European Metrology Programme for Innovation and Research

Project 18NRM01 EDC-WFD

"Metrology for monitoring endocrine disrupting compounds under the Water Framework Directive"

Deliverable D2

Report and recommendations to improve the short and long-term stability of selected estrogens in complex water matrix

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Content

1 Summary

A stability study focusing on five estrogens (17-alpha-estradiol, 17-beta-estradiol, 17-alpha-ethinylestradiol, estrone, estriol) to support the requirements of Directive 2013/39/EC, commission Directive 2009/90/EC and Commission implementation Decision (EU) 2018/840 was carried out in two different synthetic real water matrices which are representative for common European inland surface water. Water matrices with and without suspended particulate matter were tested to evaluate the stability of the mentioned estrogens under distinct storage conditions to simulate the way of the sample from the sampling site to the laboratory and to assess the stability. Additionally, different stabilisation strategies were applied to find a possible reagent to preserve the water samples. To investigate a microbial activity, model microorganisms were implemented in the experimental design.

2 Introduction

Estrogens are a very complex chemical group that is known to be relatively unstable (they undergo thermal degradation, photodegradation and biodegradation). An overview of the literature and state of the art highlight that from one study to another, controversial results are observed [1]. In fact, significant differences in the data suggest that the individual composition of tested water samples (microbial flora, pH, organic matter, minerals, etc.) within study (lack of homogeneity of the material) and between different studies may vary and thus affects kinetics of degradation. Various sample pre-treatments (filtration and freezing) and preservation techniques with both acidic and non-acidic agents have been tested with variable degrees of success [2-4].

The aim of this deliverable is to evaluate different stabilising concepts and reagents (such as different temperature conditions and addition of solvents or stabilisation agents) and implement them in a series of stability studies on representative water material. These will be focused on a short-term stability (i.e., stability under transport conditions, time from sampling until analysis) and long-term stability (few weeks).

3 Stability study without suspended particulate matter

3.1 Experimental design

Sample material

The aim of this study is the determination of the short-time stability (e.g., transport and analysis of sample in the laboratory) of five selected estrogens (Estrone (E1), 17α-Estradiol (aE2), 17β-Estradiol (bE2), Estriol (E3) and 17α-Ethinylestradiol (EE2)) in common water matrices like surface or ground water. Here, a representative synthetic real water matrix was used which consists of

mineral water, an artificial DOC (dissolved organic carbon) and the mentioned five estrogens. For preparing the individual samples for the study 100 mL Evian water at pH 7.3 was spiked with DOC (7 mg/L) and the estrogens at a concentration level of 10 ng/L for each substance. These samples were homogenised e.g., on a horizontal shaker for an appropriate time (e.g., 10 min). For the evaluation of different stabilisation reagents samples were prepared in the same manner but with the addition of individual stabilisation reagents (100 mg ascorbic acid (BAM) and 1 mL methanol as keeper (UBx). References without addition of stabilisation agents were also included (LNE, UBx and BAM).

Storage of the samples

For each sample type (without preservative, with ascorbic acid or methanol) three 250 mL amber glass bottles prepared as descript above were stored at -20°C (reference temperature). Twelve bottles of each sample type were stored at +4°C and twelve additional bottles of each sample type at room temperature (20°C). Sampling was scheduled daily after one, two, three, four, five days and on day fourteen. Then, storage of all bottles at -20°C.

Deviations from the prescribed procedure:

LNE produces a 2 L batch of Evian water spiked with DOC (7 mg/L). From this batch the individual subsamples for the stability study with a volume of 100 mL were prepared. The samples were individual spiked with the given estrogens at a concentration level of 10 ng/L.

UBx produces also a 2 L batch containing the DOC-spike, Evian water and the estrogens. Subsamples with a volume of 10 mL each were stored with and without methanol as stabilising reagent at the desired temperatures.

Analysis of the samples

After the last sampling at day fourteen all the samples were kept for two additional days at -20°C. For defreezing all the samples were allowed to store at +4°C for at least 24 hours. Then, the samples where isochronously prepared in terms of preconcentration and sample conditioning. This includes the spiking with isotopic labelled internal standard for each estrogen at a level of 10 ng/L. The measurements were performed as a triplicate plus the injection of solvent (blank) between the sample measurements. The preconcentration and the analysis of the corresponding extracts were carried out individually by each participating project partner:

BAM: HLB SPE and subsequent LC-MS/MS

LNE: SPE disk C_{18} and aminopropyl SPE. LC-MS/MS with derivatisation

UBx: HLB SPE extraction, NH_2 SPE purification and subsequent LC-MS/MS

The detailed preconcentration methods and the analytical procedures can be found in the Annex.

Data analysis

The individual recovery rates of each estrogen in the samples were calculated against the reference sample and/or were evaluated using an instrumental calibration.

LNE and BAM: using calibration for data processing and evaluation.

UBx: Reference concentration = 1 (100%), sample value = relative deviation to the reference in percentage.

Time schedule:

Two weeks for storing the samples at +4°C and room temperature plus two additional weeks for sample preparation, measurements, and data analysis.

3.2 Results

BAM

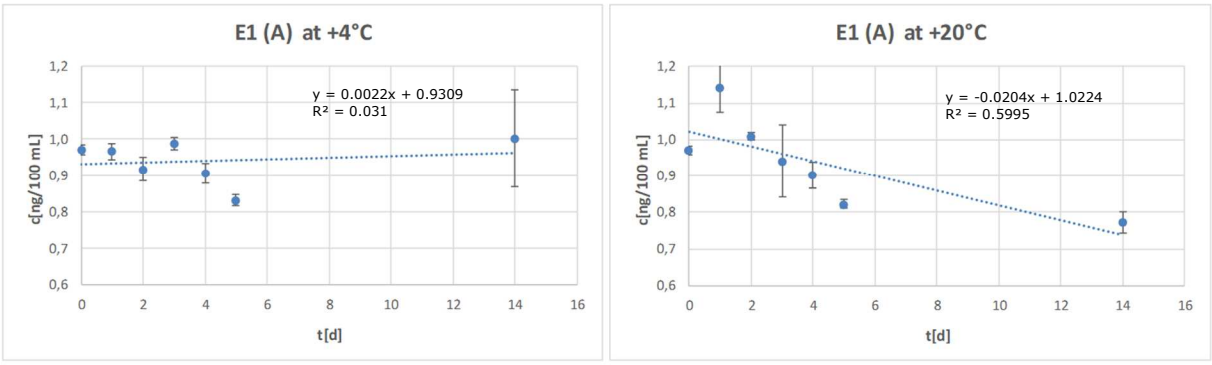

Figure 1: Left: Stability of E1 with ascorbic acid as stabilizing reagent at +4°C. Right: Stability of E1 with ascorbic acid as stabilizing reagent at +20°C.

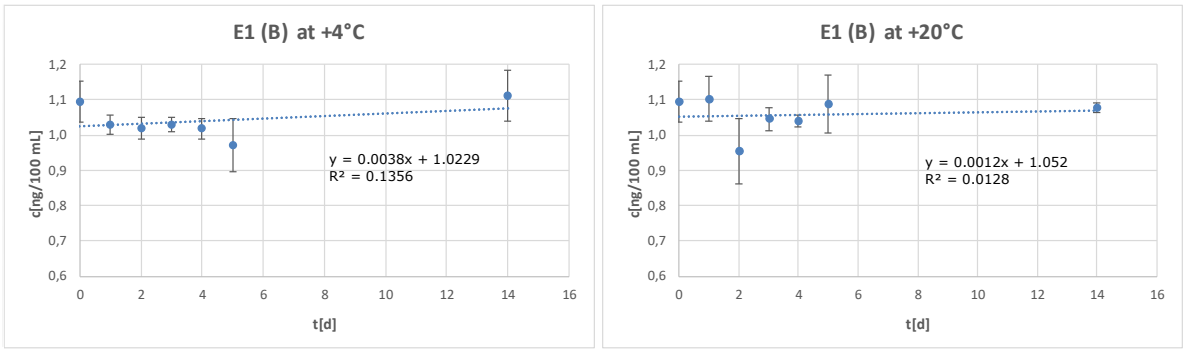

Figure 2: Left: Stability of E1 without stabilizing reagent at +4°C. Right: Stability of E1 without stabilizing reagent at +20°C.

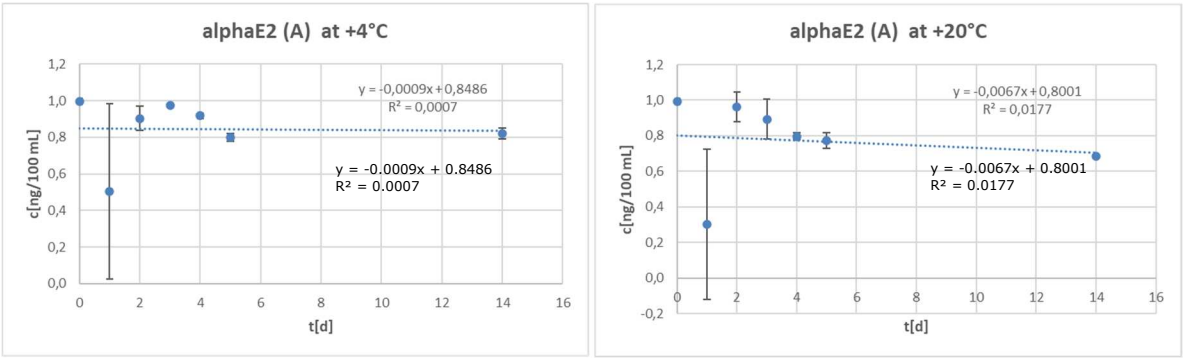

Figure 3: Left: Stability of aE2 with ascorbic acid as stabilizing reagent at +4°C. Right: Stability of aE2 with ascorbic acid as stabilizing reagent at +20°C.

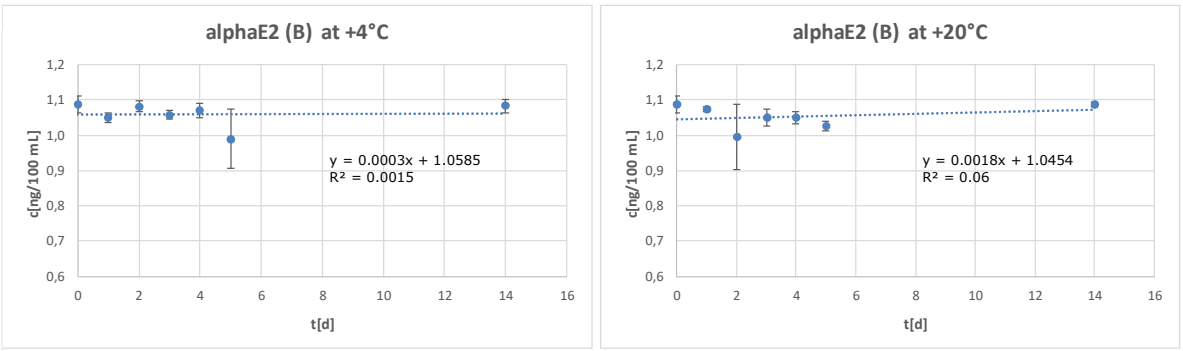

Figure 4: Left: Stability of aE2 without stabilizing reagent at +4°C. Right: Stability of aE2 without stabilizing reagent at +20°C.

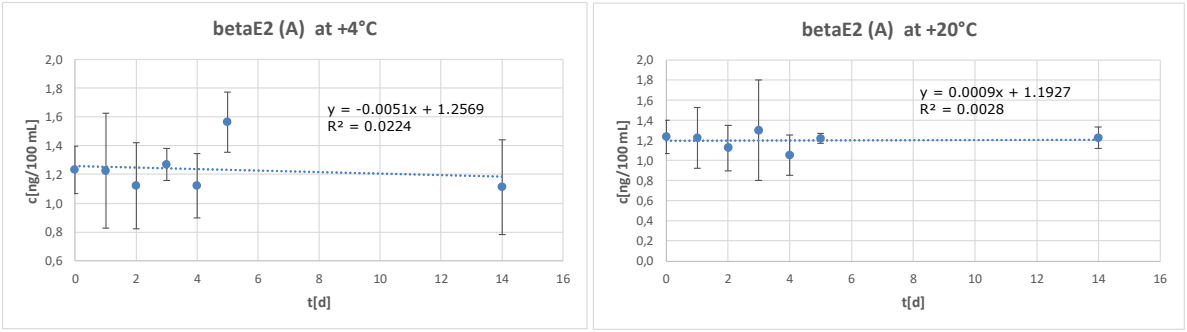

Figure 5: Left: Stability of bE2 with ascorbic acid as stabilizing reagent at +4°C. Right: Stability of bE2 with ascorbic acid as stabilizing reagent at +20°C.

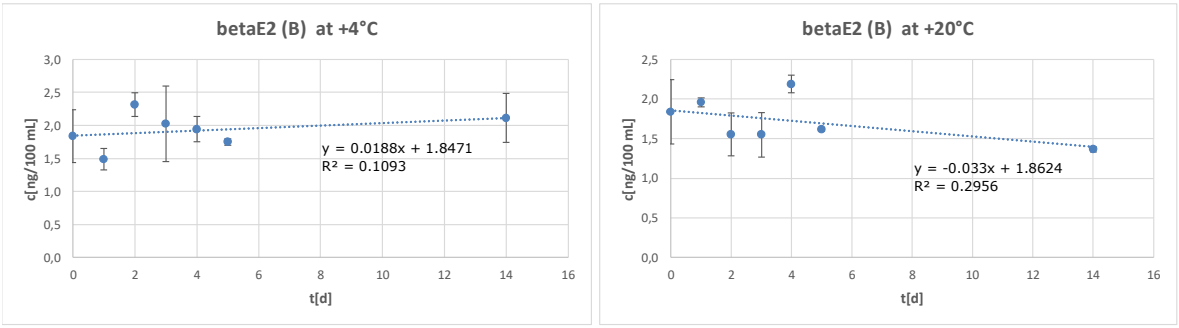

Figure 6: Left: Stability of bE2 without stabilizing reagent at +4°C. Right: Stability of bE2 without stabilizing reagent at +20°C.

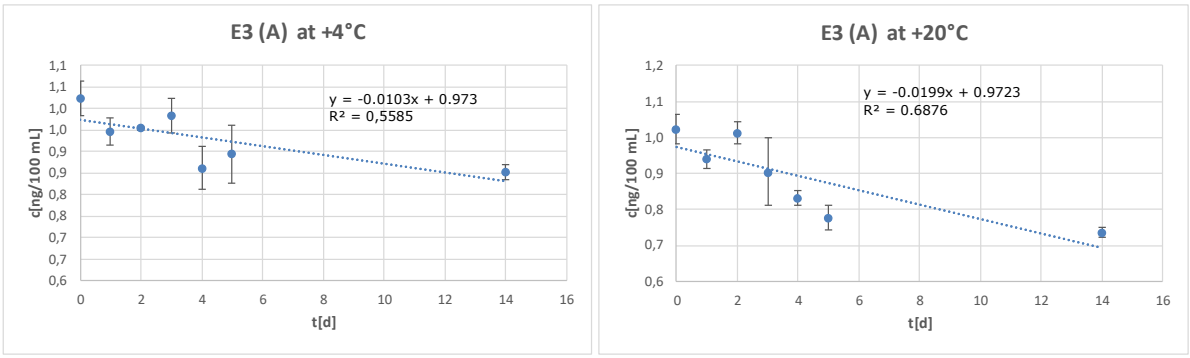

Figure 7: Left: Stability of E3 with ascorbic acid as stabilizing reagent at +4°C. Right: Stability of E3 with ascorbic acid as stabilizing reagent at +20°C.

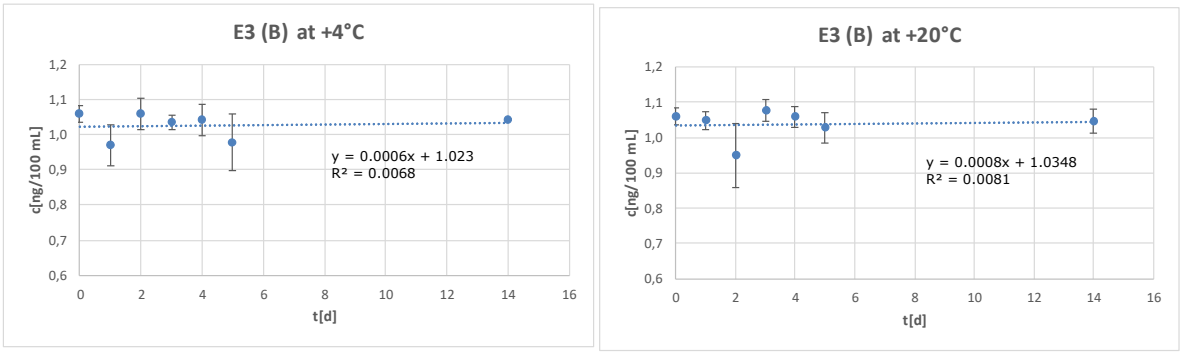

Figure 8: Left: Stability of E3 without stabilizing reagent at +4°C. Right: Stability of E3 without stabilizing reagent at +20°C.

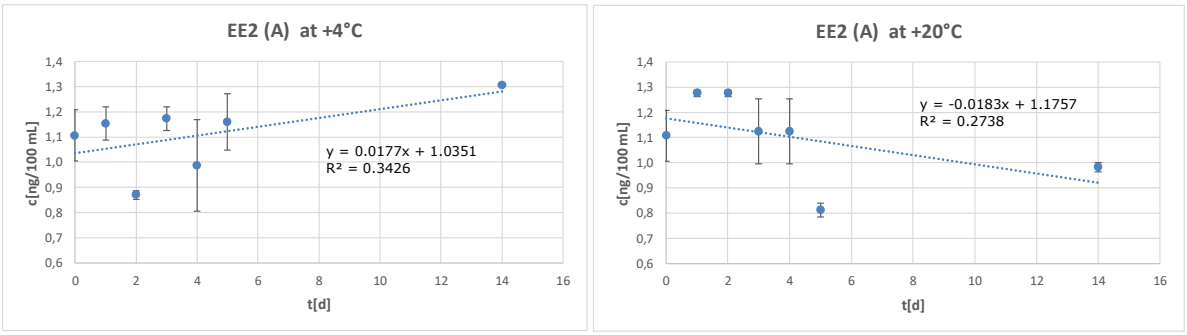

Figure 9: Left: Stability of EE2 with ascorbic acid as stabilizing reagent at +4°C. Right: Stability of EE2 with ascorbic acid as stabilizing reagent at +20°C.

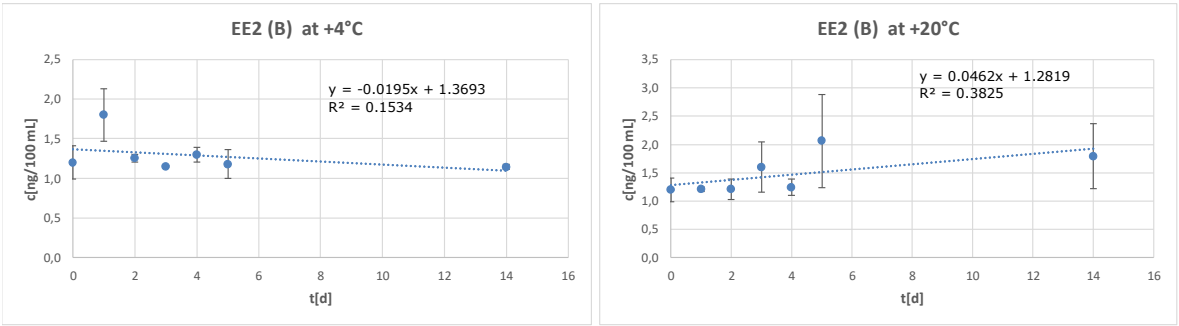

Figure 10: Left: Stability of EE2 without stabilizing reagent at +4°C. Right: Stability of EE2 without stabilizing reagent at +20°C.

LNE

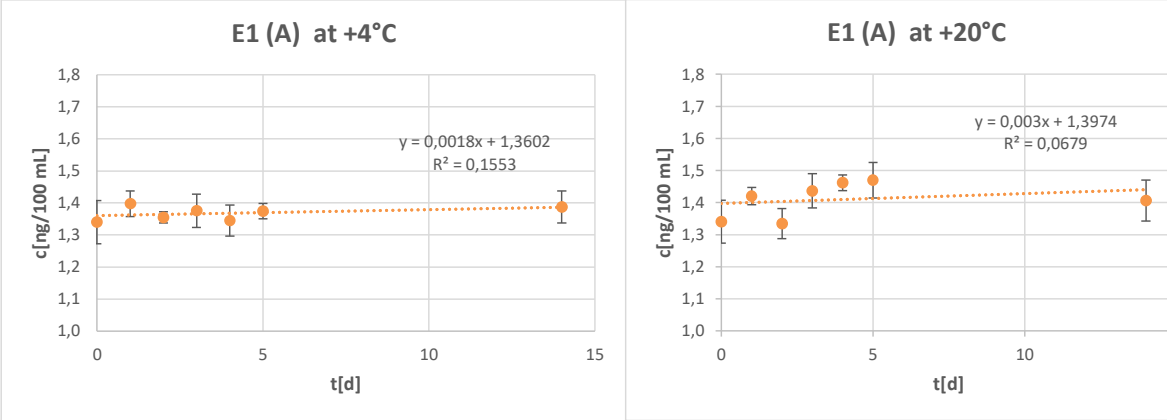

Figure 11: Left: Stability of E1 without stabilizing reagent at +4°C. Right: Stability of E1 without stabilizing reagent at +20°C.

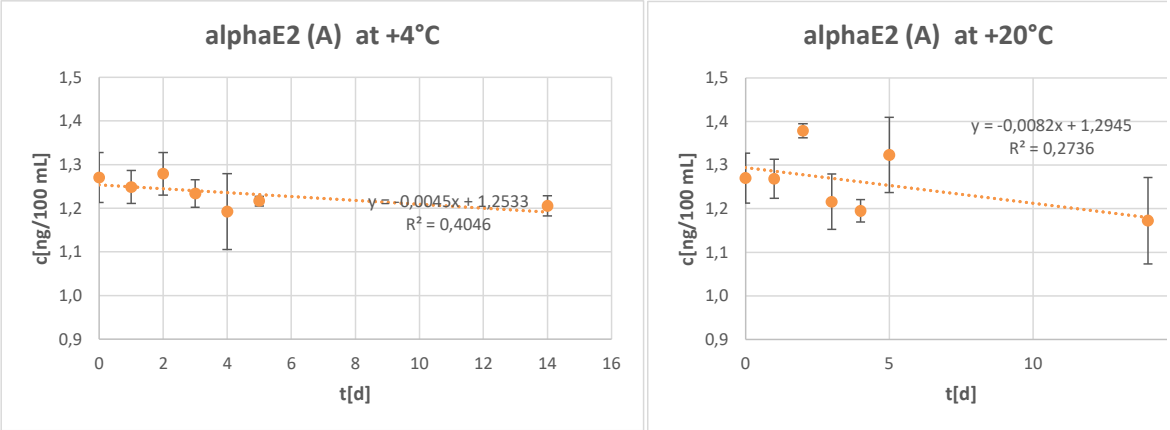

Figure 12: Left: Stability of aE2 without stabilizing reagent at +4°C. Right: Stability of aE2 without stabilizing reagent at +20°C.

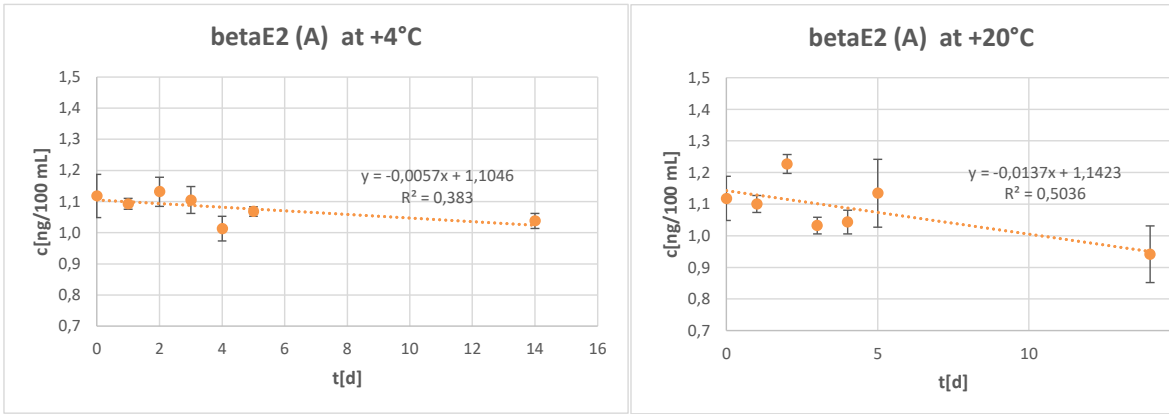

Figure 13: Left: Stability of bE2 without stabilizing reagent at +4°C. Right: Stability of bE2 without stabilizing reagent at +20°C.

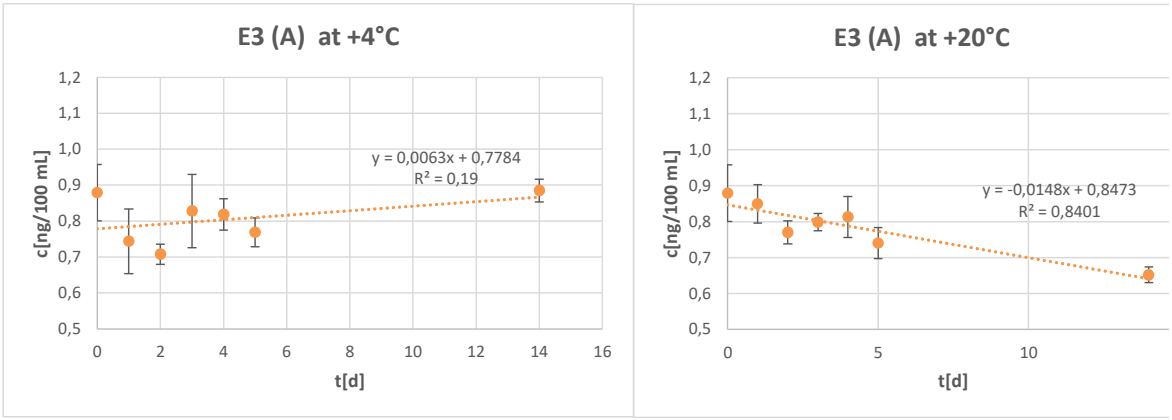

Figure 14: Left: Stability of E3 without stabilizing reagent at +4°C. Right: Stability of E3 without stabilizing reagent at +20°C.

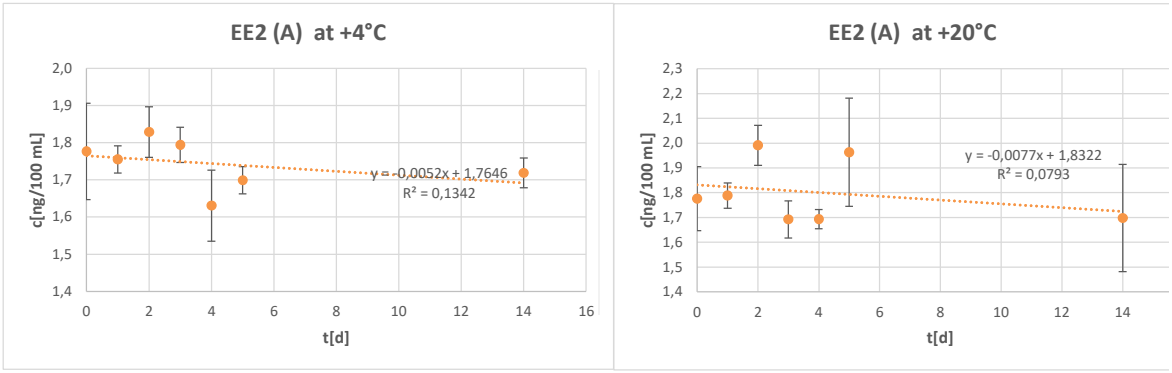

Figure 15: Left: Stability of EE2 without stabilizing reagent at +4°C. Right: Stability of EE2 without stabilizing reagent at +20°C.

UBx

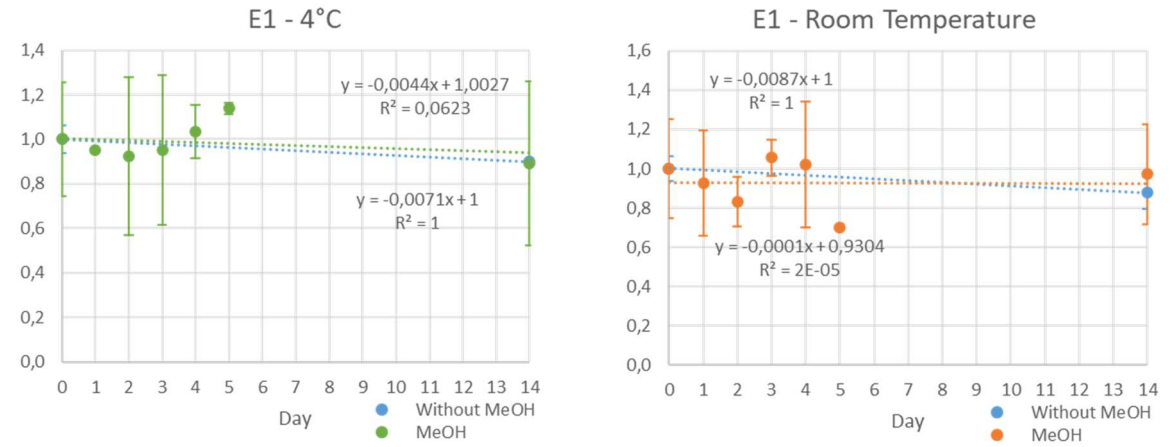

Figure 16: Left: Stability of E1 with methanol and without stabilizing reagent at +4°C. Right: Stability of E1 with methanol and without stabilizing reagent at +20°C.

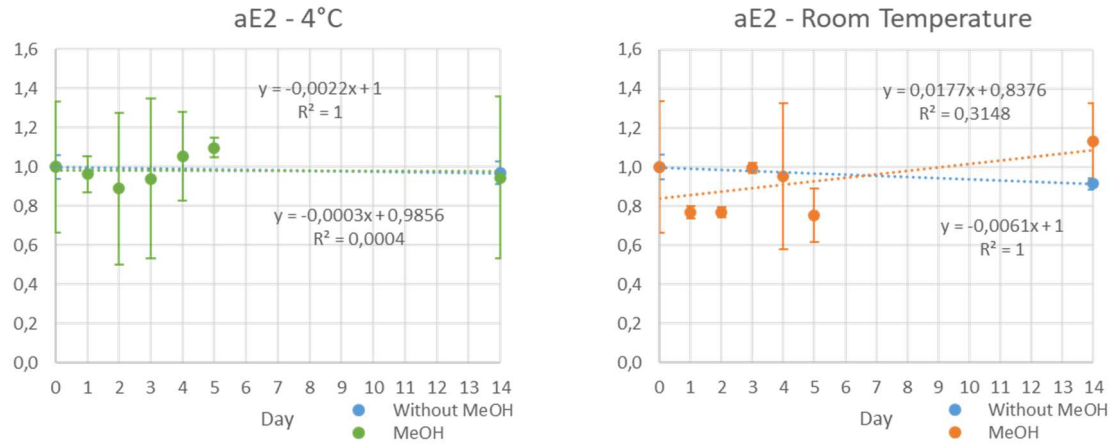

Figure 17: Left: Stability of aE2 with methanol and without stabilizing reagent at +4°C. Right: Stability of aE2 with methanol and without stabilizing reagent at +20°C.

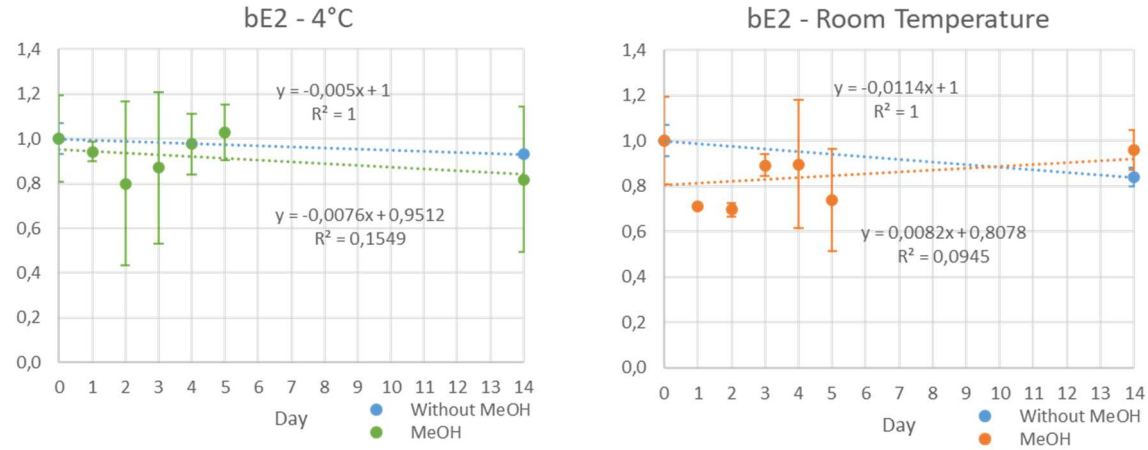

Figure 18: Left: Stability of bE2 with methanol and without stabilizing reagent at +4°C. Right: Stability of bE2 with methanol and without stabilizing reagent at +20°C.

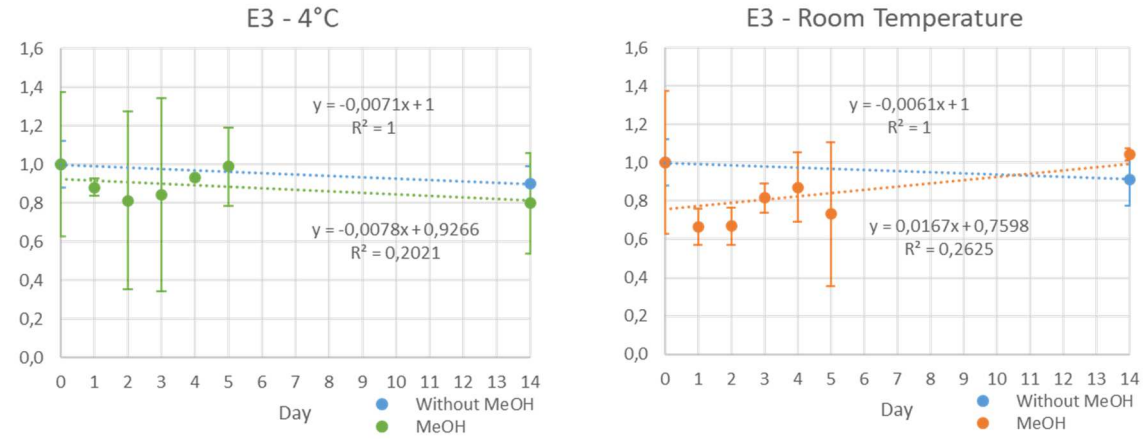

Figure 19: Left: Stability of E3 with methanol and without stabilizing reagent at +4°C. Right: Stability of E3 with methanol and without stabilizing reagent at +20°C.

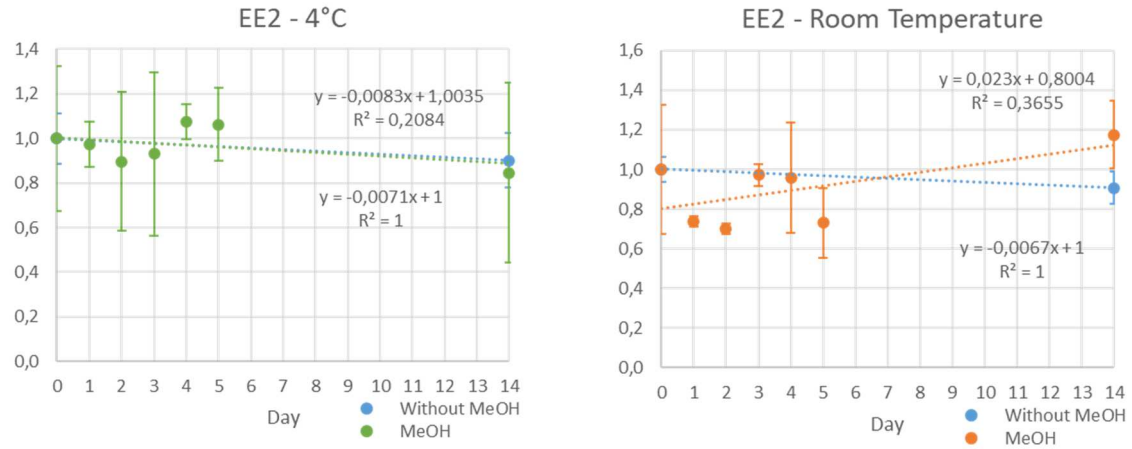

Figure 20: Left: Stability of EE2 with methanol and without stabilizing reagent at +4°C. Right: Stability of EE2 with methanol and without stabilizing reagent at +20°C.

3.3 Conclusion

In conclusion the samples stored at +4°C without stabilising reagent show no significant trends with regards to degradation or loss of the analytes during storage time. 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.

The outcomes of the first stability study suggest carrying out a second stability study, including a complex water matrix in terms of additional SPM and microbial activity, only at a single storage temperature of +4°C.

4 Stability study with suspended particulate matter

4.1 Experimental design

In a second study a more complex matrix should be evaluated with regards to a typical whole water sample. This includes a model suspended particulate matter (SPM) which is estrogen-free. Here, the experimental design was modified with respect to the results obtained from the first stability study without SPM.

Sample material

The aim of this study is the determination of the short-time stability (e.g., transport and analysis of sample in the lab) of five selected estrogens (Estrone (E1), 17α-Estradiol (aE2), 17β-Estradiol (bE2), Estriol (E3) and 17α-Ethinylestradiol (EE2)) in a complex water matrix like surface or ground water. Here, a representative synthetic real water matrix was used which consists of mineral water, an artificial DOC, the mentioned five estrogens and SPM. For preparing the individual samples for the study 100 mL Evian water at pH 7.3 is spiked with DOC (7 mg/L), 50 mg model SPM and the estrogens at a concentration level of 10 ng/L for each substance. These samples were homogenised e.g., on a

horizontal shaker for an appropriate time (e.g., 10 min). For the evaluation of different stabilization reagents, samples are prepared in the same manner but with the addition of individual components (100 mg ascorbic acid (BAM), 1 mL methanol as keeper (UBx). References without addition of stabilisation agents were also included (LNE and BAM).

Storage of the samples

For each sample type (without preservative, with ascorbic acid and methanol or microbial activity) three 250 mL amber glass bottles prepared as descript above were stored at -20°C (reference temperature). Nine bottles of each sample type were stored at +4°C. Sampling was scheduled daily after one and five days and on day fourteen. Then, storage of all bottles at -20 °C.

Deviations from the prescribed procedure:

LNE produces a batch of 2 L Evian water spiked with DOC (7 mg/L) and SPM. From this batch the individual subsamples for the stability study with a volume of 100 mL were prepared. The samples were individual spiked with the given estrogens at a concentration level of 10 ng/L.

UBx produces also a 2 L batch containing the DOC-spike, Evian water the estrogens and SPM. Subsamples with a volume of 30 mL each were stored with and without methanol as stabilising reagent at the desired temperatures.

Analysis of the samples

After last sampling at day fourteen all the samples were kept for two additional days at -20°C. For defreezing all the samples were allowed to store at +4°C for at least 24 hours. Then, the samples where isochronously prepared in terms of preconcentration and sample conditioning. This includes the spiking with isotopic labelled internal standard for each estrogen at a level of 10 ng/L. The measurements were performed as a triplicate plus the injection of solvent (blank) between the sample measurements. The preconcentration and the analysis of the corresponding extracts were carried out individually by each participating project partner:

BAM: HLB SPE and subsequent LC-MS/MS

LNE: SPE disk C_{18} and NH2 SPE purification. LC-MS/MS with derivatisation

UBx: HLB SPE extraction, NH2 SPE purification and subsequent LC-MS/MS

The detailed preconcentration method and the analytical procedure can be found in the Annex.

Data analysis

The individual recovery rates of each estrogen in the samples were calculated against the reference sample and/or were evaluated using an instrumental calibration.

LNE, BAM and UBx: using calibration for data processing and evaluation.

UBx: using also reference sample

Time schedule:

Two weeks for storing the samples at +4°C plus two additional weeks for defreezing, sample preparation, measurements, and data analysis.

4.2 Results

BAM

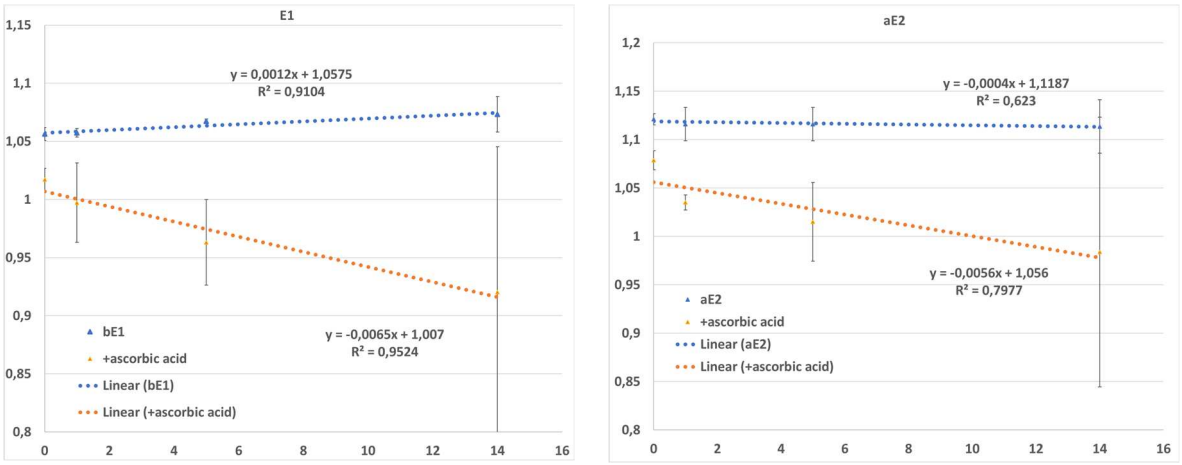

Figure 21: Left: Stability of E1 without stabilizing reagent and with ascorbic acid at +4°C. Right: Stability of aE2 without stabilizing reagent and with ascorbic acid at +4°C.

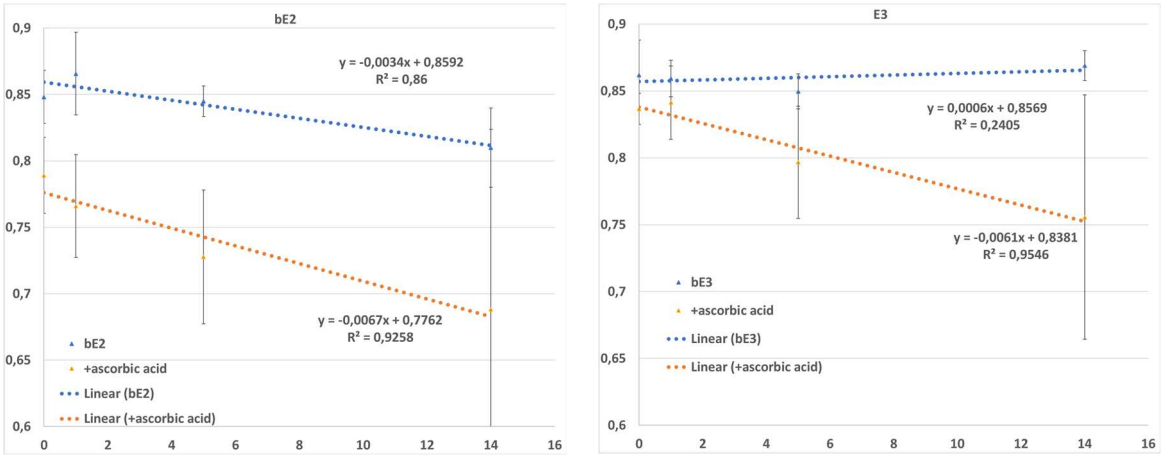

Figure 22: Left: Stability of bE2 without stabilizing reagent and with ascorbic acid at +4°C. Right: Stability of E3 without stabilizing reagent and with ascorbic acid at +4°C.

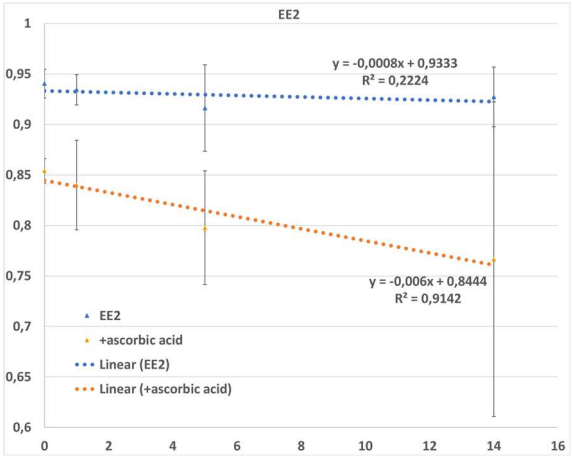

Figure 23: Stability of E3 without stabilizing reagent, with ascorbic acid and with microbial activity at +4°C.

LNE

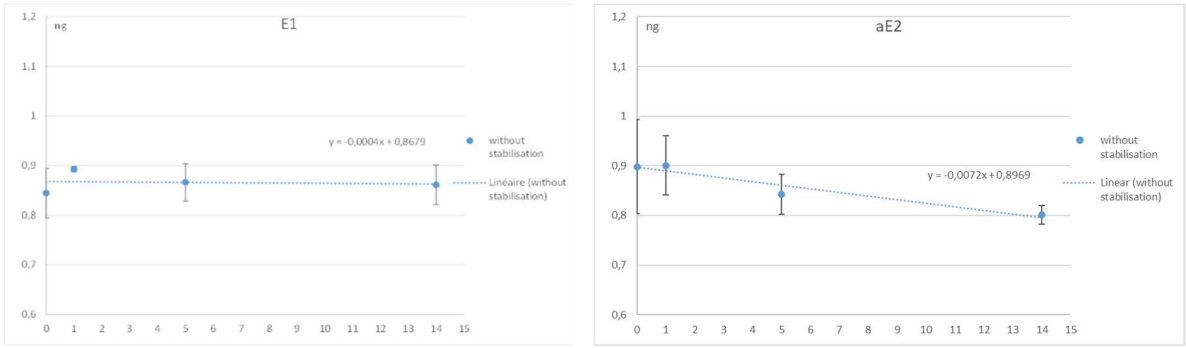

Figure 24: Left: Stability of E1 without stabilizing reagent at +4°C. Right: Stability of aE2 without stabilizing reagent at +4°C.

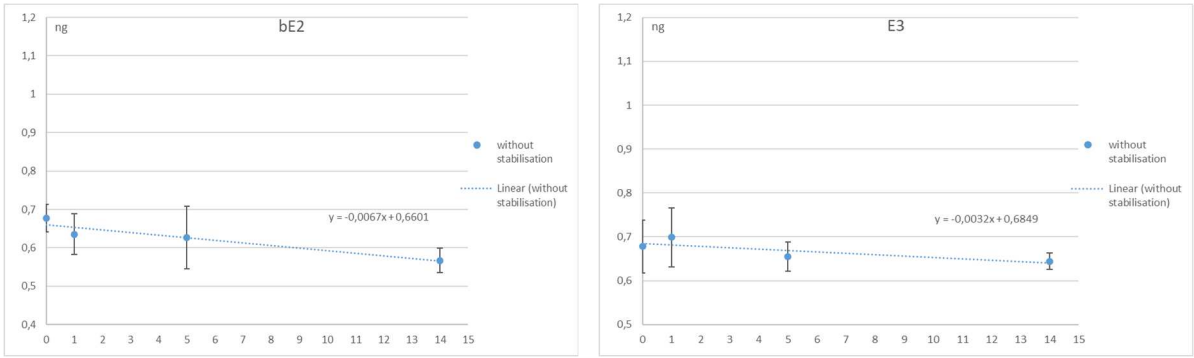

Figure 25: Left: Stability of bE2 without stabilizing reagent at +4°C. Right: Stability of E3 without stabilizing reagent at +4°C.

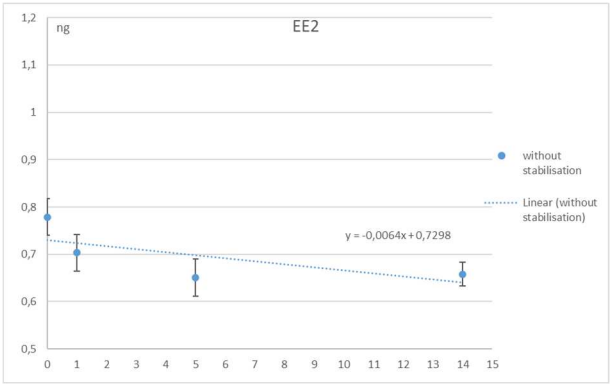

Figure 26: Stability of EE2 without stabilizing reagent at +4°C.

UBX

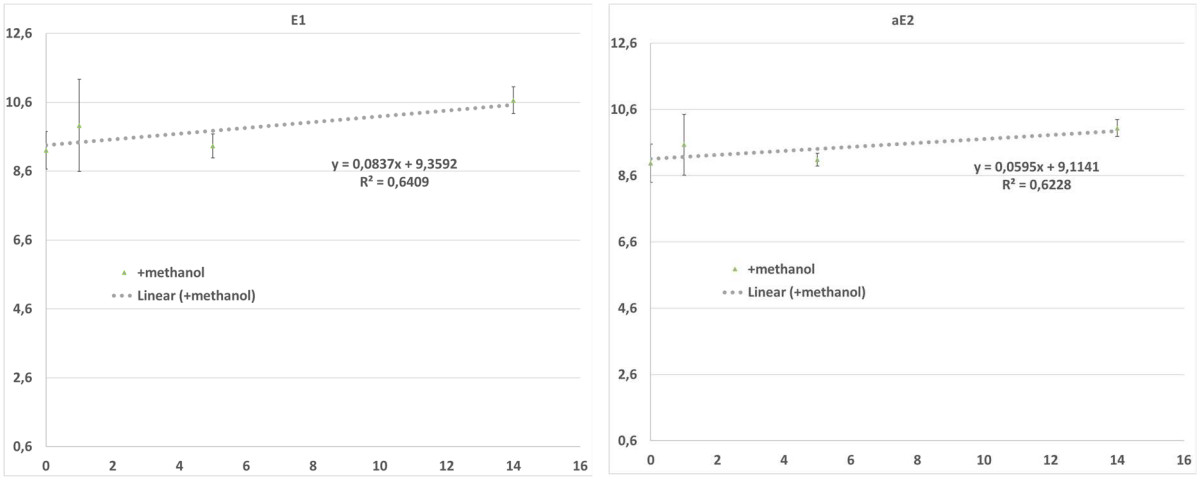

Figure 27: Left: Stability of E1 with methanol as stabilizing reagent at +4°C. Right: Stability of aE2 with methanol as stabilizing reagent at +4°C.

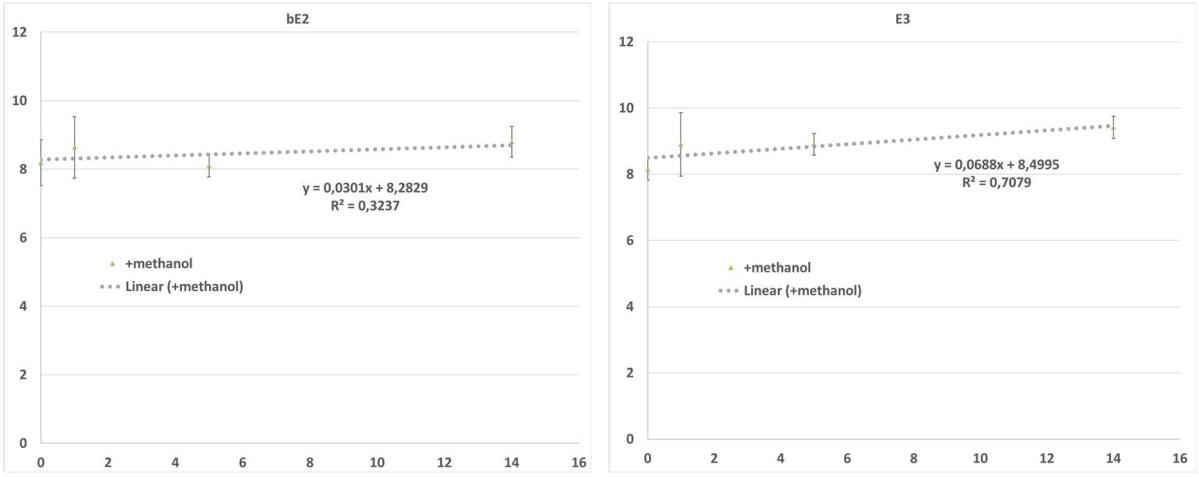

Figure 28: Left: Stability of bE2 with methanol as stabilizing reagent at +4°C. Right: Stability of E3 with methanol as stabilizing reagent at +4°C.

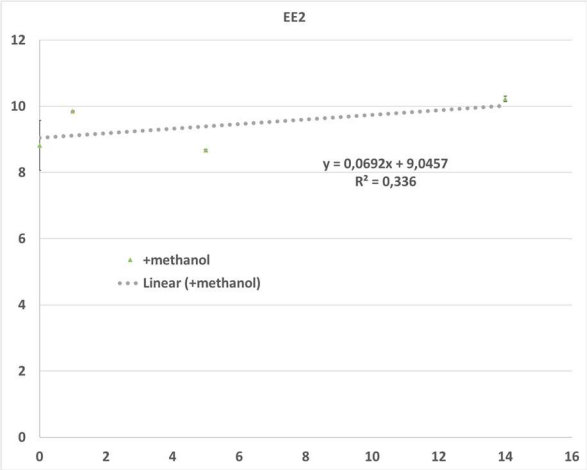

Figure 29: Stability of EE2 with methanol as stabilizing reagent at +4°C.

4.3 Conclusion

In conclusion the samples which include a complex water matrix in terms of additional SPM stored at +4 °C without stabilising reagent or with 1% methanol show no significant trend with regards to degradation or loss of the analytes during storage time. 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.

5 Impact of microbial activity on the stability

5.1 Experimental design

In a third study, a more complex matrix should be evaluated with regards to a typical whole water sample. This includes a microbiological activity in the water sample.

Sample material

The aim of this study is the determination of the short-time stability (e.g., transport and analysis of sample in the lab) of five selected estrogens (Estrone (E1), 17α-Estradiol (aE2), 17β-Estradiol (bE2), Estriol (E3) and 17α-Ethinylestradiol (EE2)) in a complex water matrix like surface or ground water. Here, a representative synthetic real water matrix was used which consists of mineral water, an artificial DOC, the mentioned five estrogens, SPM and microbial content. For preparing the individual samples for the study 100 mL Evian water at pH 7.3 is spiked with DOC (7 mg/L), 50 mg model SPM and the estrogens at a concentration level of 10 ng/L for each substance. A microorganism containing spiking solution was added to this synthetic real water samples. As model

microorganisms selected iron and manganese oxidizing bacteria were used (*Sphingomonas spec.* and *Sphaerotilus spec.*) (BAM). These samples were homogenised e.g., on a horizontal shaker for an appropriate time (e.g., 10 min). For the evaluation of different stabilization reagents and microorganism samples are prepared in the same manner but with the addition of individual components (100 mg ascorbic acid (BAM)) and reference without addition of stabilisation agents were also included (BAM).

Storage of the samples

For each sample type (without preservative, with ascorbic acid and methanol or microbial activity) three 250 mL amber glass bottles prepared as descript above were stored at -20°C (reference temperature). Nine bottles of each sample type were stored at +4°C. Sampling was scheduled daily after one and five days and on day fourteen. Then, storage of all bottles at -20 °C.

Analysis of the samples

After last sampling at day fourteen all the samples were kept for two additional days at -20°C. For defreezing all the samples were allowed to store at +4°C for at least 24 hours. Then, the samples where isochronously prepared in terms of preconcentration and sample conditioning. This includes the spiking with isotopic labelled internal standard for each estrogen at a level of 10 ng/L. The measurements were performed as a triplicate plus the injection of solvent (blank) between the sample measurements. The preconcentration and the analysis of the corresponding extracts were carried out by BAM implementing HLB SPE and subsequent LC-MS/MS.

Data analysis

The individual recovery rates of each estrogen in the samples were evaluated using an instrumental calibration for data processing and evaluation.

Time schedule:

Two weeks for storing the samples at +4°C plus two additional weeks for defreezing, sample preparation, measurements, and data analysis.

5.2 Results and conclusion

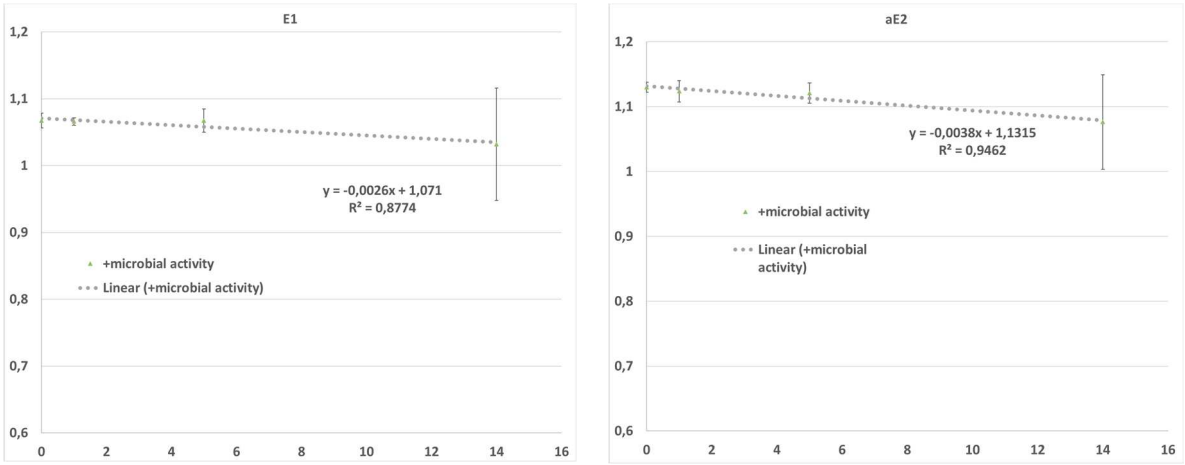

Figure 30: Left: Stability of E1 without stabilizing reagent and with microbial activity at +4°C. Right: Stability of aE2 without stabilizing reagent and with microbial activity at +4°C.

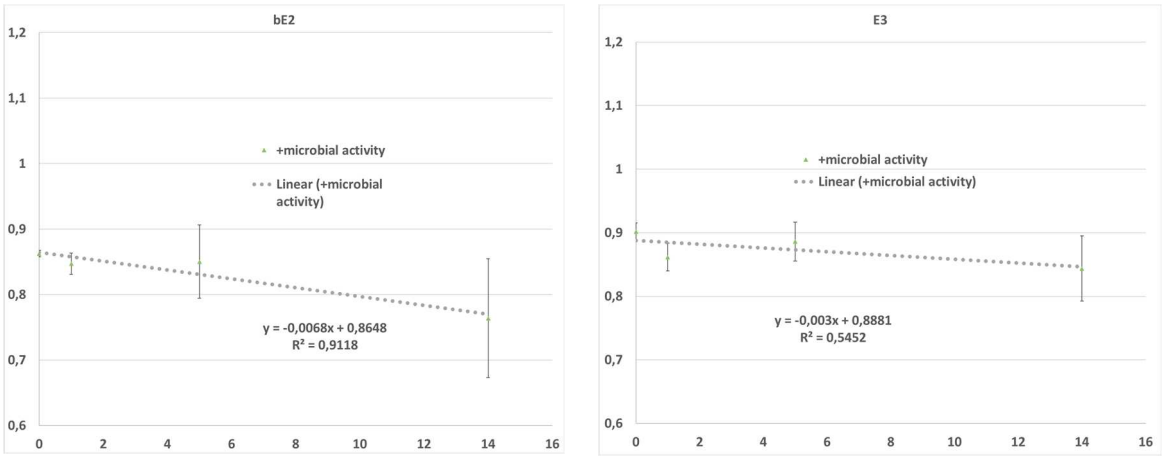

Figure 31: Left: Stability of bE2 without stabilizing reagent and with microbial activity at +4°C. Right: Stability of E3 without stabilizing reagent and with microbial activity at +4°C.

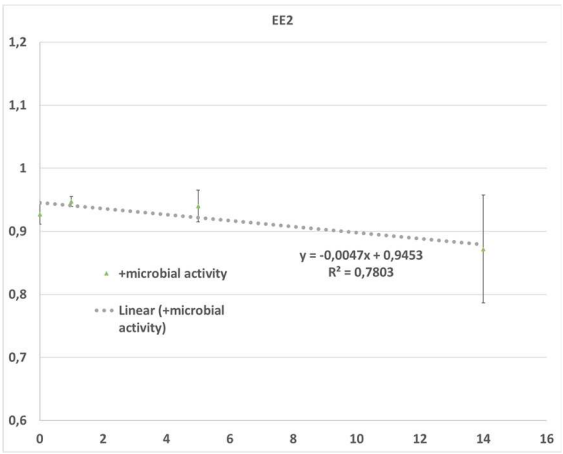

Figure 32: Stability of E3 without stabilizing reagent, with ascorbic acid and with microbial activity at +4°C.

The impact of microbial activity showed only a small influence on the decrease of the estrogen concentration within a period of fourteen days. This effect should be taken into account when calculating the uncertainty budget for the whole analytical procedure.

6 Conclusion and recommendations

Both stability studies for free estrogens in complex water matrix with or without suspended particulate matter showed good results at +4°C for at least two weeks without the use of any stabilising reagent. The microbial impact can be neglected and can be included in the uncertainty budget of the final analytical method.

7 References

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8 Annex

Preconcentration procedure – sample preparation

(1) BAM

For quality assurance a small validation of the SPE procedure was carried out. Here, each estrogen (E1, aE2, bE2, E3 and EE2) at 10 ng/L level in 1000 mL Millipore water was preconcentrated using a Waters Oasis HLB (3 mL/150 mg sorbent) as the cartridge of choice., Sufficient recovery rates within 99 to 102% using internal isotopic labelled standards at 5 ng/L level were achieved. In a second approach the Waters Oasis HLB cartridge was evaluated with the synthetic real water matrix which consists of Evian water and DOC (7 mg/L) and 50 mg SPM. Again, each estrogen (E1, aE2, bE2, E3 and EE2) at 10 ng/L level in 1000 mL. The quantification was realised by LC-MS/MS with and without internal isotopic labelled standards (aE2-d_2, E1-d_2, bE2-$^{13}C_2$, EE2-$^{13}C_2$, E3-d_2).

Procedure: A 1000 mL complex water sample was spiked with an internal standard mix at 5 ng/L. The sample was homogenised for 15 min on a horizontal shaker. The Waters Oasis HLB 3 mL/150mg cartridge was subsequently conditioned with 10 mL ACN and 15 mL H_2O. Afterwards the water sample was loaded applying a flow rate of 20 mL/min onto the cartridge followed by washing the cartridge with 10 mL H_2O, drying with N_2 for 1 min and collect a 10 mL fraction into a sample tube using ACN. This organic fraction was concentrated to 1 mL. To evaluate possible matrix effects in sense of ion suppression in the electro spray ionisation source (ESI) the resulting extracts were diluted 1:10 and 1:20.

(2) LNE

Samples are first spiked with internal isotopic labelled standards (E1-$^{13}C_3$, bE2-d_5, EE2-d_4, E3-$^{13}C_3$). and 0.1% EDTA (v/v). The extraction step was achieved on C_{18} Atlantic® ReadyDisk from Biotage (Uppsala, Sweden) on a Horizon Technology SPE-DEX® 4790. The water samples (1 L) were loaded onto C_{18} disks preconditioned with ethyl acetate (EA) followed by MeOH and finally by Evian water. After drying the disks, elution was applied with EA. Extracts were then evaporated to dryness by SpeedVac™ concentrator (Thermo Scientific, Villebon sur Yvette, France) at a temperature of 45°C and reconstituted with 2 x 0.5 mL of MeOH. Methanolic extracts were then purified with a Supelclean™ LC-NH_2 SPE (500 mg, 6 mL) cartridge (Merck, Darmstadt, Germany) previously conditioned with 4 mL of MeOH. Cartridge was then eluted with 2 mL of MeOH. The extract was evaporated to dryness by SpeedVac™ concentrator at a temperature of 45°C. Then, 200 µL of extra dry acetone and 500 µL of bicarbonate buffer (100mM, pH = 10.5) were added. After 1 minute of stirring by vortex, 500 µL of dansyl chloride solution (0.7 mg mL^{-1}, extra dry acetone) was added and the solution was stirred during 1 more minute and then heated at 60°C during 6 minutes. Then, samples were evaporated to dryness by SpeedVac™ concentrator and transfered into a new vial with 2 x 600 µL of acetonitrile in order to remove insoluble salts. Finally, samples were evaporated to dryness by SpeedVac™ Concentrator and reconstituted with 100 µL of a mixture of water/acetonitrile (50:50, v/v).

(3) UBx

Samples were first spiked with internal isotopic labelled standards (E1-$^{13}C_3$, aE2-d_2, bE2-$^{13}C_3$, EE2-d_4, E3-d_2) and 1% MeOH (v/v) then acidified at pH=5 (hydrochloride acid). The extraction step was achieved on Waters Oasis HLB (6 mL/200 mg sorbent) SPE cartridge. The water samples (200 mL Evian water and DOC 7 mg/L and SPM 50 mg/L) were loaded onto HLB cartridge preconditioned with 6 mL MeOH followed by 6 mL MQ water at pH=5. After drying the SPE cartridge, elution was applied with 2 x 5 mL MeOH. Extracts were then evaporated to 200 µL under nitrogen flow at a temperature of 50°C and reconstituted with 800 µL of EA. The organic extracts (1 mL 80:20, EA/MeOH, v/v) were then purified with a Supelco Supelclean LC-NH_2 SPE (3 mL/500 mg sorbent) cartridges previously conditioned with 2 x 2 mL EA followed by 2 x 2 mL 80:20 EA/MeOH (v/v). Cartridges were then eluted with 2 mL of 80:20 EA/MeOH (v/v). The purified extracts were then evaporated to 30 µL under nitrogen flow at a temperature of 50°C. Before analysis step, the final extracts were spiked with isotopic labelled

standards (E1-d_4, bE2-d_4) and divided as following: 35 µL reconstituted with 82 µL MQ water (30:70 MeOH/MQ Water v/v) and 25 µL MeOH.

Analytical method

(a) BAM

The resulting extracts were analysed using an Agilent 1260 HPLC and an AB SCIEX TSQ 6500 as mass selective detector.

The Agilent 1260 HPLC consists of the binary pump, autosampler, thermostatted column compartment and 1200 diode array detector coupled to an AB Sciex TSQ 6500 mass spec. The following chromatographic parameter were utilised:

HPLC-column: ZORBAX SB-Phenyl, 2.1 x 100 mm, 1.8 µm

Flow: 200 µL/min

Column temperature: 25°C

Eluents: 0.25 mM NH_4F/H_2O (A), methanol (LC-MS grade) (B) with the following gradient:

Step	Total time (min)	Flow rate (µL/min)	A(%)	B(%)
0	0.00	200	40.0	60.0
1	15.00	200	40.0	60.0
2	15.50	200	0.0	100.0
3	25.00	200	0.0	100.0
4	25.50	200	40.0	60.0
5	35.00	200	40.0	60.0

The mass spectrometric conditions and parameters are listed below:

Mass spec parameter: ESI neg., scheduled MRM (multiple reaction monitoring), target scan time 0.700 sec.

(b) LNE

Chromatographic separation was performed using a AcquityR UPLC system (Waters, Guyancourt, France) on a Cortecs Shield RP18 Waters Dp=2.7 µm 2.1 x 100 mm The column oven temperature was set at 40°C and the flow rate at 0.4 mL min^{-1}. The optimised separation conditions are presented below.

Total time (min)	Water + 0.1% formic acid (%)	Acetonitrile + 0.1% formic acid (%)
0	35.0	65.0

6.5	35.0	65.0
6.6	5.0	95.0
8.0	5.0	95.0
8.1	35.0	65.0
10.0	35.0	65.0

The UPLC system was coupled to a Xevo TQ-MSR triple quadrupole mass spectrometer (Waters, Guyancourt, France) equipped with an electrospray ionisation (ESI) source. Acquisition was performed in Multiple Reaction Monitoring (MRM) mode in positive ionisation mode. The mass spectrometer was operating with a capillary voltage set at 3 kV in positive mode. Source and desolvatation temperature were set at 150°C and 650°C, respectively. Desolvatation and cone gas were set at 1000 and 50 L h^{-1}, respectively. Collision gas (argon) pressure was controlled at a vacuum of 3.5−3 mbar.

(c) UBx

The analyses were performed on a LC/MS-MS Agilent Technologies system: 1290 Infinity II HPLC coupled to a 6495 Triple quadrupole mass spectrometer equipped with a electrospray ionisation (ESI). The chromatographic separation was performed on a Poroshell 120 Phenylhexyl column Dp = 1.9 µm 2.1 x 100 mm with guard column Dp 2.7 µm, 2.1 X 5 mm. The column oven temperature was set at 50°C and the flow rate at 0.6 mL min^{-1}. The optimised separation conditions are presented below.

Total time (min)	MQ Water + 0.1mM NH_4F (%)	65/35 MeOH/ACN (v/v) (%)
0.00	90.00	10.00
0.50	90.00	10.00
13.50	55.00	45.00
14.00	42.00	58.00
15.00	20.00	80.00
15.50	0.00	100.00
16.00	0.00	100.00
16.50	90.00	10.00
18.00	90.00	10.00

Acquisition was performed in Multiple Reaction Monitoring (MRM) mode in negative ionisation mode. The mass spectrometer was operating with a capillary voltage set at 3.5 kV. Desolvatation gas temperature and flow were set at 120°C and 16 L min^{-1}. Sheath gas temperature and flow were set at 375°C and 12 L min^{-1}. Collision gas (nitrogen) pressure was fixed by the default value system and the nozzle voltage was fixed at 300 V.

Two acquisition methodologies were performed: the first one is a multi-residue method to analyse E1, aE2, bE2, E3 and the internal isotopic labelled standards (E1-$^{13}C_3$, aE2-d_2, bE2$^{13}C_3$, E3-d_2, E1-d_4, bE2-d_4) by injecting 5 µL of methanolic extract. The second one is a specific method to analyse EE2 and EE2-d_4 by injecting 100 µL of 30:70 MeOH/MQ water (v/v) extract.