

## Interlaboratory exercise on steroid estrogens in aqueous samples

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### Abstract

An inter-laboratory comparison exercise was organized among European laboratories, under the aegis of EU COST Action 636: "Xenobiotics in Urban Water Cycle". The objective was to evaluate the performance of testing laboratories determining "endocrine disrupting compounds" (EDC) in various aqueous matrices. As the main task three steroidal estrogens: 17 $\alpha$ -ethinylestradiol, 17 $\beta$ -estradiol and estrone were determined in four spiked aqueous matrices: tap water, river water and wastewater treatment plant influent and effluent using GC-MS and LC-MS/MS. Results were then compared and discussed according to the analytical techniques applied, the accuracy and reproducibility of the analytical methods and the influence of the sample matrices. Out of a total of fourteen European laboratories that participated in the exercise, eleven laboratories submitted results.

Statistical evaluation of results revealed that the number of outliers in this exercise was low taking into account the complexity of sample matrices and the differences in analytical methods applied. Consensus values are also in good agreement with the spiked concentrations for each compound, with the exception of the wastewater influent sample for which the obtained values differed significantly. This was in agreement with standard deviation of repeatability, inter-laboratory standard deviation and standard deviation of reproducibility. Overall the results obtained in this inter-laboratory exercise reveal a high level of competence among the participating laboratories for the detection of steroidal estrogens in water samples indicating that GC-MS as well as LC-MS/MS can equally be employed for the analysis of natural and synthetic hormones.

*Key Words: Interlaboratory exercise; Estrogenic compounds; 17 $\alpha$ -ethinylestradiol; 17 $\beta$ -estradiol; estrone; tap water; surface water; wastewater; influent; effluent.*

## 1 Introduction

An important group of new emerging pollutants are those capable of disrupting the endocrine system in both humans and animals – well known as endocrine disrupting compounds (EDC). Among them, steroidal estrogens are the most potent since they show the highest affinity for the estrogen receptor. Some of them are naturally synthesized by the human body and are primarily excreted in a non-activated form. However, when they enter a wastewater treatment plant (WWTP), they are reactivated by the enzymes present in the biological treatment process. Despite that, most are readily degraded by bacteria or by abiotic processes, and only a small proportion passes through

the system into receiving waters, where, albeit in a low concentration ( $\text{ng L}^{-1}$ ), they can have negative impact on aquatic organisms such as fish and amphibians resulting in developmental and reproductive problems.

A review of the literature reveals that most of the research to date focuses on EDC pollution of wastewater, wastewater sludge and receiving waters from WWTPs (Environmental Project, 2005). There are also studies determining EDC in various matrices including surface waters, groundwater, and soil (Environmental Project, 2005). More recently the need for a European wide effort to investigate these compounds has culminated in several EU projects, among which is the EU COST Action 636: "Xenobiotics in the urban water cycle". Comparability of monitoring data, especially between different laboratories using different analytical methods, is essential for any meaningful assessment and management of the environmental risks associated with emerging pollutants. For the compounds and matrices where standard reference materials are not available, inter-laboratory exercises are the only way to compare performance of different laboratories. This is especially important when determining pollutants that appear in  $\text{sub ng L}^{-1}$  in environmental samples.

The study presented herein describes an inter-laboratory round robin test for determining steroid estrogens in aqueous samples, held in the framework of the COST Action 636. Organization and development of the activities were performed according to the ISO GUIDE 43/1 - 97. Samples of tap water, river water, WWTP influent and effluent containing known concentrations of three steroidal estrogens:  $17\alpha$ -ethinylestradiol (EE2),  $17\beta$ -estradiol (E2) and estrone (E1) were sent out to each collaborating laboratory for analysis. The main goals of the inter-laboratory comparison were to a) assess the performance of the participating laboratories, b) evaluate applied analytical methods and c) investigate the influence the sample matrices have on the results. Out of a total of fourteen laboratories invited to participate in the exercise, eleven laboratories submitted results.

## **2 Materials and methods**

### **2.1 Experimental design, sample collection and handling**

Samples were collected from the Danish WWTP in Usseørd (20 km north of Copenhagen, Denmark) and a nearby stream (Usseørd stream) by the Department of Environmental Engineering at the Technical University of Denmark. The samples were filtered ( $0.45 \mu\text{m}$  glass fibre filters), pH adjusted to 2.5 - 3 with phosphate buffer to guarantee sample stability, spiked with known amounts of the study compounds, homogenized and finally stored at  $4^\circ\text{C}$  prior to shipment.

On receiving the samples each laboratory was instructed to perform an additional filtration step ( $0.45 \mu\text{m}$ ). Stability tests were performed by the inter-laboratory test organisers and consisted of analysing under repeatable conditions at set times (0 h; 5 h; 24 h; 48 h; 168 h) sub-samples of a solution of known concentration. In all cases no significant differences in the concentration were observed and the samples were stable over a seven day period. The analytical methods were previously developed in each participant laboratory according to the available equipment and skills. The homogeneity of samples was ensured by the method applied for the preparation of samples. No common procedures were predetermined and for statistical evaluation, laboratories were asked to perform analyses in triplicate.

The analytical procedures were all based on solid-phase extraction; additionally, some laboratories applied a clean-up and derivatisation step (Table 1). For the analyses either liquid or gas chromatography coupled to a mass spectrometry (GC-MS, LC-MS/MS) were used. Five laboratories that submitted results used GC-MS (laboratories number 2, 4, 5, 7 and 9), while the remainder (laboratories number 3, 6, 10, 12, 13 and 14) used LC-MS/MS. Details about the individual laboratory methods are published in the COST Action 636 Report (Cruceu, 2008).

Table 1: Applied Pre-Treatment Techniques

Pre-treatment technique	Number of Laboratories
<b>Solid-Phase Extraction:</b> OASIS HLB 200mg/6mL; OASIS HLB 60 mg/3mL; C18 (EC); C18 1g/6 mL; Strata X 100mg; ENV 500mg	11
<b>Clean-up:</b> Fluorisil 1g/6 mL, LC-NH <sub>2</sub> cartridge, Silica gel	5
<b>Derivatisation:</b> MSTFA, BSTFA, pentafluorobenzoyl chloride	5

The deadlines for performing pre-treatment procedures, analysis and submission of the results were defined by the organiser of the scheme (Crucecu, 2008).

## 2.2 Chemicals

For sample preparation 17 $\alpha$ -ethinylestradiol (>99%), 17 $\beta$ -estradiol (>98%) and estrone (>99%) were obtained from Sigma Aldrich, Germany. Individual stock solutions (1 g L<sup>-1</sup>) used for spiking native water samples were prepared in methanol and stored at -18°C.

## 2.3 Statistical parameters

Statistical analysis included both classical and robust approach (ISO GUIDE 43/1 - 97). Outliers were eliminated using classical statistics (Cochran, Grubbs- single and double test, Mandel-h and k test) in accordance with the ISO standard ISO 5725/2 -1994. The reallocation of incorrect statistical results was achieved by robust statistic (ISO 13528-2002). The assigned value ( $\bar{X}$ ) is a consensus value of the results and was given as the mean result (in classical statistics) or as the robust mean (in robust statistics). The standard deviation ( $\sigma$ ) is used to assess the proficiency of the participating laboratories in the scheme and was calculated as the mean standard deviation/robust standard deviation. Values submitted by a laboratory in relation to all participating laboratories in a single round were expressed as a z-score according to the following equation:

$$z = \frac{(\bar{x} - \bar{X})}{\sigma},$$

where  $\bar{x}$  represents the mean of the submitted three replicate values,  $\bar{X}$  assigned value and  $\sigma$  the standard deviation of the inter-laboratory testing scheme.

## 3 Results and Discussion

The z-score values (Figure 1) suggest that the majority of laboratories are proficient in testing steroid estrogens in water samples ( $|z| \leq 2$  was obtained by all the laboratories for the analysis of tap water, 89% for surface water, 85% for wastewater influent and 95% for wastewater effluent). In case of analysis from wastewater influent (e.g. of 17 $\alpha$ -ethinylestradiol) some laboratories obtained questionable z-score ( $2 > |z| \leq 3$ ) or even unsatisfactory results ( $|z| > 3$ ). In most cases comparable z-score values were obtained using both statistical methods, but sometimes, when questionable and/or unsatisfactory results were identified, the robust statistic offered better values. Figure 1 gives such an example. For statistical evaluation of results, identified outliers were left out.

Table 2 shows that in most cases there are good agreement between the consensus values and the spiked concentrations. An exception is represented by the higher values of the consensus concentrations obtained for all the steroid estrogens in wastewater influent, where the matrix composition had a significant effect on the determined values.

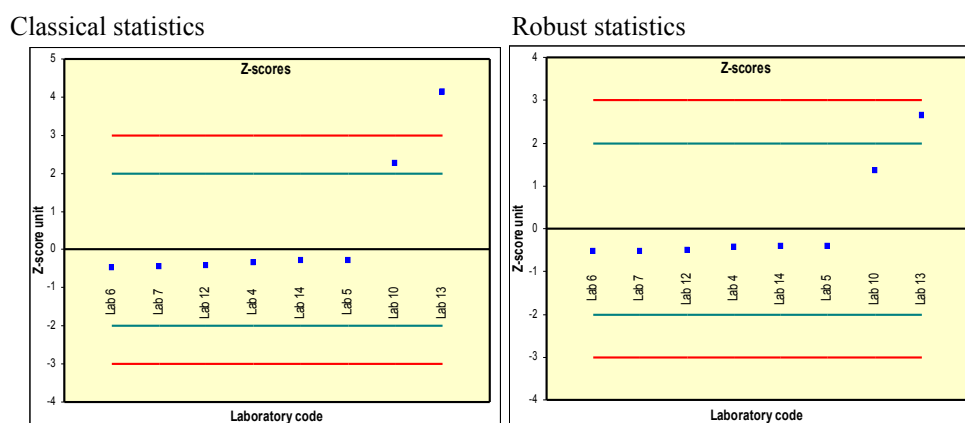
Figure 1: z-score values for 17 $\alpha$ -ethinylestradiol in wastewater influent

Table 2: Comparison of consensus values and spiked concentrations

Type of matrix	Consensus value		Spiked concentration (ng L <sup>-1</sup> )
	Classical approach (ng L <sup>-1</sup> )	Robust approach (ng L <sup>-1</sup> )	
<b>A-Tap Water</b>			
17 $\alpha$ –Ethinylestradiol	11.5	12.4	13.0
17 $\beta$ –Estradiol	14.0	14.0	15.0
Estrone	12.7	12.7	13.0
<b>B-Surface sample</b>			
17 $\alpha$ –Ethinylestradiol	2.13	2.25	2.60
17 $\beta$ –Estradiol	2.70	2.82	3.00
Estrone	4.65	4.96	4.00
<b>C-WWTP Influent</b>			
17 $\alpha$ –Ethinylestradiol	7.80	9.80	4.90
17 $\beta$ –Estradiol	46.2	42.9	28.0
Estrone	72.9	72.9	29.0
<b>D-WWTP Effluent</b>			
17 $\alpha$ –Ethinylestradiol	3.83	4.35	4.10
17 $\beta$ –Estradiol	14.2	13.8	14.0
Estrone	12.3	12.3	12.0

With respect to the compounds under investigation, the laboratory performance represented by the standard deviation of repeatability ( $s_r$ ), the interlaboratory standard deviation ( $s_L$ ) and the standard deviation of reproducibility ( $s_R$ ) was comparable (Table 3). The poorest laboratory performance resulted from analysing wastewater influent samples (Cruceru, 2008), which is due to the much more complex matrix compared with e.g. tap water.

Table 3: Performance parameters of each analyte according to different matrices

Analyte	Standard Deviation of Repeatability s(r)- (ng L <sup>-1</sup> )	Interlaboratory Standard Deviation s(L)- (ng L <sup>-1</sup> )	Standard Deviation of Reproducibility s(R)- (ng L <sup>-1</sup> )
<b>17 <math>\alpha</math> –Ethinylestradiol</b>			
A-Tap Water	1.13	3.65	3.82
B-Surface Water	0.616	1.40	1.53
C-WW Influent	1.88	13.9	14.0
D-WW Effluent	2.05	4.77	5.20
<b>17 <math>\beta</math> –Estradiol</b>			
A-Tap Water	1.40	5.83	5.99
B-Surface Water	0.365	0.827	0.904
C-WW Influent	4.70	17.6	18.2
D-WW Effluent	1.53	3.61	3.92
<b>Estrone</b>			
A-Tap Water	1.11	4.04	4.18
B-Surface Water	1.07	2.48	2.70
C-WW Influent	6.04	27.5	28.1
D-WW Effluent	1.64	3.40	3.78

Table 4 a and b compares the data obtained by GC/MS and LC-MS/MS. From results presented in these tables, it can be concluded, that even with an extra derivatisation step in the GC method, it performs slightly better in terms of repeatability and reproducibility than LC-MS/MS.

Table 4: a) Performance parameters for GC-MS method

Analyte	Standard Deviation of Repeatability s(r) (ng L <sup>-1</sup> )	Interlab. Standard Deviation s(L) (ng L <sup>-1</sup> )	Standard Deviation of Reproducibility s(R) (ng L <sup>-1</sup> )
<b>17 <math>\alpha</math> –Ethinylestradiol</b>			
A-Tap Water	1.08	3.31	3.48
B-Surface Water	0.286	0.459	0.541
C-WW Influent	1.35	1.52	1.73
D-WW Effluent	0.837	0.958	1.27
<b>17 <math>\beta</math> –Estradiol</b>			
A-Tap Water	1.50	5.23	5.49
B-Surface Water	0.419	1.14	1.21
C-WW Influent	4.75	7.75	9.09
D-WW Effluent	1.80	2.36	3.00
<b>Estrone</b>			
A-Tap Water	1.10	3.62	3.79
B-Surface Water	1.43	2.93	3.26
C-WW Influent	3.05	29.8	30.0
D-WW Effluent	1.91	3.04	3.59

Table 4 b) Performance parameters for LC-MS/MS method

Analyte	Standard Deviation of Repeatability s(r) (ng L <sup>-1</sup> )	Interlab. Standard Deviation s(L) (ng L <sup>-1</sup> )	Standard Deviation of Reproducibility s(R) (ng L <sup>-1</sup> )
<b>17 <math>\alpha</math> –Ethinylestradiol</b>			
A-Tap Water	1.35	5.49	5.65
B-Surface Water	0.822	2.03	2.19
C-WW Influent	2.12	16.5	16.6
D-WW Effluent	2.55	6.11	6.62
<b>17 <math>\beta</math> –Estradiol</b>			
A-Tap Water	1.31	4.57	4.76
B-Surface Water	0.301	0.371	0.477
C-WW Influent	4.66	22.2	22.6
D-WW Effluent	1.17	3.65	3.83
<b>Estrone</b>			
A-Tap Water	1.11	4.32	4.26
B-Surface Water	0.493	1.41	1.49
C-WW Influent	7.33	27.9	28.8
D-WW Effluent	1.34	3.12	3.40

## 4 Conclusions

Results of the inter-laboratory exercise demonstrate a high level of competence among the participating laboratories to measure steroidal estrogens in aqueous samples. The number of outliers obtained in this exercise was low taking into account the range of concentrations measured during the exercise, the complexity of the sample matrices and analytical methods used. The data obtained on the same samples show that GC-MS has a slightly better laboratory performance than LC-MS/MS. With regards to matrices, the WWTP influent sample proved most problematic showing the highest standard deviation in terms of repeatability and reproducibility, while for all other matrices under investigation satisfactory results were obtained.

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