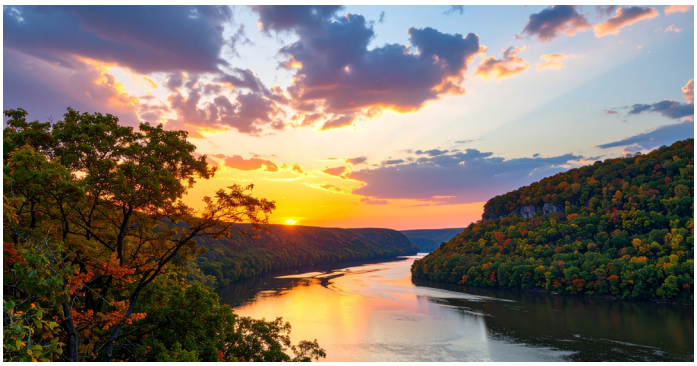
Determination of Hormones in Water by Solid-Phase Extraction (SPE) and Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)*



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EUC18156
500 mg unendcapped C18, 6 mL

Method Summary

Water samples are dechlorinated with sodium thiosulfate and protected from microbial degradation with 2-mercaptopyridine-1-oxide sodium salt during collection. Samples are fortified with surrogates and extracted using C18 cartridges before elution with methanol. The extract is concentrated to dryness with N_2 before adjusting to a 1 mL volume with 50:50 methanol:water. An aliquot is injected into an LC equipped with a C18 column interfaced to a MS/MS. Detection limits of 0.02-0.37 ng/L can be obtained using this application from samples fortified at 0.25-0.875 ng/mL.

Analytes Determined Using This Method

Analyte	CASRN
Estriol 16α-Hydroxyestradiol	50-27-1
17β-Estradiol	50-28-2
17α-Ethynylestradiol	57-63-6
Testosterone	58-22-0
Estrone 3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren-17-one	53-16-7
4-Androstene-3,17-dione	63-05-8
Equilin 3-hydroxyestra-1,3,5,7-tetraen-17-one	474-86-2

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Safety

1. The toxicity and carcinogenicity of each reagent has not been defined.
2. Each chemical should be treated as a potential health hazard and exposure minimized by the use of PPE (personal protective equipment) such as gloves, respirators and other personal safety equipment.
3. Pure standard materials and stock standard solutions of the method compounds should be handled with suitable protection.
4. Ammonium hydroxide, used during method development as a pH modifier for the HPLC mobile phase, should be fresh* and handled in a fume hood.

**NH4OH loses its strength after opening due to evaporation. A fresh bottle should be used.*

Sample Collection, Preservation, and Storage

1. Use one-liter amber glass bottles with PTFE-lined screw caps
2. If smaller sample sizes are used, adjust the amount of preservatives and surrogate/analyte fortification levels according to the sample size
3. Fill sample bottles taking care not to flush out the preservatives. Analytes are not volatile so bottles need not be headspace-free
4. When sampling from a cold water tap, remove the aerator and allow the system to flush until the water temperature has stabilized. Invert the bottles several times to mix the sample with the preservation reagents
5. Samples should be chilled during shipment and should not exceed 10° C during the first 48 hours after collection
6. In the laboratory, store samples at or below 6° C and protect from light until analysis. Do not freeze unprocessed samples
7. All compounds listed in the method have adequate stability for 28 days when collected, preserved, shipped and stored as described
8. Samples should be extracted as soon as possible for best results
9. After sample preparation, extracted samples should be stored at 0° C or less and analyzed within 28 days after extraction

Sample Preservatives

Compounds	Amount	Purpose
Sodium thiosulfate	80 mg/L	Removes free chlorine
2-mercaptopyridine-1-oxide sodium salt	65 mg/L	Microbial inhibitor

Note: Preservation reagents listed above should be added to each sample bottle prior to shipment to the field or prior to sample collection

Interferences

1. Matrix interferences may be caused by contaminants that are co-extracted from the sample and will vary from source to source
2. Humic and/or fulvic material in environmental samples may be co-extracted during SPE and can cause enhancement and/or suppression in the electrospray ionization source. Total organic carbon (TOC) is an indicator of the humic content of a sample
3. Use only high purity analogs. Depending on the source and purity, labeled analogs used as internal standards may contain a small percentage of the corresponding native analyte which may be significant when attempting to determine LCMRLs and DLs
4. Nitrile gloves should be worn at all times. Handling clean glassware may be a potential source of interference
5. It may be appropriate to include a Field Blank with the sampling bottles depending on the sampling site. The Field Blank is analyzed along with the samples to ensure that no human hormones were introduced into the samples during the collection and handling process

Internal Standards

Internal Standard	CASRN	Neat Material Cat #	Solution Standard Cat #
16α-Hydroxyestradiol-d2 (Estriol-d2)	53866-32-3	C/D/N Isotopes Cat. No. D-527	N/A
^{13}C6-Estradiol	None	None	Cambridge Isotope Labs 100 µg/mL in Methanol Cat. No. CLM-7936-1.2
^{13}C2-Ethynylestradiol	None	None	Cambridge Isotope Labs 100 µg/mL in Acetonitrile Cat. No. CLM-3375-1.2
Testosterone-d3	77546-39-5	None	Sigma Drug Std., 100 µg/mL in dimethoxyethane Cat. No. T5536

Internal Standard Stock Standards

ISSSS 500 µg/mL - Weigh 5 mg of α-hydroxyestradiol-d2 (estriol-d2) into a tared 10 mL volumetric flask and dilute to volume with methanol. The remaining internal standards can be purchased as100 µg/mL solutions

Internal Standard Primary Dilution Standard (IS-PDS)

IS PDS 1.0 – 4.0 µg/mL: The table below can be used as a guide for preparing the IS PDS. The IS PDS is prepared in acetonitrile and is stable for about six months if stored at a temperature < 6° C. Use 5 µL of the 1.0 – 4.0 µg/mL IS PDS to fortify the final 1 mL extracts. This will yield a final concentration of 5.0 – 20 ng/mL of each IS.

Internal Standards

Internal Standard	Conc IS Stock µg/mL	Volume of IS Stock, µL	Final Volume of IS PDS	Final Volume of IS PDS
16α-Hydroxyestra-diol-d2 (Estriol-d2)	500	40	10	2.0
^{13}C6-Estradiol	100	400	10	4.0
^{13}C2-Ethynylestradio	100	400	10	4.0
Testosterone-d3	100	100	10	1.0

Internal Standard Fortification Standard

5 µL of the IS PDS is difficult to accurately and reproducibly transfer into HPLC vials. Therefore, a 1-in-10 dilution of the IS PDS is prepared by transferring 1 mL of the 1.0 – 4.0 µg/mL IS PDS into a 10 mL volumetric flasks and diluting to volume with methanol. This results in a 0.1-0.4 µg/mL IS solution.

50 µL of the prepared ISPDS is added to the samples prior to analysis.

Surrogate Analytes

Two isotopically labeled surrogates are listed below. Select the surrogate that performs best under the LC-MS/MS conditions employed for the analysis.

For this application, Bisphenol A-d16 was chosen as surrogate analyte.

Surrogate Analytes

Surrogate Analyte	CASRN	Neat Materials Catalog No.
Ethynylestradiol-d_4	350820-06-3	C/D/N Isotopes, Cat. No. D-4319
Bisphenol A-d_{16}	96210-87-6	Sigma, Cat. No. 451835

Surrogate Stock Standards

1000 µg/mL - Prepare individual solutions of the surrogate standards by weighing 10 mg of the solid material into tared 10 mL volumetric flasks and diluting to volume with methanolis.

Surrogate Analyte Primary Dilution Standard

SUR PDS 2.5 – 7.0 µg/mL
Use the table below as a guide for preparation of the **SUR PDS** in methanol.

Use 10 µL of **SUR PDS** to fortify 1-L samples yielding a final concentration of 70 ng/mL ethynylestradiol-d4 or 25 ng/mL bisphenol A-d16 in the 1 mL extracts.

Surrogate Analyte Solutions

Internal Standard	Conc of SUR Stock (µg/mL)	Volume of SUR Stock (µg/mL)	Final Volume of SUR PDS (mL)	Final Conc. Of SUR PDS (µ g/mL)
Ethynylestradiol-d_4	1000	70	10	7.0
Bisphenol A-d^{16}	1000	25	10	2.5

Surrogate Fortification Standard (SUR PDS)

10 µL of the **SUR PDS** is difficult to accurately and reproducibly transfer into samples. Therefore, a 1-in-10 dilution of the **SUR PDS** is prepared by transferring 1 mL of the 2.5 µg/mL IS PDS into a 10 mL volumetric flask and diluting to volume with methanol, resulting in a 0.25 µg/mL IS solution. Use 100 µL of this fortification standard to fortify 1-L samples yielding a final concentration of 25 ng/mL bisphenol A-d16 in the 1 mL extracts.

Analyte Stock Standard Solution

Each concentration equals 1000 µg/mL - Obtain the analytes listed in Table 1 above as standard solutions or as neat materials. Prepare stock standards individually by weighing 10 mg of the solid standards into tared 10 mL volumetric flasks and diluting to volume with methanol.

Analyte Primary Dilution Standard

Prepare concentrations of the stock standard solutions between 1.0 – 3.5 µg/mL – Dilute the Analyte Stock Standard solutions into 50% methanol in reagent water. The concentrations vary based on the instrumental sensitivity. The Analyte PDS is used to prepare calibration standards, and to fortify LFBs, LFSMs, and LFSMDs with the method analyte.

Analyte Stock	Stock Concentration µg/mL	Stock Volume µL	Final Volume (ml, 50% MeOH)	Analyte PDS Concentration µg/L
Ethynylestradiol-d_4	100 0	20	10	2.0
Bisphenol A-d^{16}	100 0	20	10	2.0
Bisphenol A-d^{16}	100 0	25	10	2.5
Bisphenol A-d^{16}	100 0	35	10	3.5
Bisphenol A-d^{16}	100 0	20	10	2.0
Bisphenol A-d^{16}	100 0	10	10	1.0
Bisphenol A-d^{16}	100 0	10	10	1.0

Analyte Fortification Standard

10 µL of the Analyte PDS is difficult to accurately and reproducibly pipette into samples. Therefore, a 1-in-10 dilution of the Analyte PDS is prepared by transferring 1 mL of the 1.0 – 3.5 µg/mL Analyte PDS into a 10 mL volumetric flask and diluting to volume with 50% methanol, resulting in a 0.1 – 0.35 µg/mL solution. Use 100 µL of this fortification standard to fortify 1-L samples yielding a final concentration of 10 – 35 ng/mL in the 1 mL extracts.

An additional 1-in-10 dilution of the 0.1 – 0.35 µg/mL fortification solution is prepared in a 10 mL volumetric flask using 50% methanol. This yields a 0.01 – 0.035 µg/mL solution that is used to fortify 1-L samples yielding a final concentration of 1 – 3.5 ng/mL in the 1 mL extracts

Procedure

1) Sample Preparation

a) Add a 100-µL aliquot of SUR Fortification Standard to each 1-L sample for a final concentration of 25 ng/L bisphenol A-d16.
b) Fortify LFBs, LFSMs, or LFSMDs with an appropriate volume of Analyte Fortification Standard.
c) Cap and invert each sample several times to mix

2) Cartridge Preparation

a) Assemble a vacuum manifold. Automated extraction equipment may also be used
b) Place EUC18156 cartridge(s) on the manifold
c) Add a 10 mL aliquot of methanol to the cartridge and draw through the cartridge until dry
d) Add another 5 mL aliquot of methanol and draw through the cartridge until dry
Note: Do Not Let the Cartridge Go Dry after Starting the Following Steps
e) Add approximately 10 mL of methanol to each cartridge.
f) Draw about 1 mL of solvent through the cartridge and turn off the vacuum temporarily.
g) Let the cartridge soak for about one minute then draw most of the remaining solvent through the cartridge leaving a thin layer of methanol on the surface of the cartridge.
h) Add 10 mL of reagent water to each cartridge and draw through leaving a thin layer of liquid on the surface of the cartridge.
i) Add another 10 mL aliquot of reagent water.
j) Draw the water through the cartridge keeping the water level above the cartridge surface.
k) Turn off the vacuum.

3) Sample Extraction

a) Add the sample to the extraction reservoir containing the conditioned cartridge and turn on the vacuum (approximately 10 to 15 in. Hg). Flow of sample through the cartridge should be a fast drip. Adjust vacuum if necessary.
b) Do not let the cartridge go dry before the entire sample volume is extracted.
c) After the entire sample has been drawn through the cartridge, add a 10 mL aliquot of 15% methanol to the sample container and wash the cartridge with the rinsate from the container.
d) Using full vacuum, draw air through the cartridge by maintaining full vacuum for 10–15 minutes .
e) After drying, turn off and release the vacuum.

4) Sorbent Elution

a) Insert collection tubes into the manifold to collect the cartridge extracts. The collection tube should fit around the drip tip of the base to ensure collection of all the eluent.
b) Add 5 mL of methanol to the cartridge and draw the methanol into the cartridge to soak the sorbent.
c) Allow the cartridge to soak for about one minute.
d) Using vacuum, draw the remaining methanol slowly through the cartridge into the collection tube.
e) Elute with an additional 2 x 5 mL aliquots of methanol.
Note: The methanol can be eluted directly into a 15 mL tube (or larger). Otherwise 5 mL MeOH can be eluted into a 5 mL culture tube and evaporated to near dryness prior to adding the second 5 mL eluate. This process is repeated until all 15 mL MeOH has been added to the 5 mL culture tube.

5) Extract Concentration

a) Concentrate the extract to dryness under a gentle stream of N2 in a warm water bath (~45° C).
b) Rinse the collection tube with 950 µL of 50% methanol, vortex for 2 min and transfer the rinse into a HPLC vial
c) Add 50 µL IS Fortification Standard (ISFS) and vortex for an additional 1 min

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6) Sample Filtration

a) It is highly recommended that extracts be filtered with at least a 0.45 micron syringe filter prior to analysis to remove the particulates in a sample.

b) If filtering is incorporated as part of the sample preparation, the first lot of syringe filters should be included in the procedure to document the potential interferences that are introduced or analytes are retained on the filter.

c) Subsequent lots of syringe filters can be verified by examining CAL standards.

d) Add another 5 mL aliquot of methanol and draw through the cartridge until dry

Sample Analysis

HPLC Conditions	
HPLC instrument	Thermo Scientific Dionex UltiMate 3000 System**
Column	Thermo Scientific Accucore C18, 100 x 2.1 mm, 2.6 µm with 10 mm guard column
Column Temperature	35° C
Column Flow Rate	0.200 mL/min
Injection Volume	20 µL

Gradient		
Time (min)	**Moblie Phase A H_2O + 0.02% NH_4OH**	**Moblie Phase B MeOH +0.02% NH_4OH**
0	70	30
1	35	65
9	35	65
9.1	15	85
11	15	85
11.1	70	30
15	70	30

ESI-MS/MS Method Conditions

MS Parameters	
MS instrument	Thermo Scientific TSQ Vantage**
Polarity	$HESI^+$ & $HESI^-$
Spray Voltage V	+4500 / -3500 V
Vaporizer Temperature	350°C
Ion Transfer Capillary	300°C
Sheath Gas Pressure	45 arbitrary units
Auxiliary Gas Pressure	40 arbitrary units
Q1 and Q3 Peak Width (FWHM)	0.4 and 0.7 Da
Collision Gas and Pressure	Ar at 1.5 mTorr
Scan Type	SRM
Cycle Time	0.75 Sec

**Alternative LC-MS/MS systems may be used

LC-ESI-MS/MS Analyte Retention Times, Precursor and Product Ions, S-lens, and Collision Energy

Analyte	Ret. Time (min.)	ESI Mode	Precursor Ion	Product Ion	S-lens V	Collision Energy eV	Internal Standard
Estriol	4.99	ESI-	286.75	144.96	100	39	Estriol-d_2
Bisphenol A-d16	6.20	ESI-	240.91	223.02	60	18	$^{13}C_6$-Estradiol
Equilin	7.03	ESI-	266.80	142.97	67	37	$^{13}C_6$-Estradiol
17β-Estradiol	7.14	ESI-	270.93	144.98	67	40	$^{13}C_6$-Estradiol
Androstenedione	7.18	ESI+	286.87	96.88	76	20	Testosterone-d_3
17α-Ethynylestradiol	7.19	ESI-	294.79	244.97	80	39	$13C_2$-Ethynylestradiol
Estrone	7.24	ESI-	268.78	144.97	109	39	$13C_2$-Ethynylestradiol
Testosterone	7.76	ESI+	288.96	96.88	76	20	Testosterone-d_3

LC-ESI-MS/MS Internal Standard Retention Times, Precursor and Product Ions, S-lens, and Collision Energy

Analyte	Ret. Time (min.)	ESI Mode	Precursor Ion	Collision Energy eV	Internal Standard
Estriol-d2	5.00	288.75	146.96	100	39
$^{13}C_6$-Estradiol	7.13	276.93	146.98	67	40
$^{13}C_2$-Ethynylestradiol	7.16	296.80	144.97	80	39
Testosterone-d3	7.73	291.96	96.88	70	20

Analyte Recovery

n = 5	Estriol	Equilin	Estradiol	Androstenedione	Testosterone	Ethynylestradiol	Estrone
Fortified Conc (ng/mL)	20	20	25	10	20	10	35
Mean	97.61	82.81	96.98	92.88	90.76	96.49	93.95
SD	6.45	5.90	5.17	2.55	3.47	1.31	2.02
RSD	6.60	7.13	5.33	2.75	3.82	1.36	2.15

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n = 5	Estriol	Equilin	Estradiol	Androstenedione	Testosterone	Ethynylestradiol	Estrone
Fortified Conc (ng/mL)	2	2	2.5	1	1	3.5	2
Mean	83.05	96.34	93.68	98.31	104.63	109.45	106.10
SD	4.02	8.92	7.02	3.90	4.95	6.96	2.02
RSD	4.84	9.26	7.49	3.96	4.73	6.36	2.15

*Based on EPA Method 539, Version 1.0, November 2010, Glynda A. Smith (U.S. EPA, Office of Ground Water and Drinking Water) Alan D. Zaffiro, (Shaw Environmental, Inc.) M. L. Zimmerman (Shaw Environmental, Inc.) D. J. Munch (U.S. EPA, Office Of Ground Water And Drinking Water), Technical Support Center , Standards And Risk Management Division, Office Of Ground Water And Drinking Water, U. S. Environmental Protection Agency, Cincinnati, Ohio 45268

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