

# 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<sup>®</sup> 3  $\mu$ m NX-C18 HPLC column, which delivers stability at pH 1-12.

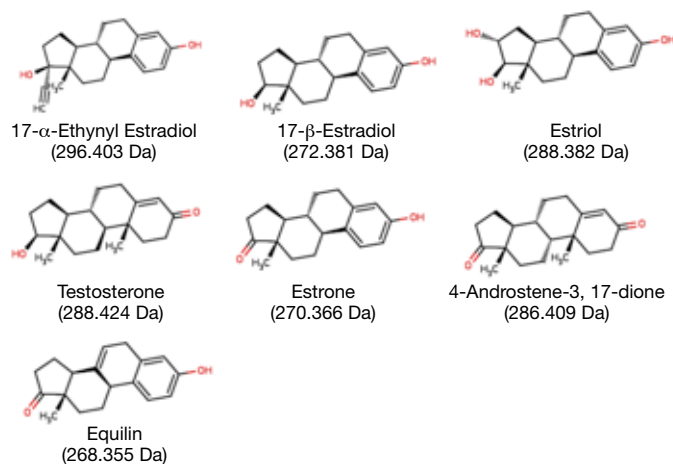
### Introduction

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.<sup>1</sup> 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).<sup>2</sup> Fewer than 20 labs across the U.S. are currently approved for this method.<sup>3</sup> 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  $\mu$ m 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 <sup>®</sup> 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 $\mu$ L of 50% methanol and transfer to a 1 mL volumetric flask. Add internal standard and adjust to 1 mL volume with 50% methanol.<br>Filtration (optional): Not necessary for drinking water sample |

#### LC/MS/MS Conditions

**Column:** Gemini 3  $\mu$ m NX-C18  
**Dimensions:** 100 x 2.0 mm  
**Part No.:** 00D-4453-B0  
**Mobile Phase:** A: 0.2% NH<sub>4</sub>OH in Water  
 B: 0.2% NH<sub>4</sub>OH 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  $\mu$ L  
**Temperature:** Ambient  
**Detection:** API 4000<sup>™</sup> (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- $\beta$ -estradiol         | 271.2 | 144.7/183.0 | 7.88     | ESI-     | 6           |
| 13C6-estradiol (I.S.)          | 277.1 | 145.0       | 7.88     | ESI-     | 7           |
| 17- $\alpha$ -Ethinylestradiol | 295.1 | 144.7/142.9 | 7.99     | ESI-     | 8           |
| Ethinylestradiol-D4 (SURR)     | 299.1 | 147.0/161.0 | 8.04     | ESI-     | 9           |
| 13C2-Ethinylestradiol (I.S.)   | 297.1 | 144.9       | 8.04     | ES-      | 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.

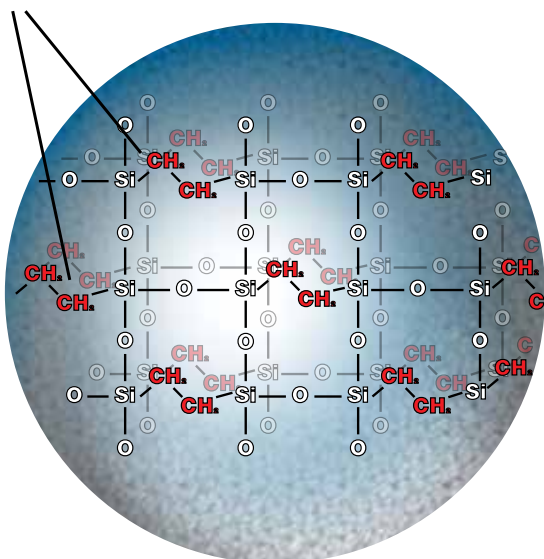
In this case, the sample extraction step was performed in accordance to EPA Method 539 using octadecyl SPE disks. However, chemists should consider using other SPE formats and more current sorbent chemistries such as Strata<sup>™</sup>-X SPE tubes to potentially improve the extraction efficiency. This 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<sup>®</sup>; **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.

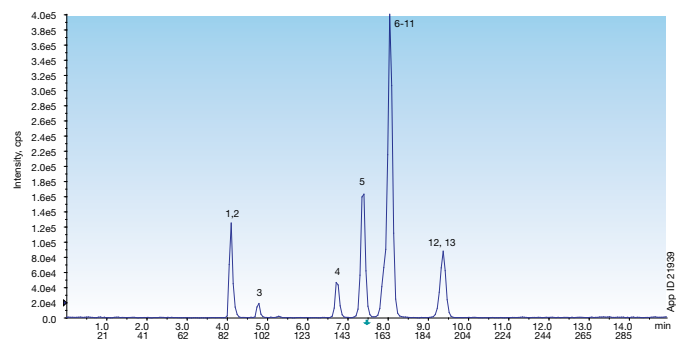
Ethane cross-linking stabilizes the silica particle providing resistance to high pH attack, while maintaining high efficiency and mechanical strength.



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  $\mu\text{m}$  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

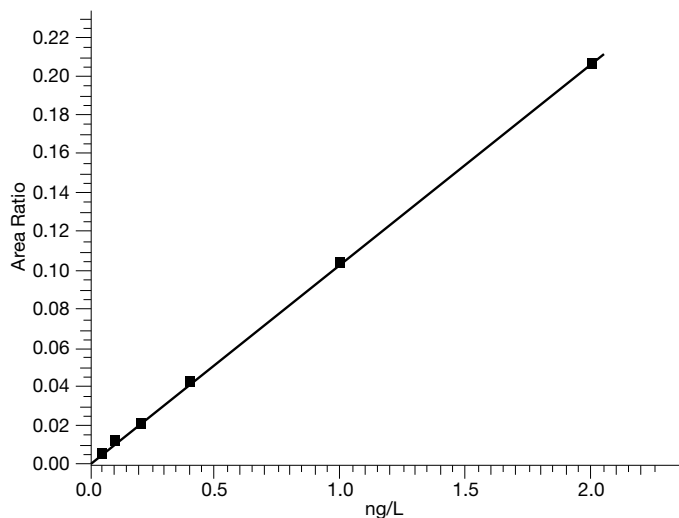
Aside from a fast analysis time, an effective analytical method also needs to be accurate with low limits of detection. **Table 2** lists the method reporting limits and linearity values for each compound. The MRLs range from 0.1 ng/L for testosterone to 4.0 ng/L for equilin. Excellent linearity values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

**Table 2.**

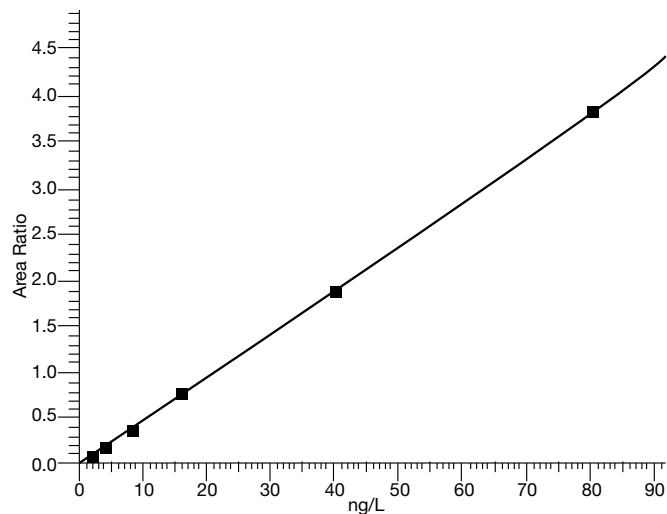
Method reporting limit and linearity for each analyte

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

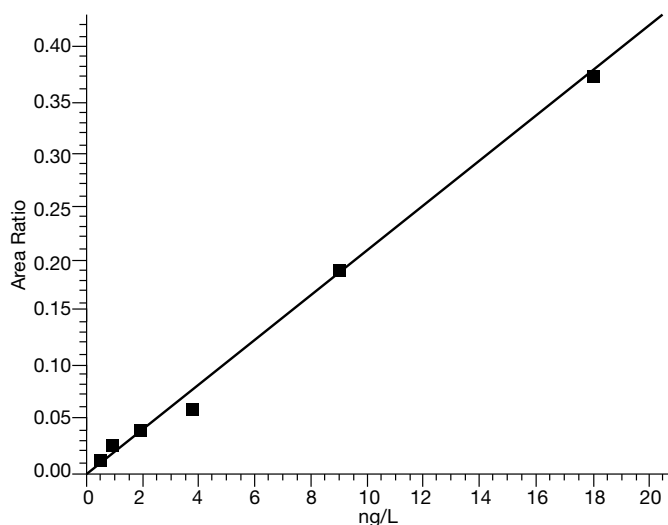
**Figure 4.**  
Calibration curve for Testosterone ranging from 0.05 to 2 ng/L in reagent water



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



**Figure 5.**  
Calibration curve for 17- $\alpha$ -Ethinylestradiol ranging from 0.9 to 18 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

1. Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. Snyder, S. A. (2009). Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environ, Sci, Technol.*, 43, p597-603.
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>.

## Acknowledgements

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# APPLICATIONS

## Ordering Information Gemini<sup>®</sup> HPLC Columns

### 3 $\mu$ m Microbore, Minibore and Narrow Bore Columns (mm)

| Phases | SecurityGuard <sup>™</sup> Cartridges (mm) |             |             |             |             |             |             |             |                    |
|--------|--------------------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------------|
|        | 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   | 4 x 2.0*           |
| 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 $\mu$ m Analytical Columns (mm)

| Phases | SecurityGuard Cartridges (mm) |             |             |             |                    |
|--------|-------------------------------|-------------|-------------|-------------|--------------------|
|        | 50 x 4.6                      | 100 x 4.6   | 150 x 4.6   | 250 x 4.6   | 4 x 3.0*           |
| NX-C18 | 00B-4453-E0                   | 00D-4453-E0 | 00F-4453-E0 | 00G-4453-E0 | AJ0-8368           |
|        |                               |             |             |             | for ID: 3.2-8.0 mm |

### 5 $\mu$ m Minibore and Narrow Bore Columns (mm)

| Phases | SecurityGuard Cartridges (mm) |             |             |             |             |             |             |          |                    |
|--------|-------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|----------|--------------------|
|        | 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   | 4 x 2.0* |                    |
| 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 $\mu$ m Analytical Columns (mm)

| Phases | SecurityGuard Cartridges (mm) |             |             |             |                    |
|--------|-------------------------------|-------------|-------------|-------------|--------------------|
|        | 50 x 4.6                      | 100 x 4.6   | 150 x 4.6   | 250 x 4.6   | 4 x 3.0*           |
| NX-C18 | 00B-4454-E0                   | 00D-4454-E0 | 00F-4454-E0 | 00G-4454-E0 | AJ0-8368           |
|        |                               |             |             |             | for ID: 3.2-8.0 mm |

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### Empore<sup>®</sup> 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<sup>™</sup>-X SPE Tubes

| Sorbent Mass                 | Part No.      | Unit           |
|------------------------------|---------------|----------------|
| <b>Tube</b>                  |               |                |
| 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)  |
| <b>Giga<sup>™</sup> Tube</b> |               |                |
| 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) |

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Gemini is patented by Phenomenex. U.S. Patent No. 7,563,367

SecurityGuard is patented by Phenomenex. U.S. Patent No. 6,162,362

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