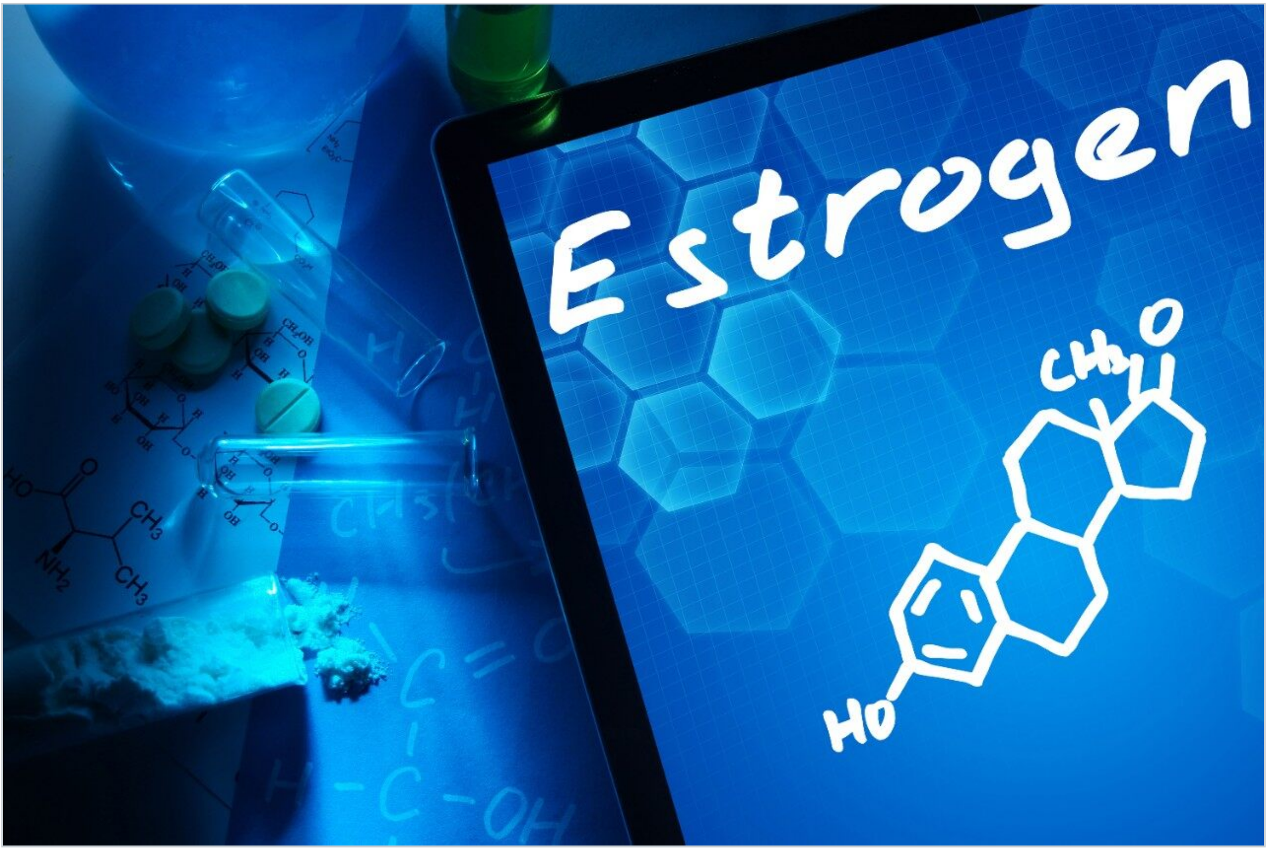
Quantitative Analysis of Natural and Synthetic Estrogens in Surface and Final Effluent Waters at Low ppq Levels Using UPLC-MS/MS

Euan Ross, Benjamin Wuyts, Angela Boag

Waters Corporation, Scottish Water



Abstract

Estrogens are routinely used either as contraceptive medicines or in hormone replacement therapy and they can enter aquatic environments via the discharge of final effluent waters. Estrogens are believed to have a negative effect on aquatic environments by disrupting the hormonal systems of fish. For the European Union, EU Directive 2013/39/EU includes 15 additional priority substances to the water framework Directive 2000/60/EC. In this update, 17α-ethinylestradiol and 17β-estradiol were not included in the priority substance list, but instead added to a watch list in order to gather further data regarding the presence of these compounds in aquatic environments, and the risks they may pose.

This application note summarizes a method for the analysis of estrone, 17α-ethinylestradiol and 17β-estradiol in surface and final effluent waters. To achieve the challenging EU LLOQ parts per quadrillion (ppq) levels required for these compounds, a combination of off-line solid phase extraction (SPE) clean-up and pre-concentration, combined with a large volume injection and triple quadrupole mass spectrometry were utilized.

Benefits

Application benefits include:

- High sensitivity to achieve EU target LLOQs.
- Baseline separation and good peak shapes for target analytes.
- Suitable linearity and repeatability data in surface water and final effluent.
- Acceptable SPE spiked recoveries and repeatability.
- Quantitation of detected residues in final effluent using standard addition in the absence of isotopically labeled internal standard.

Introduction

Estrogens are routinely used either as contraceptive medicines or in hormone replacement therapy and they can enter aquatic environments via the discharge of final effluent waters.[1] Estrogens are believed to have a negative effect on aquatic environments by disrupting the hormonal systems of fish.[1] For the European

Union, EU Directive 2013/39/EU[2] includes 15 additional priority substances to the water framework Directive 2000/60/EC.[3] In this update, 17α-ethinylestradiol and 17β-estradiol were not included in the priority substance list, but instead added to a watch list[2] in order to gather further data regarding the presence of these compounds in aquatic environments, and the risks they may pose.

This application note summarizes a method for the analysis of estrone, 17α-ethinylestradiol and 17β-estradiol in surface and final effluent waters. To achieve the challenging EU LLOQ parts per quadrillion (ppq) levels[4] required for these compounds, a combination of off-line solid phase extraction (SPE) clean-up and pre-concentration, combined with a large volume injection and triple quadrupole mass spectrometry were utilized.

Xevo TQ-XS.

Experimental

Sample Description

Extracted samples were prepared and supplied by Scottish Water. Spiked surface water and final effluent samples were first filtered, extracted, and concentrated using an off-line solid phase extraction (SPE) method [5-6] (Figure 1). After evaporation and reconstitution in LCMS grade water, the samples were then analyzed by UPLC-MS/MS using a large volume injection (100 µL).

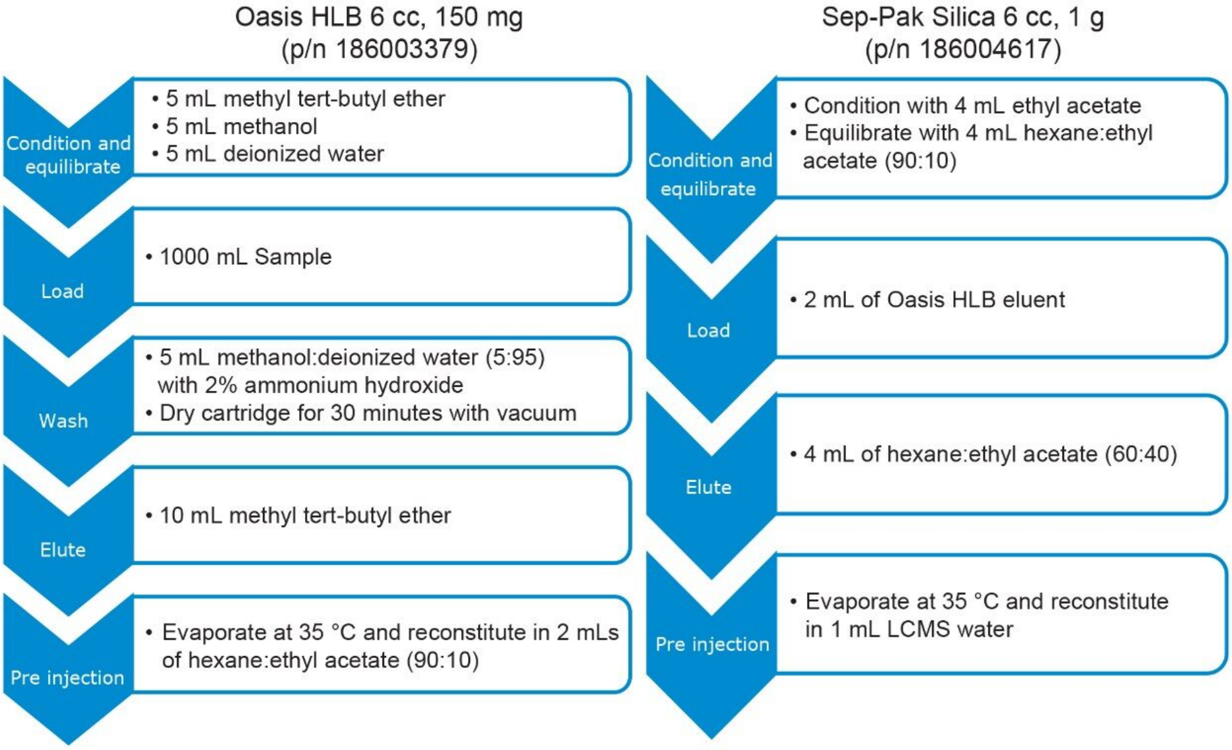


Figure 1. Solid phase extraction, clean-up, and concentration methodology, provided by Scottish Water.

LC Conditions

LC system:	ACQUITY UPLC H-Class with extension loop, needle, and syringe for large volume injection
Vials:	TruView LCMS Certified
Column:	ACQUITY UPLC BEH C_{18} 1.7 µm, 3.0 x 100 mm

Column temp.: 30 °C

Sample temp.: 10 °C

Injection volume: 100 µL

Flow rate: 0.6 mL/min

Mobile phase A: LCMS grade water with 0.01 mM NH_4F (analytical grade)

Mobile phase B: 50:50 LCMS grade acetonitrile:methanol with 0.01 mM NH_4F (analytical grade)

Gradient

Time (min)	Flow rate (mL/min)	%A	%B
Initial	0.6	70	30
1.00	0.6	70	30
3.50	0.6	5	95
5.50	0.6	5	95
5.60	0.6	70	30
8.60	0.6	70	30

MS Conditions

MS system: Xevo TQ-XS

Ionization mode: ESI

Acquisition mode: MRM

Capillary voltage: 2.00 kV

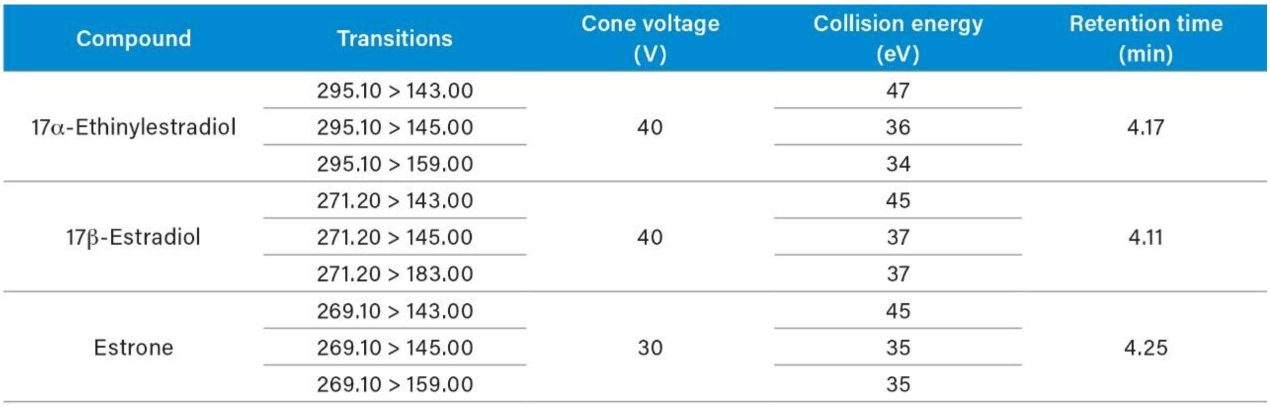
Cone gas flow: 150 L/Hr

Desolvation temp.: 600 °C

Desolvation gas flow: 1200 L/Hr

Nebulizer: 7 bar

Data management MassLynx v4.2



Compound	Transitions	Cone voltage (V)	Collision energy (eV)	Retention time (min)
17α-Ethinylestradiol	295.10 > 143.00	40	47	4.17
	295.10 > 145.00		36	
	295.10 > 159.00		34	
17β-Estradiol	271.20 > 143.00	40	45	4.11
	271.20 > 145.00		37	
	271.20 > 183.00		37	
Estrone	269.10 > 143.00	30	45	4.25
	269.10 > 145.00		35	
	269.10 > 159.00		35	

Table 1. Summary of optimized MS parameters and LC retention time for target analytes.

Results and Discussion

Method optimization was accomplished by evaluating various columns, mobile phase compositions, gradients, and MS transitions. The conditions detailed in the Experimental section provided the best overall performance of those tested. Baseline separation of the target analytes was achieved, and a example of the chromatography from a 50 ng/L solvent standard is shown in Figure 2. Due to the hydrophobic nature of the analytes, they elute in the high organic part of the gradient. As a result, it is challenging to separate the analytes away from matrix components in the final effluent used for this analysis, as shown in the RADAR scan (full scan *m/z* 100 to 1000) in Figure 3. To minimize any contamination of the MS source, the integrated fluidics system on the Xevo TQ-XS was used to divert the chromatographic region to the MS system for analysis, and unwanted regions to waste.

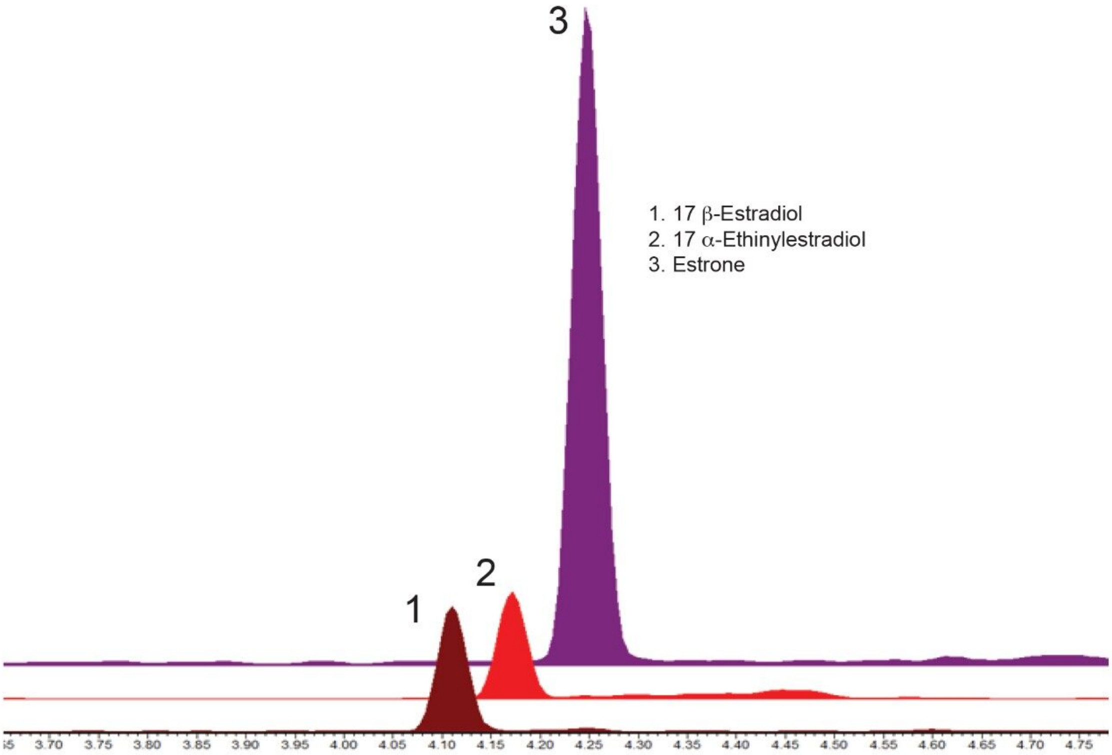


Figure 2. Example chromatography of a 50 ng/L solvent standard, separated on a 1.7 µm, 3.0 x 100 mm, ACQUITY UPLC BEH C_{18} Column, 100 µL injection.

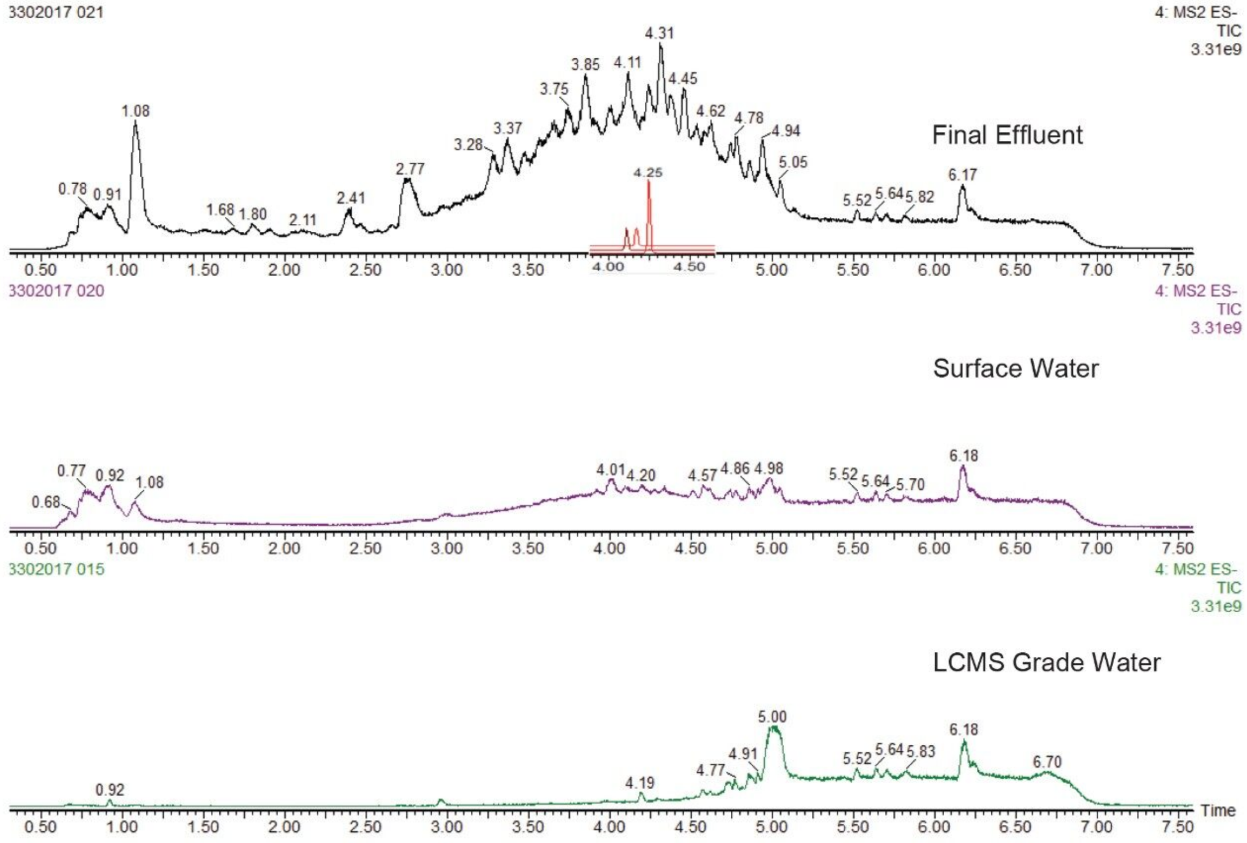


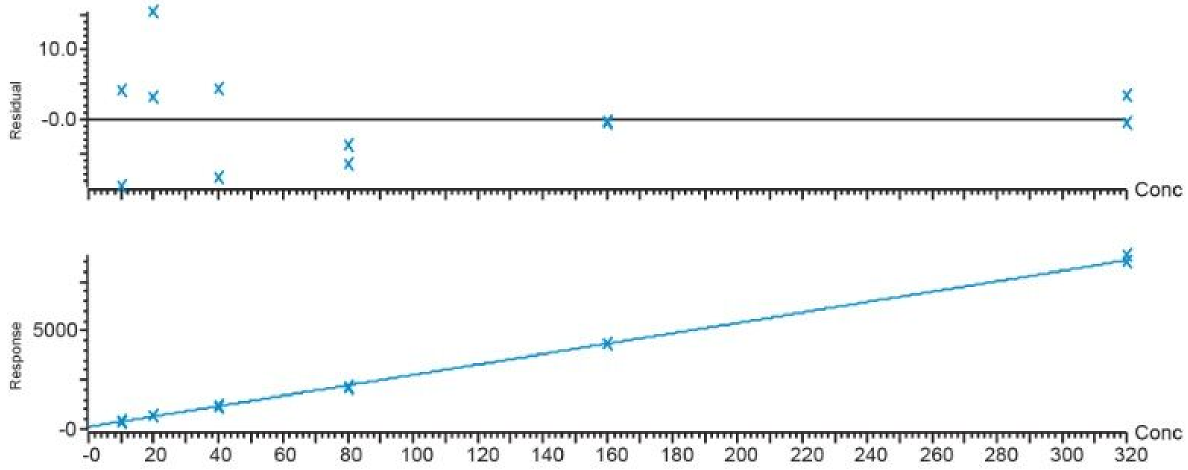
Figure 3. RADAR scan (full scan m/z 100 to 1000), on matrix samples after cleanup and concentration by SPE. Example peaks for the target compounds are shown against the final effluent RADAR scan indicate the area they elute.

Satisfactory linearity was achieved for all compounds in matrix matched (spiked post extraction) bracketed calibration curves, over the appropriate ranges. In surface water, a range of 10 to 320 ng/L for 17α-ethinylestradiol, and 62.5 to 2000 ng/L for 17β-estradiol and Estrone provided good linearity (R^2 >0.998, residuals <15%). An example of the calibration and associated residuals is shown for all three compounds in Figure 4. For final effluent, the matrix matched calibration curves ranged from 120 to 2000 ng/L for all three compounds, also giving acceptable linearity (R^2>0.997, residuals <10%). The robustness of the method was assessed using spiked water samples (n=8 for each matrix type) where %RSD values below 6% were obtained.

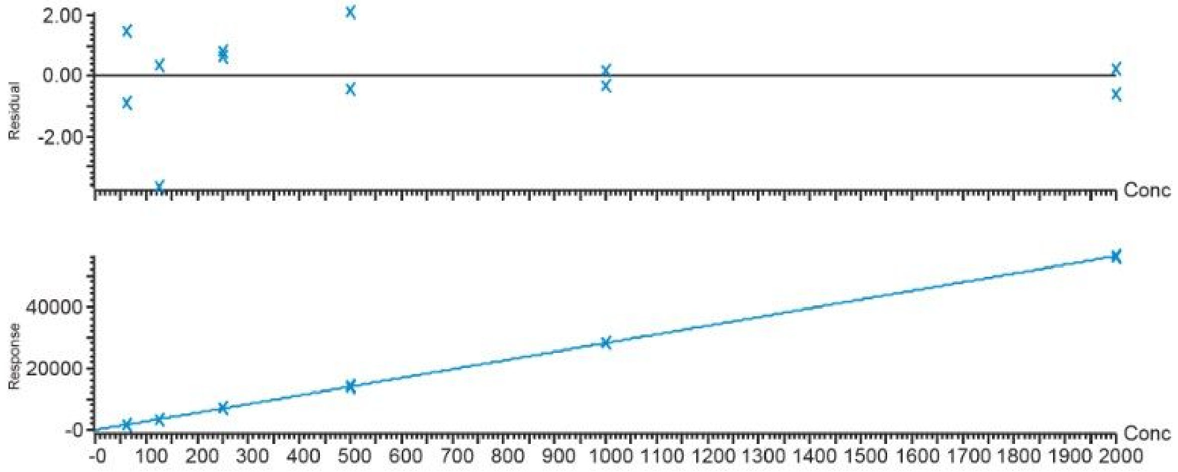
To evaluate the method performance in surface water, matrix samples were pre-spiked at suitable pg/L (ppq) levels prior to extraction, and prepared in accordance with the methodology detailed in Figure 1. An example of the chromatography and sensitivity observed for a sample pre-spiked at low ppq level in surface water, before clean-up and concentration, is shown in Figure 5.

Matrix effects were determined by quantifying post spiked surface water samples against a solvent calibration curve. For 17β-estradiol and estrone, matrix effects were calculated at ≤-22% (suppression), 17α-ethinylestradiol ≤16% (enhancement). Final effluent, even after the SPE clean-up remained a complex sample, as shown in the RADAR scan in Figure 3, this resulted in significant matrix suppression ≤-72% for all compounds. However even with this significant suppression, low levels of each compound are still detectable.

Compound name: 17 Alpha Ethynyl Estradiol
Correlation coefficient: r = 0.999001, r^2 = 0.998004
Calibration curve: 26.4155 * x + 120.806
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Compound name: 17 Beta Estradiol
Correlation coefficient: r = 0.999950, r^2 = 0.999901
Calibration curve: 28.1779 * x + -59.3078
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Compound name: Estrone
Correlation coefficient: r = 0.999910, r^2 = 0.999819
Calibration curve: 171.91 * x + 336.28
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

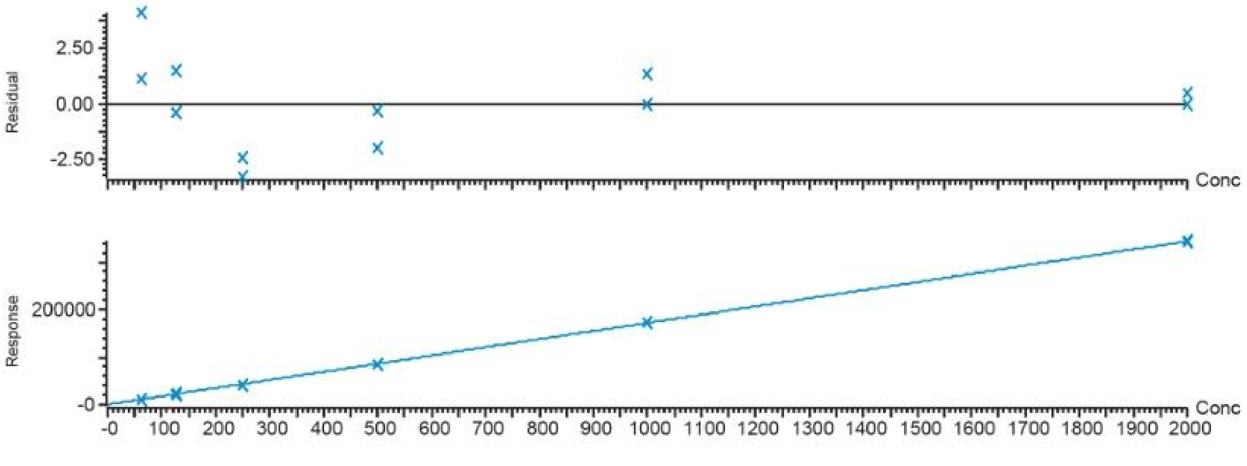
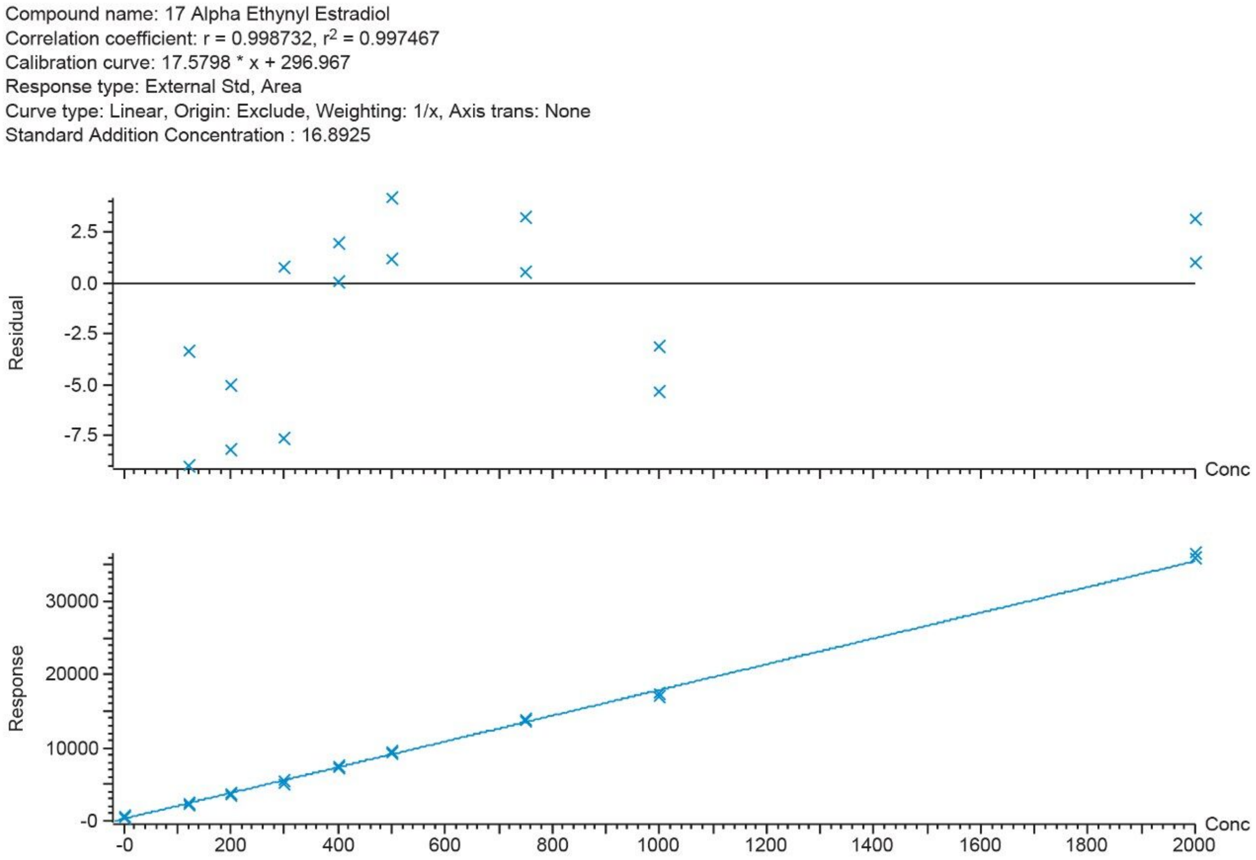


Figure 4. An example of the calibration and associated residuals (n=2) is shown for all compounds in surface

LLOQ (PtP s/n=10) levels for each compound in the surface water matrix. Low level concentrations of all compounds were detected in the final effluent matrix. A standard addition method was used to quantify the analytes present, where 17α-ethinylestradiol was measured at 16.9 pg/L, as shown in Figure 6.

Compound	17α-Ethinylestradiol	17β-Estradiol	Estrone
Spike Level 1 (pg/L) (surface water)	**30.0**	**120.0**	**400.0**
% Recovery (n=3)	80.0	99.5	92.2
% RSD (n=3)	10.8	4.0	1.6
Spike Level 2 (pg/L) (surface water)	**60.0**	**300.0**	**1000**
% Recovery (n=3)	71.3	100.6	92.6
% RSD (n=3)	12.3	4.6	1.9

Table 2. SPE method recovery and repeatability data for all analytes, spiked at two levels in triplicate into surface water matrix, pre-spiked levels are indicated in bold.



Compound name: 17 Alpha Ethynyl Estradiol
Correlation coefficient: r = 0.998732, r^2 = 0.997467
Calibration curve: 17.5798 * x + 296.967
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
Standard Addition Concentration : 16.8925

Figure 6. Standard addition method used to calculate low level concentration of 17α-ethinylestradiol in final effluent (n=2).

Conclusion

This method highlights the analysis of low ppq levels of synthetic estrogens in surface and final effluent waters. Utilizing Oasis SPE and Sep-Pak SPE sample preparation technologies, the optimized extraction method was found to provide the required concentration and clean-up, giving acceptable recoveries and repeatability in spiked surface water samples. The use of a large volume injection in combination with the ACQUITY UPLC H-Class and Xevo TQ-XS, allowed for the challenging detection requirements of this analysis to be achieved in surface water. The use of standard addition allowed for accurate quantitation of trace residues in final effluent samples.

References

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