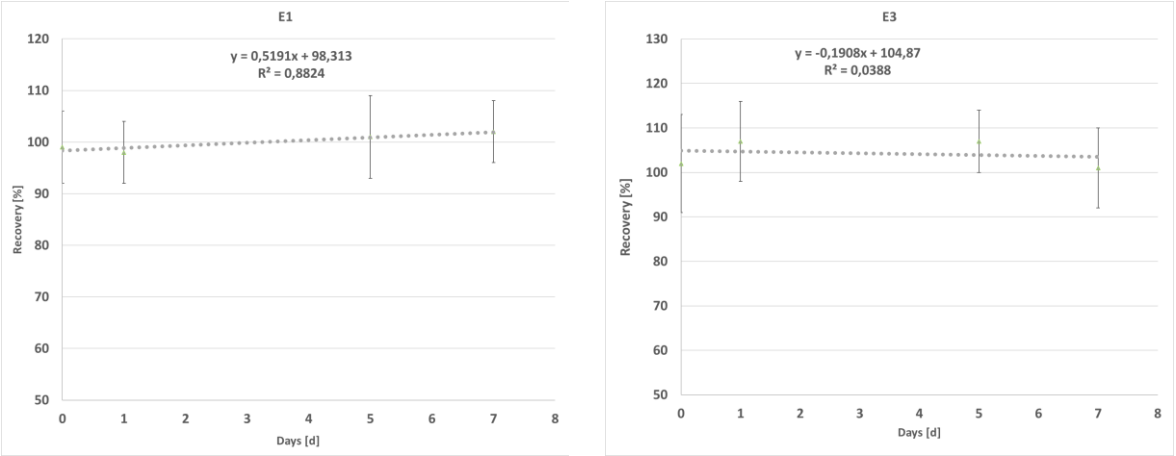


Figure 6: Left: Stability of E1 extracts. Right: Stability of E3 extracts.

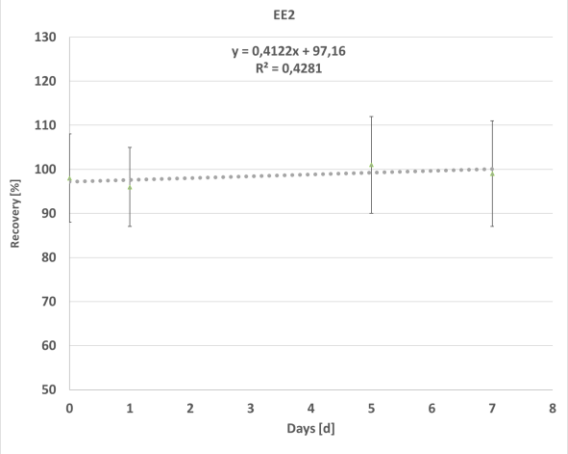


Figure 7: Stability of EE2 extracts.

In conclusion the sample extracts of the reference material candidate stored at -20°C show no significant trends with regards to degradation or loss of the analytes during storage time and sampling on different days. To elucidate this a combined BAM approach with link to ISO Guide 35 was applied. The observed increase of the analyte during the storage time is within the uncertainty of the whole experiment and represents no trend.

INSTRUCTIONS AND SAMPLE DISTRIBUTION

One kit of the study sample will be distributed to each participant. Participants were asked to return a form acknowledging receipt of the samples and to advise the co-ordinator if any obvious damage had occurred to the vials or bottles during shipping. Recipients were also asked to confirm that a

monitoring strip inside the shipping container had not registered a temperature in excess of 37 °C during the transport process.

RESULTS

The "Reporting Template" is an Excel file made of *3* sheets, as below reported. The green-color fields are already filled by the Organizer, whereas the yellow-color fields shall be filled by each laboratory. A templates for MS-based methods is provided:

Sheet 1 - "Laboratory's references": information of the laboratory (name of the organization, address, contact person, telephone/fax numbers, e-mail address);

Sheet 2- "Measurements": for each analyte to be measured, the laboratory is asked to report: the measurement results and the associated uncertainty, LOQs, recovery (R%), technical notes, and comments related to the measurement procedure;

Sheet 3 – "Uncertainty": each laboratory is asked to describe the method used for the measurement uncertainty evaluation (if implemented).

Each laboratory is asked to return back the "Reporting template" to the following e-mail address: **beatrice.lalere@lne.fr and fanny.gantois@lne.fr by 15 December 2025.**

In addition to the quantitative results, participants will be instructed to describe their analytical methods, approach to uncertainty estimation, and the Core Competencies they felt were demonstrated in this study.

Available Calibration Materials

Participants may establish the metrological traceability of their results using certified reference materials (CRMs) with stated traceability and/or commercially available high purity materials for which they determined the purity. Appendix A lists the CRMs that are available for use for this study.

USE OF EURAMET-QM-S17 COMPARISON IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in this study will demonstrate the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 170 g/mol to 500 g/mol, having low polarity pKow < -2, in mass fraction range from in a 0.01 ng/kg to 10 ng/kg in high complexity water matrix.

This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) clean-up and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC-MS, GC-MS/MS, GC-HRMS, HPLC-FLD LC-MS, etc.

Core Competency Statements and CMC support

An example of a Core Competency Table that will be used to claim competencies by the participants in this study is in the Annex B and a respective template will be provided.

APPENDIX A: **List of standards for calibration/quantification**

Institute	Standard	CAS#	Amount in Unit	CRM Code
Tubitak UME	17-beta-Estradiol	50-28-2	250 mg	UME CRM 1330
NMIJ	17-beta-Estradiol	50-28-2	300 mg	6004-a
Tubitak UME	17-alpha-Ethinylestradiol	57-63-6	250 mg	UME CRM 1331
Tubitak UME	Estrone	53-16-7	250 mg	UME CRM 1332

APPENDIX B: Core Competency Tables

CCQM OAWG: Competency Template for Analyte(s) in Matrix

CCQM-KXXX	*NMI/DI*	Title of Study

Scope of Measurement: Successful participation in this comparison will demonstrate participants' capabilities in determining low-polarity analytes (pKow < -2) with molecular mass range from 170 to 500 g/mol at mass fraction levels of 0.01 ng/kg to 10 ng/kg in high complexity water matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) value assignment of calibration solutions; (3) extraction of analyte of interest from the matrix; (4) clean-up and separation of analyte of interest from other interfering matrix or extract components; (5) separation and quantification using techniques such as GC-MS, GC-MS/MS, GC-HRMS, HPLC-FLD LC-MS, etc.

Competency	✓, ×, or N/A	Specific Information as Provided by *NMI/DI*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Indicate if you used a "pure material" or a calibration solution. Indicate its source and ID, eg CRM identifier*
Identity verification of analyte(s) in calibration material.		*Indicate method(s) you used to identify analyte(s)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).		*Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)*
For calibrants which are a calibration solution: Value-assignment method(s).		*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample		*Indicate method(s) you used to identify analyte(s) in the sample (i.e., Retention time, mass spec ion ratios, other)*
Extraction of analyte(s) of interest from matrix		*Indicate extraction technique(s) used, if any, (i.e. Liquid/liquid, Soxhlet, ASE, other)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)		*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)		*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system		*Indicate analytical system (i.e., LC-MS/MS, GC-HRMS, GC-ECD, other)*
Calibration approach for value-assignment of analyte(s) in matrix		*a) Indicate quantification mode used (i.e., IDMS, internal standard, external standard, other)* *b) Indicate calibration mode used (i.e., single-point calibration, bracketing, x-point calibration curve, other)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)		*Indicate any confirmative method(s) used, if any.*
Other		*Indicate any other competencies demonstrated.*

Instructions:

- In the middle column place a tick, cross or say the entry is not applicable for each of the competencies listed (the first row does not require a response)
- Fill in the right hand column with the information requested in blue in each row
- Enter the details of the calibrant in the top row, then for materials which would not meet the CIPM traceability requirements the three rows with a # require entries.