EDC WFD

Sample preparation strategies for estrogens with regards to the EU-WFD

Berlin, 21-22 February, 2023

EDC WFD

C1 WP1: Optimisation and evaluation of sample preparation methods

The aim of this work package is to evaluate and optimise the sample preparation for a set of various whole water samples including freshwater (surface and ground water) and a synthetic real matrix water which consists of distinct ingredients e.g. dissolved organic compound (DOC) (humic substances) and suspended particulate matter (SPM).

B1.c List of deliverables

Relevant objective (Activity delivering the deliverable)	Deliverable number	Deliverable description	Deliverable type	Partners (Lead in bold)	Delivery date
Objective 2 (A1.3.4)	D1	Report comparing sample preparation techniques for estrogens partitioning in whole water and recommendations on the most appropriate methods	Report Recommendations	**BAM**, JSI, LNE, TUBITAK, SYKE, UBX	Apr 2021 (M20)

Oct. 2021

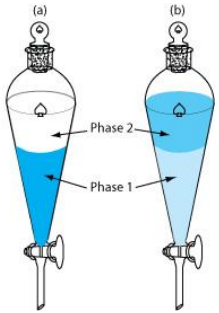
Needs for sample preparation

In general, a compromise is necessary, which addresses the whole water sample, a reasonable preconcentration factor, and the robustness of the method:

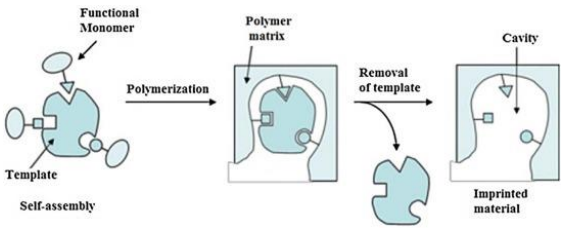
- Preconcentration of target analytes due to low EQS given by the EU-WFD
- Separation of matrix components from target analytes to prevent matrix effects on the chromatographic separation e.g., decreasing separation efficiency of the chromatographic column and enhancing or decreasing signal intensity (ion suppression in ESI source)
- Avoiding contamination of sample inlets (GC liner contamination by matrix components)

A preselection of feasible and applicable techniques was done by each project partner according to their practical knowledge and available instrumentation

Overview on preconcentration procedures



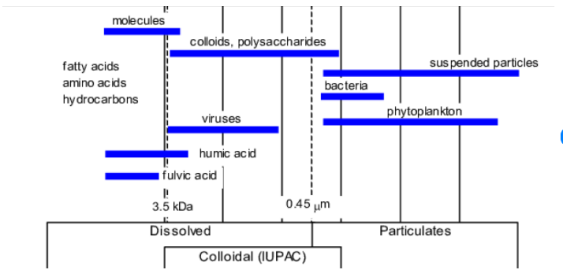
- Liquid-liquid extraction (LLE)
- Solid phase extraction (SPE) columns (off- and online)
- SPE disks
- Mi-SPE (molecular imprinted polymers)



Whole water matrix simulation

- Evian water (bottled in glass, if available)

- Addition of SPM (0 - 50 mg/l)
- Addition of DOC (0 - 14 mg/l)
- Addition of low ng/L of estrogens
- Internal standards



1. Continuum of particles, colloids and dissolved organic carbon in natural waters (Aiken and Leenheer, 1993)

Liquid-liquid extraction (LLE)

- High volumes of organic extraction solvents needed (30 – 50 mL; green chemistry)
- Limited selectivity (non-miscible organic solvents only)
- “Batch extraction” (must be repeated with portions of fresh extractions solvent)
- Not automatable (depending on who does the extraction, no extractions in parallel): not suitable for routine laboratories
- Batch extracts must be combined and evaporated

Not applicable in routine lab!

Liquid-liquid extraction (LLE)

Typical procedure:

500 mL water sample

- 3 x extraction with 30 mL DCM
- Extraction time 1 min/set 10 min

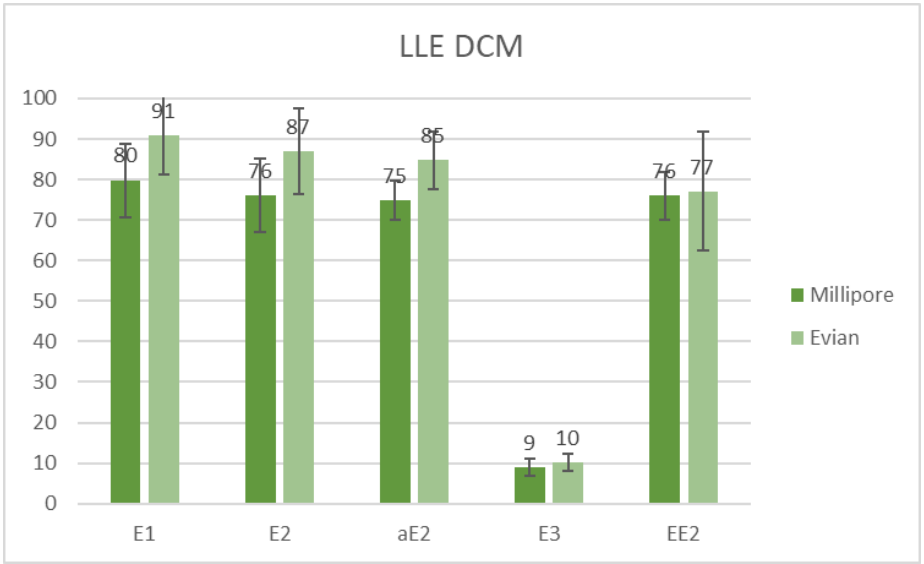
Combining organic layers

- Evaporation to dryness
- Reconstitution with 1 mL ACN

Analysis

- The ACN extract is ready for analysis

Liquid-liquid extraction (LLE)



- Sufficient absolute recoveries for E1, E2, alphaE2 and EE2 but poor for E3, independently from the type of water matrix

Solid phase extraction (SPE) off- or online

- e.g., HLB (Hydrophilic-Lipophilic Balanced) 6 or 3 mL / filled with 150 or 60 mg sorbent material. Other materials are also feasible (C_{18}, C_{18} eq, …)
- With a content of less than 50 mg L^{-1} SPM no clogging can be observed (it strongly depends on the ration of SPM to sample volume)
- Preconcentration depends not only on the target analytes, but also on enrichment of DOC content (evaluated by measuring the DOC of the sample extract)
- Problems of ion suppression when using LC-MS/MS (in most cases decrease of the signal intensity, known effect in the ESI source)

A typical SPE procedure

1000 mL water sample

- Spiked with IS-mix at desired level
- 15 min on horizontal shaker
- Oasis HLB 3 mL/100mg

Conditioning / sample load

- Conditioning with 10 mL ACN
- Conditioning with 15 mL H_2O
- Load 1000 mL water sample 20 mL/min

Washing step and elution

- Washing cartridge with 10 mL H_2O
- Dry cartridge with N_2 for 1 min
- Collect 10 mL fraction into sample tube using ACN

Concentration and analysis

- Concentrate organic fraction to 1 mL (TurboVap)
- Extract is ready for analysis by LC-MS/MS

Solid phase extraction (SPE) off- or online

- Different matrix compositions were used:
  - **Low complexity matrix** (Evian water with low DOC and without SPM)
  - **High complexity matrix** (Evian water with moderate DOC and with SPM)
- Relative and absolute recovery rates were determined to compare the different experiments with regards to their performance (applicability in routine analysis)

Solid phase extraction (SPE) off- or online

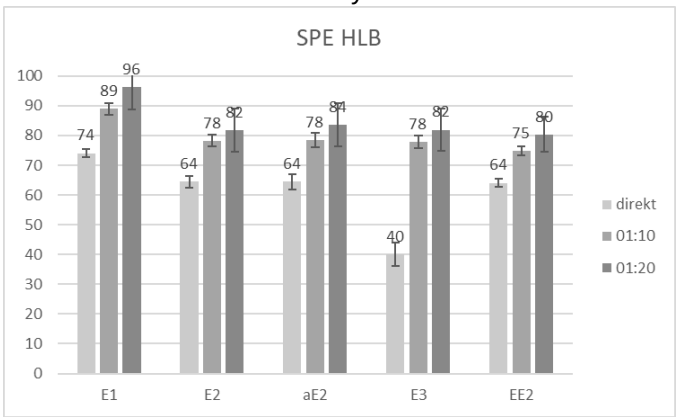
Low complex matrix (Evian water with low DOC and without SPM)

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

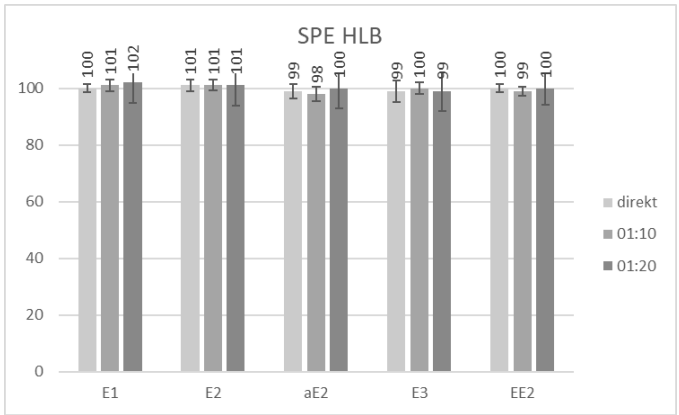
- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Ideal relative recoveries by isotope dilution calibration
- But: no SPM in the water matrix, which can cause clogging of the cartridge

High complex matrix (Evian water with moderate DOC and with SPM)

Abs. recovery



Rel. recovery



- Sufficient absolute recoveries (left) for E1, E2, alphaE2, EE2 and E3
- Matrix will also be preconcentrated; increasing signals by diluting the sample extracts (graph: 1:10 in 1:20)
- Ideal relative recoveries (righ) by isotope dilution calibration

SPE disk (e.g. combination of filtration and SPE)

- Good compromise of filtration and enrichment
- e.g., HLB, DVB or C_{18} disks with 47 mm diameter are feasible (capacity is only defined as L, H and M – not correlation to distinct amounts of SPE sorbents possible)
- Need of special manifold suitable for SPE disks
- Sufficient absolute recovery rates within 80 to 90% in complex water matrix (DOC, SPM an inorganic content)
- Applicable for SPM content higher than 50 m L^{-1} g e.g., 500 mg L^{-1}
- Preconcentration of DOC content comparable to common SPE

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A typical SPE disk procedure

1000 mL water sample

- Spiked with IS-mix at desired level
- 15 min on horizontal shaker
- SPE disk e.g., Atlantic HLB M/L or H

Conditioning / sample load

- Conditioning with 20 mL ACN
- Conditioning with 3 x 10 mL H_2O
- Load 1000 mL water sample in approx. 30 min

Washing step and elution

- Washing cartridge with 10 mL H_2O
- Drying of SPE disk by applying vacuum for 5 min
- Collect 5 x 10 mL fractions using ACN

Concentration and analysis

- Concentrate organic fraction to 1 mL (TurboVap)
- Yellowish extract is ready for analysis by LC-MS/MS

SPE disk

- Different matrix compositions were used:
  - **Low complexity matrix** (Evian water with low DOC and without SPM)
  - **High complexity matrix** (Evian water with moderate DOC and with SPM)
- Relative and absolute recovery rates were determined to compare the different experiments with regards to their performance (applicability in routine analysis)

SPE disk (combination of filtration and SPE)

Low complexity matrix (Evian water with low DOC and without SPM)

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

- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Ideal relative recoveries by isotope dilution calibration

SPE disk (combination of filtration and SPE)

High complexity matrix (Evian water with moderate DOC and with SPM)

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

- Sufficient absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Sufficient/Ideal relative recoveries by isotope dilution calibration

SPE disk (combination of filtration and SPE)

High complex matrix: unfiltered surface water

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

- Poor absolute recoveries for E1, E2, alphaE2, EE2 and E3
- Sufficient/ideal relative recoveries by isotope dilution calibration

Mi-SPE (Molecular imprinted polymers)

AFFINIMIP® SPE

Estrogens

PROTOCOL OF PURIFICATION

Sample preparation

100mL of tap water spiked with 17ß-E2-d_3 to a final concentration of 75ng/L was the loading solution.

Purification with a 3mL/100mg AFFINIMIP® SPE Estrogens cartridge

Equilibration
- 3mL Acetonitrile
- 3mL Water

Loading solution from sample preparation

Washing of interferents
- 3mL water
- 3mL Water/Acetonitrile (60/40)

Elution (E)

3mL Methanol

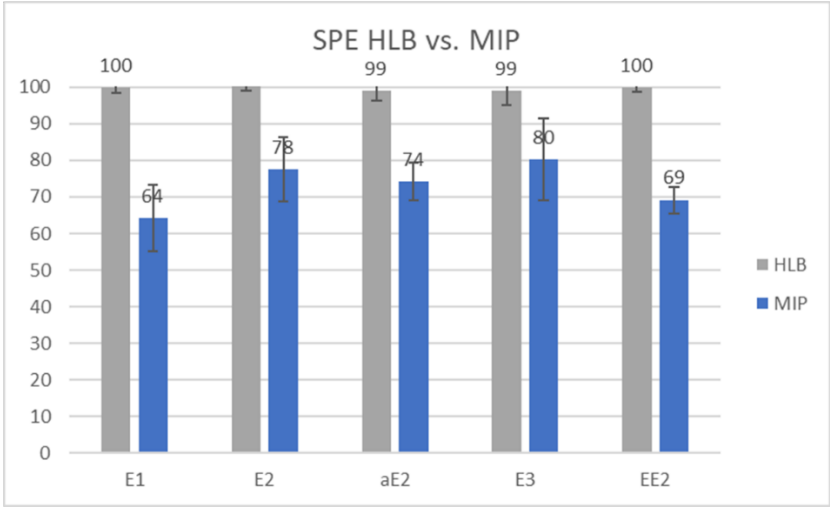
Publications

Data extracted from **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)**, D. Zacs, I. Perkons, V. Bartkevics, *Environ Monit Assess* 188, 433, 2016.

- Given protocol by the cartridge provider – limited sample volume of 100 mL – not suitable for estrogens with regards to EU-WFD
- Advantage: specific preconcentration of estrogens – key lock mode of action
- Ideal procedure to separate matrix (e.g., DOC, inorganics, ….) from estrogens (no significant ion surpression)
- Potential: implementation as purification procedure for extracts obtained from preconcentration steps

Mi-SPE (Molecular imprinted polymers)

Low complexity matrix (Evian water with low DOC and without SPM)



- Mi-SPE: Moderate relative recoveries for E1, E2, alphaE2, EE2 and E3 in comparison to common SPE;
- No high complex matrix experiments possible due to clogging of the cartridge

Overview of preconcentration methods including limitations and restrictions

(O: fulfilling , +: good, ++: excellent, -: poor, --: unsatisfactory)

Extraction method / Parameter	DI	SPE		SPE disk	LLE	Mi-SPE
		off-	online			
Preconcentration	--	++	++	++	O	--
Clean-up	--	+	+	+	O	++
Compatible to common solvents	--	+	+	+	-	O
Selectivity	--	++	++	++	O	++
Time / efficiency	++	+	++	++	+	O

Conclusions on preconcentration procedures (1: without SPM, 2: with SPM)

Parameter \ Extraction method (Typical preconc. factor)	SPE (1:1000)		SPE disk (1:1000)		MiSPE (1:100)	
	1	2	1	2	1	2
Estrone (E1)						
17α-Estradiol (aE2)						
17β-Ethinylestradiol (bE2)						
Estriol (E3)						
17α-Ethinylestradiol (EE2)						

Conclusions

- SPE and SPE disks: extraction method of choice
- **SPE is applicable for whole water samples with a SPM load less then 50 mg L-1** to avoid clogging of the cartridge
- **Higher SPM load: SPE disk is the method of choice**
- Using e.g., HLB sorbent materials (SPE or SPE disk) the DOC will be co-preconcentrated, only 50 to 70% of the initial DOC can be removed by the preconcentration
- This can interfere the analytical method e.g., in case of LC-ESI-MS ion suppression can occur (loss of sensitivity)
- **A further purification of the extract from the preconcentration procedure is recommended by the project consortium**

Overview on purification/ clean up procedures

Mi-SPE (molecular imprinted polymers)

PROTOCOL OF PURIFICATION

Sample preparation

100mL of tap water spiked with 17ß-E2-d_3 to a final concentration of 75ng/L was the loading solution.

Purification with a 3mL/100mg AFFINIMIP® SPE Estrogens cartridge

Equilibration
- 3mL Acetonitrile
- 3mL Water

Loading solution from sample preparation

Washing of interferents
- 3mL water
- 3mL Water/Acetonitrile (60/40)

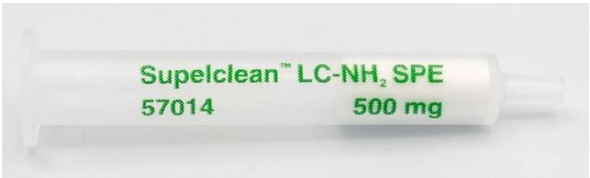
Elution (E)

3mL Methanol

- Extract from preconcentration step can be evaporated to at least 1 mL and can be diluted using MilliQ water up to 100 mL
- The volume is compatible to the max volume given by the provider SOP
- Advantage: specific preconcentration of estrogens – key lock mode of action
- Ideal procedure to separate matrix (e.g., DOC, inorganics, ….) from estrogens
- DOC in the Mi-SPE is decreased to a minimum (color changed from yellowish to a complete clear and colorless extract (evaluated by complementary DOC measurements) with sufficient recoveries

Other SPE cartridges for purification of sample extracts

- For an alternative clean up of the sample extracts a **Supelclean™ LC-NH2** SPE (500 mg, 6 mL) cartridge (Merck, Darmstadt, Germany can be used
- Silica gel based material with amino bounding functional groups
- Increase of matrix components but not as specific as MiSPE



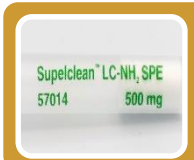
- Also mixed phase cartridges containing e.g., C_{18} and aminopropyl functionality are available (e.g., Chromabond NH2/C18). Due to limited capacity not sufficient as a two step procedure.

Overview on purification/ clean up procedures

Other SPE cartridges for purification of sample extracts

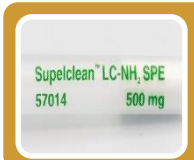
1 mL sample extract

- SPE e.g., Supelclean LC-NH2 (500 mg/6 mL)



Conditioning / sample load

- Conditioning with 4 mL MeOH
- Load sample extract



Washing step and elution

- Collect 2 mL fraction using MeOh

Concentration and analysis

- Concentrate organic fraction to desired volume (TurboVap)
- Clear extract is ready for analysis by LC-MS/MS

Two-step procedure preconcentration/purification

- **High complex matrix** (Evian water with moderate DOC and with SPM)
- Relative and absolute recovery rates were determined to compare the different experiments with regards to their performance (applicability in routine analysis)

TESTED:

1. SPE preconcentration + SPE purification: LC-NH_2
2. SPE disk preconcentration + SPE purification: LC-NH_2
3. SPE disk preconcentration + SPE purification: MiSPE

1. SPE preconcentration + SPE purification: LC-NH_2

High complex matrix (Evian water with moderate DOC and with SPM)

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		

2. SPE disk preconcentration + SPE purification: LC-NH_2

High complex matrix (Evian water with moderate DOC and with SPM)

Abs. rec.

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%

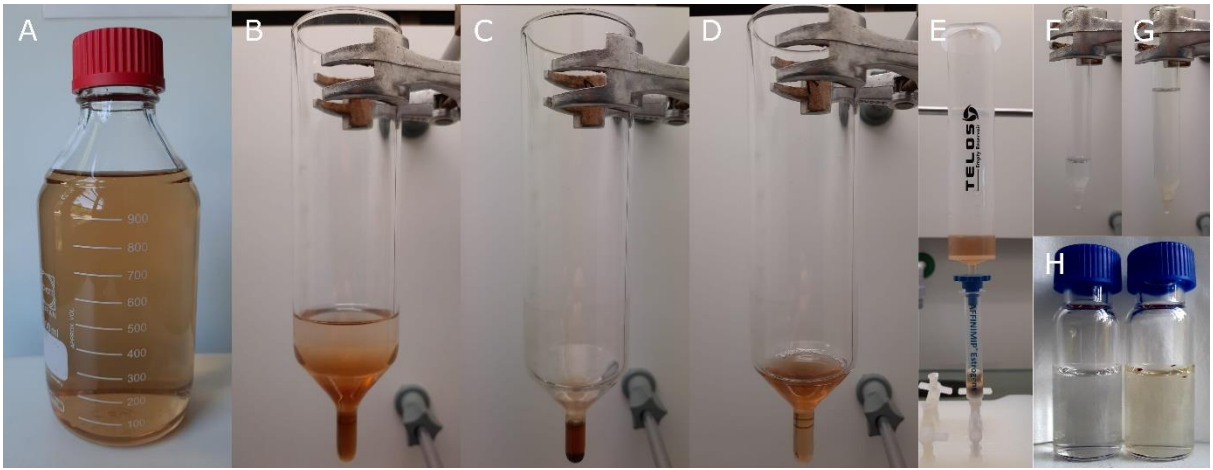
Rel. rec.

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

2. SPE disk preconcentration + SPE purification: MiSPE

High complex matrix (Evian water with moderate DOC and with SPM)

A: START



H left: FINAL

- DOC content of the preconcentrated and purified sample is less then 1% then the initial on (A: starting, H left : final)

3. SPE disk preconcentration + SPE purification: MiSPE

High complex matrix (Evian water with moderate DOC and with SPM)
1 L Evian + 7 mg L^{-1} DOC and 50 mg SPM spiked with estrogens at 0.1 ng L^{-1}

	HLB disk		MiSPE		HLB disk + MiSPE	
	Relative recovery [%]	Standard deviation [%]	Relative recovery [%]	Standard deviation [%]	Relative recovery [%]	Standard deviation [%]
Estrone	97.00	0.25	92.54	0.105	96.20	0.30
Estradiol	100.02	0.01	100.02	0.01	100.01	0.01
17α-Estradiol	100.09	0.02	100.07	0.04	100.14	0.06
Estriol	99.65	0.09	99.16	0.21	99.60	0.07
Ethinylestradiol	100.17	0.05	100.21	0.06	100.12	0.04

Take home message on sample preparation

High complexity matrix:
Preconcentration (SPE, SPE-disks) + purification (SPE, MiSPE)

Take home message on sample preparation

High complexity matrix:
Preconcentration (SPE, SPE-disks) + purification (SPE, MiSPE)

Thank you for your attention!