



## EDC-WFD ILC Final report

**FINAL MEETING**

# *Presentation overview*

- Context and aim
- ILC Structure
- Participation
- Results and evaluations
- Conclusion

## *Context and aim*

The interlaboratory comparison (ILC) was carried out within the framework of **the Project 18NRM01 EDC-WFD** “Metrology for monitoring endocrine disrupting compounds under the Water Framework Directive”.

### **OBJECTIVE**

to demonstrate the fitness for purpose of optimized and validated methods for estrogens measurements by MS-based and effect-based methods (e.g. ER $\alpha$ -Calux and A-YES), defining **performance characteristics** of the methods in terms of repeatability within laboratories and reproducibility.

# ***Background and bounds***

Definition of the ILC structure and timing

Suitable RMs (characteristic, number of units produced, transportation bounds, storage)

Preliminary reconnaissance (EDC-WFD\_ILC Pre-registration form - questionnaire)

Available laboratories (analytical capabilities, LOQs, method implementation)

# Schedule

Time	Action
by 30 <sup>th</sup> September 2022	The ILC Organizer sends the Protocol to the laboratories
by 2 <sup>nd</sup> November 2022	The test materials sent to each involved laboratory
by 23 <sup>rd</sup> November 2022 → delayed <b>30<sup>th</sup> November</b>	The involved laboratories provided the results to <a href="mailto:ilc-empir@isprambiente.it">ilc-empir@isprambiente.it</a>
by 20 <sup>th</sup> December 2022 → delayed <b>30<sup>th</sup> December 2022</b>	A Preliminary statistical evaluation sent to the involved laboratories
by 14 <sup>th</sup> January 2023	Comments from the participants
17 <sup>th</sup> February 2023	The Final Report sent to the involved laboratories.
22 <sup>th</sup> February 2023	Plenary meeting for the presentation and discussion of the results

# *Laboratories and RMs*

27 Laboratories pre-registered:

- 22 chemical measurements
- 10 EBMs

All the laboratories received the RMs (kit)

Some labs able to carry out both chemical measurements (more instrumental techniques) and EBMs

Some labs did not returned reporting templates (technical/analytical troubles, units arrived broken, not confident results, method not well implemented, LOQs too high)

# Laboratories – CHEM



ARPA FVG – Agenzia regionale per la protezione Ambientale del Friuli Venezia Giulia (Italy)

ARPA SICILIA - Agenzia regionale per la protezione Ambientale (Italy)

ARPA LOMBARDIA - Agenzia regionale per la protezione Ambientale (Italy)

BAM -Bundesanstalt für Materialforschung und -prüfung (Germany)

EAEF – Eurofins analyses pour l'environnement (France)

Ecotox Centre (Switzerland)

Université de Bordeaux, EPOC UMR5805 Equipe LPTC (France)

Eurofins Environment Testing Finland Oy (Finland)

SYKE-Finnish Environment Institute (Finland)

Institut für Energie- und Umwelttechnik e.V. (IUTA) (Germany)

JSI-Jožef Stefan Institute (Slovenia)

Laboratoire de l'Environnement et de l'Alimentation de la Vendée (France)

LANUV NRW-Landesamt für Natur, Umwelt und Verbraucherschutz (Germany)

LDAR 24-Laboratoire Départemental d'Analyses et de Recherche (France)

LNE- Laboratoire National de Métrologie et d'Essais (France)



# Laboratories - EBM's



BioDetection Systems BV (The Netherlands)

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Ecotox Centre (Switzerland)

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Federal Institute of Hydrology (Germany)

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ISPRA-Centro Nazionale per la Rete nazionale dei laboratori-Area Metrologia (Italy)

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ISSeP - Institut Scientifique de Service Public (Belgium)

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IWW Water Center (Germany)

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QuoData GmbH (Germany)

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Ruhrverband – Kooperationslabor (Germany)

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Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft (Germany)

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## ***Test materials***

- 4 bottles of natural water (2 bottles for each test material, 1L each);
- Ampoules of SPM (suspended particulate matter) and DOC (Dissolved Organic Carbon);

2 Vials (**EDC-WFD\_C1** and **EDC-WFD\_C2**, for chemical measurements; **EDC-WFD\_B1** and **EDC-WFD\_B2**, for EBMs) containing, at different concentration, 3 mL of the standard of the targeted substances in methanol

*QC unit* (3 mL target estrogens mixture in methanol) for chemical measurements

*Blank unit* for chemical measurement and EBMs

## ***RM reconstitution***

The RMs should be **reconstituted**, mixing all the constituents together to the desired whole water sample with a distinct DOC, estrogen content and SPM load (Instruction given into the Protocol).

### **Chemical measurements**

**internal standards spiked  
samples at least for 12 h at  
+4°C to ensure the  
equilibrium time**

### **Storage condition**

**Maximum two weeks at  
+4 °C**

# ***RM's composition - Chemical methods***

<b>EDC-WFD C1</b>		<b>SPM mg/L</b>	<b>DOC mg/L</b>	<b>EDC-WFD C2</b>	<b>Medium Concentration Range ng/L</b>	<b>SPM mg/L</b>	<b>DOC mg/L</b>
17βE2	0,1-0,5			17βE2	0,5-2,5		
17αEE2	0,02-0,1			17αEE2	0,1-0,5		
E1	0,2-1,0	10	2	E1	0,5-2,5	50	5
17αE2	0,1-0,5			17αE2	0,5-2,5		
E3	0,2-1,0			E3	0,5-2,5		

# RM's composition - EBM's

<b>EDC-WFD B1</b>	Low EEQ Range ng/L $\beta$ E2 eq	SPM mg/L	DOC mg/L	<b>EDC-WFD B2</b>	Medium EEQ Range ng/L $\beta$ E2 eq	SPM mg/L	DOC mg/L
Cumulative effect	0,1-1,0	10	2	Cumulative effect	0,5 -5,0	50	5

# Reference estrogens concentration

<b>EDC-WFD C1/B1</b>	Low Concentration ng/L	<b>EDC-WFD C2/B2</b>	Medium Concentration ng/L
17 $\beta$ E2	0,22	17 $\beta$ E2	1,01
17 $\alpha$ EE2	0,035	17 $\alpha$ EE2	0,210
E1	0.40	E1	1,01
17 $\alpha$ E2	0,20	17 $\alpha$ E2	1,02
E3	0,41	E3	1,01

**Gravimetric determination**

## ***Measurements: Chemical methods and EBM*s**

The RMs reconstituted were analysed in repeatability conditions in **duplicate** (independent measurements).

The overall number of samples for each kit to be analysed was four (4)

# ***Chemical Measurements (1)***

**mandatory**: Measurements of the proposed three WFD priority substances: 17-beta-estradiol (**17 $\beta$ E2**), 17-alpha-ethinylestradiol (**17 $\alpha$ EE2**), and estrone (**E1**).

**strongly recommended**: Measurement of 17-alpha-estradiol (**17 $\alpha$ E2**) and estriol (**E3**).

Measurements by GC-MS-MS, LC-MS-MS, GC-HRMS and LC-HRMS.

## ***Chemical Measurements (2)***

Pre-treatment steps were:

- i) **mandatory**: a stable isotope dilution method (i.e. isotopically labeled internal standard for each estrogen);
- ii) **mandatory**: sample preparation technique based on SPE cartridge or SPEdisk (C18, polymeric phase HLB, DVB, polymeric reversed phase);
- iii) **strongly recommended**: purification step (LC-NH<sub>2</sub>, SiOH, Florisil or selective SPE cartridges for estrogens).



## ***Effective Based Methods***

The application of ER $\alpha$ -Calux and/or A-YES was preferred, other bioassays were optional.

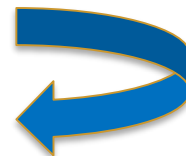
Pre-treatment steps were:

- i) **mandatory**: Sample preparation technique based on SPE cartridge or SPE disk (C18, polymeric phase (HLB, DVB, polymeric reversed phase));
- ii) **strongly recommended**: Purification step (LC-NH<sub>2</sub>, SiOH or selective SPE cartridges for estrogens).

# Laboratories participating

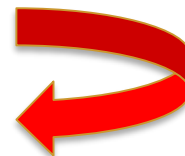
## CHEMICAL METHODS

20 Laboratories  22 templates sent  
**15 labs returned 17 templates**



## EBMs

10 Laboratories  10 templates sent  
**9 labs returned 9 templates**



# ***Data treatment & Statistical evaluation (1)***

Last Reporting template delivered (as revision) on the 7<sup>th</sup> December

Ad-hoc Data-base were implemented for Chemical methods and EBM

Verifying of the data (units, gross errors) and < LOQ values not considered.

Lab code	Measurand Unit	17β-E2 ng/L		17α-EE2 ng/L		E1 ng/L		17α-E2 ng/L		E3 ng/L	
		value	expanded uncertainty	value	expanded uncertainty	value	expanded uncertainty	value	expanded uncertainty	value	expanded uncertainty
1	unit 1	<0.3	--	<0.03	--	<0.3	--	--	--	--	--
	unit 2	<0.3	--	<0.03	--	<0.3	--	--	--	--	--
	Average value										
2	unit 1	0.281		0.029		0.288		0.310			
	unit 2	0.265		0.031		0.264		0.262			
	Average value	0.273	0.137	0.030	0.015	0.276	0.138	0.286	0.143		
3	unit 1	0.252	0.091	0.021	0.007	0.441	0.163				
	unit 2	0.290	0.104	0.016	0.005	0.442	0.164				
	Average value	0.271	0.098	0.019	0.006	0.442	0.163				
7-GC	unit 1	0.198	0.032	0.033	0.009	0.354	0.032	0.196	0.021	0.377	0.038
	unit 2	0.192	0.032	0.029	0.009	0.331	0.032	0.187	0.021	0.376	0.038
	Average value	0.195	0.032	0.031	0.009	0.343	0.032	0.192	0.021	0.377	0.038
7-LC	unit 1	0.210	0.012	0.029	0.007	0.375	0.021	0.197	0.011	0.389	0.019
	unit 2	0.202	0.012	0.031	0.007	0.355	0.021	0.205	0.011	0.392	0.019
	Average value	0.206	0.012	0.030	0.007	0.365	0.021	0.201	0.011	0.391	0.019
8	unit 1	<(LOQ)	9.0	<(LOQ)	9.0	<(LOQ)	9.0				
	unit 2	<(LOQ)	9.0	<(LOQ)	9.0	<(LOQ)	9.0				
	Average value	<(LOQ)	9.0	<(LOQ)	9.0	<(LOQ)	9.0				
9	unit 1	0.23		0.046		0.37				0.37	
	unit 2	0.25		0.051		0.43				0.36	
	Average value	0.24		0.048		0.40				0.37	
10	unit 1	0.28		0.05		0.45		0.25		0.54	
	unit 2	0.25		0.04		0.39		0.21		0.49	
	Average value	0.26		0.04		0.42		0.23		0.51	
11	unit 1	<0.4		0.059		0.467				<2	
	unit 2	<0.4		0.049		0.445				<2	
	Average value										
13	unit 1	0.230		0.030		0.410		0.230		0.410	
	unit 2	0.230		0.030		0.391		0.210		0.400	
	Average value	0.230		0.030		0.400		0.220		0.405	
15-GC	unit 1	0.28	0.03	0.02	0.01	0.34	0.03	0.25	0.02	0.45	0.08
	unit 2	0.32	0.03	<0.013		0.39	0.04	0.26	0.02	0.57	0.10
	Average value	0.30	0.03	0.02	0.01	0.36	0.04	0.25	0.02	0.51	0.09
15 LC	unit 1	0.1	0.02	0.02	0.004	0.3	0.04	0.1	0.01		
	unit 2	0.1	0.02	0.02	0.004	0.3	0.04	0.1	0.01		
	Average value	0.1	0.02	0.02	0.004	0.3	0.04	0.1	0.01		
19	unit 1	0.3	0.2	<LOQ=1.0		0.5	0.2	0.2	0.09		
	unit 2	0.3	0.2	<LOQ=1.0		0.4	0.2	0.2	0.09		
	Average value	0.3				0.4		0.2			
20	unit 1	0.22	0.13	<0.1		1.35	0.20	0.18	0.11	0.77	0.29
	unit 2	0.25	0.15	<0.1		1.40	0.21	0.22	0.13		
	Average value	0.24	0.14	<0.1		1.38	0.21	0.20	0.12	0.77	0.29
21	unit 1	0.243		0.046		0.498		0.259		0.507	
	unit 2	0.255		0.048		0.451		0.259		0.457	
	Average value	0.249		0.047		0.475		0.259		0.482	
22	unit 1			<0.2	0.1	0.4	0.2				
	unit 2			<0.2	0.1	0.4	0.2				
	Average value										
23	unit 1	0.284	0.041	0.040	0.010	0.415	0.017	0.298	0.040	0.467	0.020
	unit 2	0.233	0.041	0.036	0.010	0.413	0.017	0.324	0.040	0.465	0.020
	Average value	0.258	0.041	0.038	0.010	0.414	0.017	0.311	0.040	0.466	0.020

# EDC-WFD C1

Lab code	Measurand Unit	17β-E2		17α-EE2		E1		17α-E2		E3	
		ng/L		ng/L		ng/L		ng/L		ng/L	
		value	expanded uncertainty	value	expanded uncertainty	value	expanded uncertainty	value	expanded uncertainty	value	expanded uncertainty
1	unit 1	1,260	0,470	0,172	0,060	0,786	0,340				
	unit 2	1,227	0,470	0,165	0,060	0,829	0,350				
	Average value										
2	unit 1	0,960		0,210		0,534		1,04			
	unit 2	1,14		0,190		0,504		1,02			
	Average value	1,05	0,53	0,200	0,100	0,519	0,260	1,03	0,52		
3	unit 1	1,161	0,418	0,168	0,052	0,984	0,364				
	unit 2	1,246	0,449	0,167	0,052	1,085	0,401				
	Average value	1,204	0,433	0,168	0,052	1,035	0,383				
7-GC	unit 1	0,887	0,054	0,187	0,051	0,879	0,052	0,873	0,049	0,897	0,053
	unit 2	0,876	0,054	0,177	0,051	0,887	0,052	0,877	0,049	0,887	0,053
	Average value	0,882	0,054	0,182	0,051	0,883	0,052	0,875	0,049	0,892	0,053
7-LC	unit 1	0,997	0,041	0,195	0,045	0,967	0,043	1,002	0,051	0,977	0,047
	unit 2	0,986	0,041	0,201	0,045	0,978	0,043	0,998	0,051	0,967	0,047
	Average value	0,992	0,041	0,198	0,045	0,973	0,043	1,000	0,061	0,973	0,047
8	unit 1	<LOQ	9,0	<LOQ	9,0	<LOQ	9,0				
	unit 2	<LOQ	9,0	<LOQ	9,0	<LOQ	9,0				
	Average value	<LOQ	9,0	<LOQ	9,0	<LOQ	9,0				
9	unit 1	1,3		0,22		1,0				1,0	
	unit 2	1,3		0,22		1,0				1,1	
	Average value	1,3		0,22		1,03				1,03	
10	unit 1	1,16		0,23		1,01		1,13		1,07	
	unit 2	1,20		0,25		1,07		1,15		1,14	
	Average value	1,18		0,24		1,04		1,14		1,10	
11	unit 1	1,367		0,215		1,144					
	unit 2	1,340		0,227		1,096					
	Average value										
13	unit 1	1,20		0,197		1,10		1,10		1,00	
	unit 2	1,10		0,193		0,99		1,10		0,96	
	Average value	1,15		0,195		1,04		1,10		0,98	
15-GC	unit 1	1,32	0,12	0,30	0,08	0,85	0,09	1,27	0,09	1,13	0,19
	unit 2	1,00	0,09	<0,013	26,00	0,96	0,10	0,93	0,07	<0,2	17,00
	Average value	1,16	0,10	0,08	26,00	0,91	0,09	1,10	0,08	1,13	0,19
15-LC	unit 1	0,1	0,02	0,02	0,004	0,8	0,1	0,1	0,01		
	unit 2	0,1	0,02	0,02	0,004	0,7	0,1	0,1	0,01		
	Average value	0,1	0,02	0,02	0,004	0,7	0,1	0,1	0,01		
19	unit 1	1,1	0,5			0,8	0,3	0,8	0,4	1,0	0,3
	unit 2	1,0	0,5			0,7	0,3	0,8	0,4	1,0	0,3
	Average value	1,1				0,7		0,8		1,0	
20	unit 1	1,34	0,27	0,27	0,16	3,35	0,50	0,92	0,23		
	unit 2	1,19	0,24	0,22	0,13	3,54	0,53	0,90	0,23	1,42	0,28
	Average value	1,27	0,25	0,25	0,15	3,45	0,52	0,91	0,23	1,42	0,28
21	unit 1	1,025		0,235		1,033		1,047		1,029	
	unit 2	0,886		0,219		0,922		0,921		0,922	
	Average value	0,956		0,227		0,978		0,984		0,976	
22	unit 1	1,5	0,8	0,2	0,1	0,9	0,5				
	unit 2	0,9	0,5	0,2	0,1	0,9	0,5				
	Average value										
23	unit 1	1,330	0,072	0,230	0,018	1,047	0,068	1,308	0,072	1,164	0,078
	unit 2	1,303	0,072	0,221	0,018	1,079	0,068	1,333	0,072	1,121	0,078
	Average value	1,316	0,072	0,225	0,018	1,063	0,068	1,320	0,072	1,142	0,078

## EDC-WFD C2

# EDC-WFD – B1

Lab code	Measurand Unit	Cumulative effect	
		ng/L $\beta$ E2 eq	
		value	expanded uncertainty
5	unit 1	0,56	0,34
	unit 2	0,51	0,31
	Average value	0,54	0,33
9	unit 1	0,28	0,03
	unit 2	0,34	0,03
	Average value	0,31	0,03
12	unit 1	0,47	0,29
	unit 2	0,61	0,40
	Average value	0,54	0,34
16	unit 1	0,12	0,08
	unit 2	0,15	0,09
	Average value	0,14	0,09
17	unit 1	< LOQ	
	unit 2	< LOQ	
	Average value	< LOQ	
18	unit 1	0,006	0,006
	unit 2	0,006	0,006
	Average value	0,006	0,006
24	unit 1	0,129	0,052
	unit 2	0,195	0,078
	Average value	0,162	
25	unit 1	0,18	
	unit 2	0,31	
	Average value	0,30	
26	unit 1	0,206	
	unit 2	0,226	
	Average value	0,216	

# EDC-WFD – B2

Lab code	Measurand Unit	Cumulative effect	
		ng/L $\beta$ E2 eq	
		value	expanded uncertainty
5	unit 1	2,5	1,5
	unit 2	2,4	1,4
	Average value	2,5	1,5
9	unit 1	1,57	0,35
	unit 2	1,70	0,17
	Average value	1,63	0,35
12	unit 1	1,78	0,12
	unit 2	1,48	0,24
	Average value	1,62	0,18
16	unit 1	0,88	0,65
	unit 2	0,70	0,51
	Average value	0,79	0,58
17	unit 1	0,683	
	unit 2	0,634	
	Average value	0,658	
18	unit 1	0,233	0,022
	unit 2	0,329	0,033
	Average value	0,289	0,028
24	unit 1	0,629	0,25
	unit 2	0,784	0,31
	Average value	0,706	
25	unit 1	<LOD	
	unit 2	3,7	
	Average value		
26	unit 1	1,06	
	unit 2	1,32	
	Average value	1,19	

## ***Data treatment & Statistical evaluation (2)***

**h and k Statistics (Mandel)** according to ISO 5725-2:2019 were used to check the consistency of the data (graphical technique):

- the first (h) evaluates between-laboratory variability
- the second (k) evaluates within-laboratory variability

Examination of the h and k plots can indicate that specific laboratories exhibit patterns of results that are markedly different from the others

The critical values of the h statistic depend on the number of laboratories participating in the study while those of the k statistic depend on the number of laboratories and the number of replicates performed by each laboratory

## *Data treatment & Statistical evaluation (3)*

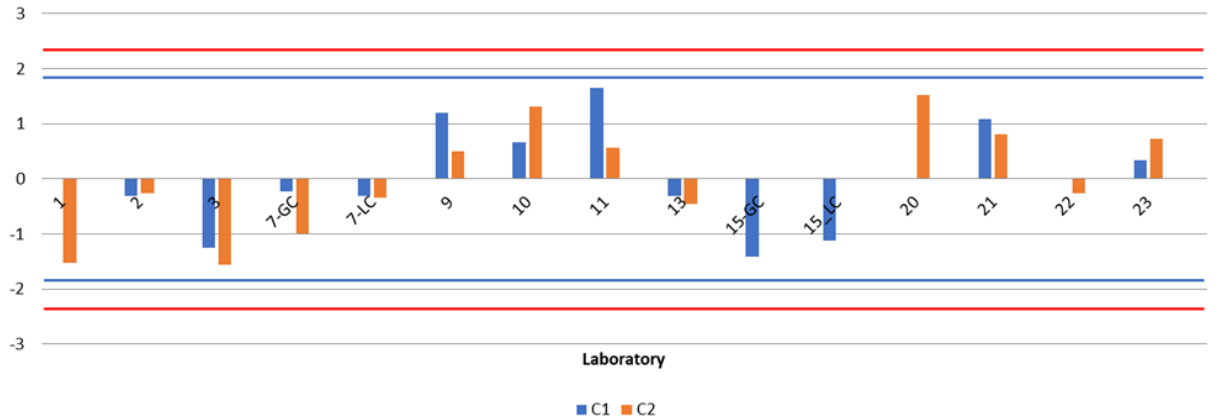
**Grubbs's test** was applied (iterative procedure) to identify “straggler” values (for test statistic  $> 5\%$  or  $\leq 1\%$  critical value) “outliers” (for test statistic  $> 1\%$  critical value).

**Outliers** values were evaluated considering the statistic outcomes.  
**Single** laboratory results (not duplicates) were not considered

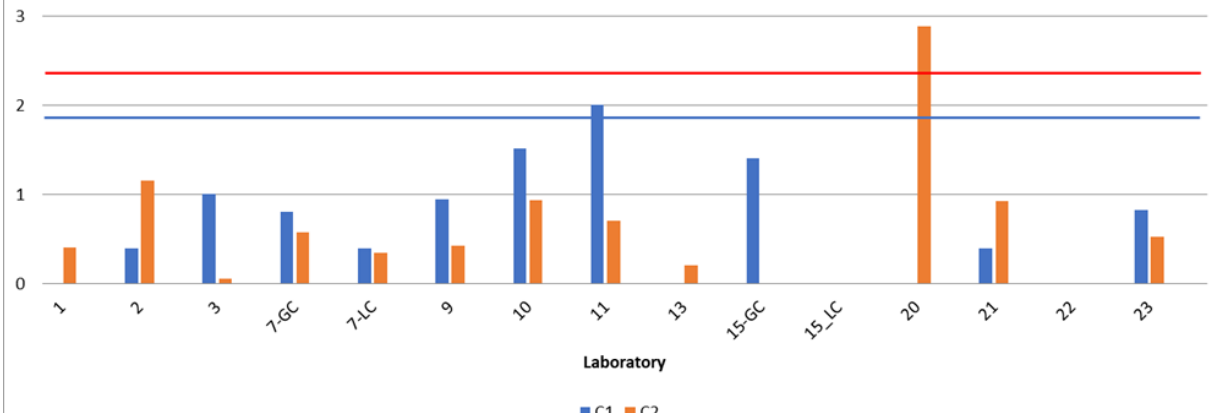
**Cochran's test** was applied for verifying the homogeneity of variance within laboratories



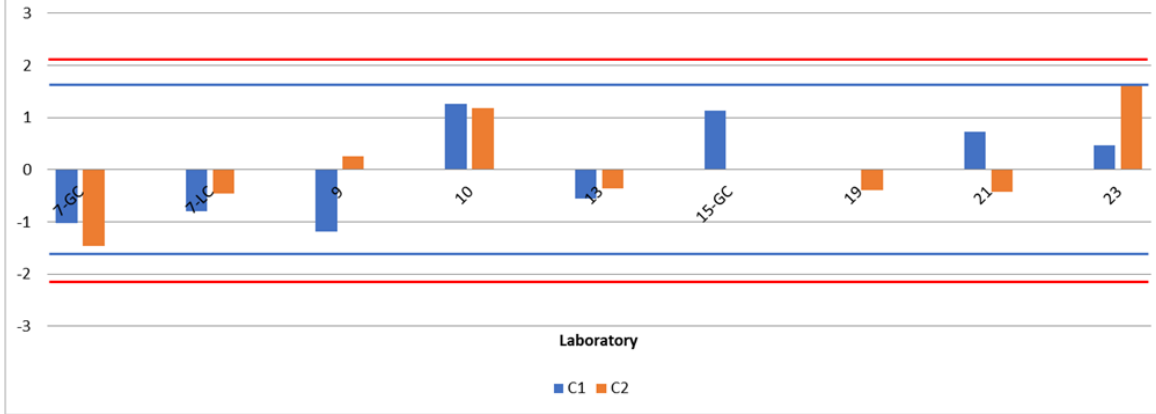
### 17 $\alpha$ -EE2 h statistic



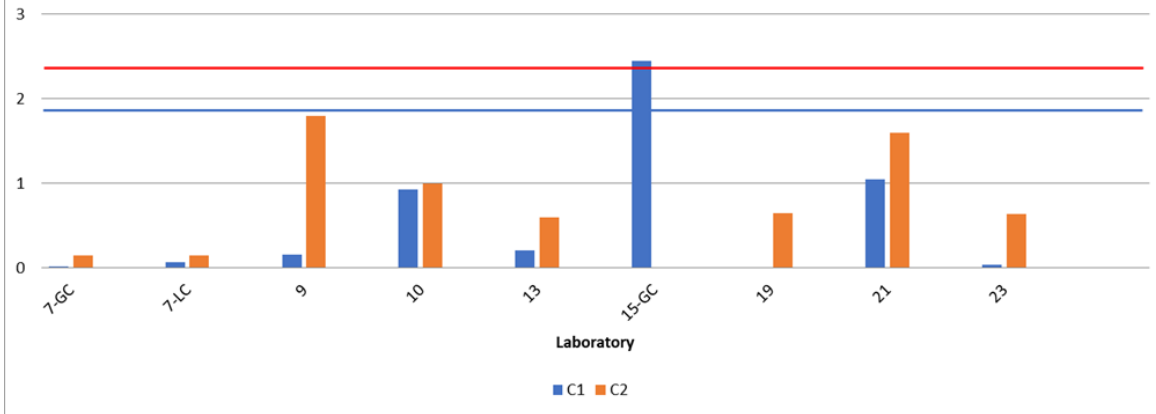
### 17 $\alpha$ -EE2 k statistic



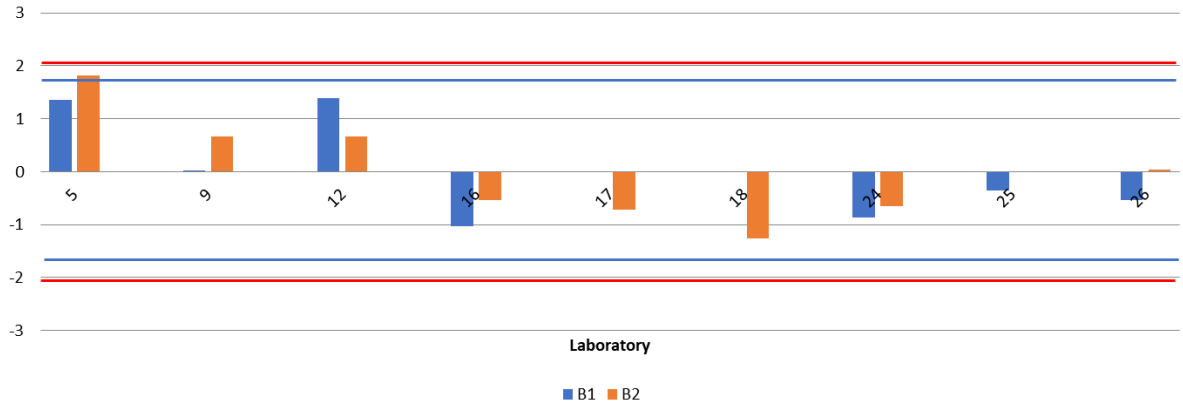
### E3 h statistic



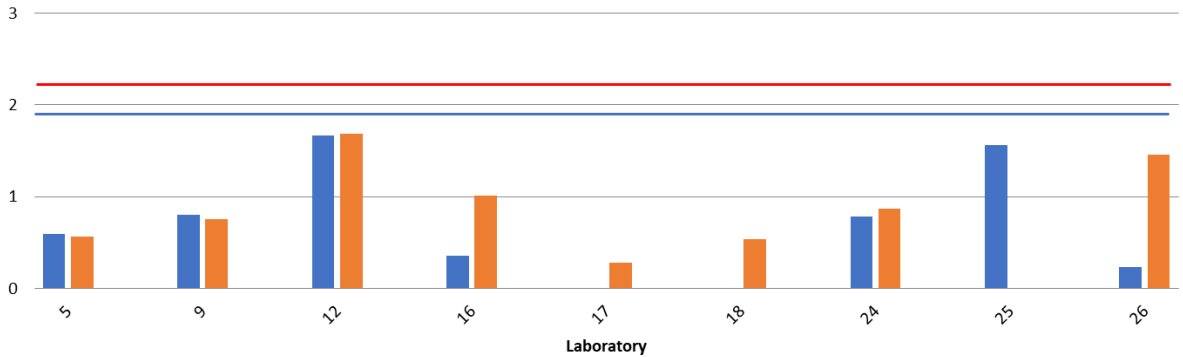
### E3 k statistic



### Cumulative Effect h statistic



### Cumulative Effect k statistic

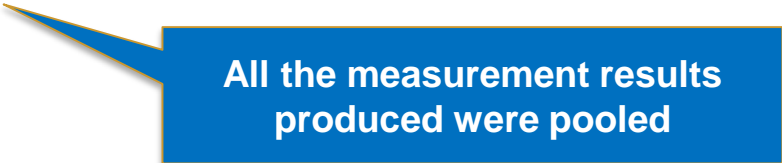


# Data treatment & Statistical evaluation (4)

## Chemical measurements

The performance characteristics of the measurement methods (**repeatability- $S_r$**  and **reproducibility- $S_R$** ) calculated according to ISO 5725-2:2019.

For each RM the mean value is calculated based on the laboratories' measurement results (the number of laboratories  $n$  and associated valid data considered for each parameter  $m$  ( $n/m$ )).



All the measurement results produced were pooled

<b>EDC-WFD C1</b>	<b>S<sub>r</sub> %</b>	<b>S<sub>R</sub> %</b>	<b>Mean value * ng/L</b>	<b>Reference value ng/L</b>
17-bE2	7,5	13,9	0,25 (12/24)	0,22
17-aEE2	10,5	36,9	0,034 (12/24)	0,035
E1	7,2	16,1	0,39 (14/28)	0,40
17-aE2	7,9	18,6	0,23 (10/20)	0,20
E3	7,7	14,8	0,44 (8/16)	0,41

\* Between brackets number of labs and valid data (i.e 12/24)

<b>EDC-WFD C2</b>	<b>S<sub>r</sub> %</b>	<b>S<sub>R</sub> %</b>	<b>Mean value * ng/L</b>	<b>Reference value ng/L</b>
17-bE2	11,8	14,8	1,16 (15/30)	1,01
17-aEE2	5,9	12,8	0,21 (13/26)	0,21
E1	5,7	18,3	0,92 (15/30)	1,01
17-aE2	7,9	16,0	1,02 (10/20)	1,02
E3	4,7	8,6	1,01 (8/16)	1,01

# Data treatment & Statistical evaluation (5)

## Effective Based Methods

The performance characteristics of the measurement methods (**repeatability- $S_r$**  and **reproducibility- $S_R$** ) calculated according to ISO 5725-2:2019.

Considering the number of valid data for EBMs, not sufficient to give reliable  $S_r$  and  $S_R$  values, two different evaluation approach were carried out

Measurement results were **grouped** by the two main different bioassays (Era-Calux and A-YES), providing **only indicative  $S_r$  and  $S_R$  values** if statistically possible

Measurement results obtained by different bioassays (ISO 19040-1, ISO 19040-2, ISO 19040-3 and p-YES) were **pooled**

## EDC-WFD B1

	$S_r$ %	$S_R$ %	mean value ng/L $\beta$ E2 eq
Cumulative effect	<b>19,4</b>	<b>56,4</b>	<b>0,31 (7/14)</b>

\* *Between brackets number of labs and valid data (i.e 12/24)*

## EDC-WFD B2

	$S_r$ %	$S_R$ %	mean value ng/L $\beta$ E2 eq
Cumulative effect	<b>10,8</b>	<b>60,8</b>	<b>1,17 (8/16)</b>

\* *Between brackets number of labs and valid data (i.e 12/24)*

Bioassay		Reproducibility (%)	Repeatability (%)	Mean value (*) ng/L $\beta$ E2 eq
EDC-WFD B1	<u>ER<math>\alpha</math>-Calux</u>	61,8	11,1	0,33 (3-6)
	<u>A-YES</u>	29,1	29,0	0,21 (3-6)
EDC-WFD B2	<u>ER<math>\alpha</math>-Calux</u>	74,2	7,3	1,29 (4-8)
	<u>A-YES</u>	37,8 (CV%)	16,0 (CV%)	0,95 (2-4)

Cumulative  
effect

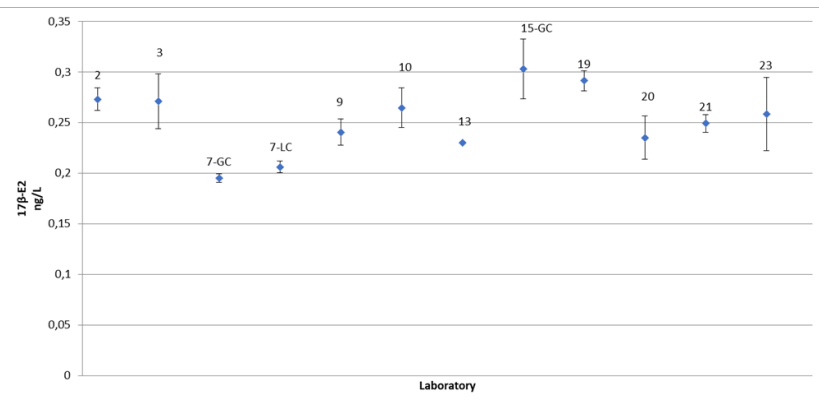
\* Between brackets number of labs and valid data (i.e 12/24)

**Indicative values**  
**Insufficient valid data**

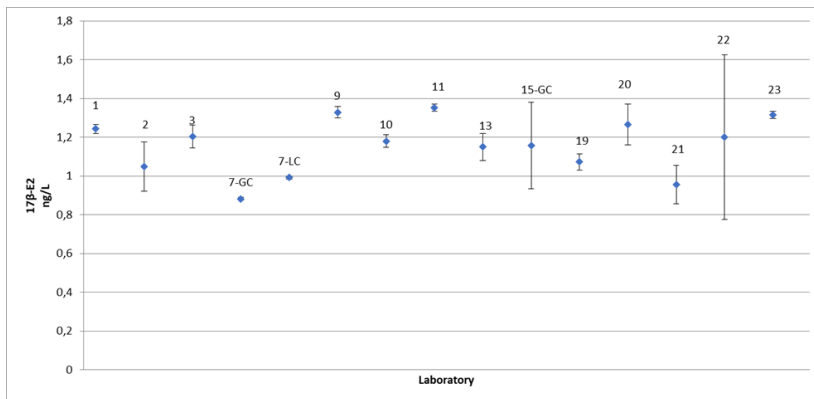


# Chemical measurements and repeatability

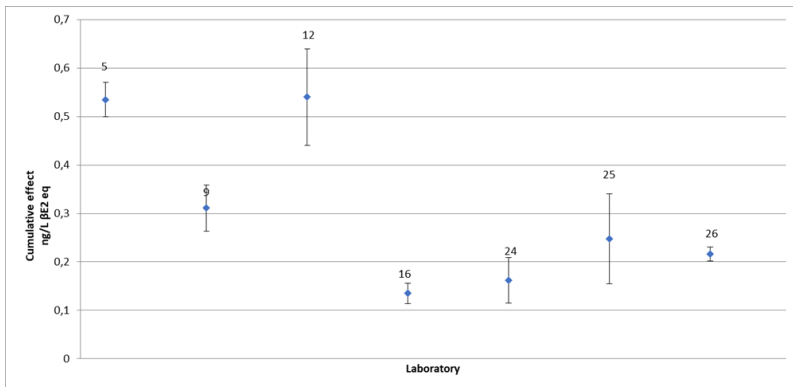
**EDC-WFD C1  
17-bE2**



**EDC-WFD C2  
17-bE2**

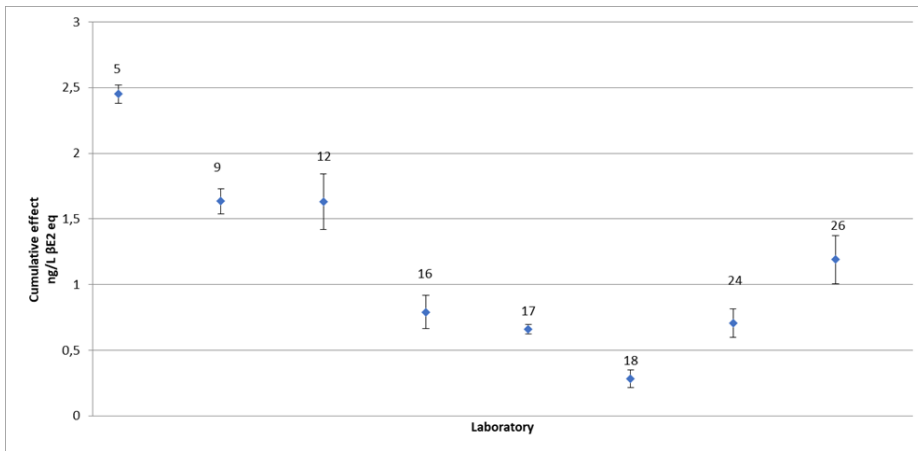


# EBMs measurements and repeatability



**EDC-WFD B1  
Cumulative effect**

**EDC-WFD B1  
Cumulative effect**

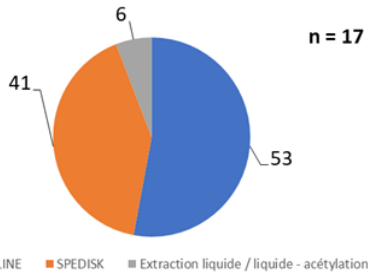


# Chemical measurement procedures

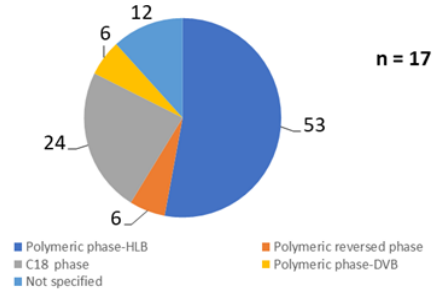
Lab code	Extraction technique	Extraction sorbent	Purification sorbent	Instrumental analysis technique	NOTE/COMMENTS:
1	SPE offline	Polymeric phase-HLB		LC-HRMS	
2	SPE offline	Polymeric phase-HLB		LC-MS/MS	
3	SPE offline	Polymeric phase-HLB	SPE online su C18	LC-MS/MS	DOC spiking solution added it to the samples without filtering
7-GC	Speedisk	Polymeric phase-HLB	MISPE	GC-MS/MS	
7-LC	Speedisk	Polymeric phase-HLB	MISPE	LC-MS/MS	
8	SPE offline	Polymeric phase-HLB	LC-NH2	LC-MS/MS	
9	SPE offline	Polymeric reversed phase		LC-MS/MS	Agilent 1290 Infinity II - Agilent G6495A Triple Quadrupole (QQQ)
10	SPE offline	Polymeric phase-HLB	LC-NH2	LC-MS/MS	2 analytical methodologies LC/MSMS. First one to analyse 17 E2, 17βE2, E1, E3, 17 E2-d2, 17βE2-13C3, E1-13C3 and E3-d2; organic extract conditioned in 100% MeOH with 5 L injected. The second one, more specific, to analyse 17 EE2 and 17 EE2-d4; organic extract conditioned in 70:30 MQ water / MeOH(v/v) with 100 L injected.
11	SPE offline			LC-MS/MS	
13	Speedisk	C18 phase	LC-NH2	LC-MS/MS	
15-GC	Speedisk	C18 phase	SIOH	GC-MS/MS	
15-LC	Speedisk	C18 phase	SIOH	LC-MS/MS	
19	Speedisk	Polymeric phase-DVB		GC-MS/MS	
20	SPE offline	Polymeric phase-HLB		LC-MS/MS	
21	SPE offline	Polymeric phase-HLB	SIOH	LC-MS/MS	Enrichment Factor: 1000
22	Extraction liquide / liquide - acétylation			GC-MS/MS	Standard extracted under the same conditions as the samples
23	Speedisk	C18 phase	LC-NH2	LC-MS/MS	

Lab code	Extraction technique	Extraction solvent	Specify Other	Purification sorbent	Bioassay	Data Treatment	NOTE/COMMENTS:
5	SPE offline	Polymeric phase-HLB		none	Era-Calux	PCx model. Effect level at 5% 3 Parameters Logistic Function	
9	SPE offline	Polymeric reversed phase	Strata XL (Phenomenex)	none	Era-Calux	PCx model. Effect level at 10% 4 Parameters Log Logistic Function.	For further information on data treatment see also Simon et al (2022) Evaluation of Three ISO Estrogen Receptor Transactivation Assays Applied to 52 Domestic Effluent Samples. Environmental Toxicology and Chemistry -Volume 41, Number 10 - pp. 2512-2526 as well as International Organization for Standardization. (2018c). ISO 19040-3 Water quality—Determination of the estrogenic potential of water and waste water—Part 3: In vitro human cell-based reporter gene assay and International Organization for Standardization. (2022). ISO 23136 Water quality—Calculation of biological equivalence (BEQ) concentrations
12	SPE offline	Polymeric phase-HLB		none	P-YES	Reverse Function Model (for Individual estrogen). 5 Parameters Logistic Function.	p-YES allow in addition to the effect based analysis the quantification of estrogenic compounds such as E1, EE2, bE2 and E3.  REP were determined by quantification of compound signals against the E2-Calibration to express the signal as E2-Equivalence. The REP are the quotient of the calculated E2-Equivalence divided by the amount of the compound exposed.  Quantifications for individual compounds (mean value with n=3 +/- CI 35%) E1, beta-E2 and EE2 are reported in ng/L. alpha-E2 is reported in the beta-E2 equivalence concentration in ng/L: EDC-WFD-B1.1: beta-E2: 0.27 +/- 0.17; EE2: 0.06 +/- 0.03; E1: 0.68 +/- 0.12; alpha-E2: 0.655 +/- 0.02 EDC-WFD-B1.2: beta-E2: 0.24 +/- 0.03; EE2: 0.08 +/- 0.05; E1: 0.73 +/- 0.34; alpha-E2: 0.11 +/- 0.16 EDC-WFD-B2.1: beta-E2: 1.07 +/- 0.23; EE2: 0.27 +/- 0.13; E1: 1.47 +/- 0.29; alpha-E2: 0.18 +/- 0.06 EDC-WFD-B2.2: beta-E2: 0.92 +/- 0.24; EE2: 0.22 +/- 0.08; E1: 1.08 +/- 0.59; alpha-E2: 0.14 +/- 0.02
16	Speedisk	Polymeric phase-HLB		MISPE	Era-Calux	PCx model. Effect level at 10% 4 Parameters Logistic Function	
17	SPE offline	Polymeric reversed phase	Polystyrene/divinyl benzene	none	YES	4 Parameters Logistic Function. Effect level not specified	For B2 (unit 1 and unit 2) we didn't have enough DOC spiking solution. So instead of adding 4540 µl to the sample we added only 3500 µl.
18	SPE offline	Polymeric phase-HLB		none	Era-Calux	According to B03 calculation sheet 4 Parameters Logistic Function.	EDC-WFD B2, unit 2: only 0,72 L instead of 1 L could be extracted via SPE due to clogging of the cartridge.
24	SPE offline	C18 phase			A-YES	BioVAL® software (new_diagnostics GmbH), calculations according to ISO 19040-2:2018. 4 Parameters Logistic Function. Effect level at 10%	Enrichment factor for each sample was 250.
25	SPE offline	Polymeric reversed phase		none	A-YES	BioVAL® software (new_diagnostics GmbH), calculations according to ISO 19040-2:2018. 4 Parameters Logistic Function.	Due to an error in sample preparation, the sample composition to be set for the test of sample B1, unit 2, was diluted with drinking water. Sample B2 unit 1 did not re-dissolve well after SPE.
26	SPE offline	C18 phase		none	A-YES	BioVAL® software (new_diagnostics GmbH), calculations according to ISO 19040-2:2018. 4 Parameters Logistic Function.	After SPE with C18 phase, extracts are dried in N2 stream and then dissolved in DMSO. The DMSO extract is diluted with H2O for the A-YES assay to a final DMSO percentage of 5%. Method recently established; clean-up step for the extract was not possible.

### Extraction

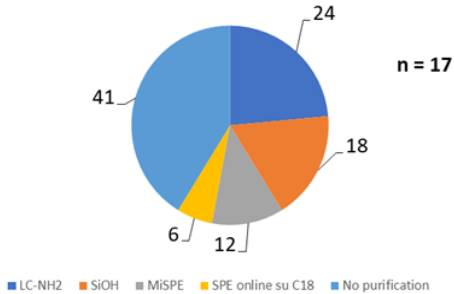


### Extraction sorbent

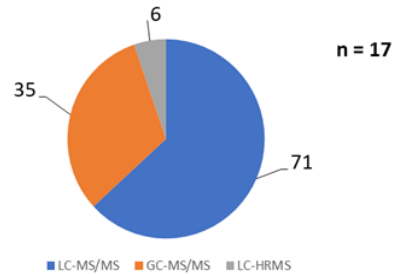


1

### urification sorbent



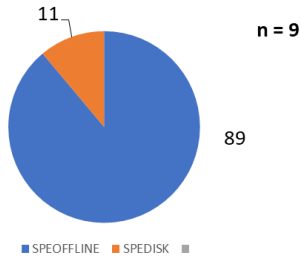
### Instrumental techniques



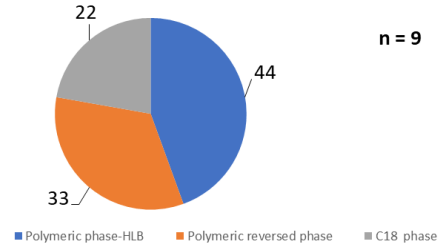
# EDC-WFD C1/C2

Including labs not considered in the statistics

Extraction

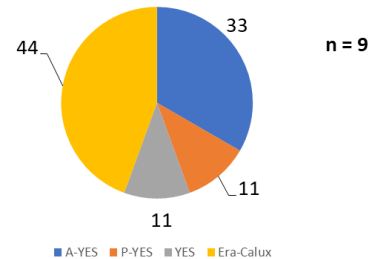


Extraction sorbent



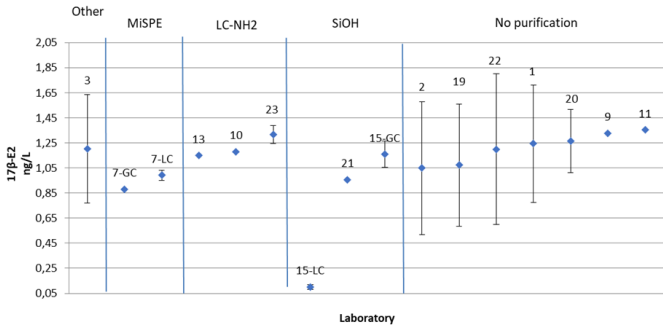
Only 1 lab  
performed  
purification  
step

Bio-assays

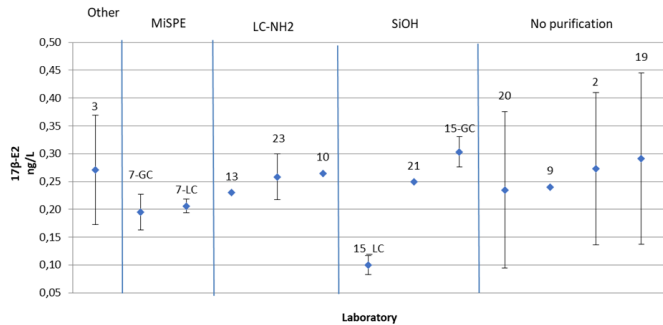


## EDC-WFD B1/B2

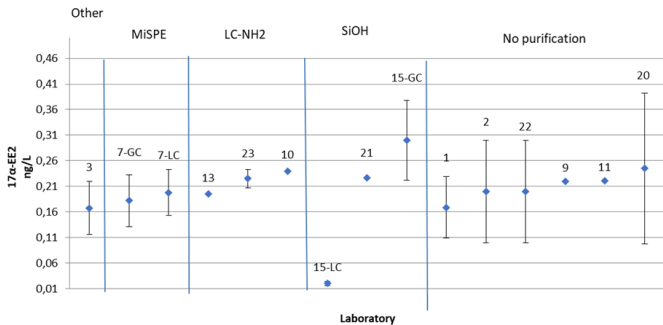
### Purification sorbent



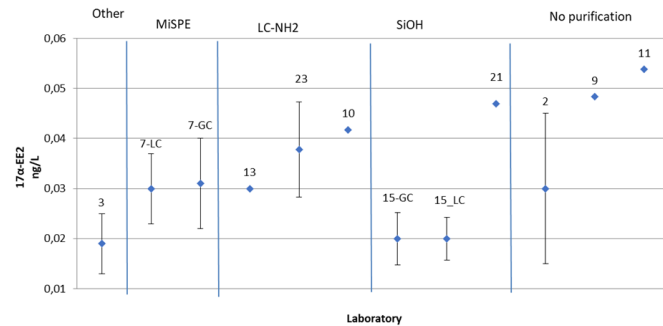
### Purification sorbent



### Purification sorbent



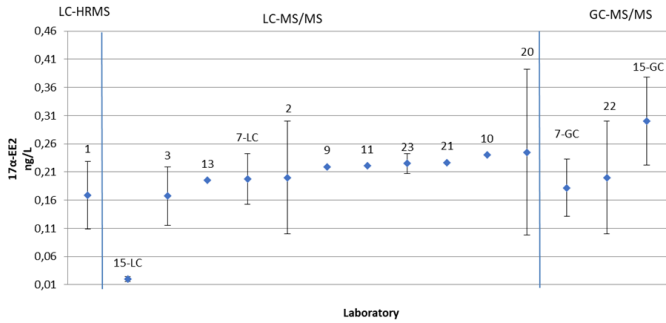
### Purification sorbent



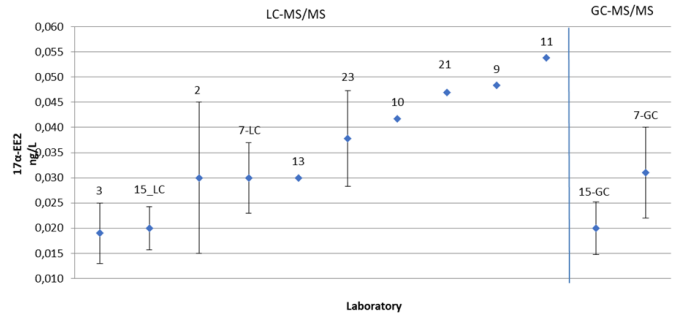
C1

C2

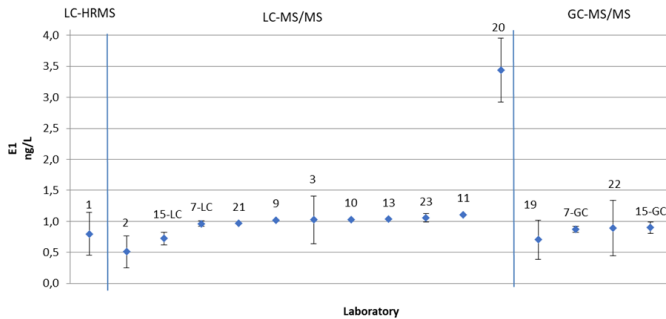
### Instrumental analysis technique



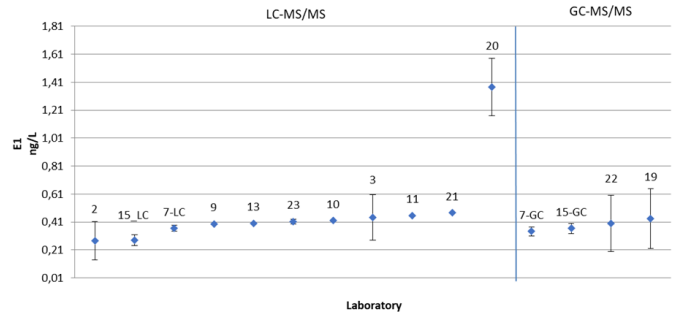
### Instrumental analysis technique



### Instrumental analysis technique



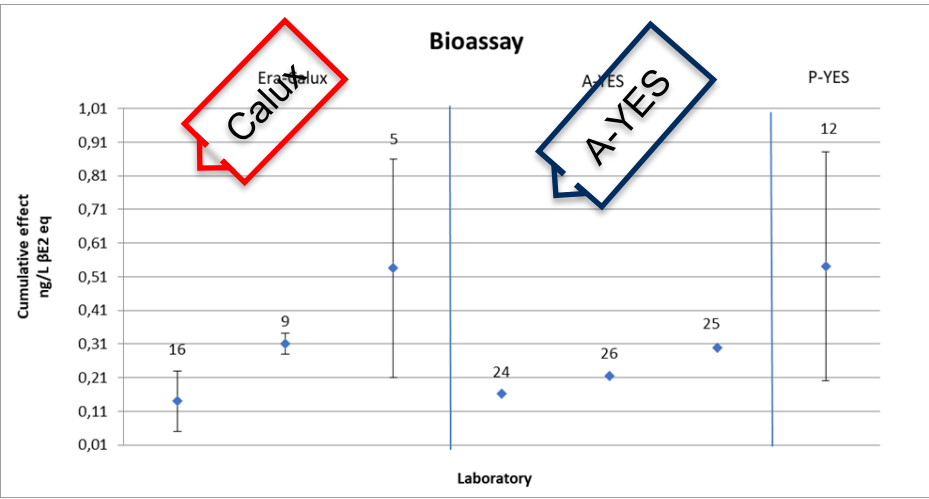
### Instrumental analysis technique



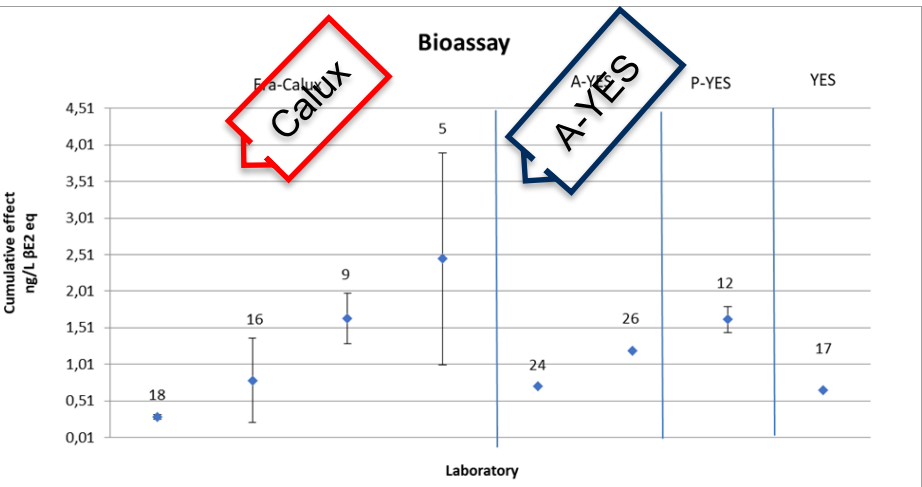
C1

C2





**B1**



**B2**

## ***Some considerations (1)***

- Very good agreement between the reference concentration of 5 estrogens in the RMs, gravimetrically determined, and the mean values calculated based on the laboratories chemical measurement results.
- Good application of the measurement procedures by the labs within the ILC context, including also the demanding reconstitution step.

## ***Some considerations (2)***

- General good performance characteristics are observed for both level of concentration.
- Repeatability within the laboratories below 12 %, while reproducibility values fall below 19%; the only exception is 17 $\alpha$ EE2 in EDC-WFD C1 (reproducibility around 37%) probably due to the lower and challenging concentration of 17 $\alpha$ EE2 (0,035 ng/L)
- Not possible to separate performance characteristic by technical instrument

## ***Some considerations (3)***

The evaluation of performance characteristics for EBM is conditioned by the low number of labs and valid data.

Outputs interesting, considering the characteristic of the RMs, mimicking real samples:

- Concentration significantly lower than those tested in ILC reported in ISO 19040-2 (A-YES) and ISO 19040-3 (ER $\alpha$ -Calux): the lowest concentrations in ISO were respectively 6,4 ng/L  $\beta$ E2 eq and 1,7 ng/L  $\beta$ E2 eq.
- the reduced number of laboratories and valid data for each bioassay may provide only an indicative information on repeatability and reproducibility

## ***Some considerations (4)***

Different data treatment used by the ER $\alpha$ -Calux laboratories (e.g. different fitting and effect levels in percentage) might partially justify the higher variability observed in EBM measurement results.

A-YES laboratories, applying the same data evaluation (BIOVAL), are characterized by lower variability.

EDC-WFD ILC data ( $S_r$  and  $S_R$ ) could also be compared with values observed in ISO 19040-2 (A-YES) and ISO 19040-3 (Calux);

- 20-25 valid data,  $S_r=7,5-21,4\%$  and  $S_R=16,6-28,0\%$ .
- 22-25 valid data,  $S_r=13-18\%$ ,  $S_R=18-33\%$ .

## ***Some considerations (5)***

Labs applied in general the indication given by the ILC protocol. SPE-Offline extraction phase was used with higher frequency together with Polymeric-phase HLB as sorbent. Higher percentage of labs used LC-MS/MS.

In EBMs only one laboratory carried out a purification of the extract

# ***Conclusion (1)***

Laboratories able to apply the demanding reconstitution step.

RMs tested fit for purpose in EDC-WFD ILC and in future experiences

EDC-WFD ILC show good application and performance characteristics of the tested methods for chemical measurements, facing with low concentration of the selected estrogens, close to the laboratories LOQ, with variable content of the interferents

.

## ***Conclusion (2)***

For EBMs, the low number of laboratories in EDC-WFD ILC suggests the need of new interlaboratory trials characterized by:

- challenging samples with low concentration of estrogenic substances;
- higher number of laboratories (public and private), coming from different European countries



***Thank you for the participation and  
for your attention***