European Metrology Programme for Innovation and Research

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

Deliverable D1

Report on the comparison of sample preparation techniques for estrogens partitioning in whole water and recommendations on the most appropriate methods

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1 Summary

Different extraction and pre-concentration methods have been published for endocrine disrupting chemicals and selected estrogens like Estrone, 17β-Estradiol and 17α–Ethinylestradiol in water, however only very few publications discuss and analyses the concentration level regarded in this project. Moreover, many of these methods did not demonstrate the efficient separation of matrix and analyte and they did not handle with whole water samples (Piechotta et al., in preparation). 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. Here, a set of complementary extraction methods was evaluated and optimised. In parallel this study was used to optimise chemical measurement methods compatible with the requirements of the Directive 2009/90/EC "QA/QC" implementing various detection methods- hyphenated MS techniques like LC-MS/MS and GC-MS(/MS)- routinely used by laboratories involved in the monitoring of priority and emerging substances. Isotope dilution mass spectrometry (IDMS) is considered to provide analytical results of highest metrological degree regarding accuracy and precision as well as the smallest uncertainties. For these reasons IDMS is the most suitable as reference method and will be applied.

2 Introduction

Estrogen measurements are carried out differently in European countries. An overview of the literature and state of the art measurements capabilities has shown that laboratories 1) develop a variety of measurement methods (sample preparation, detection methods) and 2) implement a variety of strategies to evaluate their performances in terms of Limit of quantification LOQ and accuracy. The capacity of the methods to effectively and accurately provide compliant measurements is questionable, especially regarding Directive 2009/90/CE.

EQS (environmental quality standards) values refer to the whole water sample, i.e., they include contaminants, which are associated to suspended solids present in natural water. The distribution of contaminants in different compartments of aquatic systems is important because it determines their transport, fate and possible toxic effects. Most studies in the literature focus on measurement of estrogens in filtered water samples to achieve the lowest LOQ (limit of quantification) and also because it is easier to manage. When studied, mass balance for total estrogens showed that the SPM (suspended particulate matter) phase could not be neglected even if partitioning processes are not enough understood. Petrovic [1] pointed out that whole water analysis as required by WFD was one of the main analytical challenges. This issue has also been discussed by Ademollo [2]. The authors recommended that total concentration should be obtained by direct analysis of the whole-water sample (without filtration) or by separate determinations of filterable (dissolved) and solid phases (particulate). In other words, the strategy to address whole water is totally dependent of the sample preparation strategy and methods. Since there is no RM (reference

material) available, it is complicated to evaluate the compliance of different sample preparation methods with respect to the whole water requirement.

Figure 1: This scheme shows all aspects that have to be considered and followed in the analysis of a whole water sample with regards to the Water Framework Directive.

This deliverable will present the optimisation and comparison of a wide range of sample preparation techniques focusing on extraction and enrichment. All the selected sample preparation techniques are available within the testing laboratories and are already standardised. To make the whole set of experiments more comparable within the project consortium, synthetic real water matrix was used as simulation of representative inland surface water. This synthetic water matrix can be setup independently by each laboratory using Evian mineral water (or equivalent) as a major constituent and a defined composition of inorganic anions and cations. This matrix can be spiked with a concentrated solution containing a dissolved organic carbon simulant which consists of a commercially available humic acid in a distinct concentration dissolved in Evian water. The pH value was maintained to 7.3. In a second approach, this complex water matrix was expanded into a whole water sample by adding suspended solids in terms of suspended particulate matter to this matrix. Here, a common load of 50 mg which is representative for surface water was added. These representative waters can be spiked with the desired amounts of the five selected estrogens to evaluate and validate the most promising and robust sample preparation procedure and preconcentration method.

3 General aspects and realization

Material

A representative water matrix is necessary to be used during the whole period of the project with a defined and constant composition. Here, a "synthetic real water matrix" was the candidate of choice, as developed in ENV08. A survey of European monitoring data from France, Germany and Finland was realized to fix criteria to define a representative material especially regarding the following parameters: dissolved organic carbon (DOC), suspended particulate matter (SPM), pH, ionic strength.

A defined composition, which consists of commercially available mineral water (Evian™ water) with known ingredients (inorganics, pH, and "one source water") and a simulated DOC: commercially available humic acid (CAS-No. 68131-04-4, Sigma-Aldrich) at different concentration (between 1-7 mg/L level) was used. The pH-value is given by the mineral water 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/L. In comparison to naturally contaminated water, this synthetic whole water matrix can be spiked by using an estrogen solution containing all five estrogens at desired concentration level (e.g., EQS).

Stability

The stability of the whole water samples from sampling until the sample preparation and analysis in terms of degradation, metabolism or transformation of the analytes has to be ensured. This is descripted and investigated in the deliverable D2 "Report and recommendations to improve the short and long-term stability of selected estrogens in complex water matrix".

Sample preparation/preconcentration

The most specific sample preparation/preconcentration with highest recovery rates must evaluated by comparing different methods and procedures.

Analysis

The most sensitive and specific mass spectrometric (MS) based detection method in combination with a chromatographic separation (liquid chromatography (LC), gas chromatography (GC)) has to be evaluated by comparing different chromatographic approaches and different mass spectrometric based detection methods.

4 Stability and behaviour of whole water samples during sample preparation

4.1 Evaluation of possible deconjugation of present estrogen conjugates

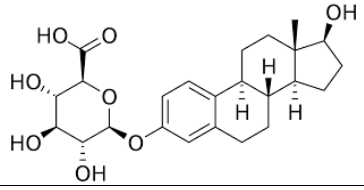
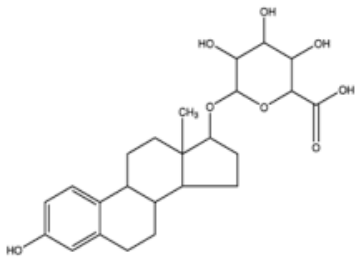
Estrogens are naturally synthetized by mammals but are widely used in human and veterinary therapies. They are partially metabolized and excreted as complex mixtures of both parent compounds and metabolites mainly in their sulphate and/or glucuronide conjugate forms, which are not totally degraded or removed by wastewater treatment plants. Consequently, they are chronically discharged into the receiving waters.

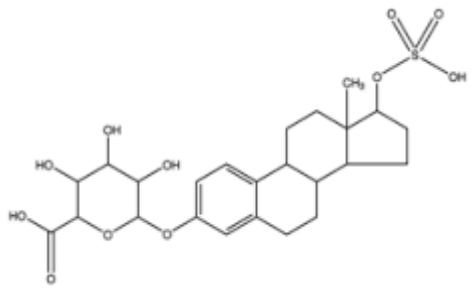
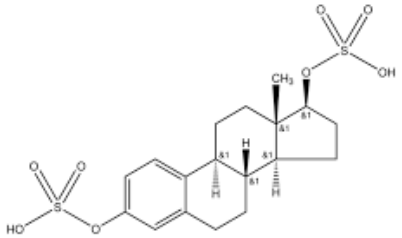
To ensure the stability of possible present estrogen conjugates in a taken water sample for monitoring purpose, a separate study on this issue was designed and carried out. Here, it should be evaluated that the sampling, the sample preparation, and the analysis of the conjugate containing water samples generates no further free estrogens during these procedures in terms of chemical or physical induced decomposition.

4.1.1 Design of experiments to evaluate possible deconjugation

For the evaluation of the stability of estrogen conjugates three common conjugate species were selected as shown in the following table 1. These are the most relevant conjugated forms of 17β-estradiol. The separate stability study for these estrogen conjugates were carried out in synthetic water matrix (e.g., 200 ng/L of each conjugate with DOC of 7 mg/L, SPM 50 mg/L and pH 7.3, without free estrogens). The determination of possible free estrogens generated during storage time and sample preparation was performed using the best evaluated sample preparation technique developed and described in deliverable D1 "Report on the comparison of sample preparation techniques for estrogens partitioning in whole water and recommendations on the most appropriate methods".

Table 1: Model conjugates for the evaluation of the stability of possible present estrogen conjugates in complex water matrix.

Conjugate	Molecular structure
17 β estradiol 3-(β-D-glucuronide)	
17 β estradiol 17-(β-D-glucuronide)	

17 β estradiol 3-(β-D-glucuronide) 17-sulfate	
17 β estradiol 3,17-disulfate	

4.1.2 Results and conclusion

Table 2: Concentrations of free estrogens after storage and sample preparation of spiked complex water matrix using the three model conjugates at four different concentration levels.

Free estrogen	Spiked conjugates 10 ng/L	Spiked conjugates 20 ng/L	Spiked conjugates 100 ng/L	Spiked conjugates 200 ng/L
17 beta Estradiol (bE2)	0.0135	0.012	0.1136	0.157
17 alpha Estradiol (aE2)	n.d.	n.d.	n.d.	n.d.
Estrone (E1)	n.d.	n.d.	n.d.	n.d.
Estriol (E3)	n.d.	n.d.	n.d.	n.d.
17 alpha Ethinylestradiol (EE2)	n.d.	n.d.	n.d.	n.d.

As shown in table 2 it can be demonstrated that an effect of deconjugation could be observed during storage and sample preparation. Calculated on the average molar mass of these three conjugates approximately 0.16% of the used model conjugates was deconjugated during the whole analytical procedure. It was considered as negligible with respect to inland waters monitoring.

4.2 Evaluation of the internal standards during sample preparation

The effect of dissolved humic acids and SPM on the recovery of organic compounds [13], here the five targeted estrogens from water samples will be investigated using a commercially available humic acid preparation and a model SPM and a spiking solution containing the used isotopically labelled internal standards. The

presence of humic acid and SPM reduced the extraction efficiency down to between 10 and 75% and the recovery of the internal standards depending on the equilibrium time. An analytical protocol will therefore be developed for the accurate determination of estrogens in the presence of humic acids and SPM based on isotope dilution mass spectrometry. The procedure should compensate the losses due to sorption of estrogens and can be used for the determination of the total estrogens concentration in whole water. To obtain reliable estimates it is essential to allow a certain time for equilibration between the isotope spike and the aqueous matrix.

4.2.1 Design of experiments to find the best equilibrium time

Samples consisting of Evian water at pH 7.3 were spiked with DOC (7 mg/L) and 50 mg of estrogen-free SPM. Isotopically labelled estrogens (Deuterated or C13 labelling, see Annex) at a concentration level of 10 ng/L or individual by each partner for each compound is used for spiking the complex water matrix.

Day one: Setting up of two separate complex water samples by spiking 1 L of Evian water with DOC spike (7 mg/L), 50 mg estrogen-free SPM and internal standard mix using the individual concentration of each partner. Homogenization on horizontal shaker for at least 5 to 10 min. Spiking time at the end of a working day. Storage at +4°C for at least 12h.

Day two: Setting an additional complex water sample by spiking 1 L of Evian water with DOC spike (7 mg/L), 50 mg estrogen-free SPM and with internal standard mix (using the individual concentration of each partner). Homogenization on horizontal shaker for at least 5 to 10 min. Storage at +4°C for at least 1 h. Sample one from day one is analysed first followed by the sample from day two and the second sample from day one (3 h later than sample one from day one).

After extracting all the three samples the extracts were spiked with a mixture of unlabelled estrogens at the same concentration level as the expected isotopic labelled estrogens. Measurements were carried out as a triplicate with additional injection of solvents between sample measurements.

The calculation of recovery rates of each isotopic labelled estrogen was done either against the reference and/or an instrumental calibration.

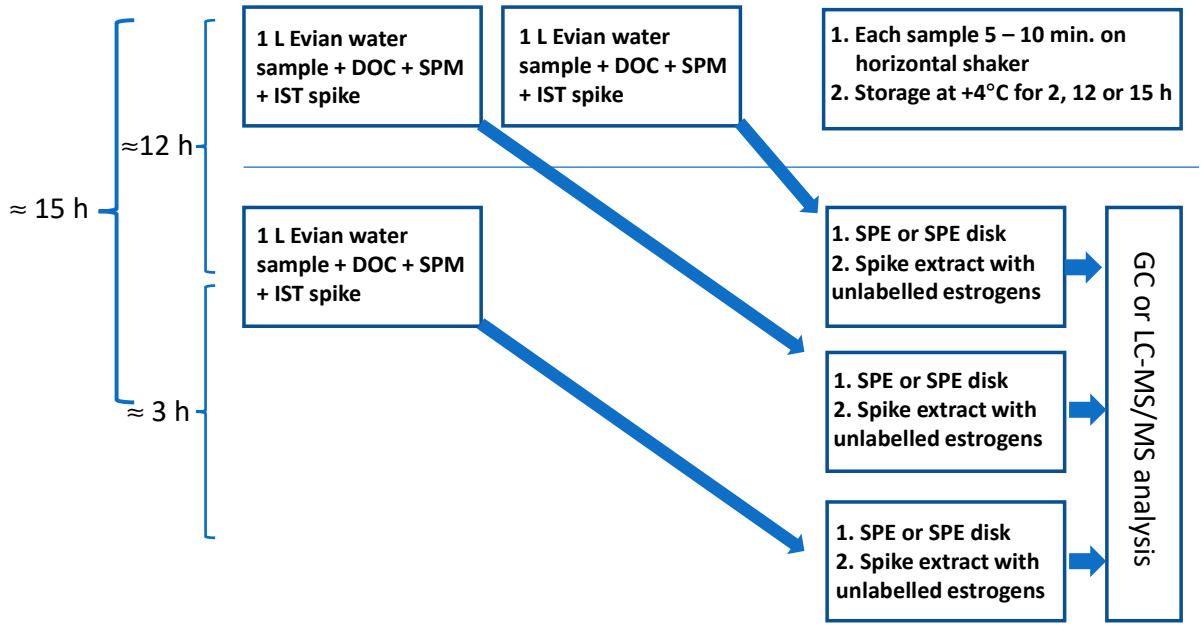


Figure 2: This scheme shows experimental design of the equilibrium time of the individual internal standards used for quantifying the five target estrogens.

4.2.2 Results and conclusion

For the different labelled estrogens, an equilibrium time of at least 15 h is suggested. No difference is observed between the different types of internal standards (Deuterated, C13). In consequence, the samples must be spiked with the isotopically labelled standards at the end of a working day to store them overnight (15 h) at +4°C.

5 Presentation of the selected sample preparation methods

In a next step of a set of complementary extraction methods should be evaluated and optimised. The focus is set here on the liquid-liquid extraction (LLE), solid phase extraction (SPE) cartridges (offline), SPE disks and MiSPE (molecular imprinted polymer SPE). T

To obtain the highest preconcentration factor and cleanest extracts in terms of matrix load: different sorbents, extraction solvents and washing steps should be selected and optimised. In the following table, all methods are compared and evaluated with regard to direct injection.

Table 3: An overview of the extraction and enrichment methods considered in the project with respect to the limitations and restrictions. With DI: direct injection, SPE: solid phase extraction (cartridges) SPE disk: a disc based solid phase extraction, LLE: liquid-liquid-extraction and MISPE: solid phase extraction based on molecular imprinted polymers. +: good, ++: excellent, -: poor, --: unsatisfactory.

Parameter \ Extraction method	DI	SPE	SPE disk	LLE	MISPE
Preconcentration	--	++	++	-	++
Clean-up	--	+	+	-	++
Compatible to common solvents	--	+	+	-	-
Selectivity	--	++	++	-	++
Time / efficiency	++	+	++	+	+

Based on preliminary results the direct injection, the Online SPE, and the liquid-liquid-extraction are rejected due to poor preconcentration and insufficient matrix reduction.

6 Performances of the selected methods on low complexity water

6.1 Experimental design

BAM – LLE

Each estrogen (E1, aE2, bE2, E3 and EE2) was spiked at 10 ng/L level in 1000 mL Millipore or Evian water with 7 mg/L DOC at pH 7.3. These matrices were extracted using different organic solvents which are not miscible with water (dichloromethane (DCM), ethyl acetate (EA)). The absolute recovery rates were determined by analysing the resulting organic extracts by LC-MS/MS.

BAM – MiSPE

Each estrogen (E1, aE2, bE2, E3 and EE2) was spiked at 10 ng/L level in 1000 Evian water with 7 mg/L DOC at pH 7.3. These matrices were extracted according to the fixed protocol provided by the cartridge supplier Affinisep (Paris, France). The quantification was realised by LC-MS/MS with (relative recovery) and without (absolute recovery) internal isotopic labelled standards (aE2-d_2, E1-d_2, bE2-$^{13}C_2$, EE2-$^{13}C_2$, E3-d_2).

SYKE – SPE disk

Each estrogen (E1, aE2, bE2, E3 and EE2) and their mass-labelled surrogates ($^{13}C_3$-E1, $^{13}C_3$-bE2, $^{13}C_3$-E3, $^{13}C_2$-EE2) were spiked at 10 ng/L level in 1000 mL Millipore or Evian. These matrices were extracted using C_{18} disks (Atlantic® C_{18} Disks, 47 mm) using ethyl acetate as the extraction solvent. The recovery rates were determined by analysing the resulting organic extracts by LC-MS/MS.

LNE – SPE disk – SPE purification

Each estrogen (E1, aE2, bE2, E3 and EE2) and their mass-labelled surrogates (E1-$^{13}C_3$, bE2-d_5, EE2-d_4, E3-$^{13}C_3$) were spiked at 10 ng/L respectively level in 1000 mL Mont-Roucous or Evian (with EDTA-Na_2). The optimisation of the methods leads to the following final conditions. These matrices were extracted using C18 disks using ethyl acetate as the extraction solvent followed by LC-NH2-SPE purification step (MeOH). The extracts are then dansylated before to be analysed by LC-MS/MS. The quantification was realised by LC-MS/MS with (relative recovery) and without (absolute recovery) internal isotopic labelled standards (E1-$^{13}C_3$, bE2-d_5, EE2-d_4, E3-$^{13}C_3$).

JSI - SPE

The method optimization was conducted at 5 ng/L levels of each estrogen (E1, aE2, bE2, E3, and EE2) in 100, 250, and 500 mL Millipore water with 7 mg/L DOC was pre-concentrated using a Waters Oasis Prime HLB (3 mL/60 mg sorbent) as the cartridge of choice. With the final sample volume of 500 mL, we achieved the instrumental LOQs and sufficient recovery rates within 98 to 101% using internal isotopic labelled standards at 10 ng/L level (αE2-d_2, E1-d_2, βE2-$^{13}C_2$, EE2-$^{13}C_2$, E3-d_2). In a second approach, the Waters Oasis Prime HLB cartridges with different amounts of sorbent (3 mL/60 mg, 6ml/150mg, 6ml/500mg) were evaluated with the synthetic real water matrix which consists of Millipore water, 7 mg/L DOC and 25 mg SPM. However, we were not able to load the final volume (V=500 mL) of the whole water sample even in the case of using the cartridges with the highest amount of sorbent (clogging of the cartridges).

6.2 Results

BAM – LLE

Table 4: Absolute recovery rates of the five selected estrogens in synthetic real water matrix and Millipore water using DCM as organic solvent of choice with. Sufficient recoveries for E1, bE2, aE2 and EE2 but poor for E3 independently from the type of water matrix.

Extraction method / Parameter	LLE with estrogens at 10 ng/L level			
	absolute recovery in % (Milipore water)	RSD in %	absolute recovery in % (Evian water)	RSD in %
Estrone (E1)	80	5	91	5
17α-Estradiol (aE2)	75	6	85	5
17β-Estradiol (bE2)	76	3	87	4
Estriol (E3)	9	2	10	2
17α-Ethinylestradiol (EE2)	76	3	77	7

BAM – MiSPE

Table 6: Absolute recovery rates of the five selected estrogens in synthetic real water matrix using AffiniMiP SPE cartridges (3 mL / 100 mg sorbent material). No significant ion suppression effect can be observed when analysing the diluted extracts. The recovery rate ranging from 64 – 110% but no matrix effect can be observed independently from the dilution factor or the direct injection of the extract. The high recovery of E3 was caused by an impurity.

Extraction method / Parameter	MiSPE with estrogens at 10 ng/L level					
	absolute recovery in % (direct)	RSD in %	absolute recovery in % (1:10)	RSD in %	absolute recovery in % (1:20)	RSD in %
Estrone (E1)	64	4	84	4	110	3
17α-Estradiol (aE2)	74	4	81	3	88	3
17β-Estradiol (bE2)	78	3	82	2	90	3
Estriol (E3)	80	4	135	5	192	2
17α-Ethinylestradiol (EE2)	69	2	71	2	79	2

SYKE – SPE disk

Table 7: Absolute and relative recovery rates of targeted estrogens in low complex Evian water matrix with SPE disk preconcentration.

Extraction method / Parameter	Atlantic® C-18 Disks, 47 mm, Evian without additives (1000 mL) and estrogens at 10 ng/L level; no sample clean-up or derivatization			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	77,2	12,6	90,0	0,3
17α-Estradiol (aE2)	NA	NA	86,8	0,4
17β-Ethinylestradiol (bE2)	88,1	16,3	84,5	10,0
Estriol (E3)	82,8	9,8	88,1	1,4
17α-Ethinylestradiol (EE2)	72,6	10,2	93,8	5,6

LNE – SPE disk – SPE purification

Table 8: Average absolute recoveries for the extraction (C18 disk, elution with ethyl acetate) and the purification step (Supelclean™ LC-NH2 SPE, elution with methanol) (Evian water spiked at 10 ng L^{-1}, n=3)

Estrogen	Average absolute extraction recovery SPE disk (%) (n=3)	RSD (%)	Average absolute purification recovery NH2 (%) (n=3)	RSD (%)
EE2	81	5	97	9
E3	78	3	83	10
aE2	79	7	100	8
bE2	62	1	95	7

E1	85	2	97	7

JSI – SPE

Table 9: Absolute and relative recovery rates of targeted estrogens in low complex Evian water matrix with SPE preconcentration.

Extraction method / Parameter	Waters Prime HLB size 3cc/60mg, Evian water (500ml) + DOC (7 mg/L) and estrogens at 5 ng/L level			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	111,8	5,0	101,3	0,6
17α-Estradiol (aE2)	132,8	5,7	98,5	6,5
17β-Ethinylestradiol (bE2)	110,5	6,3	97,9	0,6
Estriol (E3)	118,4	6,9	101,0	5,9
17α-Ethinylestradiol (EE2)	110,9	4,6	98,3	4,1

6.3 Conclusions

For low complex matrix containing water samples like mineral, tap or demineralised water solid phase extraction and SPE disk are the procedures of choice. Here, the variety of sample volume (e.g., between 100 and 1000 mL) has not effect on the absolute recovery rates of the target analytes. Together with the use isotopically labelled standard compounds the relative recovery rates are in a comfortable range between 83 and 102% with a sufficient standard deviation depending on the analytical method. Both chromatographic approaches, liquid and gas chromatography are able to separate all five targeted estrogens especially aE2 and bE2. The more specific MiSPE is limited with regards to the sample volume of 100 mL in maximum. To achieve the mentioned EQS for all selected estrogens a larger volume is necessary.

7 Performances of the selected methods on high complexity water

7.1 Experimental design

BAM - SPE

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 (relative recovery) and without

(absolute recovery) internal isotopic labelled standards (aE2-d_2, E1-d_2, bE2-$^{13}C_2$, EE2-$^{13}C_2$, E3-d_2).

SYKE – SPE disk

Each estrogen (E1, aE2, bE2, E3 and EE2) and their mass-labelled surrogates ($^{13}C_3$-E1, $^{13}C_3$-bE2, $^{13}C_3$-E3, $^{13}C_2$-EE2) were spiked at 10 ng/L level in 1000 mL surface water. The samples were extracted using C_{18} disks (Atlantic® C_{18} Disks, 47 mm) using ethyl acetate as the extraction solvent. The recovery rates were determined by analysing the resulting organic extracts by LC-MS/MS.

LNE - SPE disk – SPE purification

Here, different tests were realised implementing synthetic real matrix of various complexity. Different level of DOC (1, 5 and 7 mg/L) were evaluated as well as different levels of SPM (50 and 150 mg/L) and different level of the concentrations of the targeted estrogens (10 ng/L and EQS/PNEC). The same optimised method was implemented. The quantification was realised by LC-MS/MS with (relative recovery) and without (absolute recovery) internal isotopic labelled standards (E1-$^{13}C_3$, bE2-d_5, EE2-d_4, E3-$^{13}C_3$).

UBx - SPE – SPE purification

Samples were first spiked with internal isotopic labelled standards, MeOH and were acidified at pH = 5. The extraction was done on Waters Oasis HLBSPE cartridge. The water samples (200 mL Evian water and DOC 7 mg/L and SPM 50 mg/L) were loaded onto the preconditioned HLB cartridge. After drying the SPE cartridge, elution was applied methanol. The resulting extracts were evaporated to a distinct volume under nitrogen flow and reconstituted of ethyl acetate. Subsequent purification was applied using a Supelco Supelclean LC-NH2 SPE. Before analysis step, the final extracts were spiked with isotopic labelled standards.

JSI – SPE Disk

The 500 mL of synthetic real water matrix, consisting of 500 mL Millipore water with 7 mg/L DOC and 25 mg SPM was successfully concentrated using an Atlantic® DVB Disks, 47 mm. Again, the sample preparation was optimised at 5 ng/L level of each estrogen (E1, aE2, bE2, E3 and EE2) and 10 ng/L of internal standards (aE2-d_2, E1-d_2, bE2-$^{13}C_2$, EE2-$^{13}C_2$, E3-d_2). In this case, we were able to obtain the instrumental LOQs of all target estrogens as well as sufficient recovery rates within 88 to 99%.

7.2 Results

BAM – SPE

Table 10: (a): Absolute recovery rate of the five selected estrogens in synthetic real water matrix using Waters Oasis HLB cartridges (3 mL/150 mg sorbent). An ion suppression effect can be observed when analysing the diluted extracts. At a dilution of 1:20 the matrix effect could be eliminated. (b): The relative recovery rates of the five selected estrogens were

here determined by including the internal isotopically labelled standards. The recovery rate ranging from 98 – 102% and the matrix effect can be observed independently from the dilution factor or the direct injection of the extract.

Extraction method / Parameter	(a) HLB with estrogens at 10 ng/L level in complex water matrix					
	absolute recovery in % (direct)	RSD in %	absolute recovery in % (1:10)	RSD in %	absolute recovery in % (1:20)	RSD in %
Estrone (E1)	74	1	89	1	96	2
17α-Estradiol (aE2)	64	1	78	1	84	2
17β-Estradiol (bE2)	64	1	78	1	82	2
Estriol (E3)	40	1	78	1	82	2
17α-Ethinylestradiol (EE2)	64	1	75	1	80	2

Extraction method / Parameter	(b) HLB with estrogens at 10 ng/L level in complex water matrix					
	relative recovery in % (direct)	RSD in %	relative recovery in % (1:10)	RSD in %	relative recovery in % (1:20)	RSD in %
Estrone (E1)	100	1	101	1	102	2
17α-Estradiol (aE2)	99	1	98	1	100	3
17β-Estradiol (bE2)	101	1	101	1	101	3
Estriol (E3)	99	2	100	1	99	2
17α-Ethinylestradiol (EE2)	100	1	99	1	100	2

SYKE – SPE disk

Table 11: Absolute and relative recovery rates of targeted estrogens in high complex surface water matrix with SPE disk preconcentration.

Extraction method / Parameter	Atlantic® C-18 Disks, 47 mm, unfiltered surface water (1000 mL) and estrogens at 10 ng/L level; no sample clean-up or derivatization			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	25,3	6,0	90,8	4,3
17α-Estradiol (aE2)	NA	NA	78,4	9,7
17β-Ethinylestradiol (bE2)	29,4	4,8	90,5	5,0
Estriol (E3)	23,4	10,4	87,8	0,6
17α-Ethinylestradiol (EE2)	20,7	4,4	104,5	2,6

LNE - SPE disk – SPE purification

Table 12: Absolute recovery rates of targeted estrogens in high complex surface water matrix with SPE disk preconcentration and SPE purification.

Estrogen	Evian® + SPM (50 mg L^{-1})	Evian® + DOC (5 mg L^{-1})	Evian® + DOC (5 mg L^{-1}) + SPM (150 mg L^{-1})
EE2	68 ± 22%	67 ± 3%	88 ± 1%
E3	64 ± 14%	62 ± 6%	76 ± 4%
aE2	68 ± 20%	70 ± 2%	85 ± 2%
bE2	67 ± 19%	69 ± 9%	90 ± 2%
E1	68 ± 22%	66 ± 1%	91 ± 4%

Table 13: Relative recovery rates of targeted estrogens in high complex surface water matrix with SPE disk preconcentration and SPE purification.

Estrogen	Evian® + SPM (50 mg L^{-1}) (%)		Evian® + DOC (5 mg L^{-1}) (%)		Evian® +DOC (5 mg L^{-1}) + SPM (150 mg L^{-1}) (%)	
EE2	109	5	93	5	84	1
E3	97	8	90	9	86	6

aE2	98	1	99	4	98	0,40
bE2	96	2	93	4	92	4
E1	105	1	98	2	96	0,50

UBx – SPE – SPE purification

Table 14: Absolute and relative recovery rates of targeted estrogens in low complex Evian water matrix with SPE preconcentration and SPE purification.

Extraction method / Parameter	SPE extraction OASIS **HLB 500mg/6cc** + SPE purification **LC-NH2 500mg/3cc**, **200 mL** Evian water + SPM (50mg/L) + DOC (7 mg/L) and estrogens at **0,4 ng/L level excepted 17α-ethynylestradiol at 0,035ng/L**). Sample acidification at pH = 5, 1% MeOH added.			
	absolute recovery in %	RSD in %	Isotope dilution recovery in %	RSD in %
Estrone (E1)			98	2
17α-Estradiol (aE2)			98	15
17β-Estradiol (bE2)			108	11
Estriol (E3)			116	7
17α-Ethinylestradiol (EE2)			109	10
Estrone-13C3 (E1-13C3)	83	7		
17α-Estradiol-d2 (aE2-d2)	74	4		
17β-Estradiol-13C3 (bE2-13C3)	81	6		
Estriol-d2 (E3-d2)	80	8		
17α-Ethinylestradiol-d4 (EE2-d4)	79	5		

JSI – SPE disk

Table 15: Absolute and relative recovery rates of targeted estrogens in low complex Evian water matrix with SPE disk preconcentration.

Extraction method / Parameter	Atlantic® DVB Disks, 47 mm , Evian water (500ml) + 25 mg SPM + DOC (7 mg/L) and estrogens at 5 ng/L level			
	Absolute recovery (%)	RSD (%)	Relative recovery (%)	RSD (%)
Estrone (E1)	92,6	4,0	95,0	7,5
17α-Estradiol (aE2)	92,4	8,8	88,1	6,5
17β-Ethinylestradiol (bE2)	90,8	9,7	88,1	1,4
Estriol (E3)	99,6	4,4	92,5	4,2
17α-Ethinylestradiol (EE2)	97,1	10,7	99,0	2,6

7.3 Conclusions

For high complex matrix like surface or ground water with a load of suspended particulate matter, solid phase extraction and SPE disk are the procedures of choice. Here, the variety of sample volume (e.g., between 100 and 1000 mL) has not effect on the absolute recovery rates of the target analytes but the SPE is limited with respect to the SPM load. To avoid clogging of the cartridges only a restricted SPM load is tolerable depending on the type of cartridge. Thanks to the implementation of isotopically labelled standard compounds, the relative recovery rates are in a comfortable range between 76 and 116% with a sufficient standard deviation depending on the analytical method. The more specific MiSPE is limited with regards to the sample volume of 100 mL in maximum and the load of SPM. To achieve the mentioned EQS for all selected estrogens a larger volume is

necessary. Both chromatographic approaches, liquid and gas chromatography are able to separate all five targeted estrogens especially aE2 and bE2.

8 Conclusions and recommendations

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. Furthermore, this last technique could be an issue in a standardization process.

Table 13: **An overview of the extraction and enrichment methods considered within the project with respect to the recovery rates and preconcentration factors. With SPE: solid phase extraction, SPE disk: a disc based solid phase extraction, and MISPE: solid phase extraction based on molecular imprinted polymers. Complex water matrix without (1) and with (2) SPM. Red not applicable, green applicable, yellow applicable only for a small volume.**

Extraction method (Typical preconc. factor) / Parameter	SPE [4, 7-10] (1:1000)		SPE disk [11, 12] (1:1000)		MiSPE [5, 6] (1:100)	
	1	2	1	2	1	2
Estrone (E1)						
17α-Estradiol (aE2)						
17β-Ethinylestradiol (bE2)						
Estriol (E3)						
17α-Ethinylestradiol (EE2)						

9 References

[1] Petrovic, M. (2014). "Methodological challenges of multi-residue analysis of pharmaceuticals in environmental samples." Trends in Environmental Analytical Chemistry 1: E25-E33.

[2] Ademollo, N., et al. (2012). "The analytical problem of measuring total concentrations of organic pollutants in whole water." Trac-Trends in Analytical Chemistry 36: 71-81.

[3] Ben Fredj, S., et al. (2015). "Removal of estrone (E1), 17 beta-estradiol (E2), and 17 alpha-ethinylestradiol (EE2) from wastewater by liquid-liquid extraction." Chemical Engineering Journal 262: 417-426.

[4] Miege, C., et al. (2009). "Method validation for the analysis of estrogens (including conjugated compounds) in aqueous matrices." Trac-Trends in Analytical Chemistry 28(2): 237-244.

[5] Lucci, P., et al. (2011). "Solid-phase extraction using molecularly imprinted polymer for selective extraction of natural and synthetic estrogens from aqueous samples." Journal of Chromatography A 1218(30): 4828-4833.

[6] Zacs, D., et al. (2016). "Determination of steroidal oestrogens in tap water samples using solid-phase extraction on a molecularly imprinted polymer sorbent and quantification with gas chromatography-mass spectrometry (GC-MS)." Environ Monit Assess 188(7): 433.

[7] Barreca, S., et al. (2019). "Determination of estrogenic endocrine disruptors in water at sub-ng L-1 levels in compliance with Decision 2015/495/EU using offline-online solid phase extraction concentration coupled with high performance liquid chromatography-tandem mass spectrometry." Microchemical Journal 147: 1186-1191.

[8] Lardy-Fontan, S., et al. (2018). "Toward the determination of estrogenic compounds in the framework of EU watch list: validation and implementation of a two-step solid phase extraction-liquid phase chromatography coupled to tandem mass spectrometry method." Accreditation and Quality Assurance 23(5): 285-295.

[9] Li, Y. J., et al. (2021). "Determination of estrogens and estrogen mimics by solid-phase extraction with liquid chromatography-tandem mass spectrometry." Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 1168.

[10] Simon, E., et al. (2019). "Solid-phase extraction of estrogens and herbicides from environmental waters for bioassay analysis-effects of sample volume on recoveries." Analytical and Bioanalytical Chemistry 411(10): 2057-2069.

[11] Cesen, M. and E. Heath (2017). "Disk-based solid phase extraction for the determination of diclofenac and steroidal estrogens E1, E2 and EE2 listed in the WFD watch list by GC-MS." Science of the Total Environment 590: 832-837.

[12] Glineur, A., et al. (2020). "Development And Validation Of A Method For Determining Estrogenic Compounds In Surface Water At The Ultra-Trace Level Required By The EU Water Framework Directive Watch List." Journal of Chromatography A 1624.

[13] Bercaru, O., Ulberth, F., Emons, H., and Vandecasteele, C. (2006). "Accurate quantification of PAHs in water in the presence of dissolved humic acids using isotope dilution mass spectrometry." Analytical and bioanalytical chemistry, 384(5): 1207–1213.

10 Annex

Sample preparation - Preconcentration

(1) BAM – LLE

Each estrogen was spiked at a 10 ng/L level in 500 mL Evian water with 7 mg/L DOC or in Millipore water. These 500 mL water sample were extracted 3 times with 30 mL DCM and an extraction time of one min by shaking the separation funnel. Combining organic layers, evaporation to dryness and reconstitution with 1 mL ACN. This ACN extract is ready for analysis by LC-MS/MS. The absolute recoveries without internal standards were determined and no further cleanup was applied. In sum ten independent extractions were carried out.

(2) BAM - SPE

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

(3) BAM – MiSPE

For MiSPE sample preparation the provided protocol of purification was applied. Here, each estrogen (E1, aE2, bE2, E3 and EE2) was spiked at 10 ng/L level in 1000 mL Evian water with 7 mg/L DOC at pH 7.3 with or without 50 mg SPE per litre. These matrices were extracted according to the fixed protocol provided by the cartridge supplier Affinisep (Paris, France). For purification a 3mL/100mg AFFINIMIP® SPE Estrogens cartridge was utilised. For equilibration 3 mL acetonitrile and 3 mL Water were subsequently used. The loading of the water samples was applied using a flow rate of 1 mL/min. A washing step as applied by using 3 mL water and 3 mL water/acetonitrile (60/40, v/v). The final elution of the preconcentrated estrogens was done with 3 mL methanol. The elution fraction was then evaporated to 1 mL under a stream of nitrogen. The quantification was realised by LC-MS/MS with (relative recovery) and without (absolute recovery) internal isotopic labelled standards (aE2-d_2, E1-d_2, bE2-$^{13}C_2$, EE2-$^{13}C_2$, E3-d_2).

(4) JSI – SPE disk

Procedure: The Atlantic® DVB Disks, 47 mm were subsequently conditioned with 10 mL EtAC, 10 mL of MeOH, and 10 mL of H2O. Afterward, the 500 mL complex water sample was loaded applying a flow rate of 5 mL/min onto the SPE disk followed by washing the cartridge with 10 mL 30% MeOH in Millipore water and drying under vacuum (−1.33 kPa) for 45 min. The elution step was performed using EtAc (3 × 4 mL) into 20mL glass serum. The collected solvent was completely

dried under a gentle stream of nitrogen at 40 °C. The dried sample was reconstituted with EtAc (3 x 0.6 mL) into a 2 mL glass vial for GC-MS/MS analysis and dried completely. Derivatisation was performed with 25 µL of TMSI and 25 µL of pyridine for 1 h at 90 °C prior to analysis.

(5) LNE – SPE disk – SPE purification

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-NH2 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). The workflow of the optimized method allows to reach a 10000 preconcentration factor.

(6) UBx – SPE – SPE purification

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-NH2 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: 35µL reconstituted with 82 µL MQ water (30:70, MeOH/MQ, Water v/v) and 25µL MeOH.

(7) JSI – SPE

Procedure: A 500 mL complex water sample was loaded on the Waters Oasis Prime HLB cartridge applying a flow rate of 3 mL/min. After loading and washing the cartridges with 3 mL 30% MeOH in Millipore water, the sorbent was dried under vacuum (−1.33 kPa) for 45 min. The elution step was performed using MeOH/MeCN (3 × 0.6 mL). The solvent was removed under a gentle stream of nitrogen at 40 °C. Derivatisation was performed with 25 µL of TMSI and 25 µL of pyridine for 1 h at 90 °C before GC-MS/MS analysis.

(8) SYKE – SPE disk

A 1000 mL samples of Evian and unfiltered surface water were first spiked with 10 ng/L each estrogen (E1, aE2, bE2, E3 and EE2) and their mass-labelled surrogates ($^{13}C_3$-E1, $^{13}C_3$-bE2, $^{13}C_3$-E3, $^{13}C_2$-EE2). These matrices were extracted with SPE-DEX® 4790 (Horizon Technology, Inc.) automated extraction device using C_{18} SPE disks (Atlantic® C_{18} Disks, 47 mm). Disks were prewetted with MeOH and MQ-water, before loading the sample. After air drying the disks for 10 min, samples were eluted with approximately 15 ml of ethyl acetate. Samples were evaporated to dryness with Genevac EZ-2 Envi centrifugal evaporator (Genevac Ltd), reconstituted to 200 µl with mixture of MeOH and MQ-water and finally filtered with 0.2 µm Captiva Premium cellulosa syringe filter (Agilent Technologies) before analysing extracts by LC-MS/MS.

Analytical methods

(9) LNE – LC-MS/MS

Chromatographic separation was performed using a Acquity® 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.

Table 14: Gradient for the separation of targeted estrogens

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.

Table 15: Summarised MS parameters and SRM transitions for the selected estrogens and isotopes.

	Parent (*m/z*)	CV (V)	Quantifier ion (*m/z*)	CE (eV)	Qualifier ion (*m/z*)	CE (eV)
Estrone (E1)	504.3	40	171.5	34	156.0	54
Estriol (E3)	522.2	40	171.1	38	156.1	56
17α-estradiol (aE2)	506.3	50	171.1	36	156.0	54
17β-estradiol (bE2)	506.3	50	171.1	36	156.1	54
17α-ethinyl-estradiol (EE2)	530.1	50	171.1	36	156.1	56
Estrone-$^{13}C_3$	507.3	40	171.1	32	156.1	56
17α-ethinyl-estradiol-d_4	534.3	30	171.1	36	156.0	56
17β-estradiol-d_5	511.3	40	171.1	36	156.1	56
Estriol-$^{13}C_3$	525.3	24	171.1	34	156.1	54

(10) BAM – LC-MS/MS

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, thermostated column compartment and 1200 diode array detector coupled to an AB Sciex TSQ 6500 mass spectrometer. 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

Injection volume: 10 µL

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

Table 16: Gradient for the separation of targeted estrogens.

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 specrometer parameter: ESI negative, scheduled MRM (multiple reaction monitoring), target scan time 0.700 sec.

(11) UBx – LC-MS/MS

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 1.9µm, 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.

Table 17: Gradient for the separation of targeted estrogens.

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.

(12) JSI – GC-MS/MS

Extracts were analysed on a GC-MS/MS system: 7890B series GC coupled to an 7000 series triple quad MS/MS, (Agilent Technologies, USA). Briefly, the chromatographic separation was achieved using 30 m × 0.25 mm Ø x 0.25 µm i.d. µm DB-5 MS capillary column (Agilent Technologies). The carrier gas was He, operated in constant flow mode (1 mL min^{-1}) at an average velocity of 26 cm sec^{-1}, and the collision gas was N_2 (1.5 mL min^{-1}). Samples were injected (2 µL) in the spitless mode at 250°C. The GC oven temperature program was as follows: 145°C (0 min), 30°C min^{-1} to 290 °C (6 min), 30°C min^{-1} to 310°C (0.5 min). The total runtime was 9.33 min.

The mass spectrometer was operated in EI mode at 70 eV in multiple reaction monitoring (MRM) mode. The source temperature was 250°C. Retention times (RT) and MS/MS optimised conditions are summarised in table 18.

Table 18: Summarised MS parameters and MRM transitions for the selected native and labelled estrogens.

Compound	RT	MRM transition	CE [eV]	DT [ms]
aE2	8.07	285-->72.9	60	30
		285-->205	30	15
		416-->285	30	9
aE2-d_2	8.07	418-->287	30	15
		287-->207	30	15
E1	8.22	218->203	30	9
		342->244	60	12
		342-->257	30	15
E1-d_2	8.22	344-->259	30	12
		344-->246	30	12
bE2	8.39	232-->217	60	12
		285-->73	30	30
		416-->285	30	9
bE2-^{13}C	8.07	287-->207	30	9
		418-->287	30	15
EE2	9.31	205-->115	30	15
		232-->217	60	12
		425-->193	30	15
EE2-^{13}C	9.31	427-->195	30	15
		442-->427	30	3
E3	10.02	270-->225	60	9
		311-->282	30	15
		296-->281	30	12
E3-d_2	10.02	298-->283	30	9
		506-->416	30	3

(13) SYKE – LC-MS/MS

Chromatographic separation and tandem mass spectrometric analysis of organic extracts was performed on a Waters Acquity UPLC® coupled with Waters Xevo TQMS instrument and equipped with an electrospray ionisation (ESI) source. UPLC column was from Waters (BEH C18, 2.1 x 50 mm, 1.7 µm), eluent flow rate was 0.4 mL min^{-1} and injection volume was 7.5 µL. Column oven was set at 40 °C while sample tray was cooled to 7.0 °C. Gradient and eluents used are listed below.

Table 19: Gradient for the separation of the estrogens.

Time (min)	0.2 % NH_3 (Aq)	100 % MeOH
0.00	60.00	40.00
0.50	60.00	40.00
1.60	35.00	65.00
3.50	0.10	99.90
4.50	0.10	99.90
5.00	60.00	40.00
6.50	60.00	40.00

Acquisition of estrogens was performed using scheduled Multiple Reaction Monitoring (MRM) and negative ionization modes. Other instrument parameters were: Capillary 2.00 kV, Source Temperature 150 °C, Desolvation Temperature 500 °C, Cone Gas Flow 20 L Hr^{-1}, Desolvation Gas Flow 1000 L Hr^{-1} and Collision Gas Flow 0.18 mL min^{-1}. Analyte precursors, fragments and used cone voltages and collision energies are listed below.

Table 20: Summarised MS parameters and SRM transitions for the selected estrogens and isotopes

Compound	ES	MRM	Cone (V)	Collision energy (V)
Estrone (E1)	-	269.10 > 144.90 *269.10 > 131.00*	54 54	40 40
Estrone $^{13}C_3$	-	272.20 > 148.00	54	40
Estriol (E3)	-	287.00 > 171.00 *287.00 > 144.70*	45 45	35 40
Estriol_$^{13}C_3$	-	290.00 > 174.00	45	35
17b_Estradiol (bE2)	-	271.28 > 145.09 *271.17 > 182.90* *271.17 > 238.90*	60 60 60	35 30 40
17b_Estradiol_$^{13}C_3$	-	274.20 > 148.10	60	35
17a-ethinyl-estradiol (EE2)	-	295.24 > 145.07 *295.17 > 158.90* *295.17 > 269.00*	52 52 52	38 40 35
17a-ethinyl-estradiol _$^{13}C_2$	-	297.20 > 145.10	52	38