APPLICATIONS

A Fast and Effective Approach to Analyzing Hormones in Drinking Water Using SPE and LC/MS/MS

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A method is illustrated for steroid hormone analysis from drinking water using an optimized LC/MS/MS method, reducing analysis time from 50 minutes down to about 15 minutes while maintaining excellent linearity (0.99 R^2 value or greater for each compound) and low minimum reporting level MRLs. The method presented uses a Gemini® 3 µm NX-C18 HPLC column, which delivers stability at pH 1-12.

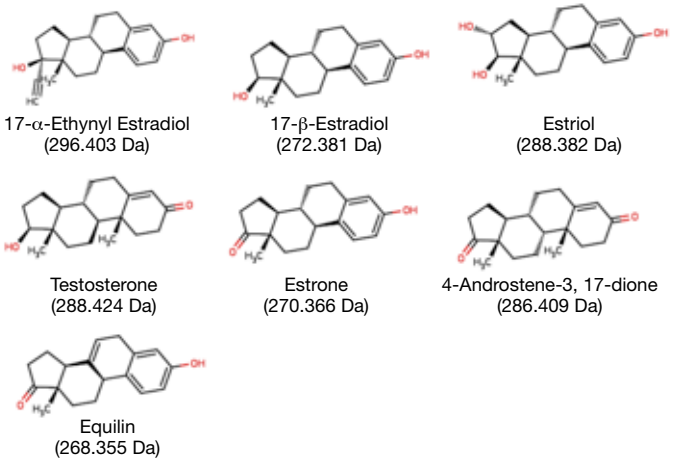
Introduction

Hormones have been found in a wide range of water supplies throughout the world. Compounds such as ethynylestradiol (**Figure 1**), the active ingredient in a commonly prescribed birth-control medication, can cause detrimental affects to both aquatic life and humans.[1] Due to this public health risk, there is a rapidly growing interest in monitoring these compounds, and within the United States, EPA Method 539 was specifically developed to monitor this growing problem.

This study follows EPA Method 539, which is a challenging analysis because not only does it require very low detection limits (0.1 part per trillion for some compounds), but it also requires mass-spectrometer analysis in both positive and negative modes. Despite these challenges, more than 1,000 utilities across the United States are required to have samples analyzed by this method under the Unregulated Contaminant Monitoring Rule (UCMR3).[2] Fewer than 20 labs across the U.S. are currently approved for this method.[3] However, laboratories throughout many regions of the globe are monitoring these compounds with similar methods. Due to the widespread concern and the test's popularity, it is extremely important to have a fast, accurate, and reproducible analytical testing method.

The study offers an optimized LC/MS/MS method that utilizes a high pH mobile phase in order to maximize the LC/MS/MS response. Since conventional silica-based HPLC media is not stable under alkaline conditions, an organo-silica hybrid column (Gemini 3 µm NX-C18; Phenomenex, Inc. Torrance, CA, USA) was used to perform these analyses.

Figure 1.
Structures of hormones



Material and Methods

Sample Preparation

Pre-treatment:	Per the EPA method protocol, 1 L water samples are dechlorinated, preserved, collected and stored. Surrogate standards are then added prior to solid phase extraction.
SPE disk:	47 mm Octadecyl sorbent (Empore® p/n, AH0-2540)
Disk cleaning:	Rinse disk with 10 mL of methanol followed by 5 mL of methanol
Conditioning:	Apply 10 mL of methanol followed by 10 mL of water
Load:	Load entire (1 L) sample under vacuum, without letting the disk go dry
Wash:	Apply 10 mL of 15 % methanol
Dry:	Under full vacuum for 10-15 min
Elution:	Apply 3 x 5 mL of methanol (allow time for the disk to soak for 1 min during first elution step)
Evaporation:	Under nitrogen gas at 45 °C
Reconstitute:	Add 500 µL of 50 % methanol and transfer to a 1 mL volumetric flask. Add internal standard and adjust to 1 mL volume with 50 % methanol. Filtration (optional): Not necessary for drinking water sample

LC/MS/MS Conditions

Column: Gemini 3 µm NX-C18
Dimensions: 100 x 2.0 mm
Part No.: 00D-4453-B0
Mobile Phase: A: 0.2 % NH_4OH in Water
B: 0.2 % NH_4OH in Methanol
Flow Rate: 0.2 mL/min
Gradient:

Time (min)	% B
0.00	35
0.01	35
0.6	65
7.5	65
8.5	85
13.0	85
13.01	35
15.0	35

Injection Volume: 10 µL
Temperature: Ambient
Detection: API 4000™ (AB SCIEX) Tandem Mass Spec MS/MS
Backpressure: 108 bar
Sample: 1 mL of drinking water extract

Table 1.
LC/MS/MS parameters

Compound	Q1	Q3	RT (min)	Polarity	Peak Number
Estriol	287.1	170.8/144.7	4.09	ESI+	1
Estriol-D2 (I.S.)	289.1	173.1	4.09	ESI+	2
Bisphenol A-D16 (SURR)	241.1	223.1/142.1	4.78	ESI+	3
Equilin	267.1	142.7/223.1	6.73	ESI+	4
Estrone	268.9	144.7/143.0	7.37	ESI+	5
17-β-estradiol	271.2	144.7/183.0	7.88	ESI+	6
13C6-estradiol (I.S.)	277.1	145.0	7.88	ESI+	7
17-α-Ethynylestradiol	295.1	144.7/142.9	7.99	ESI+	8
Ethynylestradiol-D4 (SURR)	299.1	147.0/161.0	8.04	ESI+	9
13C2-Ethynylestradiol (I.S.)	297.1	144.9	8.04	ESI+	10
4-Androstene-3,17-dione	287.2	97.1/109.1	8.08	ESI-	11
Testosterone	289.2	97.2/109.1	9.33	ESI-	12
Testosterone-D3 (I.S.)	292.2	97/109.1	9.4	ESI-	13

Results and Discussion

The purpose of this investigation was to develop a fast and reproducible method for analyzing hormones in drinking water using LC/MS/MS. These goals were achieved while maintaining the required linearity and method reporting limit (MRL) values of EPA Method 539.

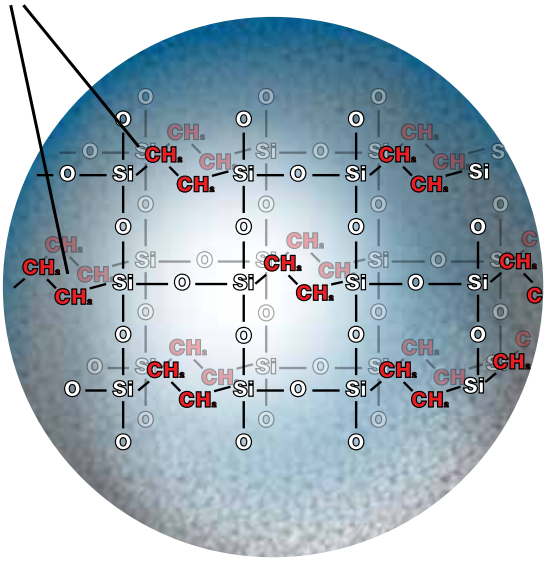
In this case, the sample extraction step was performed in accordance to EPA Method 539 using octadecyl SPE disks. However, chemists should consider using other SPE formats and more current sorbent chemistries such as Strata™-X SPE tubes to potentially improve the extraction efficiency. This can, in turn, bring benefits such as faster processing, increased sensitivity, consistency, and extraction efficiency.

Following extraction, EPA Method 539 requires LC/MS/MS analysis using an alkaline mobile phase. The high pH mobile phase greatly improves the ionization of some of the target compounds, resulting in improved LC/MS/MS sensitivity. Specifically, the compounds containing phenol functionality are deprotonated at high pH, allowing them to be analyzed in the negative polarity mode.

However, running high pH mobile phase severely limits the choice of HPLC columns that can be used, as typical silica-based HPLC media is not stable under alkaline conditions. The use of an organo-silica based HPLC media (Gemini®; **Figure 2**), specially designed to withstand alkaline mobile phase conditions, allows such mobile phases to be used without compromising column lifetime.

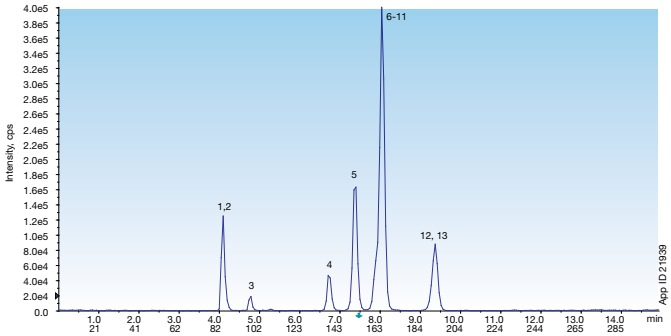
Figure 2. Structure of the organo-silica Gemini NX-C18 particle.

Ethane cross-linking stabilizes the silica particle providing resistance to high pH attack, while maintaining high efficiency and mechanical strength.



The LC/MS/MS procedure in the EPA Method 539 monograph requires a total run time of about 50 minutes. This study has modified the EPA Method 539 protocol and optimized it to effectively analyze all of the target compounds in only 15 minutes (**Figure 3**). The fast analysis time on the Gemini 3 µm NX-C18 column is very beneficial because laboratories are required to analyze a multitude of check standards aside from actual drinking water samples. These check standards include calibration data, MRL standards, and QC samples. Over time, a reduced analysis time allows a laboratory to be significantly more productive.

Figure 3.
Total ion chromatogram (TIC) of a representative standard injection



Full compound list can be found in Table 1

Aside from a fast analysis time, an effective analytical method also needs to be accurate with low limits of detection. **Table 2** lists the method reporting limits and linearity values for each compound. The MRLs range from 0.1 ng/L for testosterone to 4.0 ng/L for equilin. Excellent linearity values were achieved with all compounds exceeding R^2 values of 0.99 (**Figures 4, 5** & **6**).

Table 2.
Method reporting limit and linearity for each analyte

Compound	MRL (ng/L)	Linearity (R^2)
Estriol	0.8	0.9979
Equilin	4.0	0.9998
Estrone	2.0	1.0000
17-β-estradiol	0.4	0.9988
17-α-Ethynylestradiol	0.9	0.9980
4-Androstene-3,17-dione	0.3	0.9993
Testosterone	0.1	0.9994

Figure 4.
Calibration curve for Testosterone ranging from 0.05 to 2 ng/L in reagent water

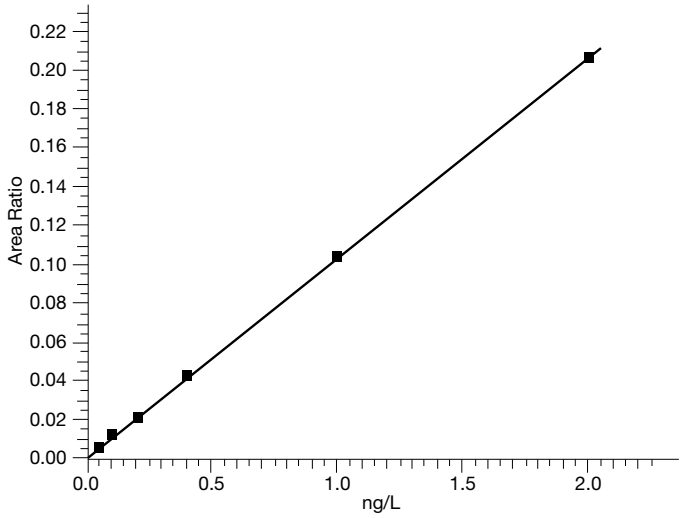


Figure 5.
Calibration curve for 17-α-Ethynylestradiol ranging from 0.9 to18 ng/L in reagent water

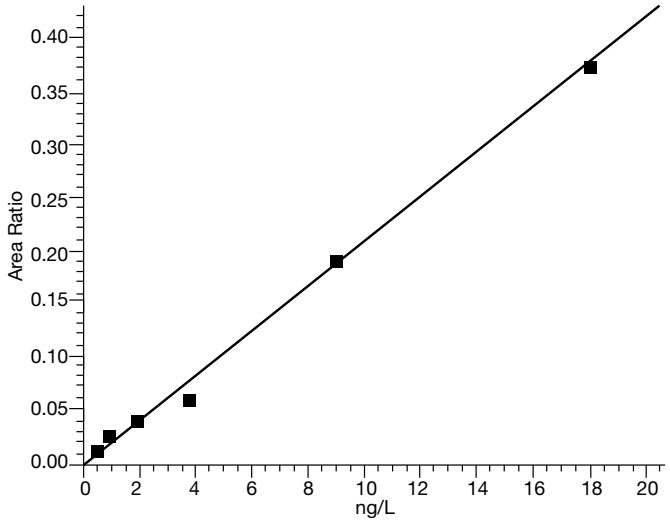
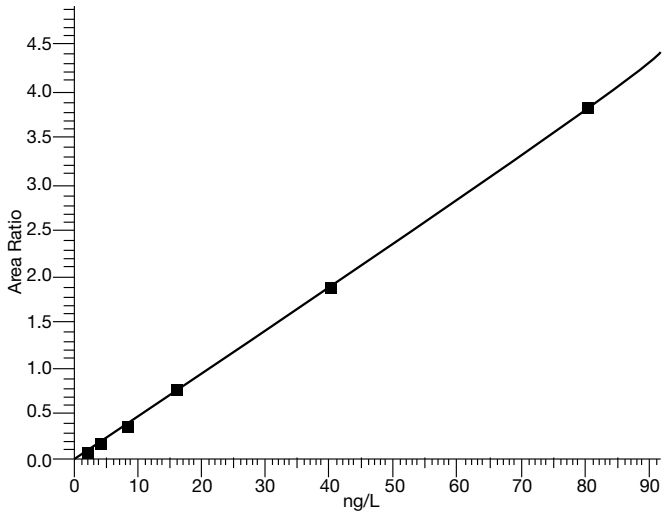


Figure 6.
Calibration curve for Equilin ranging from 2 to 80 ng/L in reagent water



Conclusion

This work presents an optimized chromatographic method for analyzing hormones in drinking water. Analysis time was significantly reduced from 50 to 15 minutes with the use of a Gemini NX-C18 column, while maintaining excellent linearity and low MRLs. This improved method should greatly increase lab efficiency and productivity.

References

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2. Revisions to the Unregulated Contaminant Monitoring Regulation (UCMR 3) for Public Water Systems; Final Rule, Federal Register, 77, Fed. Reg. 85 (May 2, 2012). Rules and Regulations, Environmental Protection Agency, 40 C.F.R. pts. 141 and 142.

3. United States Environmental Protection Agency. (December 21, 2012) EPA Approved Laboratories for UCMR 3. Retrieved: October 13, 2013, from http://water.epa.gov/lawsregs/rulesregs/sdwa/ucmr/ucmr3/upload/lablist-ucmr3-pdf.

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APPLICATIONS

Ordering Information
Gemini® HPLC Columns

3 µm Microbore, Minibore and Narrow Bore Columns (mm)

Phases	20 x 2.0	30 x 2.0	50 x 2.0	100 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard™ Cartridges (mm) 4 x 2.0*
									/10pk
NX-C18	00M-4453-B0	00A-4453-B0	00B-4453-B0	00D-4453-B0	00F-4453-B0	00B-4453-Y0	00D-4453-Y0	00F-4453-Y0	AJ0-8367

for ID: 2.0-3.0 mm

3 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
					/10pk
NX-C18	00B-4453-E0	00D-4453-E0	00F-4453-E0	00G-4453-E0	AJ0-8368

for ID: 3.2-8.0 mm

5 µm Minibore and Narrow Bore Columns (mm)

Phases	30 x 2.0	50 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	250 x 3.0	SecurityGuard Cartridges (mm) 4 x 2.0*
								/10pk
NX-C18	00A-4454-B0	00B-4454-B0	00F-4454-B0	00B-4454-Y0	00D-4454-Y0	00F-4454-Y0	00G-4454-Y0	AJ0-8367

for ID: 2.0-3.0 mm

5 µm Analytical Columns (mm)

Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	SecurityGuard Cartridges (mm) 4 x 3.0*
					/10pk
NX-C18	00B-4454-E0	00D-4454-E0	00F-4454-E0	00G-4454-E0	AJ0-8368

for ID: 3.2-8.0 mm

Ordering Information
Empore® Extraction Disks

Part No.	Description	Unit
AH0-2540	Empore Extraction Disks, Octadecyl (C18) 47 mm	20/pk
AH0-2541	Empore Extraction Disks, Octadecyl (C18) 90 mm	10/pk
AH0-2543	Empore Extraction Disks, Octadecyl (C8) 47 mm	20/pk

Recommended Alternative: Strata™-X SPE Tubes

Sorbent Mass	Part No.	Unit
Tube		
10 mg	8B-S100-AAK	1 mL (100/Box)
30 mg	8B-S100-TAK**	1 mL (100/Box)
30 mg	8B-S100-TBJ	3 mL (50/Box)
60 mg	8B-S100-UAK	1 mL (100/Box)
60 mg	8B-S100-UBJ**	3 mL (50/Box)
100 mg	8B-S100-EBJ	3 mL (50/Box)
100 mg	8B-S100-ECH	6 mL (30/Box)
200 mg	8B-S100-FBJ	3 mL (50/Box)
200 mg	8B-S100-FCH	6 mL (30/Box)
500 mg	8B-S100-HBJ	3 mL (50/Box)
500 mg	8B-S100-HCH	6 mL (30/Box)
Giga™ Tube		
200 mg	8B-S100-FDG	12 mL (20/Box)
500 mg	8B-S100-HDG	12 mL (20/Box)
1 g	8B-S100-JDG	12 mL (20/Box)
1 g	8B-S100-JEG	20 mL (20/Box)
2 g	8B-S100-KEG	20 mL (20/Box)
5 g	8B-S100-LFF	60 mL (16/Box)
10 g	8B-S100-MFF	60 mL (16/Box)
20 g	8B-S100-VFF	60 mL (16/Box)
50 g	8B-S100-YSN	150 mL (8/Box)

** Tab-less tubes available. Contact Phenomenex for details.

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