Publishable Summary for 18NRM01 EDC-WFD
Metrology for monitoring endocrine disrupting compounds under the Water Framework Directive

Overview

Natural and pharmaceutical estrogens are key Endocrine Disrupting Chemicals (EDC) which are monitored differently depending on the country, and for which standardised reference methods were not yet available. The overall objective of the project was to develop reliable and harmonised measurement methods for estrogens, to comply with the Water Framework Directive requirements (Directive 2013/39EC, Commission Directive 2009/90/EC and Commission Implementation Decision (EU) 2018/840). The outcomes of this project, in particular the validated mass spectrometry (MS) based reference methods, were disseminated to CEN/ TC 230 and ISO/ TC 147 to be fed into the documentary standards they develop. Accredited analysis laboratories will be able to implement the standard and provide accurate and reliable data. And thus, the project will enable public authorities to provide data with high level of confidence, to ensure an efficient and comparable implementation of the WFD between Member States, and to inform European citizens who have clearly demonstrated their concern about EDC.

Need

It is known that estrogens 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. Despite occurring at ultra-trace levels (below ng/L), it is believed that they are, due to their estrogenic potency, contributing to the feminisation of fish and other endocrine disruptive effects. Moreover, they may be a factor in biodiversity loss. Therefore, appropriate measurement methods are needed allowing determination and monitoring of estrogen levels below the environmental quality standard (EQS) and in order, even more importantly, to show if a water body is at risk.

- The objectives of this project were derived from the need expressed by different communities. For example: CEN/ TC 230 “Water analysis” has agreed that there is a lack of standardised analytical methods to monitor three relevant estrogenic substances (17-beta-estradiol, 17-alpha-ethinylestradiol and estrone) in conditions that meet the requirements of the WFD and its derivatives. There were no methods yet available to guarantee the integrity of samples between sampling and analysis, nor quality control tools to ensure reliability. In addition, there were no CEN or ISO standards available to address the measurement of EDCs by conventional chemical analysis, and reference materials for validating in-house methods and establishing quality assurance and control measures are not available. Some promising effect-based methods for EDC were under development at ISO but they just complemented the classical approach of quantitative methods.
- ASLAE (ASsociation des directeurs et cadres des Laboratoires publics Agréés pour les analyses d'Eau), an association representing 50 testing laboratories in the field of water analysis, has highlighted that many of its members face difficulties in developing and validating estrogens measurement capabilities and have failed to achieve the very low limits of quantification that are required by WFD.
- In addition, for a substance to be added to a regulatory monitoring list, the French Ministry of the environment requires a reliable reference method to be available.

Objectives

The overall objective of this project was to develop traceable measurement methods for endocrine disrupting chemicals, with a specific focus on three estrogens of the first watch list (17-beta-estradiol (17βE2), 17-alpha-ethinylestradiol (17αEE2), and estrone (E1)). Estrogens 17-alpha-estradiol (17αE2) and estriol (E3) have been included to demonstrate the reliability of the developed methods and to support the requirements

of Directive 2013/39/EC, Directive 2009/90/EC and Commission Implementation Decision (EU) 2018/840, hence improving the comparability and compatibility of measurement results within Europe.

The specific objectives of the project were to:

1. Optimise and validate traceable aqueous reference mass spectrometry-based methods for the analysis of 5 estrogenic compounds prioritising 3 selected estrogenic compounds 17-beta-estradiol, 17-alpha-ethinylestradiol, and estrone, in whole water samples at environmental quality standard (EQS) levels. Methods will have limit of quantification (LOQ) not exceeding 30 % EQS with a measurement uncertainty of ≤50 % at EQS.
2. Evaluate the capability of developed methods to address the different fractions of matrix (whole water and dissolved concentrations of estrogens).
3. Develop production methods for aqueous reference materials (RM), which are as close as possible to real water samples, with proven homogeneity, short- and long-term stability.
4. Improve the comparability of estrogen measurements with selected Effect-Based Methods (EBM) in whole water samples at EQS level. Methods that have been correctly calibrated and information on uncertainty were provided.
5. Organise and perform an interlaboratory comparison (ILC) to demonstrate the performance of the developed methods using the reference material (RM) for the selected estrogen substances.
6. Contribute to the work of key European and international standardisation organisations e.g. CEN TC 230 and ISO TC 147 ensuring that the outputs of the project were aligned with needs, communicated quickly to those developing the standards and to those who will use them to support the implementation of directives, and in a form that can be incorporated into the standards at the earliest opportunity.

Progress beyond the state of the art

As a result of the lack of validated measurement methods for estrogens (in terms of LOQ, accuracy, and uncertainty), the comparability and reliability of measurement results has been found to be inadequate. The partitioning (distribution) of estrogens between suspended particulate matter (SPM) and dissolved phase of water is not yet fully understood and the resulting problems in analysis are widely ignored. This knowledge is critical for the comparability of measurements between laboratories and for the evaluation of the chemical status that will be based on whole water measurements. A look in the international reference material database COMAR (International reference material database) three years ago revealed that no representative RM (reference material) existed for estrogens. This can be explained by the estrogens' lack of stability in real matrix and missing fit for purpose measurement methods. EBM (Effect-based method) used for identifying estrogen receptor (ER) mediated risk can overcome the LOQ problems encountered with other analytical techniques, e.g. MS-based methods such as LC-MS/MS and GC-MS/MS. These are suitable screening methodologies for the identification and prioritisation of waterbodies requiring further examination, as well as for measuring the ecotoxicological status in relation to receptor-mediated estrogenicity. Proficiency tests for estrogens are rarely offered and there is low confidence in the quality of estrogen measurements.

Fully validated MS-based reference methods for the detection of estrogen (objective 1):

This project optimised and validated MS-based reference methods for the analysis of the 5 estrogenic compounds, prioritising 3 selected ones. This included complementary sample preparation techniques, stabilisation of samples, extraction, purification and re-concentration to enable reliable measurements of estrogens. To maximise the impact of the project, the performance of the methods for 17βE2, 17αEE2, and E1was aligned to the requirements of Directive 2009/90/EC in such a way that the methods will have a LOQ not exceeding 30 % EQS, with a measurement uncertainty ≤ 50 % at EQS.

Evaluation of the capability of developed methods to address the different fractions of matrix (whole water and dissolved concentrations of estrogens) (objective 2):

Many of extraction and pre-concentration methods that have been published for endocrine disrupting chemicals and selected estrogens did not demonstrate the efficient separation of matrix and analyte and they did not handle with whole water samples. This study was designed to develop an extraction and preconcentration method for providing an enrichment of the analytes of about four orders of magnitude to achieve the low levels 30% EQS to EQS for these selected estrogens as required by the Water Framework directive WFD.

SPE and SPE disk are suggested for the analysis of whole water samples with individual sample volume. The common SPE is limited by the SPM load and must be evaluated before use. The MiSPE cannot be recommended while the sample volume is given by the provided protocol.

Reference material preparation (objective 3):

Two types of certified reference materials have been developed during the project: pure compounds to assure the traceability of the measurements and aqueous reference materials (RM) to evaluate the accuracy of analytical methods. Their implementation during an inter laboratory comparison show a correct use by the laboratories (good accuracy of the results).

Well characterised bioassays methods (objective 4):

An in-house validation strategy for the EBMs optimized within the Project (i.e. A-YES and ERα-CALUX) was developed and agreed by the partners. The method validation design was planned to improve the comparability of estrogen measurements.

The methods performance characteristics were assessed through ad-hoc experiments on six matrices (i.e. three synthetic waters and three natural waters chosen by the partners) at three different concentrations level each (i.e. low, medium and high concentration).

Matrix, interferents and DOC do not impact on the samples analyses in terms of results and dose-response curves when the implemented procedure is applied as preparation procedure of the samples.
And at concentrations close to the EQS, CALUX bioassay showed better results in terms of precision and bias component, whereas A-YES provided the lowest uncertainties when higher concentrations were considered.

Standardisation and intercomparison as knowledge transfer to end-users (objective 5):

This project established strong links with CEN/ TC 230, ISO/ TC 147 and national standardisation bodies and provide them documents and reports with the outcomes of this project. The interlaboratory comparison organised by this project reached end-users, in particular testing (accredited) laboratories, which will facilitate the quick uptake of the developed methods and knowledge by testing laboratories and regulatory bodies.

Results

Fully validated MS-based reference methods for the detection of estrogen (objective 1):

Each partner has optimised their methods to try to reach the target LOQ. In total, twelve sample preparation methods have been developed based on several extraction protocols, e.g., solid phase extraction (SPE) offline using cartridge or disk, SPE online, high volume SPE, liquid-liquid extraction. Considering the potencies to reach the requirements of the Directive QA/QC in terms of LOQ and uncertainty, the most promising ones have been carried out leading to a selection of five methods to be validated (HRMS and LRMS GC-MS/MS, LC-MS/MS and methods with IDMS technique).

On the basis of an overview European database, the selection of 3 representative water samples (synthetic real matrix water) with distinct amounts of SPM (solid particulate matter) and DOC (dissolved organic carbon) has been discussed and agreed. Partners agreed that the validation of the methods would have also been completed by addressing three matrix of their choice.

In order to guarantee the effective recognition of the results of the project by standardisation, Partners agreed that the validation of the methods would have been performed following the guidance of the CEN/ TS 16800 and ISO 21253-1 standards. Furthermore, all the partners evaluated uncertainty according to ISO 11352, additionally NMI applied GUM approach. To support the demonstration, experimental plans have been discussed and agreed between partners.

The data validation show that for all other compounds, the LOQs and associated uncertainties are of the same order of magnitude regardless of the laboratory or method. This objective has been fully and successfully completed.

Evaluation of the capability of developed methods to address the different fractions of matrix (whole water and dissolved concentrations of estrogens). (objective 2):

Because of the very low concentrations of estrogens that have to be measured, the high complexity to display of high volume of water sample with significant concentrations of estrogens (upper to limit of quantification) and significant content of SPM, the partners recommend to have an equilibrium time of at least 12 h after the addition of the internal standard ((labelled estrogens). Furthermore, SPE and SPE disk are extraction method of choice. The common SPE is applicable for whole water samples with a SPM load less then 50 mg L-1 to

avoid clogging of the cartridge limited by the SPM load and for samples with higher SPM content, the SPE disk is highly recommended. A further purification of the extract from the preconcentration procedure is recommended by the project consortium. This objective has been fully and successfully completed.

Reference material preparation (objective 3):

Two types of certified reference materials have been developed during the project:

- Pure compounds to assure the traceability of the measurements: RMs of five analytes listed on the project (17βE2, 17αEE2, E1, 17βE2 and E3) have been produced according to ISO 17034. The RMs have been dispatched to project partners after certification.
- Aqueous reference materials (RM), which were as close as possible to real water samples were provided as a kit that consisted of Evian water, a DOC-spiking solution, SPM, and an estrogen spiking solution at the desired concentration level. A standard operating procedure was given to the customer to set up the reference material under comparable conditions

An inter laboratory comparison, implementing the reference materials, has been organised. The general accurate application of RMs reconstitution procedure by the laboratories positively influenced the very good agreement of the measurements results with the reference concentration of the RMs. Moreover, the results show good methods reproducibility, particularly for low levels: from 14 % for 17βE2 (0,25 ng/L) to 37 % for 17αEE2 (0,034 ng/L) for MS based methods. This objective has been fully and successfully completed.

Well characterised bioassays methods (objective 4):

Outcomes of EBMs validation study were the following recommendations and conclusions:

- the analysis as sample of a βE2 concentration-response curve independently prepared from the calibration curve is helpful in the assessment of the calibration curve validity over batches.
- the preparation of more than a reference plate decreases the risk of discharging all the other samples plates in case the reference curve does not fulfil the acceptance criteria.
- sensitivity to different compounds is not always stable over the time, thus it is recommended that the laboratory should determine relative potencies and periodically check them.
- EBMs validation can be carried out following an Experimental Design in accordance with CEN/TS 16800:2020. The resulting validation will be more aligned to MS based Methods validation.
- Matrix, interferents and DOC do not impact on the samples analyses in terms of results and concentration-response curves when the selected implemented procedure within the Project is applied as preparation procedure of the samples. Therefore, the validated methods have been correctly calibrated and the uncertainty estimation was carried out in accordance with ISO 11352.

Finally, at concentrations close to the former EQS, CALUX bioassay showed better results in terms of precision and bias component, whereas A-YES provided the lowest uncertainties when higher concentrations were considered. This objective has been fully and successfully completed.

Standardisation and intercomparison as knowledge transfer to end-users (objective 5):

Based on the methods and aqueous reference materials developed during this project, an inter laboratory comparison was organised. The general accurate application of RMs reconstitution procedure by the laboratories, positively influenced the very good agreement of the measurements results with the reference concentration of the RMs, Moreover, the results show good methods reproducibility, particularly for low levels: from 14 % for 17βE2 (0,25 ng/L) to 37 % for 17αEE2 (0,034 ng/L) for MS based methods.

For bioassays methods, whatever the level, the reproducibility is around 50-60 %. This value decreases if the two EBMs are looked separately.

These results demonstrated the performance of the developed methods using the reference material (RM) for the selected estrogen substances and will be included in the draft standard ISO CD (Committee Draft) to support the validation. The PR ISO 13646 (X) : Water quality — Determination of selected oestrogens in whole

water samples — Method using solid phase extraction (SPE) followed by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) detection has been reviewed during the meeting at the ISO meeting of TC 147 on 17th April 2023. This objective has been fully and successfully completed.

Impact

The website of the project has been created. http://projects.lne.eu/jrp-edc-wfd/

Nine presentations of the project have been realised at Eurachem Workshop - Uncertainty from sampling and analysis for accredited laboratories (Berlin, 19-20 November 2019); ICRAPHE 2nd International conference on risk assessment of pharmaceuticals in the environment (Barcelona, 28/-29 November 2019); SETAC (Dublin, 3-7 May 2020); Goldschmidt 2021 (Lyon; 4-9 July 2021); CIM 2021 (Lyon, 6-9 September 2021), EuChemS 2022 (Lisbon, 28 August-1 September 2022); IMEKO TC11&TC24 (Croatia, 17-19 October 2022); Maastricht IMSC22 (Netherlands, 27 August-2 September 2022) and CIM 2023 (Lyon 7-10 march 2023).

One published open access papers present the evaluation, comparison and combination of molecularly imprinted polymer solid phase extraction and classical solid phase extraction for the preconcentration of endocrine disrupting chemicals from representative whole water samples.

Two interactive online training courses for members' consortium about method validation were organised (November 2020 and February 2021).

An online workshop for external audience on "Solutions to tackle WFD requirements for estrogen determination in water" was organised on the (7-9 September 2022).

A final meeting was organised face to face for members' consortium (21 February 2023) and on line for user community (22 February 2023).

An advisory group composed by: Ulrich BORCHERS (IWW water center CHIEF STAKHOLDER of the project, TC CHAIR CEN/TC230 « Water Quality»), Mario CARERE (National Institute of Health ISS, researcher, expert for WG Chemical EC), Pierre François STAUB (French Office for Biodiversity OFB; head project water pollution and metrology), Olivier PERCEVAL (French Office for Biodiversity OFB; head project ecotoxicology), Marina RICCI (Join Research Center) has been implemented and was kept informed on the project regularly. Their recommendations from the first meeting 8th October 2019, second meeting 29th November 2021 and 31st January 2022 have been discussed, amended and implemented by the project partners. Some of them have participated in the final meeting.

Impact on industrial and other user communities

This project enabled harmonised monitoring of endocrine disrupting compounds in water in response to European water policies. Regulatory acceptance of emerging technologies is a slow process, and currently hampers the use of such modern bioassays for compliance testing and regulatory purposes. The outcomes of this project will facilitate the adoption of such technologies. Testing (accredited) analytical laboratories will be targeted to benefit from this project, therefore supporting the provision of services. This has been fostered by the webinar training that was organised in September 2022 and at the final meeting in February 2023.

Impact on the metrology and scientific communities

This project will supported the metrology community in handling the long-standing scientific problem of environmental monitoring and risk assessment. This project have direct impact on different metrology committees, especially the EURAMET Technical Committee of Metrology in Chemistry (TC-MC) and the Organic Analysis Working Group (OAWG) of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) of the BIPM. CCQM-OAWG is responsible for the CMC (Calibration and Measurement Capabilities) entries related to chemistry. It contributes to the visibility of EURAMET and its leadership in chemical metrology for environment to the wider metrology and scientific communities. The interlaboratory comparison that was organised within the project has been included in the OAWG strategic document 2021-2027 as Track C comparison. The results of the project will form the basis for developing calibration and measuring capabilities (CMC) entries related to estrogens and comparable substances in water.

The project is a positive answer of the EURAMET 2030 strategy and a demonstrator to support the European Metrology network Pollution Monitoring (EMN POLMO).

The project communicates the results and scientific knowledge gained in the project to the scientific environmental analytical community via open access publications in peer-reviewed journals, workshops and training. It increases the awareness of a wider community on quality assurance and quality control (QA/QC) issues as well as metrology concepts that are often misunderstood or misapplied. As measurements are often instrumental for research in diverse scientific fields, the methods developed provides scientists with guidance to make their measurements metrologically sound.

Impact on relevant standardisation bodies

Dialogue with **Standardization Organisations** AFNOR, SFS, DIN, CEN and ISO has been strengthened and lead to the establishment of formal liaisons. In its annual meeting on the 27th of October 2021, ISO TC 147 SC2 in its Resolution 419 (WebEx-20) states "*SC 2 appreciates the presentation of Sophie Lardy-Fontan and agrees to register a Preliminary Work Item on "Monitoring estrogens" as soon as the working draft is forwarded to the secretariat.*

Once the methods were validated, the former project coordinator, Sophie LARDY-FONTAN, proposed a draft standard at the European Standardisation Technical Committee on water analysis (CEN TC 230). In parallel, the German standardisation group also prepared a draft standard. The two delegations decided therefore to submit a single project to ISO TC 147 "Water quality - SC2 "Physical, chemical and biochemical methods".

A working group was then created: ISO/TC 147/SC 2/WG 84 "Estrogens using MS based methods" whose coordinator is Mrs Lardy-Fontan (ANSES) and whose secretariat is provided by AFNOR (Arnaud Gaudrier). The project ISO 13646 was initiated 'Water quality — Determination of selected oestrogens in whole water samples — Method using solid phase extraction (SPE) followed by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) detection'

This working group met on 26 September 2022 to discuss the the JRP outcome document, first draft of ISO 13646. 4 countries were present: Finland, France, Germany and United Kingdom.

It was agreed that:

- The drafting committee consists of M Gaudrier, Mme Lardy-Fontan and M Türk.
- ISO/CD 13646 prepared the CD version before the end of October 2022, based on the discussion on the comments of the meeting. This new version circulated – for checking – about October 15, 2022 during about one week. As there was no disapproval, the project was submitted to the ISO TC 147 SC2 secretariat for the launch of the CD consultation (Committee Draft). This consultation took place between October 28 and December 23, 2022. The results of the ISO CD ballot vote were :
  - ✓ 10 countries did not comment
  - ✓ 7 countries submitted a comment form
  - ✓ 14 abstained
- WG 84 members decided to propose to register the project in the work program of CEN TC 230 "Water Analysis" (WG 1 – "Physical and biochemical methods") and to activate the Vienna agreements (ISO lead). The secretary of WG 84 will communicate this decision to the secretary of ISO TC 147 SC2 as well as to the secretariat of CEN TC 230 "Water Analysis" to start the procedure. The takeover by CEN TC 230 WG1 of the ISO 13646 project is currently being validated (consultation in progress until April 14, 2023). If the consultation validates this recovery, the project will become an international, European and French project standard; NF EN ISO 13646.

The coordinator and the WG 84 working group reviewed the document for consideration at the WG 84 on March 14 in a hybrid meeting (Afnor/Zoom) and during the meeting at the ISO week meeting of TC 147; on 17th April 2023:

The project will become an international, European and French project standard; NF EN ISO 13646.

Longer-term economic, social and environmental impacts

The outcomes of this project will improve the assessment of human and environmental risks related to the occurrence of endocrine disrupting chemicals in the environment through more accurate and reliable measurement data. The project will enable public authorities to provide data with high level of confidence, to ensure an efficient and comparable implementation of the WFD between Member States, and to inform European citizens who have clearly demonstrated their concern about EDC. By providing measurement results with full uncertainty budgets at very low level of concentration, the project will contribute to better decision making by European policy makers and, as a consequence, to a better protection of human health, aquatic environment and biodiversity. Furthermore, the comparability of data will enable an indirect financial impact by reducing the costs of monitoring and prevention of incorrect decision-making.

With the implementation of the methods developed in this JRP and summarised and validated in the draft project ISO 13646 'Water quality — Determination of selected oestrogens in whole water samples — Method using solid phase extraction (SPE) followed by gas chromatography (GC) or liquid chromatography (LC)

coupled to mass spectrometry (MS) detection'. Testing laboratories will be able to provide reliable results with full uncertainty budgets at very low level of concentration. Public authorities will thus be able to ensure an efficient and comparable implementation of the WFD.

Publications:

1) Evaluation, comparison and combination of molecularly imprinted polymer solid phase extraction and classical solid phase extraction for the preconcentration of endocrine disrupting chemicals from representative whole water samples, L.B.E. Steinhaeuser, T. Westphalen, K. Kaminski, C. Piechotta; Talanta Open Volume 6, December 2022, 100163; https://doi.org/DOI:10.1016/j.talo.2022.100163

This list is also available here: https://www.euramet.org/repository/research-publications-repository-link/

Project start date and duration:	01 September 2019, 42 Months	
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Chief Stakeholder Organisation: CEN/ TC 230 “Water Analysis”	Chief Stakeholder Contact: Ulrich Borchers (Chair TC 230)	
Internal Funded Partners: 1. LNE, France 2. BAM, Germany 3. NIC, Slovenia 4. SYKE, Finland 5. TUBITAK, Türkiye	External Funded Partners: 6. ISPRA, Italy 7. JSI, Slovenia 8. UBX, France	Unfunded Partners:
Linked Third Parties: 9. CNRS, France (Linked to UBX)		
RMG: -		