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Increased Capabilities for the Analysis of Hormones in Drinking and Waste Water using SPE and LC/MS/MS

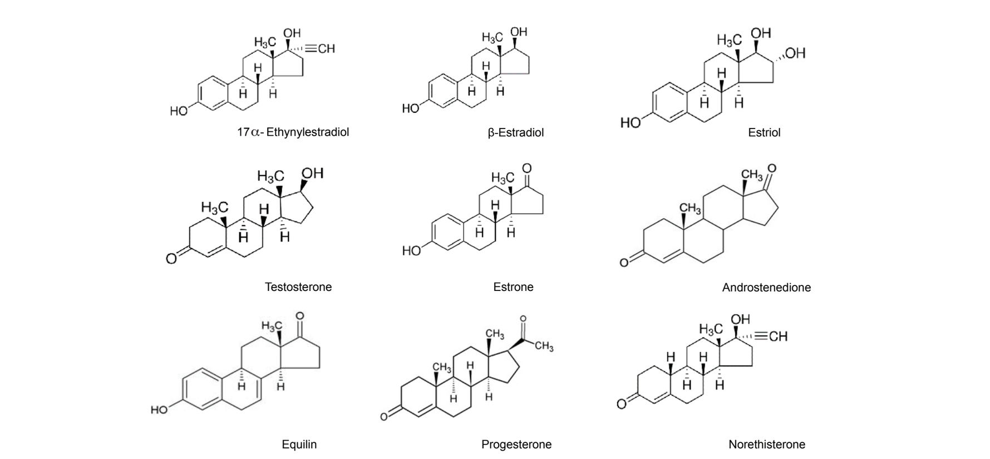
Introduction

The presence of various hormones in worldwide drinking water supplies has been of public concern for some time. As the scientific community tries to identify acceptable exposure limits, many new endogenous and synthetic hormones are being discovered. Compounds such as ethynylestradiol, the active ingredient in a commonly prescribed birth control medication, are known to cause detrimental effects to both aquatic and human life. Many other compounds are currently being investigated. Due to this public risk, there is a rapidly growing interest in monitoring these compounds. Within the United States, EPA Method 539 was specifically developed to monitor this growing problem.

This study builds on the foundation of EPA 539 by providing an expanded list of target analytes to include compounds of current interest (e.g. progesterone) and a more modern extraction and separation method, all while shortening the run time to less than 14 minutes. We expand the sample preparation technique to employ a more versatile format for ease of use in a variety of water matrices. By utilizing a SPE tube format and exploring various particle sizes, differing water sources can be scaled and processed more easily. In addition, the final extract concentration levels can be easily monitored and controlled to be more appropriate for a variety of different detectors, depending on the required sensitivity.

This study also offers an optimized LC/MS/MS method that explores ionization polarities and utilizes a high pH mobile phase in order to maximize LC/MS/MS response. Because conventional silica-based HPLC media is not stable under alkaline conditions, a core-shell organo-silica column was used to perform these analyses.

Figure 1. Structures of Hormones



Experimental Conditions

Sample Preparation

Pretreated Samples:

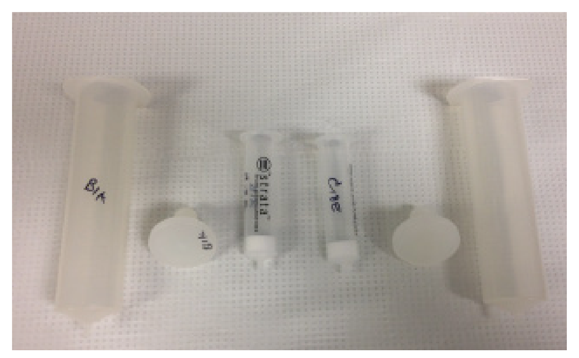
Per the EPA 539 method protocol, 1L water samples are dechlorinated, preserved, collected and stored.
All standards and QCs are freshly prepared in 50 % Methanol in water containing 20 ng/mL of working internal standards.

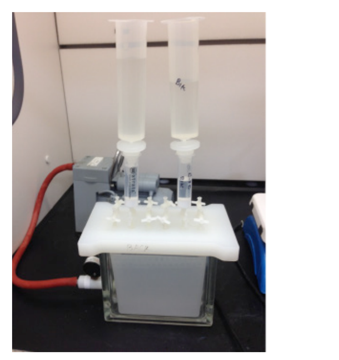
We evaluated silica-based (Strata® C18-E) and polymer-based (Strata™-X) SPE sorbents. Strata C18-E provides recoveries of 91.1 – 103 % across all compounds **(Table 3)**.

Optimized Solid Phase Extraction Method

Cartridge: Strata C18-E, 1 g/20 mL
Part No.: 8B-S001-JEG
Condition: 10 mL Methanol
Equilibrate: 10 mL Water
Load: Pretreated samples
Wash: 10 mL 15 % Methanol in water
Dry: 5 – 10 minutes under 10" Hg vacuum
Elute: 2 x 6 mL Methanol
Dry down: Evaporate completely under a stream of nitrogen @ 50 °C
Reconstitute: Add 1.0 mL of 50 % Methanol in water containing 20 ng/mL of working internal standards.

SPE Accessories





LC/MS/MS Conditions

Column: Kinetex® 5 µm EVO C18
Dimensions: 100 x 2.1 mm
Part No.: 00D-4633-AN
Mobile Phase: A: 0.2% NH_4OH in Water
B: 0.2% NH_4OH in Methanol
Gradient:

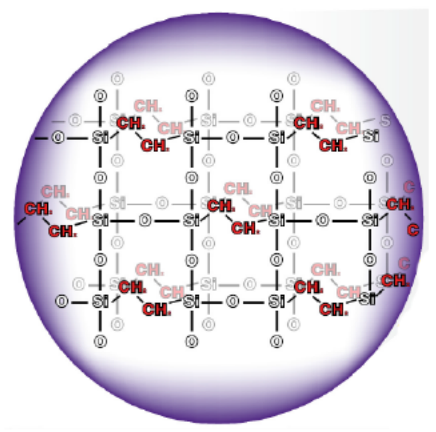
Time (min)	% B
0	30
1	65
6	65
6.5	85
10.5	85
11	30
13.5	30

Flow Rate: 200 µL/min
Injection Volume: 30 µL
Instrument: Shimadzu® 30-AD and Agilent® 1260 LC
Detection: AB Sciex API 4000™ QTRAP®
AB Sciex API 4500™

Sample:
1. Estriol
2. Equilin
3. Estrone $^{13}C_3$
4. Estrone
5. 17β – Estradiol D_5
6. β – Estradiol
7. 17α - Ethynylestradiol
8. Norethisterone
9. Androstenedione
10. Testosterone D_3
11. Testosterone
12. Progesterone D_9
13. Progesterone

Note: The ionization source was ESI with rapid polarity switching in positive and negative ion mode. Shimadzu 30-AD LC series, interfaced with an AB Sciex API 4000 QTRAP, was for lowest detection level test only; all other experiments were done on Agilent 1260 LC series with an AB Sciex API 4500 mass spectrometer.

Figure 2. Structure of the Organo-Silica Kinetex EVO Particle



Kinetex EVO: a patented organo-silica grafting process which incorporates uniform stabilizing ethane cross-linking to provide resistance to high pH attack while maintaining mechanical strength of the core-shell particle.

Stable to pH ~12

Table 1. MRM Transitions

Negative Ions

ID	Q1	Q3	RT (min)	Dwell	DP	EP	CE	CXP
Beta-estradiol 1	271.1	145	6.34	75	-92	-10	-51	-14
Beta-estradiol 2	271.1	183.3	6.34	75	-92	-10	-51	-15
Estriol 1	287.1	145.2	4.21	75	-90	-10	-58	-17
Estriol 2	287.1	171	4.21	75	-90	-10	-50	-17
Equilin 1	267.1	143	5.80	75	-99	-12	-46	-20
Equilin 2	267.1	223.1	5.80	75	-90	-12	-48	-12
Estrone 1	269	145.1	6.14	75	-83	-10	-54	-23
Estrone 2	269	143.2	6.14	75	-83	-10	-70	-23
17α-Ethynylestradiol 1	295.3	145	6.46	75	-110	-10	-58	-17
17α_Ethynylestradiol 2	295.3	183.3	6.46	75	-110	-10	-58	-17
17 Beta-estradiol D_5 1	276.3	147	6.29	75	-90	-10	-55	-15
18 Beta-estradiol D_5 2	276.3	187.1	6.29	75	-90	-10	-55	-15
Estrone $^{13}C_3$ 1	272.2	148.1	6.13	75	-80	-10	-50	-20
Estrone $^{13}C_3$ 2	272.2	162.1	6.13	75	-80	-10	-54	-20

Positive Ions

ID	Q1	Q3	RT (min)	Dwell	DP	EP	CE	CXP
Androstenedione 1	287.1	97.1	6.56	150	89	9	31	8
Androstenedione 2	287.1	109	6.56	150	89	9	35	10
Testosterone 1	289.1	97.2	7.20	150	81	9	37	8
Testosterone 2	289.1	109.1	7.20	150	81	9	35	8
Testosterone-D_3 1	292.2	97	7.19	150	91	9	33	6
Testosterone-D_3 2	292.2	109	7.19	150	91	9	37	8
Progesterone 1	315.4	109.1	9.67	150	86	9	35	10
Progesterone 2	315.4	97.2	9.67	150	86	9	35	10
Norethisterone 1	299.1	109	6.61	150	78	9	38	10
Norethisterone 2	299.1	231	6.61	150	78	9	27	10
Progesterone_D_9 1	324.4	100	9.61	150	87	9	30	10
Progesterone_D_9 2	324.4	113	9.61	150	87	9	30	10

Table 2. Mini Validation Results

ID	Final concentration (ng/L)	Accuracy%								
		Testosterone	Androstenedione	Progesterone	Norethisterone	17β-estradiol	Estriol	Equilin	Estrone	17α-Ethynylestradiol
STD 1	0.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
STD 2	0.5	83.1	100	95.0	94.9	97.8	95.8	91.6	94.8	92.4
STD 3	1.0	105	100	94.8	100	98.2	92.6	94.5	94.8	89.0
STD 4	2.0	103	104	99.0	96.5	98.5	96.9	91.9	99.9	90.9
STD 5	10	104	107	102	100	99.8	94.0	95.7	94.8	102
STD 6	40	98.9	99.5	103	102	99.8	102	103	103	101
STD 7	50	100	98.8	97.2	98.2	100	100	99.2	98.8	99.8
QC L	2	104	105	97.2	101	101	103	90.8	102	93.0
QC M	20	103	103	93.5	93	103	99.7	96.9	99.6	102

STD 2 is Method Reported Limit (MRL)

Table 3. Extracted Matrix Recovery

Strata C18-E (55 µm, 1g/20 mL) Extracted Matrix Recovery

ID (Neg ions)	500 ng/L (DI water)		2 ng/L (Tap water)	
	Recovery %	CV %	Recovery %	CV %
17 β-estradiol	99.7	2.93	88.1	6.53
Estriol	99.9	3.60	109	1.00
Equilin	91.1	2.75	72.8	8.46
Estrone	99.3	2.17	89.6	7.23
17α-Ethynylestradiol	103	3.67	91.6	3.15

ID (Pos ions)	500 ng/L (DI water)		2 ng/L (Tap water)	
	Recovery %	CV %	Recovery %	CV %
Testosterone	99.9	0.10	100	1.24
Androstenedione	99.2	0.46	108	5.09
Progesterone	91.8	1.56	113	5.07
Norethisterone	101	3.78	104	2.07

Samples (n=3) were collected on 26 March 2015 in Torrance, CA 90501, USA.

Tap water contains all the possible contaminants, so the recoveries of analytes are various, but within ±30% of acceptance criteria, respectively.

Figure 3. Representative of TIC Chromatogram

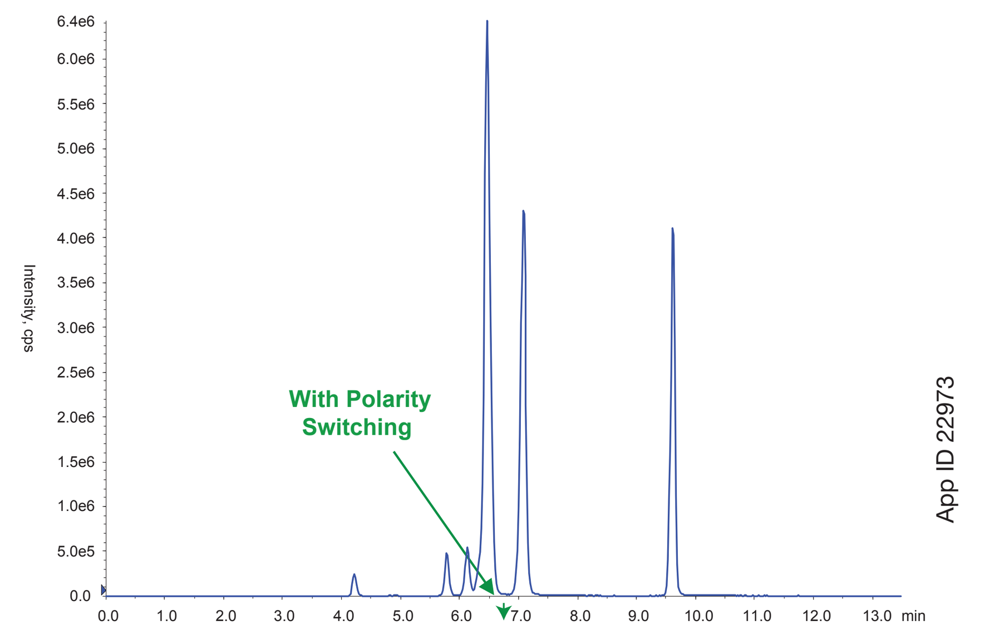


Figure 4. Representative of Positive Ion Chromatogram – 2.0 ng/L

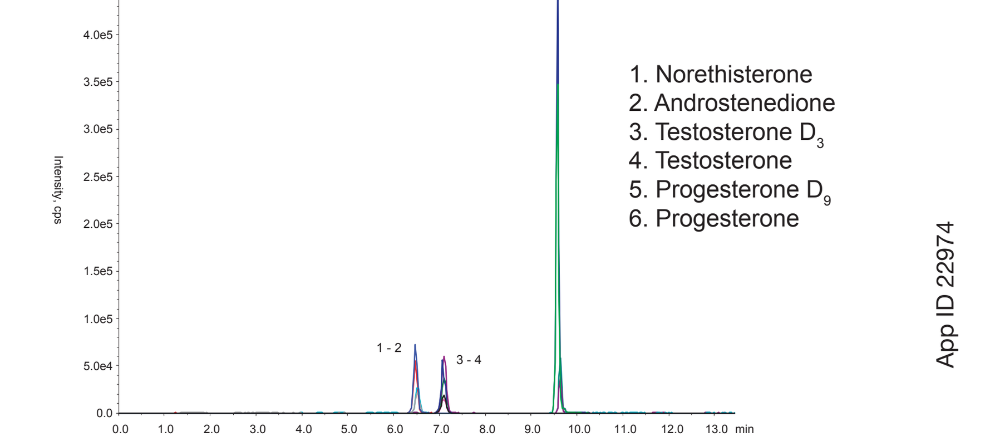


Figure 5. Representative of Negative Ion Chromatogram – 2.0 ng/L

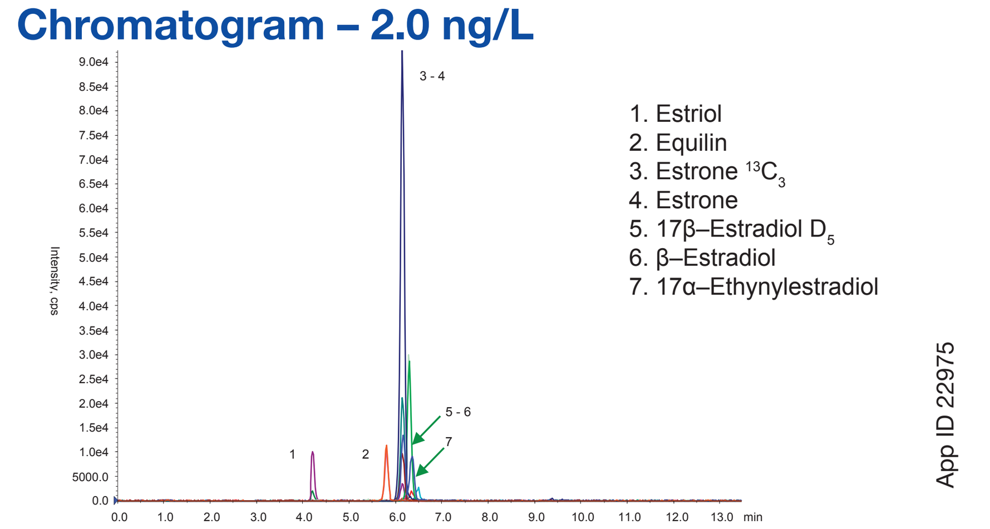


Table 4. Method Limits and Linearity for Each Analyte

Negative Ions

ID	MRL (ng/L)	LOD (ng/L)	Linearity (R^2)
Beta-estradiol	0.50	0.05	1.0000
Estriol	0.50	0.05	0.9997
Equilin	0.50	0.05	0.9997
Estrone	0.50	0.05	0.9996
17α-Ethynylestradiol	0.50	0.10	0.9998

Positive Ions

ID	MRL (ng/L)	LOD (ng/L)	Linearity (R^2)
Androstenedione	0.50	0.05	0.9997
Testosterone	0.50	0.05	0.9998
Progesterone	0.50	0.05	0.9996
Norethisterone	0.50	0.10	0.9998

MRL - Method reporting limit
LOD - Lowest of detection level
Linear Regression at 1/X

Figure 6. Representative of Curve – Testosterone

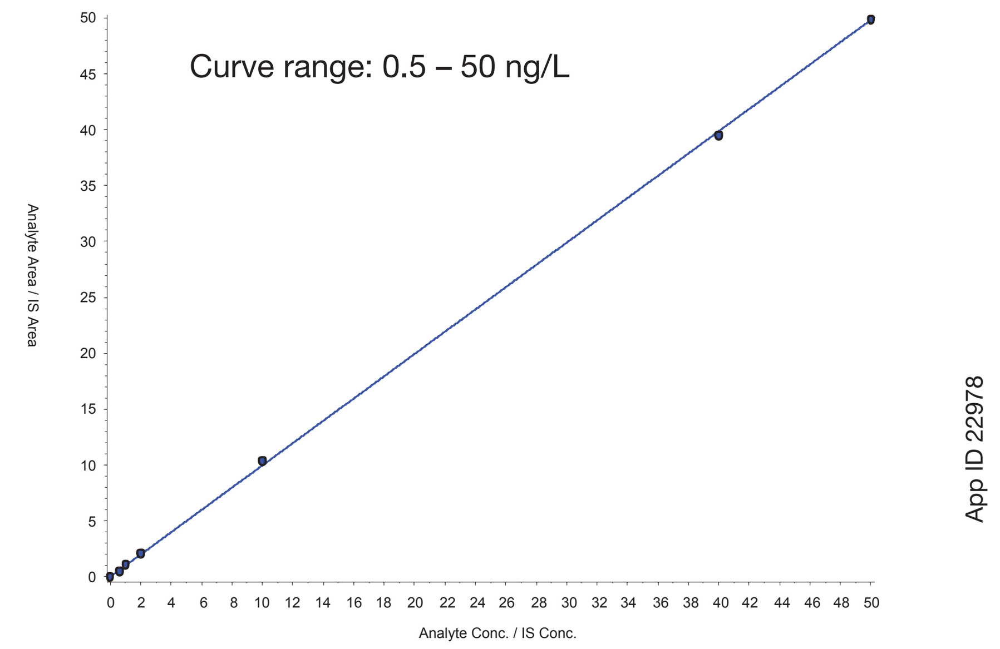
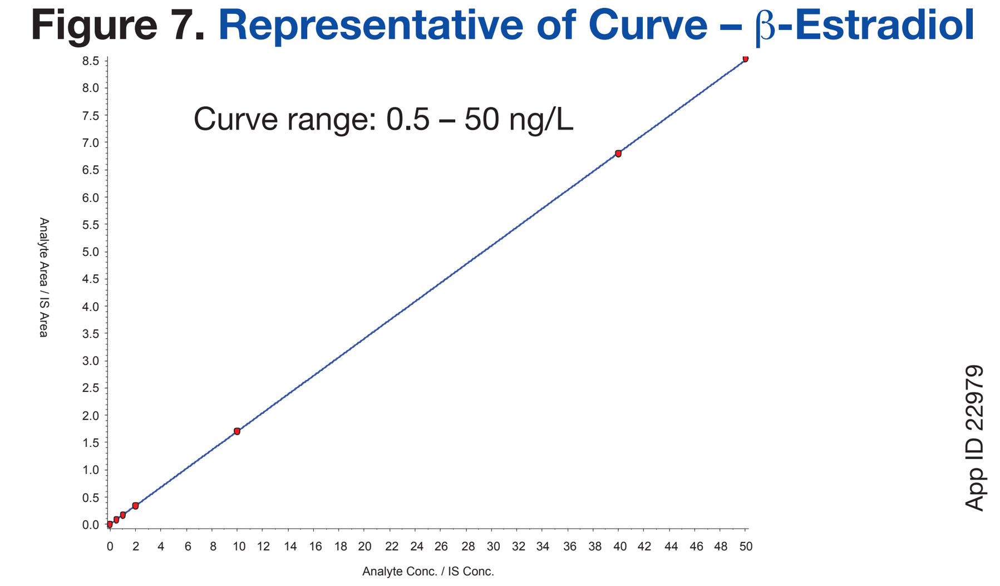


Figure 7. Representative of Curve – β-Estradiol



Results and Discussion

On the foundation of EPA Method 539, a fast and reproducible SPE method for analyzing an expanded hormones list **(Table 1)** in drinking and waste water using LC/MS/MS is developed. In this study, we use Strata C18-E SPE in tube format to bring benefits such as faster processing, increased sensitivity, consistency, and extraction efficiency **(Tables 2 and 3)**.

The high pH mobile phase greatly improves the ionization of some of the target compounds, resulting in improved LC/MS/MS sensitivity. Because conventional silica-based HPLC media is unstable under alkaline conditions, the choice of analytical columns is very limited. In this study, we selected a Phenomenex Kinetex 5 µm EVO C18 100 x 2.1 mm column, a core-shell organo-silica particle which incorporates uniform stabilizing ethane cross-linking and provides resistance to high pH attack, all while maintaining mechanical strength **(Figure 2)**. These benefits allow an alkaline mobile phase to be used without compromising column lifetime, and increases compound sensitivity significantly. The lowest detection limits on an AB Sciex 4000 QTRAP can be reached at 0.05 ng/L for most analytes **(Table 4)**.

To improve sample analysis for high throughput in the lab, we have optimized LC/MS/MS procedures to effectively analyze all target compounds in one injection within 13.5 minutes **(Figures 3, 4 and 5)**, with mass spectrometer polarity switching. The representatives of linearity of analytes across all positive and negative analytes are shown in **Figures 6** and **7**.

Conclusion

EPA Method 539 is a liquid chromatography, electrospray ionization, tandem mass spectrometry method for the determination of hormones in finished drinking water. This work presents an optimized method with an expanded list of hormones that includes compounds of current interest (e.g. progesterone and norethisterone) to successfully monitor a growing problem in our scientific community. An SPE extraction method using Phenomenex Strata C18-E provides excellent recovery and reproducibility. LC/MS/MS analysis time was significantly reduced from 55 minutes to 13.5 minutes with a Kinetex 5 µ EVO C18 100 x 2.1 mm column, which delivers stability under high pH mobile phase, while maintaining excellent linearity and low detection levels. This method will significantly increase laboratory productivity, efficiency, and throughput.

Acknowledgements

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