Determination of Hormones in Drinking Water by LC/MS/MS Using an Agilent InfinityLab Poroshell HPH Column (EPA 539)

Application Note

Environmental

Authors

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Abstract

A method for the simultaneous determination of the seven hormones in finished water was developed and validated according to EPA Method 539 [1]. The analytes were extracted and cleaned by Agilent SPEC 47 mm C18AR disc solid phase extraction (SPE), separated on Agilent InfinityLab Poroshell HPH-C18, 4 µm and 2.7 µm HPLC columns under a basic mobile phase with a high pH of 10.5, and quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/MS/MS). The dynamic calibration range for the analytes was obtained from 0.5 to 70 ng/L. The limits of detection (LODs) for the method analytes fortified into reagent water ranged from 0.03 to 0.7 ng/L. The overall recoveries ranged from 82.6 to 105.6%, with RSD values between 2.7 and 6.0%.

Introduction

Hormones have been found in a wide range of water supplies throughout the world. Many hormones are active ingredients for birth-control medication. Due to this public health risk, there is a rapidly growing interest in monitoring these compounds. EPA Method 539 was specifically developed to monitor this growing problem. Figure 1 shows details of the analytes listed in the EPA Method 539. This is a challenging analysis because not only does it require low detection limits (0.1 parts per trillion (ppt) in water sample for some compounds), but it also requires mass spectrometer analysis in both positive and negative modes. To maximize the LC/MS/MS negative response, a high pH mobile phase was used.

Since conventional silica-based HPLC media is not stable under alkaline conditions, Agilent InfinityLab Poroshell HPH-C18 columns packed with superficially porous materials based on the Agilent Poroshell 120 family were used for this method. The column is designed to be stable in high pH mobile phases by integrating organic material into the porous outer silica layer, thus resisting dissolution under extreme high pH and high temperature conditions.

This study follows EPA Method 539, and determines the seven hormones in finished water using an Agilent 6460 Triple Quadrupole LC/MS system, an Agilent SPEC 47 mm C18AR disc SPE, and an Agilent InfinityLab Poroshell HPH-C18 column.

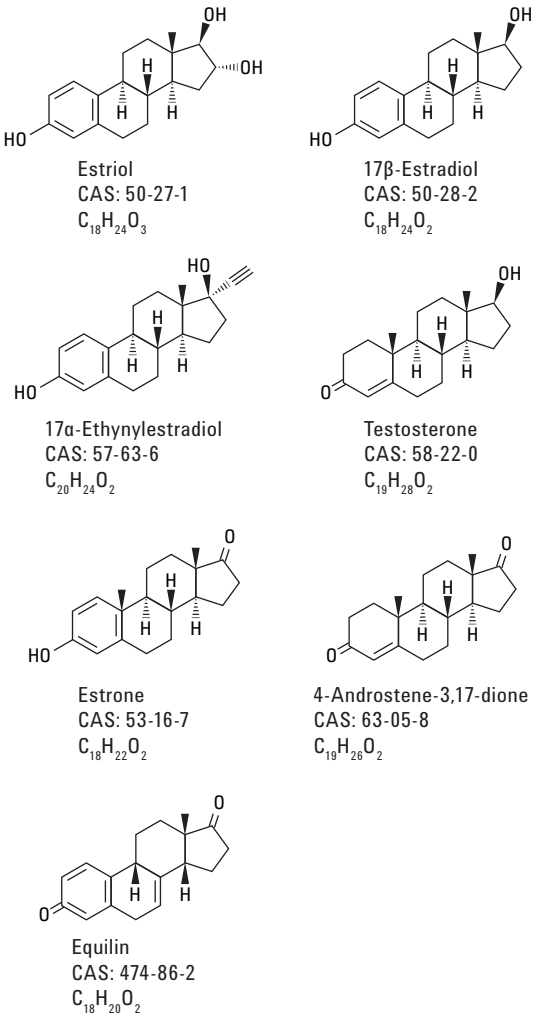


Figure 1. Hormones used in this study.

Materials and Methods

Reagents and chemicals

All reagents were MS, HPLC, or analytical grade. Methanol and ammonium hydroxide were purchased from J and K Co. Ltd. (Beijing, China), and water was made using an ELGA water purification system (UK). The standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Analyte stock standard and internal standard solutions were made according to EPA 539 at a temperature ≤ 6 °C.

Equipment and materials

- Agilent 1290 Infinity LC and Agilent 6460 Triple Quadrupole LC/MS system
- Agilent SPEC 47 mm C18AR disc (p/n A74819)
- Agilent SPEC environmental disc holder (p/n A713)
- Agilent SPEC flask (p/n A714)
- Agilent InfinityLab Poroshell HPH-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-502)
- Agilent InfinityLab Poroshell HPH-C18, 3.0 × 100 mm, 4 µm (p/n 695970-502)
- Eppendorf minispin plus centrifuge (Brinkmann Instruments, Westbury, NY, USA)
- Digital vortex mixer (VWR International, LLC, Radnor, Pennsylvania, USA)

Sample preparation

Each 1 L water was extracted using solid phase extraction (SPE) on a SPEC 47 mm C18AR disc (p/n A74819), as described in EPA Method 539, eluted with methanol, followed by concentration under a gentle stream of nitrogen to near dryness (Figure 2), brought to 1 mL with 50% methanol, and centrifuged at 14,000 rpm for 1 minute before analysis.

The analytes were monitored in positive or negative mode by the 6460 Triple Quadrupole LC/MS system [2]. Table 1 shows the multiple-reaction-monitoring details. As noted, the mobile phase applied in EPA Method 539 is 0.02% ammonium hydroxide in water and 0.02% ammonium hydroxide in methanol, which gives a pH of 10.5.

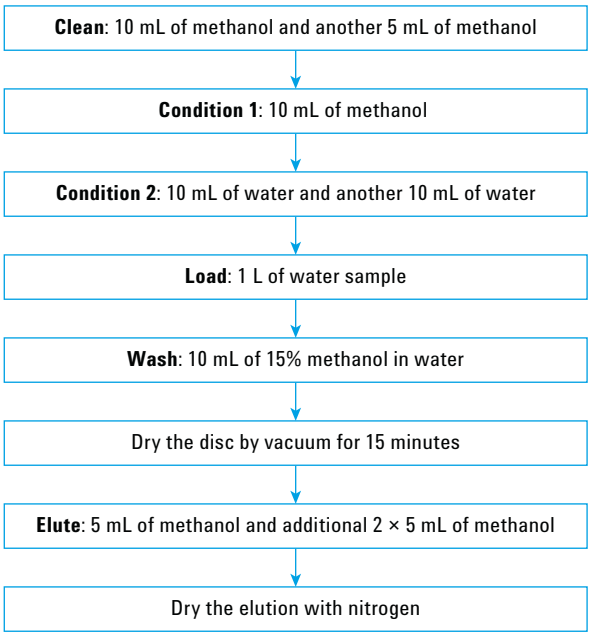


Figure 2. SPE procedure for water treatment using an Agilent SPEC 47 mm C18AR disc SPE.

Instrument conditions

HPLC conditions	
Instrument:	Agilent 1290 Infinity LC and Agilent 6460 Triple Quadrupole LC/MS system
Columns:	Agilent InfinityLab Poroshell HPH-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-502) Agilent InfinityLab Poroshell HPH-C18, 3.0 × 100 mm, 4 µm (p/n 695970-502)
Flow rate:	0.4 mL/min
Column temp:	25 °C
Injection volume:	20 µL
Mobile phase:	A) 0.02% ammonium hydroxide in water B) 0.02% ammonium hydroxide in methanol
Gradient:	Time (min) A (%) B (%) 0 50 50 10 10 90
Post run:	1.5 min
MS conditions	
Gas temp:	300 °C
Gas flow:	5 L/min
Nebulizer:	25 psi
Sheath gas temp.:	375 °C
Sheath gas flow:	11 L/min
Nozzle voltage:	Positive, 500 V; Negative, 1,500 V
Capillary:	Positive, 4,000 V; Negative, 4,500 V

Table 1. Masses monitored in the MRM for the seven hormones and internal standards.

Analyte	Retention time (min)	ESI mode	Precursor ion	Product ion	Fragmentor (V)	Collision energy (V)
Estriol-d_2 (IS)	2.68	Negative	289.1	173	180	32
Estriol	2.7	Negative	287.2	145	159	44
Estriol	2.7	Negative	287.2	171.2	159	36
Androstenedione	5.43	Positive	287.2	97.1	107	20
Androstenedione	5.43	Positive	287.2	109.1	107	24
Equilin	5.47	Negative	267.1	143.1	136	40
Equilin	5.47	Negative	267.1	265.1	136	20
Estrone	5.7	Negative	269.1	143.2	136	56
Estrone	5.7	Negative	269.1	145	136	40
$^{13}C_2$-Ethynylestradiol (IS)	5.76	Negative	277.2	186.1	170	38
17β-Estradiol	5.76	Negative	271.2	145.1	171	44
17β-Estradiol	5.76	Negative	271.2	183.2	171	40
$^{13}C_6$-Estradiol (IS)	5.77	Negative	297.2	144.9	175	38
17α-Ethynylestradiol	5.77	Negative	295.2	145	139	36
17α-Ethynylestradiol	5.77	Negative	295.2	199	139	36
Testosteone-d_3 (IS)	6.01	Positive	292.2	109.1	116	24
Testosterone	6.04	Positive	289.2	109.1	116	24
Testosterone	6.04	Positive	289.2	97.1	116	20

Most silica-based columns are not recommended for use in a mobile phase with a pH higher than 8 or 9. In this work, InfinityLab Poroshell HPH columns were used. They are designed to be stable in up to pH 11 mobile phase. This is achieved by integrating organic into the porous outer silica layer, which resists degradation under high pH conditions.

By using these columns, chromatographers can obtain the ultrahigh efficiency of these superficially porous particles, and enhance column lifetime even at high pH and temperature. The chromatograms of LC/MS/MS transitions for EPA Method 539 analytes and internal standards are shown in Figure 3 and Figure 4.

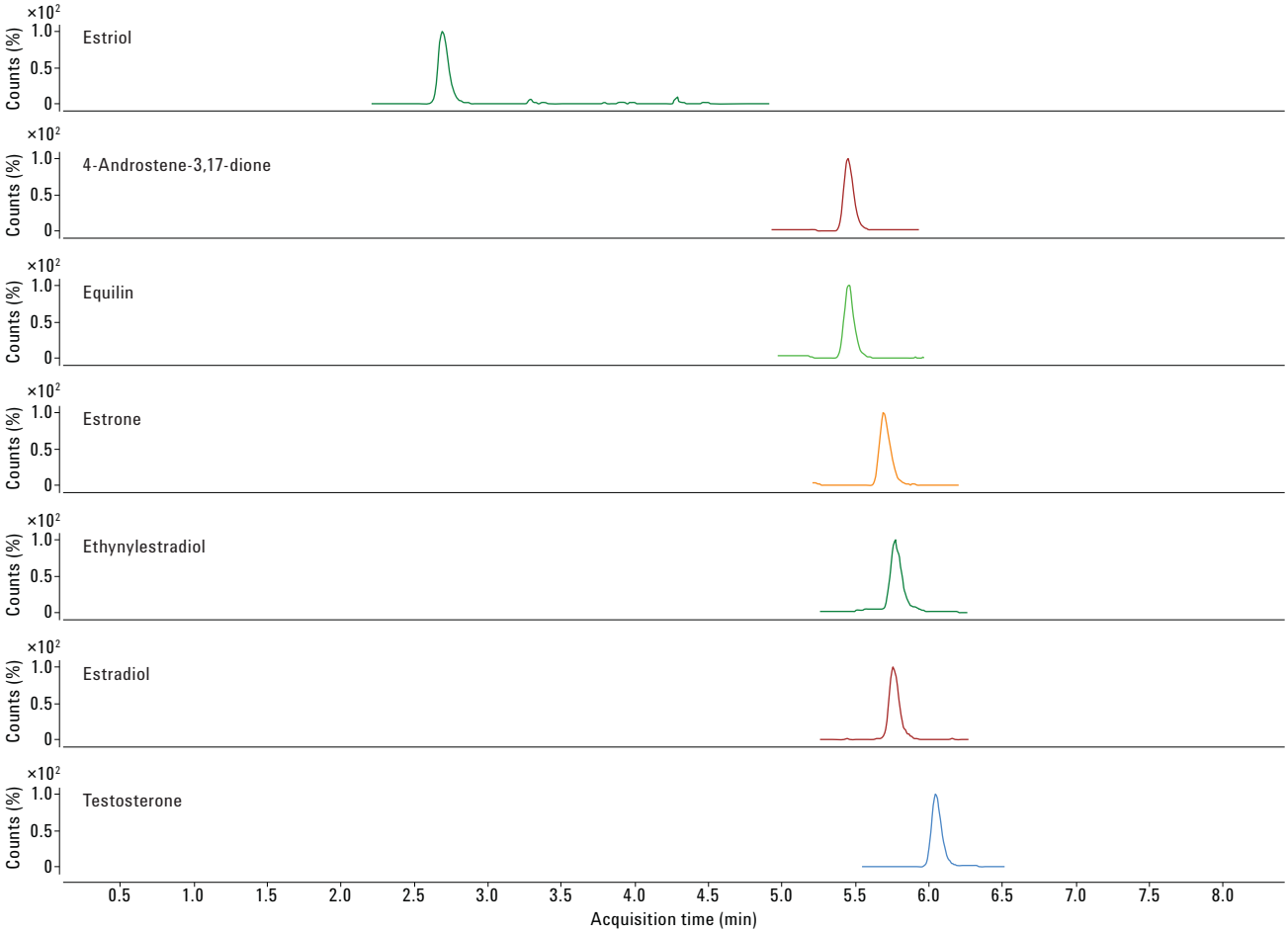


Figure 3. Chromatogram of LC/MS/MS transitions for EPA method 539 analytes using an Agilent InfinityLab Poroshell HPH-C18, 3.0 × 100 mm, 2.7 µm column.

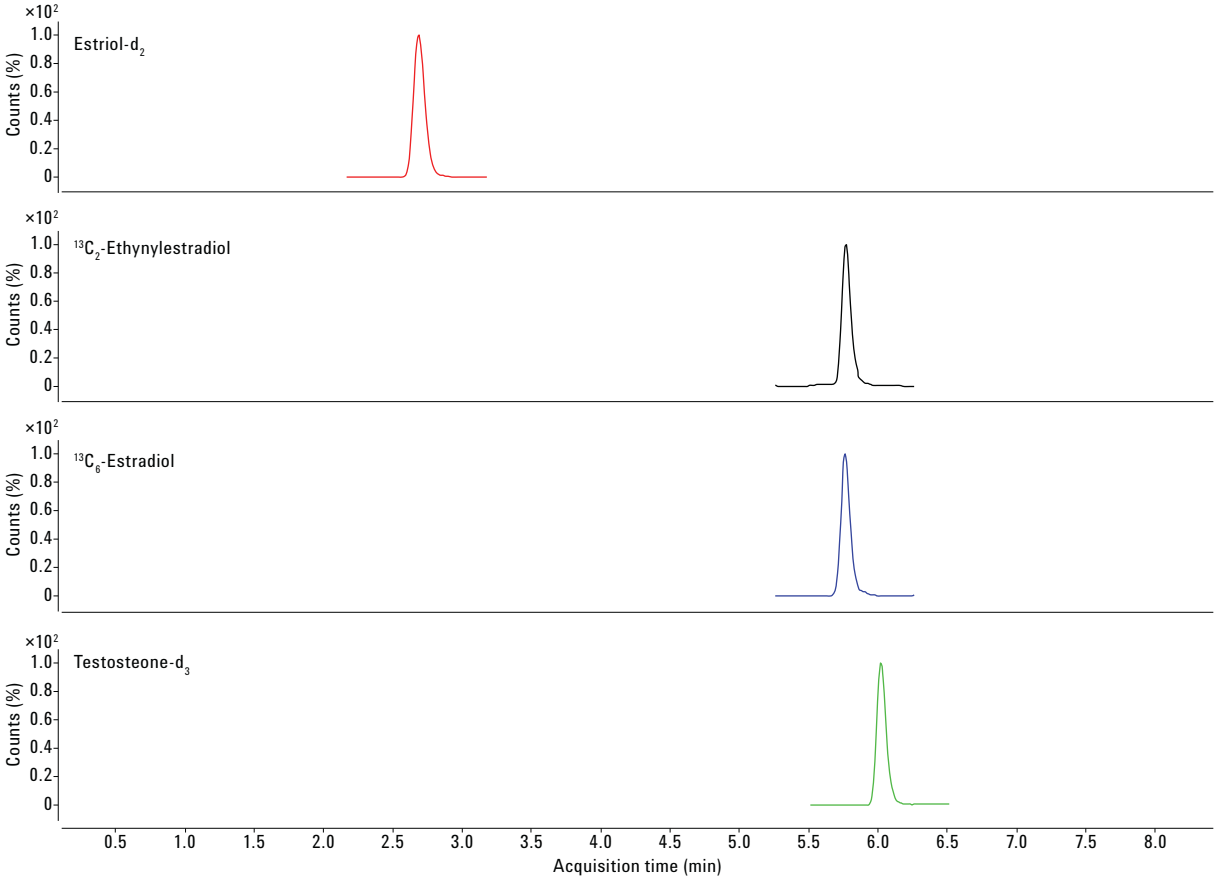


Figure 4. Chromatogram of LC/MS/MS transitions for EPA method 539 internal standards using an Agilent InfinityLab Poroshell HPH-C18, 3.0 ×100 mm, 2.7 µm column.

Results and Discussion

Linearity and limit of detection (LOD)

Six calibration standards from a combined stock solution were prepared to spike matrix blanks (Table 2). Matrix blanks were created by taking 1 L of reagent water through the entire sample preparation procedure. Calibration curves were constructed for all hormones, as shown in Table 3, and correlation coefficients (R^2) were ≥ 0.99. The data of LODs were calculated with a signal-to-noise ratio (S/N) of 3 by injecting the post spiked water matrix blanks at the concentrations shown in Table 2. The LODs for the analytes were from 0.03 to 0.7 ng/L, which was within the requirements of EPA Method 539 (from 0.04 to 2.9 ng/L).

Recovery and precision

The recovery and precision of the method were determined for all seven hormones, using six blanks at the fortified concentration. Table 4 shows the recovery and precision data. The data show good recovery and precision for all the analytes.

Table 2. Calibrator concentrations used for the seven hormones (ng/mL).

Analyte	Cal L1 (ng/mL)	Cal L2 (ng/mL)	Cal L3 (ng/mL)	Cal L4 (ng/mL)	Cal L5 (ng/mL)	Cal L6 (ng/mL)
17α-Ethynylestradiol	1.75	3.5	7	14	35	70
17β-Estradiol	1.25	2.5	5	10	25	50
4-Androstene-3,17-dione	0.5	1	2	4	10	20
Equilin	1	2	4	8	20	40
Estriol	1	2	4	8	20	40
Estrone	1	2	4	8	20	40
Testosterone	0.5	1	2	4	10	20

Table 3. Linearity and LOD of hormones in water.

Analyte	Regression equation	R^2	Range of linearity (ng/L)	LOD fortified concentration (ng/L)	LOD (ng/L)
17α-Ethynylestradiol	y = 1.177274x + 0.043932	0.996	1.75–70	1.75	0.72
17β-Estradiol	y = 1.399269 x – 0.020048	0.996	1.25–50	1.25	0.35
4-Androstene-3,17-dione	y = 1.022892 x – 0.010667	0.9992	0.5–20	0.5	0.035
Equilin	y = 4.067829 x – 0.065557	0.997	1–40	1	0.28
Estriol	y = 1.241231 x + 0.012070	0.998	1–40	1	0.24
Estrone	y = 5.242245 x – 0.034049	0.998	1–40	1	0.37
Testosterone	y = 2.859187 x – 0.017231	0.9997	0.5–20	0.5	0.031

Table 4. Recoveries and accuracy of hormones in water (n = 6).

Analyte	Fortified concentration (ng/L)	% Recovery	% RSD (n = 6)
17α-Ethynylestradiol	17.5	86.2	2.7
17β-Estradiol	12.5	87.8	6.7
4-Androstene-3,17-dione	5	82.6	3.7
Equilin	10	89.1	6.0
Estriol	10	91.7	4.6
Estrone	10	93.7	5.5
Testosterone	5	105.6	5.8

Comparison between 2.7 and 4 µm columns

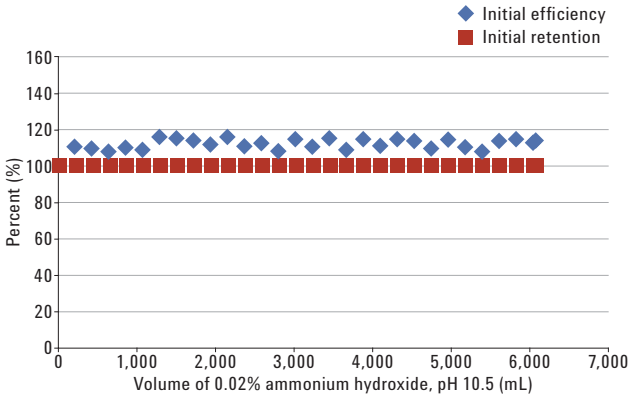
The method was run on 4 µm and 2.7 µm columns with the same 4.6 × 100 mm dimensions, and the same phase of Poroshell HPH-C18. As expected, the 2.7 µm column gave better S/N than the 4 µm column, and provided better LODs (Table 5). However, pressure on the 4 µm column decreased by 45%, and yielded adequate LODs, which were still within the EPA Method 539 requirements. The 4 µm is better suited for use on a 400 bar HPLC, whereas the 2.7 µm column is suitable for 600 bar and even higher pressure UHPLC.

Column lifetime test

The stability of an HPLC column is one of the critical factors that determines the success of a method. One of the most important things that chromatographers consider is how long the column will last under a specific set of analysis conditions. In our study, the lifetime of an InfinityLab Poroshell HPH-C18 was evaluated with a modified version of the above developed hormone analysis method using a high pH mobile phase. This 0.02% ammonium hydroxide mobile phase is not typically used with standard silica HPLC columns. The test was carried on a InfinityLab HPH-C18, 2.1 × 100 mm column, and the column was tolerant for over 6,000 mL of delivered mobile phase volume. The volume of mobile phase eluted during the test and the initial efficiency and retention factor of internal standard Testosteone-d_3 were plotted, and are shown in Figure 5. The retention factor was unchanged, and the efficiency had some drift, but did not show any decline during the entire test. These data demonstrated that the InfinityLab Poroshell HPH-C18 column had a long lifetime even with a high pH mobile phase.

Table 5. LODs comparisons between 4 and 2.7 µm columns.

Analyte	LOD fortified concentration (ng/L)	LOD (ng/L) 2.7 µm	S/N 2.7 µm	LOD (ng/L) 4 µm	S/N 4 µm
17α-Ethynylestradiol	1.75	0.72	5.93	1.29	4.71
17β-Estradiol	1.25	0.35	13.83	0.49	10.71
4-Androstene-3,17-dione	0.5	0.035	39.92	0.58	29.41
Equilin	1	0.28	21.91	0.51	19.06
Estriol	1	0.24	9.38	0.22	0.62
Estrone	1	0.37	42.22	0.47	35.35
Testosterone	0.5	0.031	126.47	0.044	95.48



Test conditions

Column:	Agilent Poroshell HPH-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-702)
Flow rate:	0.3 mL/min
Column temp.:	25 °C
Injection volume:	2 µL (hormones standards mixture)
Mobile phase:	A) 0.02% ammonium hydroxide in water B) 0.02% ammonium hydroxide in methanol
Gradient:	Time (min) A (%) B (%) 0 50 50 6 10 90
Post run:	1 min

Figure 5. Stability of an Agilent InfinityLab Poroshell HPH-C18 in 0.02% ammonium hydroxide with a pH of 10.5 mobile phase.

Conclusions

The method using an Agilent 6460 Triple Quadrupole LC/MS System and Agilent SPEC 47 mm C18AR disc SPE and an Agilent InfinityLab Poroshell HPH-C18 column provides LODs and recovery that meet or, in some cases, greatly exceed EPA requirements. Most of the LC/MS conditions remained the same as EPA Method 539, but with a short run time using a superficially porous particle column. The InfinityLab Poroshell HPH column not only maintains the advantages of superficially porous particles, but also provides chemical stability under high pH mobile phase conditions. A smaller particle 2.7 µm gave a better S/N than the 4 µm column, while both are suitable for the EPA 539 method.

References

1. EPA Method 539 – Determination of Hormones in Drinking Water by Solid Phase Extraction (SPE) and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS), EPA Document No. 815-B-10-001, November **2010**.
2. R. Hindle. *Improved Analysis of Trace Hormones in Drinking Water by LC/MS/MS (EPA 539) using the Agilent 6460 Triple Quadrupole LC/MS*; Application note, Agilent Technologies, Inc. Publication number 5991-2473EN, **2013**.

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