APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

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Estrogen Monitoring in River Water by LC/MS/MS with Online SPE

Introduction

Endocrine disrupting chemicals (EDCs) have been found to have harmful effects on the health of both humans and wildlife. Of these EDCs, endogenous estrogens, such as estriol, 17ß-estriadiol, and estrone are of particular interest due to their potency/physiological activity even at very low levels[1-5] (their structures are shown in Figure 1). This makes them an important environmental target, as hormonal drugs are frequently discarded by flushing them down the drain, their contents making their way into rivers and lakes. These hormones are particularly challenging to monitor, as concentrations are typically expected to be in the low ng/mL (ppb) to low pg/mL (ppt) range.

To improve sensitivity, this work utilizes the addition of on-line solid phase extraction (SPE) coupled to an LC/MS/MS system for sample enrichment and quantitation. This approach allows for significant/efficient analyte concentration, obviating the need for elaborate and time-consuming sample preparation procedures. Due to enrichment, compared to other methods, large sample volumes (≥ ½ liter) are no longer needed to reach part per trillion levels for estrogen monitoring in river water.

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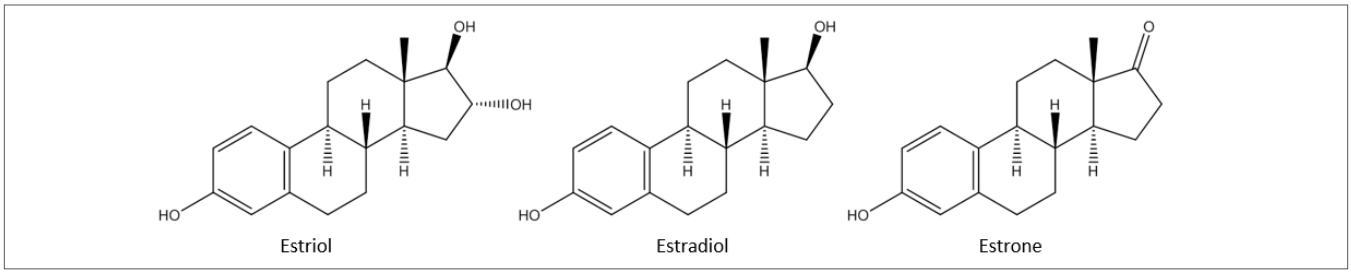

Figure 1. Chemical structures of the three estrogens analyzed in this study.

Experimental

Hardware/Software

Online analyte pre-concentration/enrichment, chromatographic separation, and quantitation were accomplished with a PerkinElmer QSight® SP50 Online SPE System and QSight 220 MS/MS Detector. All instrument control, analysis and data processing were performed using the Simplicity™ 3Q software platform.

Online SPE is accomplished through two six-port valves on the autosampler and a high-pressure dispenser (HPD). Per Figure 2, valve A is dedicated to SPE, while valve B allows for the flexible switching from direct injection to online SPE mode.

The system was configured with a 10 µL stainless steel needle, 1 mL sample loop, 1 mL syringe and 2 mL buffer tubing. Conditioning and equilibration solvents are delivered via the HPD and are directed to waste upon passing through the SPE cartridge. The sample is then aspirated into the sample loop using the autosampler syringe and subsequently transferred via a load solvent from the loop to the SPE cartridge. For this application, for somewhat saline water matrices, this was followed by an optional water rinse, allowing any residual salts to be eluted off the cartridge. It should be noted that initial testing indicated that there were no significant analyte recovery differences with and without this rinse; however, to protect against possible matrix salinity, this step was included here. Analytes are then eluted off the SPE cartridge and onto the analytical column using the LC gradient. There is no separate SPE elution step needed, as the focused analytes on the SPE cartridge are eluted right onto the analytical column, as part of the chromatographic run.

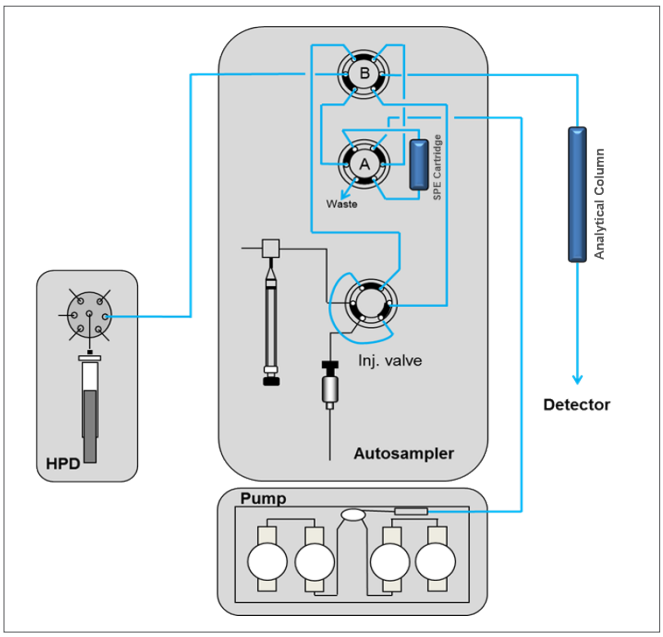

Figure 2. Schematic of QSight SP-50 Automated Sample Handler with fixed online SPE.

For this method, sample enrichment was accomplished by loading a total of 2 mL of sample onto the SPE cartridge via a 2 x 1000-uL stacked loading process. The SPE parameters for this method are shown in Table 1.

A 24-vial tray was used, accommodating 10-mL sample vials (Part# N9300922; 100-vial/caps with integrated septa kit).

Method Parameters

The SPE, LC and MS/MS method parameters are shown in Tables 1-4.

Table 1. SPE Parameters.

SPE Cartridge	PerkinElmer Brownlee Spheri-5 C18, 5 µm, 2.1 x 30 mm (Part# 07110014, 2/pkg)
SPE Solvents	Methanol (conditioning); HPD port# 2 Water (equilibration/loading); HPD port# 3
SPE Program	(2x Stacked Sample Mode; Flow Rate = 1.5 mL/min for all Steps)

Step	Step Type	Methanol (mL)	Water (mL)	Sample (mL)
1	Wash/Conditioning	1.0	-	-
2	Equilibration	-	1.0	-
3	Sample Loading into 1-mL Loop	-	-	1.5*
4	Sample Loading onto SPE cartridge	-	1.5	-
5	Sample Loading into 1-mL Loop	-	-	1.5*
6	Sample Loading onto SPE cartridge	-	1.5	-
7	Wash	-	1.0	-

** The total sample load volume onto SPE cartridge is 2.0 mL (a fixed-loop injection mode is used, in which 1 mL of the 3-mL sample load goes to waste).*

Table 2. LC Parameters.

Analytical Column	PerkinElmer Brownlee SPP C18, 2.7 µm, 3.0 x 100 mm (Part# N9308410)
Mobile Phase	Solvent A: Water with 0.1% Formic Acid Solvent B: Acetonitrile with 0.1% Formic Acid Solvent Program:

Step	Time (min.)	Flow Rate (mL/min.)	%A	%B
1	0.0	0.6	70	30
2	2.0	0.6	30	70
3	3.0	0.6	30	70
4	3.5	0.6	70	30
5	7.0	0.6	70	30

Analysis Time	3.5 min.; Equilibration Time: 3.5 min.
Pressure	3500 psi/233 bar maximum
Oven Temp.	30 °C

Table 3. MS/MS Parameters.

Compound	ESI Mode	Ret. Time (min)	Time-Managed MRM™ Group	Precursor Ion	Frag. Ion 1 (Quantifier)	EV1	CCL2	CE1	Frag. Ion 2 (Qualifier)	EV1	CCL2	CE1
Estriol	+	1.36	0.0 – 2.0 min	271.3	133.1	15	-100	-38	253.2	15	-65	-13
Estradiol	+	2.29	1.5 – 3.5 min	255.3	159.1	15	-56	-28	133.1	15	-68	-24
Estrone	+	2.58	1.5 – 3.5 min	271.3	133.1	15	-70	-28	253.2	15	-65	-16

Table 4. MS/MS Source Parameters.

Parameter	Setting
Ionization Mode	ESI; Positive
Drying Gas	120
HSID Temperature (°C)	310
Nebulizer Gas	240
Electrospray Voltage (V)	5000
Source Temperature	400
Detection Mode	Time-Managed MRM™

Solvents, Standards and Samples

All solvents were HPLC grade and the river water sample was filtered using a 0.45-µm nylon filter.

The estradiol, estrone and estriol standards were obtained from Sigma-Aldrich®, Inc (Allentown, PA). A 10-ppm estrogen stock standard solution was prepared using a 50:50 methanol/water diluent.

A river water sample was collected from the lower end of the Housatonic River in Bridgeport, Connecticut. This water was first filtered using a 0.45-µm filter and then stabilized by adding 0.5% acetonitrile.

A 10-ppb working standard solution was then prepared from the 10-ppm stock standard, using filtered river water as diluent. All calibrants were prepared via serial dilution from the working standard, using the filtered river water as diluent. The calibration levels ranged from 0.05 to 5.0 ppb for estriol and 0.01 – 5.0 ppb for estradiol and estrone.

Results and Discussion

Figure 3 shows the chromatographic separation of the 2.5-ppb estrogen standard mix.

Per Figure 4, chromatographic repeatability was found to be exceptional, here shown via the chromatographic overlay of ten replicate 2.5-ppb standard mix injections.

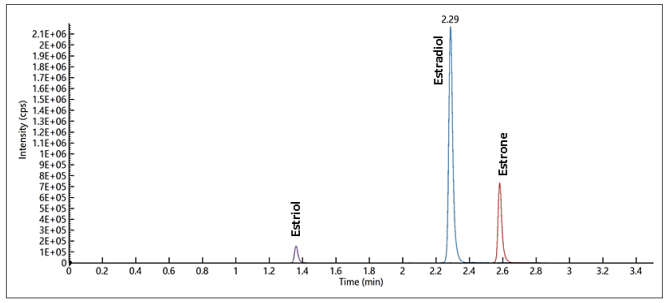

Figure 3. Overlaid EIC chromatograms, showing the separation of the 2.5-ppb estrogen standard mix.

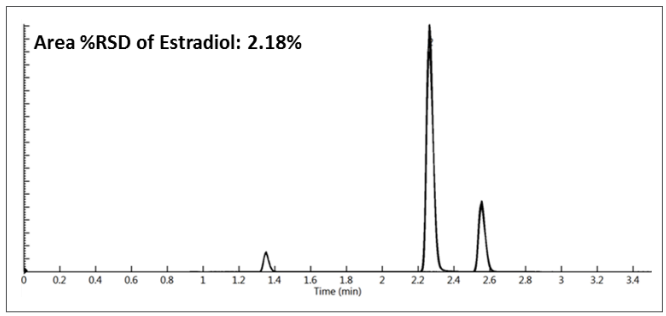

Figure 4. Overlay of ten replicates of the 2.5-ppb estrogen mix spiked into river water.

The linearity plots for the three estrogens are shown in Figure 5, with R^2 values all above 0.999.

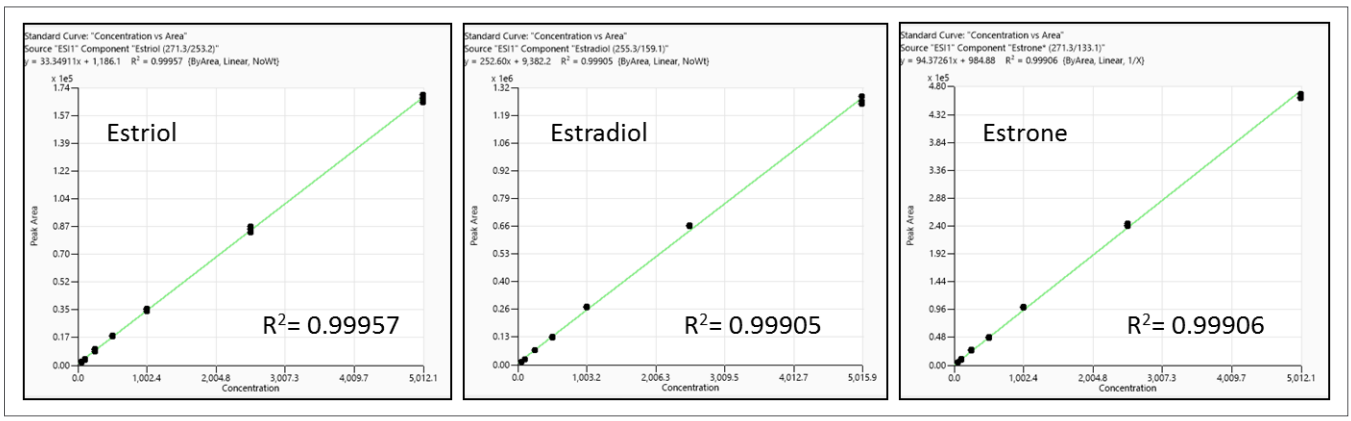

Figure 5. Linearity plots for the three estrogens.

As listed in Table 5, LOQ (limit of quantitation, S/N = 10) levels were established for each estrogen, based upon their averaged Level 1 calibration standard response. The representative Level 1 MRM chromatograms are shown in Figure 6.

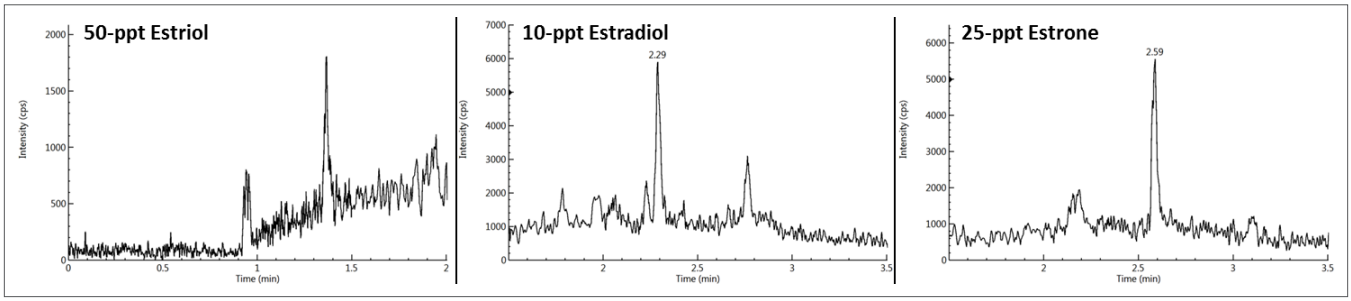

Figure 6. EIC chromatograms of the Level 1 standard for each estrogen.

Table 5. LOQs for the three analytes.

Analyte	Calculated LOQ (ppb)
Estriol	0.050
Estradiol	0.008
Estrone	0.015

Figure 7 shows the results of a 50-ppt, 50-uL direct injection (top) compared to 50-ppt 2 x 1000-uL SPE loading (bottom). Using a 50-uL direct injection, only estradiol can be reliably detected in the river water sample. By comparison, the 2 x 1000-uL SPE load allows for the detection and quantitation of all three estrogens at 50-ppt in river water, with a 30-fold increase in peak area for estradiol.

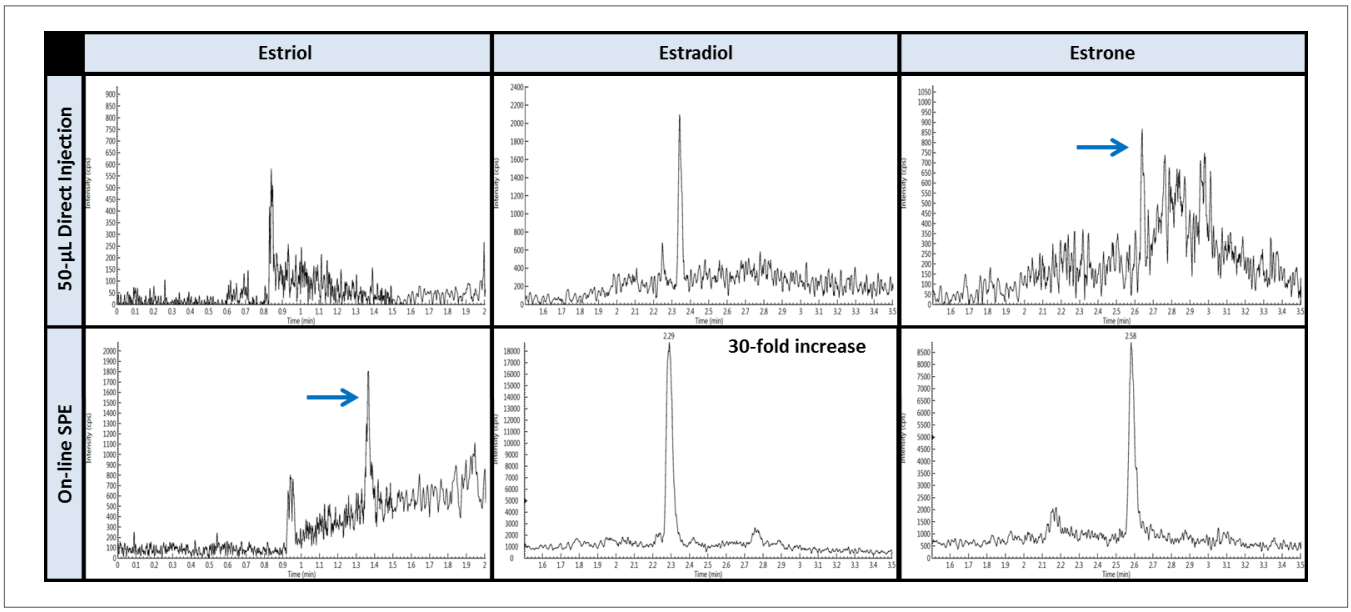

Figure 7. Comparative results of 50-ppt 50-uL direct injection (top) and 50-ppt 2 x 1000uL SPE loading (bottom), both in river water.

SPE sample loading performance is highlighted in Figure 8, showing the chromatographic results of triplicate injections of the 50-ppt spiked into river water. Even down at the LOQ level of estriol (left), exceptional reproducibility is demonstrated.

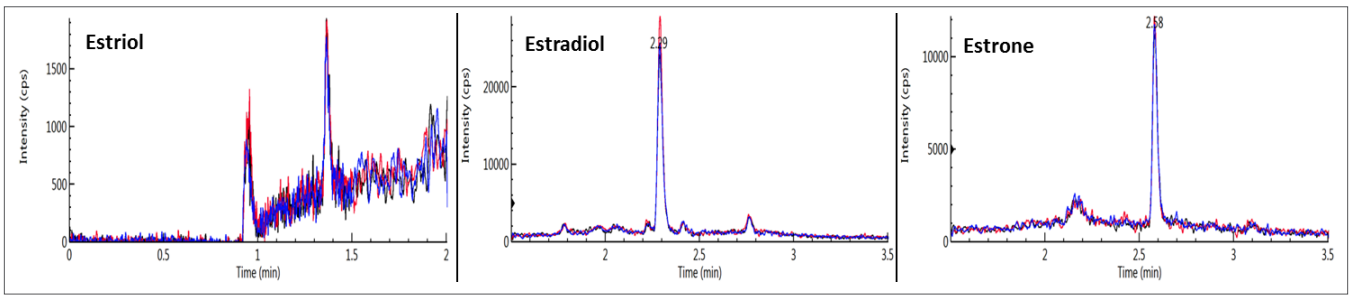

Figure 8. MRM chromatograms of triplicate injections of 50-ppt std spiked in river water.

As an unspiked blank test, a 2 x 1000-uL river water sample was loaded by SPE and injected. As shown by the results in Figure 9, none of the three estrogens were detected.

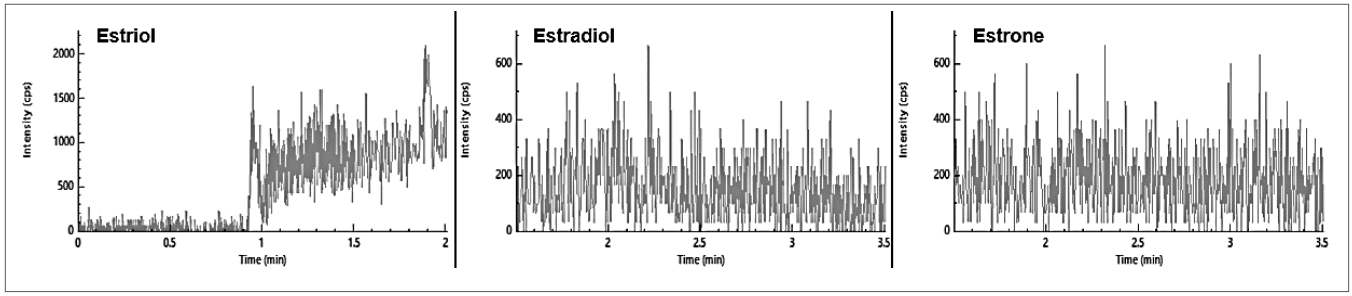

Figure 9. MRM chromatograms of unspiked river water blank.

Taking estrone as an example, sample loading repeatability, recovery performance and reliable analyte confirmation are demonstrated in Table 6, per *Sample Accuracy % (Area), CV%* (Area) and *Ion Ratio* columns, across all calibrant levels. As highlighted in green, all values were well within acceptable tolerances. Though not shown, the results for estradiol and estriol were equally impressive. As seen at the bottom of the table, the results also confirmed the absence of any detectable estrone in the Housatonic River water sample. Both estriol and estradiol were also not detected in the river water sample.

Table 6. Sampling performance and analyte confirmation results for estrone.

Sample	Peak Area	Sample Accuracy % (Area)	CV% (Area)	Ion Ratio (253.2/ 133.1) Area	Expected Ion Ratio (253.2/ 133.1) Area Range
50 ppt Estrogens Std	5727	103.96	4.73	1.96	1.56, 2.33
50 ppt Estrogens Std	5383	97.083	4.73	1.84	1.56, 2.33
50 ppt Estrogens Std	5223	93.885	4.73	1.88	1.56, 2.33
100 ppt Estrogens Std	9744	92.154	4.185	2.02	1.56, 2.33
100 ppt Estrogens Std	9957	94.286	4.185	1.98	1.56, 2.33
100 ppt Estrogens Std	10558	100.301	4.185	1.93	1.56, 2.33
250 ppt Estrogens Std	26022	102.171	0.835	1.91	1.56, 2.33
250 ppt Estrogens Std	26306	103.313	0.835	1.84	1.56, 2.33
250 ppt Estrogens Std	25878	101.591	0.835	1.93	1.56, 2.33
500 ppt Estorgens Std	47958	95.333	1.654	2.03	1.56, 2.33
500 ppt Estorgens Std	48829	97.095	1.654	1.95	1.56, 2.33
500 ppt Estorgens Std	47243	93.886	1.654	1.96	1.56, 2.33
1 ppb Estrogens Std 1	99081	99.782	0.738	1.96	1.56, 2.33
1 ppb Estrogens Std_1	100385	101.122	0.738	1.94	1.56, 2.33
1 ppb Estrogens Std_1	100330	101.065	0.738	1.97	1.56, 2.33
2.5 ppb Estrogens Std	242693	100.25	1.263	1.95	1.56, 2.33
2.5 ppb Estrogens Std	246659	101.956	1.263	1.95	1.56, 2.33
2.5 ppb Estrogens Std	248822	102.887	1.263	1.94	1.56, 2.33
5 ppb Estrogens Std_2	467011	100.257	0.726	1.97	1.56, 2.33
5 ppb Estrogens Std_2	460563	98.758	0.726	1.95	1.56, 2.33
5 ppb Estrogens Std_2	462130	99.122	0.726	1.95	1.56, 2.33
10 ppb Estrogen Std	874761	102.049	1.526	1.94	1.56, 2.33
10 ppb Estrogen Std	858706	99.802	1.526	1.96	1.56, 2.33
10 ppb Estrogen Std	848720	98.415	1.526	1.95	1.56, 2.33

Conclusion

- This work has demonstrated the effective and robust online SPE sample loading, chromatographic separation, and quantitation of estrogens using the PerkinElmer QSight SP50 Online SPE System coupled to a QSight 220 MS/MS Detector.
- Due to the unique high-capacity SPE cartridge and the high enrichment factor, large sample sizes are not required, saving time and improving throughput.
- As the Simplicity 3Q CDS automatically provides for work-ahead flow as part of the online SPE/chromatography process, online SPE only extends the injection-to-injection analysis time by 2.5 minutes per sample.
- This procedure allows for relatively low solvent consumption (≤ 5 mL per sample) as part of the SPE preparation phase.
- The method provides exceptional online sample preparation/pre-concentration and chromatographic repeatability and affords LOQs of 8 ppt for estradiol, 15 ppt for estrone and 50 ppt for estriol.
- The method/procedure defined herein can be expected to fulfill the essential task of monitoring for low-level estrogens in river water.

References

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