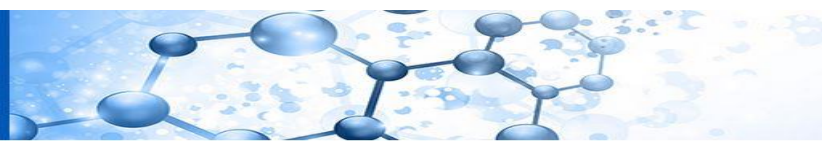


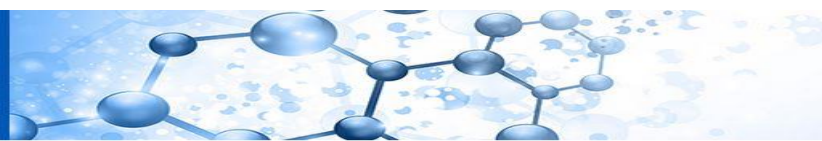
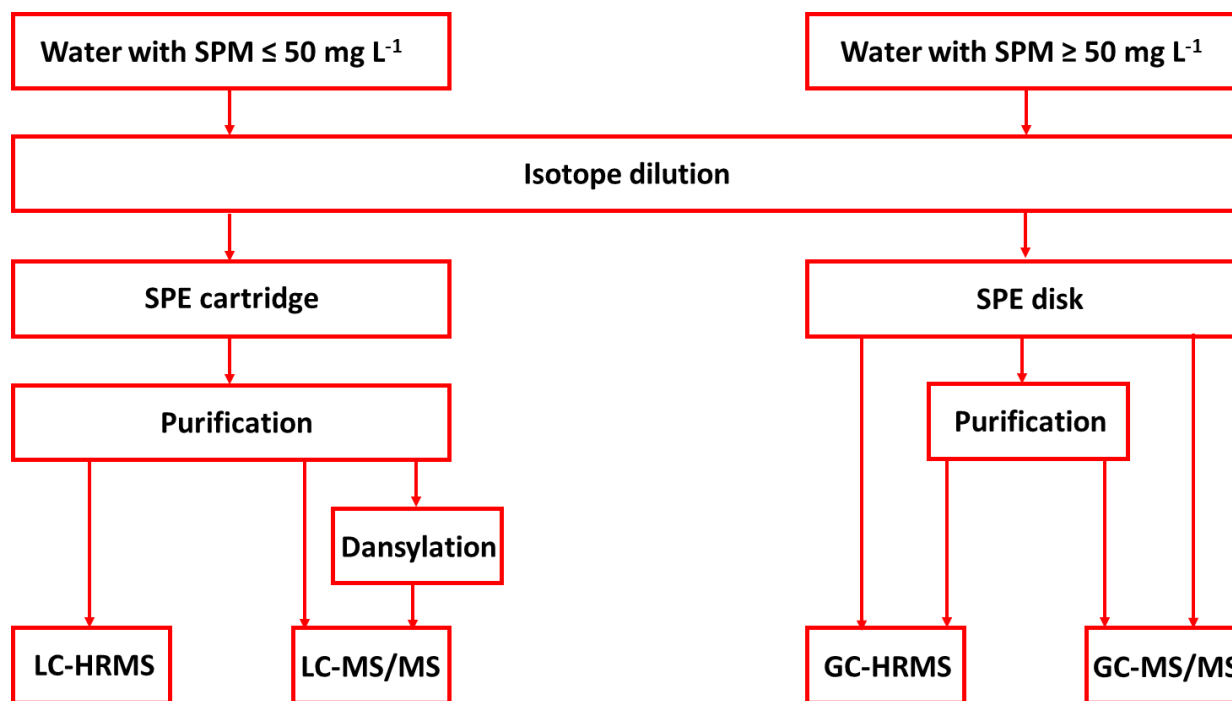
# “Validation of MS-based methods”

22<sup>nd</sup> February 2023



# General aspects

Institute	Volume [L]	Pre-concentration	Purification	Derivatization	LC-MS/MS	GC-MS/MS
UBx	0.2	HLB SPE	LC-NH2 SPE	none	X	
BAM	1	HLB SPE disk	MISPE	none	X	
JSI	0.5	DVB SPE disk	none	TMSI + pyridine		x
LNE	1	C <sub>18</sub> SPE disk	LC-NH2 SPE	none	X	
SYKE	1	C <sub>18</sub> SPE disk	none	none	X	

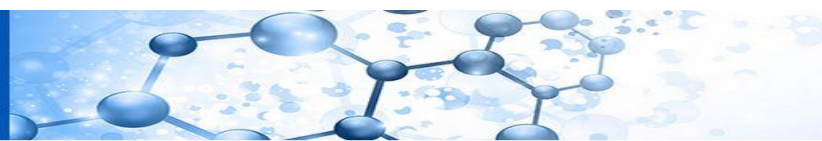


# Validation study

## Scope

- The experimental design summarizes the in-house validation strategy for the MS-based methods optimized within the project.
- Validation of the individual methods in accordance with CEN/TS 16800:2020.
- The methods performance characteristics will be assessed through ad-hoc experiments on six matrices (three synthetic waters and three natural waters) at three different concentrations level each (LOQ-V, medium and high concentration).
- LOQ-V (Verified Limit of Quantification) is the lowest concentration of a measurand that can be determined with acceptable accuracy under the stated conditions of the test.

	MS Based Methods
Measurand	E1, $\alpha$ E2, $\beta$ E2, $\alpha$ EE2, E3
Unit	ng L <sup>-1</sup>
Matrix	Inland surface waters and ground waters Options: marine waters and drinking waters
Fraction of the matrix	Whole water
Application range	LOQ-V to tens ng/L



# Validation study

Matrix	EDC levels of concentration tested (ng/L)
In-house reference materials containing EVIAN+DOC 1 mg L <sup>-1</sup> as matrix. Three levels, each spiked with different amount of estrogens	LOQ-V
	3LOQ-V
	10LOQ-V
In-house reference materials containing EVIAN+DOC 7 mg L <sup>-1</sup> as matrix. Three levels, each spiked with different amount of estrogens	LOQ-V
	3LOQ-V
	10LOQ-V
In-house reference materials containing EVIAN+DOC 7 mg L <sup>-1</sup> + SPM 50 mg L <sup>-1</sup> as matrix. Three levels, each spiked with different amount of estrogens	LOQ-V
	3LOQ-V
	10LOQ-V
1st Natural sample spiked at three different levels of concentration	LOQ-V
	3LOQ-V
	10LOQ-V
2nd Natural sample spiked at three different levels of concentration	LOQ-V
	3LOQ-V
	10LOQ-V
3rd Natural sample spiked at three different levels of concentration	LOQ-V
	3LOQ-V
	10LOQ-V

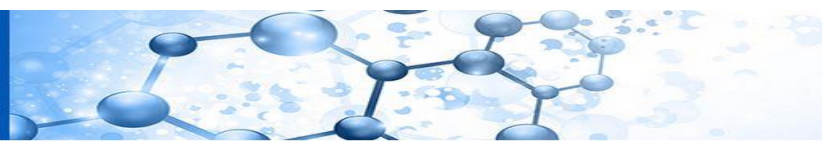


# Validation study

## Parameter

- Methods performance characteristics which will be assessed within the method validation:

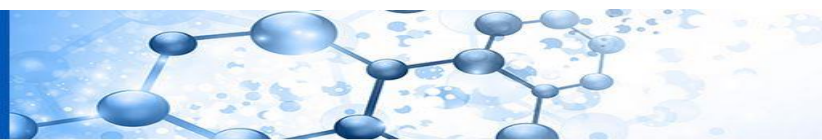
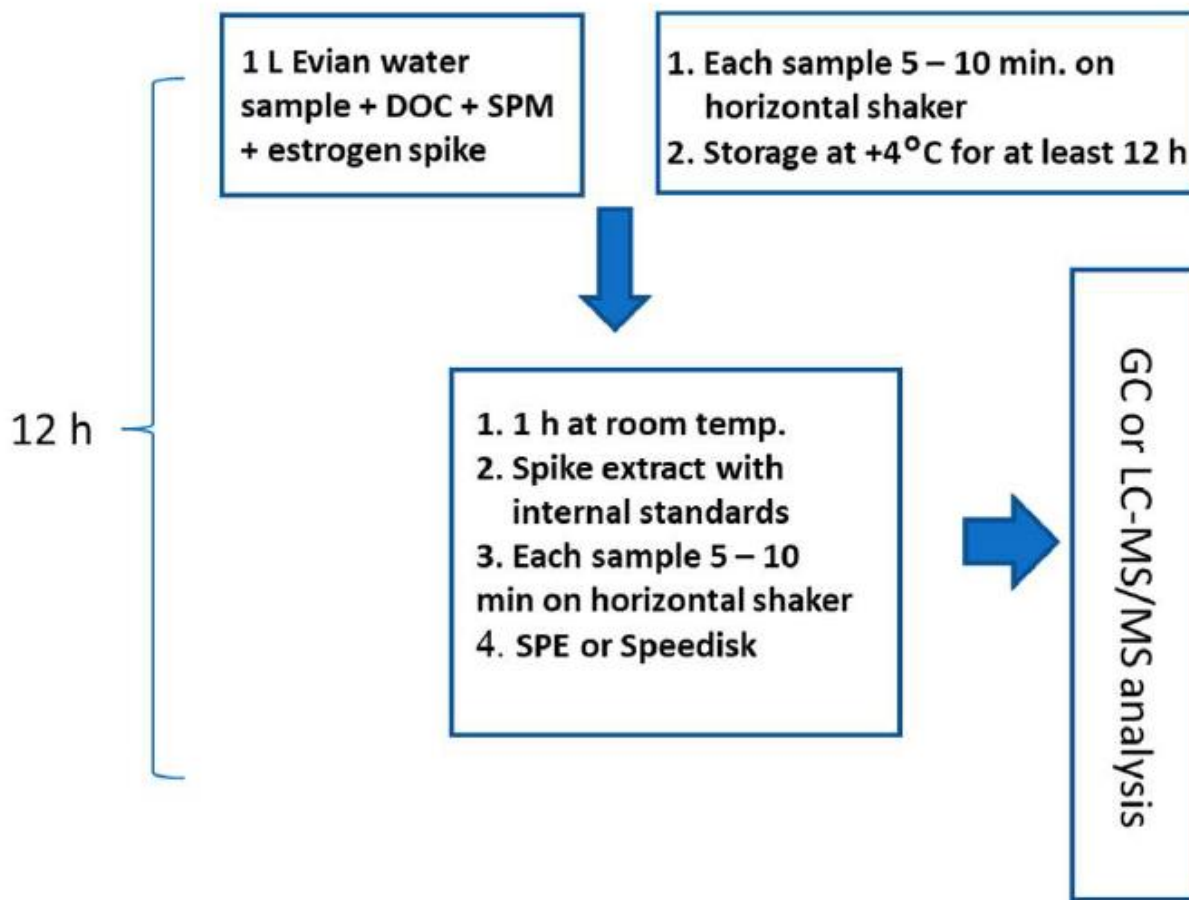
	MS-based methods
Linearity	X
Application range	X
Limit of detection	-
Limit of quantification	-
Verified limit of quantification	X
Selectivity	X
Sensitivity	X
Precision	X
Trueness (bias)	X
Method recovery	X
Measurement uncertainty	X
Robustness	-



# Samples used for validation

## Sample material

- RM candidate with selected compositions (SPM, DOC and estrogen concentrations) + three individual natural water



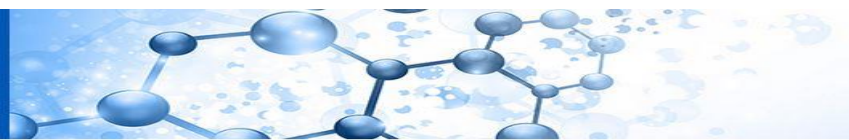
# Validation parameter

## Linearity

- In order to evaluate the linearity, the measuring system should be properly calibrated. The calibration is carried out according to ISO/CD 8466-1. Calibration is crucial in traceability establishment; therefore, calibration standards should be obtained by dilution of appropriate CRMs.

## Application range

- The application range, as defined in CEN/TS 16800:2020, is the range of concentrations routinely measured by a method. Thus, the lower limit of the application range is the LOQ-V and the upper limit of the optimized methods under validation is given by the measurand corresponding to the highest calibration level



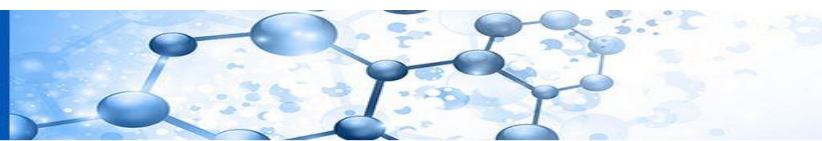
# Validation parameter

## Selectivity

- The target analytes should be identified in accordance with ISO 21253-1 and observing a non-significant bias of the methods can be considered as an indirect way to demonstrate the methods selectivity. Moreover, past experience during the implementation and optimization step by means of ad-hoc experiments (e.g. interferences spiking studies) are valuable to assess the selectivity of the method.

## Sensitivity

- Sensitivity is the change in the measuring system response which determines a change in the measured quantity. As indicated in CEN/TS 16800 it should be evaluated via calibration. Moreover, within the method validation, the matrix effect is studied. In fact, matrix effect can influence the method sensitivity. Ad-hoc experiments with spiked samples must be carried out following the post-extraction approach.





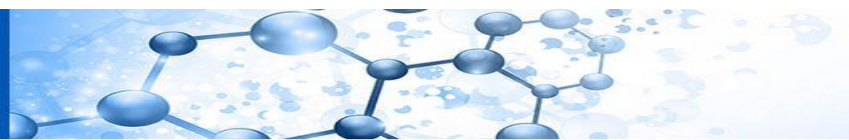
# Validation parameter

## Precision (repeatability and intermediate precision)

- Four factors which might impact the method precision are: a) time b) calibration c) operator and d) equipment.
- Within the method validation, both repeatability and intermediate precision will be assessed.

## Trueness (bias)

- According to ISO 11352, method and laboratory bias can be assessed by applying three different approaches: a) analyses of suitable reference materials b) participation in interlaboratory comparisons c) recovery experiments.
- Within the method validation, ad-hoc recovery experiments will be performed. The six selected matrices will be spiked at three different concentrations levels of the target analytes.



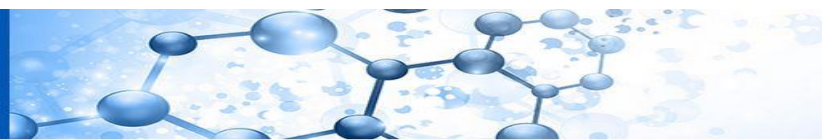
# Validation parameter

## Method recovery

- Absolute recovery will be determined in each matrix by comparing the peak areas of spiked samples prior and post extraction. Relative recoveries will be calculated in each matrix by comparing the ratios of the peak areas of the target analytes to the corresponding peak areas of the internal standards in spiked samples prior extraction and post extraction.

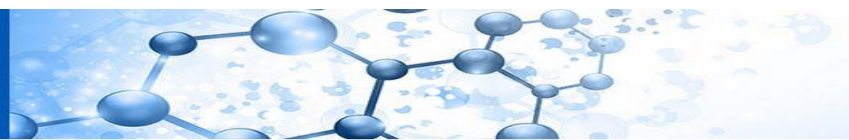
## Measurement uncertainty

- The evaluation of the measurement uncertainty (U) is in accordance with ISO 11352:2012. Method bias and within laboratory reproducibility are the two main contributions to the uncertainty budget. The corresponding relative standard uncertainties are combined according to the propagation law of the uncertainties in order to obtain the relative combined standard uncertainty.
- The calculation of the relative expanded uncertainty is obtained using a coverage factor of  $k=2$  (confidence interval of 95%).



# Validation reporting form

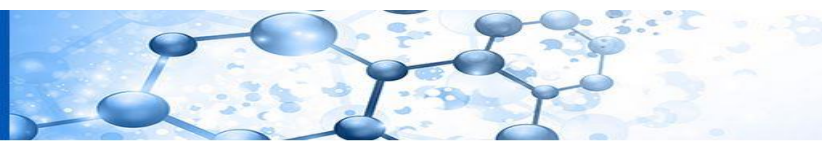
<b>Partner contacts details</b> (i.e. Name, Surname, Institute name)	
<b>Method name</b>	
<b>Method description</b>	
<b>Measurands:</b>	
<b>Unit of measurands</b>	
<b>Concentration range</b>	
<b>Matrices description:</b>	
1	
2	
3	
4	
5	
6	
<b>Additional information</b>	
Selected Within Laboratory Reproducibility Approach	



# Validation reporting form

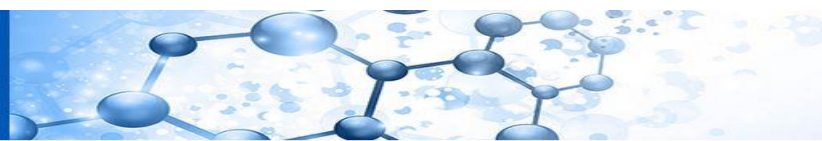
Matrix	Compound	EDC level	Date of Extraction	Date of Analysis	EDC Background (ng/L)	Replicate 1 (ng/L)	Replicate 2 (ng/L)	Replicate 3 (ng/L)	Mean (ng/L)	EDC Spiked Reference value (ng/L)	(**) Uncertainty of the standard solution concentration (%)	(**) Standard uncertainty of the volume added (%)	Standard uncertainty associated to the EDC addition (%)	(**) Standard Solution Concentration (ng/L)	(**) Approximated Final Concentration (ng/L)	(**) Amount of standard (uL)
DOC 1 mg/L	β-E2	LOQ														
DOC 7 mg/L	β-E2	LOQ														
DOC 7 mg/L+SPM	β-E2	LOQ														
Natural 1*	β-E2	LOQ														
Natural 2*	β-E2	LOQ														
Natural 3*	β-E2	LOQ														
DOC 1 mg/L	β-E2	3 LOQ														
DOC 7 mg/L	β-E2	3 LOQ														
DOC 7 mg/L+SPM	β-E2	3 LOQ														
Natural 1*	β-E2	3 LOQ														
Natural 2*	β-E2	3 LOQ														
Natural 3*	β-E2	3 LOQ														
DOC 1 mg/L	β-E2	10 LOQ														
DOC 7 mg/L	β-E2	10 LOQ														
DOC 7 mg/L+SPM	β-E2	10 LOQ														
Natural 1*	β-E2	10 LOQ														
Natural 2*	β-E2	10 LOQ														
Natural 3*	β-E2	10 LOQ														

Matrix	Compound	EDC level	Date of Extraction	Date of Analysis	EDC Spiked Reference value (ng/L)	Matrix effect (%)	Absolute recovery (%)	Relative recovery (%)
DOC 1 mg/L	β-E2	3 LOQ						
DOC 7 mg/L	β-E2	3 LOQ						
DOC 7 mg/L+SPM	β-E2	3 LOQ						
Natural 1*	β-E2	3 LOQ						
Natural 2*	β-E2	3 LOQ						
Natural 3*	β-E2	3 LOQ						



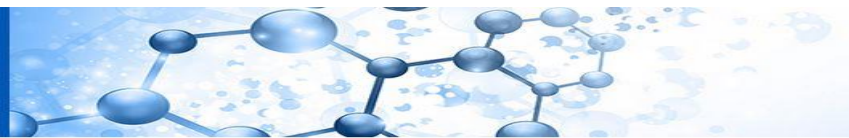
# Validation results

	Compound Concentration level	17β-estradiol			17α-estradiol			17α-ethinyl estradiol			Estrone			Estriol		
		LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ	LOQ	3 LOQ	10 LOQ
Partner 1	C (ng/L)	0,123	0,275	0,958	0,111	0,247	0,859	0,008	0,017	0,060	0,134	0,298	1,037	0,112	0,250	0,869
	Bias (%)	15	8	10	17	13	10	31	26	20	13	10	14	69	33	12
	Precision (%)	20	11	10	11	17	12	20	15	12	14	12	11	22	8	10
	U (k=2) (%)	50	26	29	41	43	32	74	60	47	37	31	35	140	68	32
Partner 2	C (ng/L)	0,117	0,303	1,011	0,124	0,320	1,070	0,132	0,342	1,141	0,153	0,396	1,321	0,126	0,326	1,088
	Bias (%)	15	13	13	13	7	5	15	7	6	24	19	17	25	8	6
	Precision (%)	6	3	2	13	2	0	5	2	1	7	1	1	12	4	2
	U (k=2) (%)	32	26	26	37	14	9	33	14	13	51	38	34	56	19	12
Partner 3	C (ng/L)	0,099	0,274	0,931	0,094	0,267	0,909	0,849	2,455	8,349	0,096	0,280	0,951	0,948	2,764	9,397
	Bias (%)	23	17	18	22	31	28	28	30	32	23	24	20	20	15	16
	Precision (%)	13	5	13	7	10	7	9	6	3	9	5	5	9	2	4
	U (k=2) (%)	53	35	44	47	65	57	58	61	63	48	49	41	43	31	34
Partner 4	C (ng/L)	0,056	0,163	0,560	0,057	0,165	0,567	0,016	0,047	0,161	0,114	0,332	1,139	0,113	0,331	1,133
	Bias (%)	18	22	29	8	13	14	39	14	22	25	31	31	10	9	8
	Precision (%)	23	16	15	9	4	6	21	15	17	9	6	7	9	6	7
	U (k=2) (%)	59	55	65	24	28	31	89	41	56	53	62	64	27	22	20
Partner 5	C (ng/L)	0,120	0,364	1,331	0,122	0,370	1,353	0,087	0,265	0,969	0,128	0,387	1,414	0,183	0,553	2,023
	Bias (%)	40	15	6	26	11	12	11	11	5	95	86	14	109	29	6
	Precision (%)	40	8	3	18	6	3	6	3	3	30	30	3	96	18	3
	U (k=2) (%)	110	34	14	63	26	25	24	22	11	200	180	29	290	69	14



# Conclusion and recommendation

- The applied experimental design for the validation study has proven to be fit for purpose of MS-based method validation.
- Matrix, interferents and DOC may affect the analyses of the samples in terms of sensitivity and background.
- Preconcentration and purification of the resulting extracts are strongly recommended.
- It is mandatory to apply Isotope dilution calibration by using isotopically labelled standards (deuterated or  $^{13}\text{C}$ ).
- SPE disk should be used even when the water samples contain more than 50 mg of SPM.
- LC-MS/MS and GC-MS/MS with derivatization show comparable performances.



# References used for validation study

## References

- [1] ISO 21253-2: “Water quality — Multi-compound class methods — Part 2: Criteria for the quantitative determination of organic substances using a multi-compound class analytical method”
- [2] Eurachem Guide – “Metrological Traceability in Chemical Measurement - A guide to achieving comparable results in chemical measurement” 2<sup>nd</sup> edition 2019
- [3] Eurachem Guide – “The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics” 2<sup>nd</sup> edition 2014.
- [4] C. Miège, P. Bados, C. Brosse, M. Coquery, Trends in Analytical Chemistry, 28, 2, 2009, “Method validation for the analysis of estrogens (including conjugated compounds) in aqueous matrices”.
- [5] E. Mirmont, A. Boeuf, M. Charmel, S. Vaslin-Reimann, B. Lalère, O. Laprèvote, S. Lardy-Fontan, Journal of Chromatography B 1175 (2021) 122732, “Development and implementation of an analytical procedure for the quantification of natural and synthetic steroid hormones in whole surface waters”.
- [6] ISO 5725-3:1994: “Accuracy (trueness and precision) of measurement methods and results — Part 3: Intermediate measures of the precision of a standard measurement method”
- [7] NF T90-210:2018 – “Qualité de l'eau - Protocole d'évaluation initiale des performances d'une méthode dans un laboratoire”
- [8] Nordtest NT TR 537 edition 4:2017 – “Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories”



