Determination Of Hormones In Drinking Water By Solid Phase Extraction (SPE) and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)*

EPA Method 539 Version 1.0

UCT Product: **ECUNIC18** (83 mL cartridge with 1100 mg C18)

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Background

Method 539 is a liquid chromatography, electrospray ionization, tandem mass spectrometry (LC-ESI-MS/MS) technique for the determination of hormones in finished drinking water. It requires the use of MS/MS in Multiple Reaction Monitoring (MRM) mode to enhance selectivity

Analytes Covered in Method 539

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

Table 1

Method Summary

Water samples are dechlorinated and protected from microbial degradation during collection. Samples are then fortified with surrogates and passed through solid phase octadecyl C18 cartridges which are equivalent to disk SPE media.** Compounds are eluted using a small amount of methanol. The extract is then 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. Analytes are identified by comparing the mass spectra and retention times to reference spectra for calibration standards acquired under identical LC-MS/MS conditions. The concentration of each analyte is determined using the internal standard technique.

Safety

- The toxicity and carcinogenicity of each reagent has not been defined
- Each chemical should be treated as a potential health hazard and exposure minimized
- Pure standard materials and stock standard solutions of the method compounds should be handled with suitable protection
- Ammonium hydroxide, used during method development as a pH modifier for the HPLC mobile phase, should be handled in a fume hood

Sample Collection, Preservation, and Storage

- Use one-liter amber glass bottles with PTFE-lined screw caps
- Smaller sample volumes (e.g., 500-mL) may be used if the laboratory demonstrates acceptable performance in meeting the required MRLs. Adjust the amount of preservatives and surrogate/analyte fortification levels according to size
- Grab samples must be collected using conventional sampling practices
- Fill sample bottles taking care not to flush out the preservatives. Analytes are not volatile so bottles need not be headspace-free
- 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.
- Samples must be chilled during shipment and must not exceed 10° C during the first 48 hours after collection
- Samples must be confirmed to be at or below 10° C when they are received at the laboratory
- In the laboratory, store at or below 6° C and protected from light until analysis. Do not freeze samples
- All compounds listed in the method have adequate stability for 28 days when collected, preserved, shipped and stored as described
- Samples should be extracted as soon as possible for best results, but must be extracted within 28 days
- Extracts must be stored at 0° C or less and analyzed within 28 days after extraction

Preservation reagents listed below are added to each sample bottle prior to shipment to the field or prior to sample collection

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

Interferences

- All glassware must be meticulously cleaned. Wash glassware with detergent and tap water, rinse with tap water, followed by reagent water
- Non-volumetric glassware can be heated in a muffle furnace at 400° C for two hours or solvent rinsed instead
- Never heat volumetric glassware in an oven above 120° C. Use solvent rinse
- Method interferences may be caused by contaminants in solvents, reagents (including reagent water), sample bottles and caps, and other sample processing hardware
- All laboratory reagents and equipment must be routinely demonstrated to be free from interferences (less than 1/3 the MRL for the target analytes) under the conditions of the analysis
- Analyze laboratory reagent blanks (LRB) by processing exactly as a sample including exposure to all glassware, equipment, solvents and reagents, sample preservatives, surrogates and internal standards that are used in the extraction
- Subtracting blank values from sample results is not permitted
- Matrix interferences may be caused by contaminants that are co-extracted from the sample and will vary from source to source
- 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
- 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
- Nitrile gloves should be worn at all times. Handling clean glassware may be a potential source of interference
- 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-*d*$_2$ (Estriol-*d*$_2$)	53866-32-3	C/D/N Isotopes Cat. No. D-5279	N/A
$^{13}C_6$-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-*d*$_3$	77546-39-5	None	Sigma Drug Std., 100 µg/mL in dimethoxyethane Cat. No. T5536

Internal Standard Stock Standards

ISSS 500 µg/mL - Weigh 5 mg of α-hydroxyestradiol-d_2 (estriol-d_2) into a tared 10-mL volumetric flask and dilute to volume with methanol. The remaining internal standards can be purchased as 100 µg/mL solutions

Internal Standard Primary Dilution Standard

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. Analysts are permitted to use other IS PDS concentrations and volumes provided all extracts and calibration standards contain the same final concentration of the internal standards and adequate signal is obtained to maintain precision.

Internal Standard Concentrations

Internal Standard	Conc IS Stock µg/mL	Volume of IS Stock, µL	Final Volume of IS PDS, mL	Final Conc. of IS PDS (µg/mL)
16α-Hydroxyestradiol-*d*$_2$ (Estriol-*d*$_2$)	500	40	10	2.0
$^{13}C_6$-Estradiol	100	400	10	4.0
$^{13}C_2$-Ethynylestradiol	100	400	10	4.0
Testosterone-*d*$_3$	100	100	10	1.0

Surrogate Analytes – Two isotopically labeled surrogates are acceptable and are listed below. Only one surrogate is required with each extraction batch. The analyst is permitted to select the surrogate that performs best under the LC-MS/MS conditions employed for the analysis

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 methanol

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. Compounds are stable for about six months when stored at a temperature < 6° C. Use 10 µL of SUR PDS to fortify 1-L samples yielding a final concentration of 70 ng/mL ethynylestradiol-d_4 or 25 ng/mL bisphenol A-d_{16} in the 1-mL extracts

Surrogate Analyte	Conc of SUR Stock (µg/mL)	Volume of SUR Stock (µg/mL)	Final Volume of SUR ODS (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

Method Analyte Standard Solutions

Analyte Stock Standard Solution

1000 µg/mL - Obtain the analytes listed in Table 1 above as ampoulized 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

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 59% MeOH)	Analyte PDS Concentration µg/L
Estriol, 16α-Hydroxyestradiol	1000	20	10	2.0
Estrone	1000	20		2.0
17β-Estradiol	1000	25		2.5
17α-Ethynylestradiol	1000	35		3.5
Equilin	1000	20		2.0
4-Androstene-3,17-dione	1000	10		1.0
Testosterone	1000	10		1.0

Analysis Procedure

1. Sample Preparation

- Add a 10-µL aliquot of SUR PDS to each 1-L samples for final concentrations of 25 ng/L bisphenol A-d_{16} and 70 ng/L ethynylestradiol-d_4
- Fortify LFBs, LFSMs, or LFSMDs, with an appropriate volume of Analyte PDS
- Cap and invert each sample several times to mix

2. SPE Procedure

a) Assemble an all glass manifold **(UCT ECUCTVA1, ECUCTVA3 or ECUCTVA6---**1, 3 or 6 stations). Automated extraction equipment may also be used

b) Place **ECUNIC18 cartridge(s)** on the manifold

c) Add a 10-mL aliquot of methanol to the cartridge and draw through the cartridge until dry. 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

d) Add approximately 10 mL of methanol to each cartridge.
e) Draw about 1 mL of solvent through the cartridge and turn off the vacuum temporarily
f) 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
g) Add 10 mL of reagent water to each cartridge and draw through leaving a thin layer of liquid on the surface of the cartridge
h) Add another 10-mL aliquot of reagent water
i) Draw the water through the cartridge keeping the water level above the cartridge surface
j) 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) Cartridge Elution

a) Insert collection tubes into the manifold to collect the cartridge extracts. The collection tube must 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 enough 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 additional 2 x 5-mL aliquots of methanol

5) Extract Concentration

a) Concentrate the extract to approximately dryness under a gentle stream of N_2 in a warm water bath (~45° C)
b) Rinse the collection tube with 500 µL of 50% methanol, and transfer the rinsate to a 1-mL volumetric
c) Add IS PDS solution, and adjust to the 1-mL volume with 50% methanol

6) Extract Filtration

a) It is highly recommended that extracts be filtered prior to analysis as finished water matrices may contain particulates
b) If filtering is incorporated as part of the sample preparation, the first lot of syringe filters must be included in the procedure to that interferences are introduced or method analytes are retained in the filter. Subsequent lots of syringe filters can be verified by examining CAL standards
c) Filter aliquots of at least two prepared CAL standards and compare the filtered samples to the unfiltered CAL standards
d) The filtered and unfiltered area counts should agree within 15% of each other. If the difference is greater than 15%, another lot of syringe filters should be obtained

Sample Analysis

- Establish LC-ESI-MS/MS operating conditions equivalent to those summarized in Table 2-5 below

HPLC Conditions

Column: Waters Xterra® MS C18, 2.1 x 150 mm, 3.5µm d_p or equivalent			
Column Temperature: 35° C			
Column Flow Rate: 0.200 mL/min			
Injection Volume: 50 µL			
Gradient			
Time (min)	**% RW**	**%MeOH**	**%NH_4OH (0.2% v/v)**
0	40	50	10
16.5	40	50	10
17.5	25	65	10
30	25	65	10
31	5	85	10
35	5	85	10
35.1	40	50	10
50	40	50	10

Table 2

ESI-MS/MS Method Conditions

MS Parameter	HPLC/MS/MS
Polarity	ESI+ & ESI-
Capillary Voltage kV	3.0
Source Temperature °C	120
N_2 Desolvation Temperature °C	350
N_2 Desolvation Gas Flow L/hr	900
Cone Gas Flow L/hr	50
Extractor Lens V	2
RF Lens V	0.1
Collision Cell Pressure mbar	3.4 e^{-3}

Table 3

LC-ESI-MS/MS Analyte Retention Times, Precursor and Product Ions, Cone Voltage, and Collision Energy

Analyte	Ret. Time (min.)	ESI Mode	Precursor Ion	Product Ion	Cone Voltage V	Collision Energy eV	Internal Standard
Estriol	4.99	ESI-	287	144.7	55	40	Estriol-d_2
Bisphenol A-d16	9.31	ESI-	241.1	223	40	18	$^{13}C_6$-Estradiol
Equilin	16.92	ESI-	267.1	142.7	35	32	$^{13}C_6$-Estradiol
Estrone	19.49	ESI-	268.9	144.7	55	40	$^{13}C_6$-Estradiol
Androstenedione	19.97	ESI+	287.1	96.6	30	20	Testosterone-d_3
17β-Estradiol	20.84	ESI-	271.2	144.7	55	40	$^{13}C_6$-Estradiol
17α-Ethynylestradiol	22.74	ESI-	295.1	144.7	50	35	$^{13}C_2$-Ethynylestradiol
Ethynylestradiol-d4	22.57	ESI-	299	144.7	55	40	$^{13}C_2$-Ethynylestradiol
Testosterone	24.24	ESI+	289.1	96.8	35	25	Testosterone-d_3

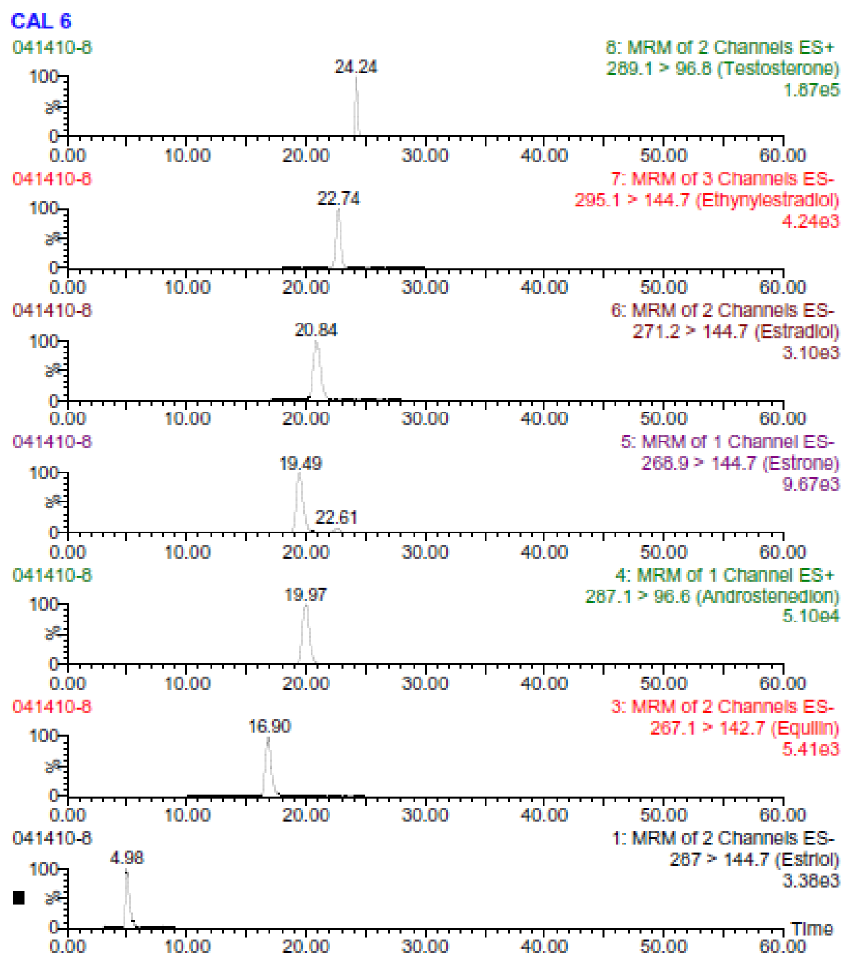
Table 4

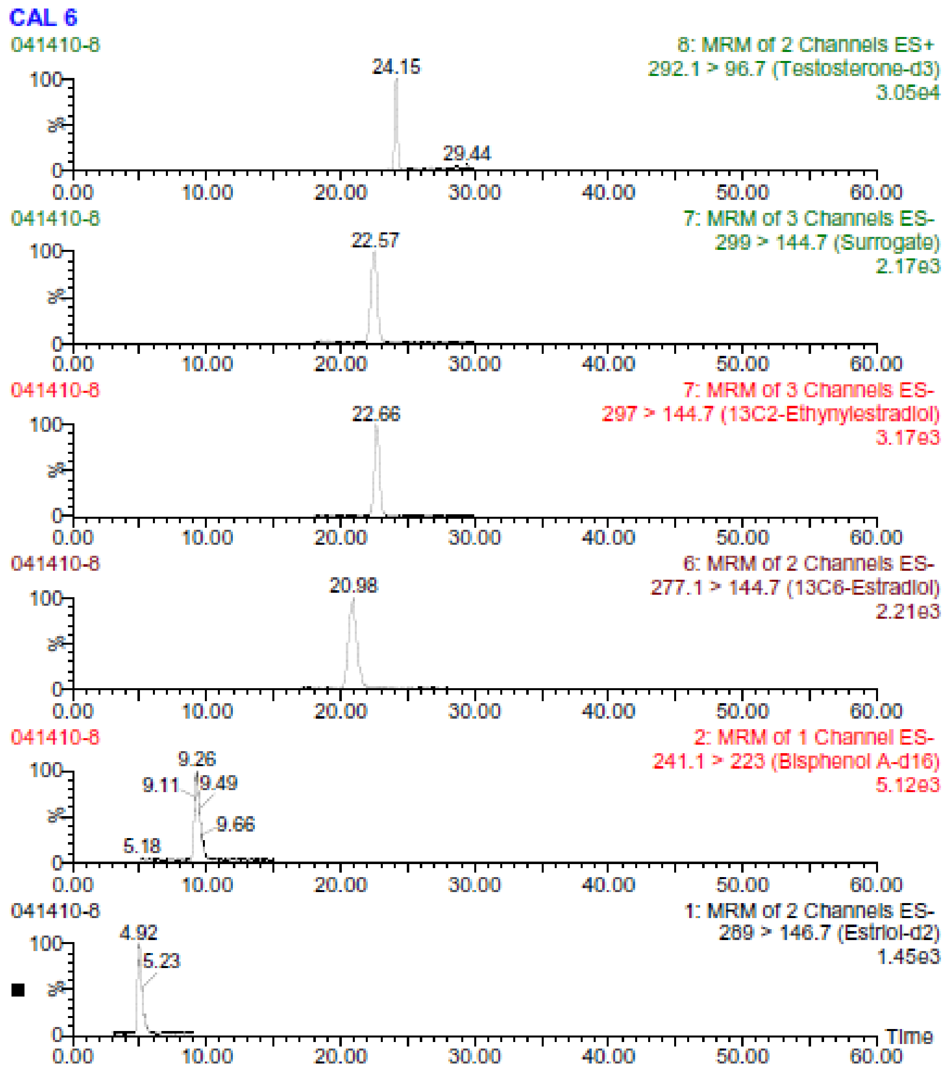
LC-ESI-MS/MS Internal Standard Retention Times, Precursor and Product Ions, Cone Voltage, and Collision Energy

Internal Standard	Ret. Time (min.)	Precursor Ion	Product Ion	Cone Voltage V	Collision Energy eV
Estriol-d_2	4.94	289	146.7	55	40
$^{13}C_6$-Estradiol	20.98	277.1	144.7	55	40
$^{13}C_2$-Ethynylestradiol	22.70	297	144.7	55	38
Testosterone-d_3	24.15	292.1	96.7	35	26

Table 5

Examples Chromatogram of LC-ESI-MS/MS Transitions For Method 539 Analytes





*The analyst should refer to 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

** Section 6.9.1 of Method 539 permits equivalent substitution

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