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)

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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)

1. SCOPE AND APPLICATION

1.1 METHOD – Method 539 is a liquid chromatography, electrospray ionization, tandem mass spectrometry (LC-ESI-MS/MS) method for the determination of hormones in finished drinking water. Method 539 requires the use of MS/MS in Multiple Reaction Monitoring (MRM) mode to enhance selectivity. This method is intended for use by analysts skilled in the performance of solid phase extraction, the operation of LC-ESI-MS/MS instrumentation, and in the interpretation of the associated data. Method 539 is applicable for the measurement of the following analytes:

Analyte	Chemical Abstracts Services Registry Number (CASRN)
16α-Hydroxyestradiol (Estriol)	50-27-1
17β-Estradiol	50-28-2
17α-Ethynylestradiol	57-63-6
Testosterone	58-22-0
Estrone	53-16-7
4-Androstene-3,17-dione	63-05-8
Equilin	474-86-2

1.2 SUPPORTING DATA

1.2.1 Precision and accuracy data have been generated for the detection of the method analytes in reagent water and finished drinking water from both ground water and surface water sources (Sect. 17, Tables 6 to 9).

1.2.2 Single laboratory lowest concentration minimum reporting levels (LCMRLs) for the analytes in this method ranged from 0.06 to 4.0 nanograms per liter (ng/L) (Section 17, Table 5). The LCMRL is the lowest spiking concentration such that the probability of spike recovery in the 50% to 150% range is at least 99%. The procedure used to determine the LCMRL is described elsewhere.[1] Laboratories using this method are not required to determine LCMRLs, but they must demonstrate that the Minimum Reporting Level (MRL) for each analyte meets the requirements described in Section 9.2.4.

1.2.3 Determining detection limits (DL) for the method analytes is optional (Sect. 9.2.6). The DL is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than zero.[2] DLs for method analytes fortified into reagent water ranged from 0.04 to 2.9 ng/L (Table 5).

1.3 METHOD FLEXIBILITY – The laboratory is permitted to change LC columns, LC conditions, internal standards or surrogate standards, and MS conditions different from those utilized to develop the method. Changes may not be made to sample collection and preservation (Sect. 8), the quality control (QC) requirements (Sect. 9), or the extraction and elution steps (Sect 11). Single quadrupole instruments are not permitted. Method modifications should be considered only to improve method performance. Modifications that are introduced in the interest of reducing cost or sample processing time, but result in poorer method performance, may not be used. Analytes should be adequately resolved chromatographically in order to permit the mass spectrometer to dwell on a minimum number of compounds eluting within a retention time window. Instrumental sensitivity can decrease if too many compounds are permitted to elute within a retention time window. In all cases where method modifications are proposed, the analyst must perform the procedures outlined in the Initial Demonstration of Capability (IDC, Sect. 9.2), verify that all QC acceptance criteria in this method are met (Tables 11 and 12), and verify method performance in a real sample matrix (Sect. 9.4.2).

2. SUMMARY OF METHOD

Samples are dechlorinated with sodium thiosulfate and protected from microbial degradation using 2-mercaptopyridine-1-oxide sodium salt during sample collection. Samples are fortified with surrogates and passed through solid phase extraction (SPE) disks containing octadecyl (C18) functional groups in order to extract the method analytes and surrogates. The compounds are eluted from the solid phase with a small amount of methanol. The extract is concentrated to dryness with nitrogen in a heated water bath, and then adjusted to a 1-mL volume with 50:50 methanol:water after adding the internal standards. An aliquot of the sample is injected into an LC equipped with a C18 column that is interfaced to a MS/MS. The analytes are separated and identified by comparing the acquired mass spectra and retention times to reference spectra and retention times for calibration standards acquired under identical LC-MS/MS conditions. The concentration of each analyte is determined using the internal standard technique.

3. DEFINITIONS

3.1 ANALYSIS BATCH – A set of samples that is analyzed on the same instrument during a 24-hour period that begins and ends with the analysis of the appropriate Continuing Calibration Check (CCC) standards. Additional CCCs may be required depending on the length of the analysis batch and the number of field samples.

3.2 CALIBRATION STANDARD – A solution of the method analytes, surrogate analytes and internal standards prepared from the Primary Dilution Standards (Sect. 3.18). The calibration standards are used to calibrate the instrument response with respect to analyte concentration.

3.3 CONTINUING CALIBRATION CHECK (CCC) – A calibration standard that is analyzed periodically to verify the accuracy of the existing calibration.

3.4 DETECTION LIMIT (DL) – The minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero. This is a statistical determination (Sect. 9.2.6), and accurate quantitation is not expected at this level.

3.5 EXTRACTION BATCH – A set of up to 20 field samples (not including QC samples) extracted together by the same person(s) during a work day using the same lot of solid phase extraction devices, solvents, surrogate solution, and fortifying solutions. Required QC samples include a Laboratory Reagent Blank (Sect. 3.12), a Laboratory Fortified Blank (Sect. 3.9), a Laboratory Fortified Sample Matrix (Sect. 3.10), and either a Field Duplicate (Sect. 3.6) or a Laboratory Fortified Sample Matrix Duplicate (Sect. 3.11).

3.6 FIELD DUPLICATES (FD) – Separate samples collected at the same time, shipped and stored under identical conditions. Method precision, including the contribution from sample collection procedures, is estimated from the analysis of FDs. For the purposes of this method, Field Duplicates are necessary to conduct repeat analyses if the original field sample is lost, or to conduct repeat analyses in the case of QC failures associated with the analysis of the original field sample. Field Duplicates are used to prepare Laboratory Fortified Sample Matrix (Sect. 3.10) and Laboratory Fortified Sample Matrix Duplicate (Sect. 3.11) QC samples.

3.7 INTERNAL STANDARD (IS) – A pure compound added to all standard solutions and sample extracts in a known amount and used to measure the relative response of other method analytes that are components of the same solution. The internal standard must be a chemical that is structurally similar to the method analytes, has no potential to be present in water samples, and is not a method analyte.

3.8 ION SUPPRESSION/ENHANCEMENT – An observable decrease or increase in analyte response in complex (field) samples as compared to the response obtained in standard solutions.

3.9 LABORATORY FORTIFIED BLANK (LFB) – A volume of reagent water, containing method preservatives, to which known quantities of the method analytes are added. The LFB is used during the IDC (Sect. 9.2) to verify method performance for precision and accuracy. The LFB is also a required QC element with each extraction batch. The results of the extracted LFB verify method performance in the absence of sample matrix.

3.10 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) – A preserved field sample to which known quantities of the method analytes are added in the laboratory. The LFSM is processed and analyzed as a sample, and its purpose is to determine whether the sample matrix contributes bias to the analytical results.

3.11 LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (LFSMD) – A Field Duplicate of the sample used to prepare the LFSM which is fortified and analyzed identically to the LFSM. The LFSMD is used instead of the Field Duplicate to assess method precision

when the method analytes are rarely found at concentrations greater than the MRL (Sect. 3.15).

3.12 LABORATORY REAGENT BLANK (LRB) – A volume of reagent water or other blank matrix that is processed 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 and analysis batches. The LRB is used to determine if the method analytes or other interferences are present in the laboratory environment, the reagents, or the apparatus.

3.13 LOWEST CONCENTRATION MINIMUM REPORTING LEVEL (LCMRL) – The single laboratory LCMRL is the lowest true concentration for which the future recovery is predicted to fall between 50% to 150% with 99% confidence.[1]

3.14 MATERIAL SAFETY DATA SHEETS (MSDS) – Written information provided by vendors concerning a chemical's toxicity, health hazards, physical properties, fire and reactivity data, storage instructions, spill response procedures, and handling precautions.

3.15 MINIMUM REPORTING LEVEL (MRL) – The minimum concentration that can be reported by a laboratory as a quantified value for the method analyte in a sample following analysis. This concentration must meet the criteria defined in Section 9.2.4 and must be no lower than the concentration of the lowest calibration standard for each method analyte.

3.16 MULTIPLE REACTION MONITORING (MRM) – A mass spectrometric technique in which a precursor ion is first isolated, then subsequently fragmented into a product ion(s). Quantitation is accomplished by monitoring a specific product ion. As described in Section 10.1.2, MS parameters must be optimized for each precursor ion (Sect. 3.17) and product ion (Sect. 3.19).

3.17 PRECURSOR ION – The precursor ion is the gas-phase species corresponding to the method analyte produced in the ESI interface. In MS/MS, the precursor ion is mass selected and fragmented by collision-activated dissociation to produce distinctive product ions of smaller mass/charge (*m/z*) ratio.

3.18 PRIMARY DILUTION STANDARD (PDS) – A solution containing the method analytes, internal standards, or surrogate analytes, which is prepared from Stock Standard Solutions (Sect. 3.22). The PDS solutions are diluted to prepare calibration standards and sample fortification solutions.

3.19 PRODUCT-ION – For the purpose of this method, a product ion is one of the fragment ions produced in MS/MS by collision-activated dissociation of the precursor ion.

3.20 QUALITY CONTROL SAMPLE (QCS) – A solution containing the method analytes at a known concentration that is obtained from a source external to the laboratory and different from the source of calibration standards. The purpose of the QCS is to verify the accuracy of the primary calibration standards.

3.21 REAGENT WATER (RW) – Purified water that does not contain any measurable quantity of the method analytes or interfering compounds at or above 1/3 the MRL.

3.22 STOCK STANDARD SOLUTION (SSS) – A concentrated standard solution that is prepared in the laboratory using assayed reference materials or that is purchased from a commercial source with a certificate of analysis.

3.23 SURROGATE ANALYTE (SUR) – A pure chemical which is unlikely to be found in any sample, and which is added to a sample volume in a known amount before extraction. Surrogates are evaluated using the same procedures as other sample components. Because surrogates are present in every sample, they provide a means of assessing method performance for each sample extraction.

4. INTERFERENCES

4.1 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 ^{o}C for two hours or solvent rinsed. Volumetric glassware should be solvent rinsed and never heated in an oven above 120 ^{o}C.

4.2 Method interferences may be caused by contaminants in solvents, reagents (including reagent water), sample bottles and caps, and other sample processing hardware. These interferences may lead to discrete artifacts and/or elevated baselines in the chromatograms. 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. This may be accomplished by analyzing LRBs as described in Sections 3.12 and 9.3.1. **Subtracting blank values from sample results is not permitted**.

4.3 Matrix interferences may be caused by contaminants that are co-extracted from the sample. Matrix components may directly interfere by producing a signal at or near the retention time of an analyte peak. The extent of matrix interferences will vary considerably from source to source, depending on the nature of the water. 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. Analysis of LFSMs (Sect. 3.10 and 9.3.6) provides evidence for the presence (or absence) of matrix effects.

4.4 Solid phase extraction media may be a source of interferences. The analysis of LRBs can provide important information regarding the presence or absence of such interferences. Each brand and lot of SPE devices should be tested to ensure that contamination does not preclude analyte identification and quantitation.

4.5 Depending on the source and purity, labeled analogs used as internal standards may contain a small percentage of the corresponding native analyte. Such a contribution may be significant when attempting to determine LCMRLs and DLs. The labeled internal standards must meet the purity requirements stated in the IDC (Section 9.2.1).

4.6 The method involves the extraction and concentration of trace levels of human hormones. As such, a potential source of interference lies with the analyst. Nitrile gloves should be worn at all times while handling clean glassware and throughout the extraction process.

4.7 Depending on the sampling site, it may be appropriate to include a Field Blank with the sampling bottles. At the lab, fill a sample bottle with reagent water and preservatives, seal, and ship it to the sampling site along with the other sample bottles. Also, include an empty sealed bottle. At the sampling site, open the bottle containing the preserved reagent water and pour it into the empty bottle (thus exposing it to the sampling environment). Seal and label as "Field Blank". The Field Blank is shipped back to the lab along with the samples and analyzed along with the samples to ensure that no human hormones were introduced into the samples during the collection and handling process. In general, if the Field Blank shows analytes present at a level >1/3 the MRL, the samples should be flagged accordingly and new samples collected if possible.

5. SAFETY

5.1 The toxicity and carcinogenicity of each reagent used in this method has not been precisely defined. Each chemical should be treated as a potential health hazard and exposure to these chemicals should be minimized. Each laboratory is responsible for maintaining an awareness of OSHA regulations regarding safe handling of chemicals used in this method.[3] A reference file of MSDSs should be made available to all personnel involved in the chemical analysis.

5.2 Pure standard materials and stock standard solutions of the method compounds should be handled with suitable protection for skin, eyes, etc.[4]

5.3 Concentrated ammonium hydroxide was used during method development as a pH modifier for the HPLC mobile phase. Concentrated ammonium hydroxide should only be handled in a chemical fume hood.

6. EQUIPMENT AND SUPPLIES

References to specific brands or catalog numbers are included as examples only and do not imply endorsement of the product. Such reference does not preclude the use of other vendors or suppliers.

6.1 SAMPLE CONTAINERS – 1000-mL amber glass bottles fitted with polytetrafluoroethylene (PTFE) lined screw caps. (Alternate sample container volumes may be used as discussed in Section 8.1.1)

6.2 AUTOSAMPLER VIALS – Amber glass vials with PTFE/silicone septa.

6.3 MICRO SYRINGES – Suggested sizes include 5, 10, 25, 50 and 100 microliters (µL).

6.4 ANALYTICAL BALANCE – Capable of weighing to the nearest 0.0001 gram (g).

6.5 DISPOSABLE PASTEUR PIPETTES – 5 ¾-inch or 9-inch borosilicate glass, used to transfer samples to autosampler vials and for sample preparation (Fisher Cat. No. 13-678-20B, 13-678-20C, or equivalent).

6.6 DISPOSABLE SYRINGES (*Optional*) – 3-mL, polypropylene, Luer Lock syringes for use in filtering standards and extracts (Aldrich Cat. No. Z248002, Fisher Cat No. 14-817-27, or equivalent).

6.7 FILTERS – 0.22 µm, 47 mm PVDF disk filters for filtering LC mobile phase components (Millipore Durapore Cat. No. GVWP04700, or equivalent).

6.8 SYRINGE FILTERS (*Optional*) – 13 mm, 0.2 µm pore size GHP filters (Waters Corp. Part No. WAT097962, or equivalent).

6.9 SOLID PHASE EXTRACTION APPARATUS FOR USING SPE DISKS

6.9.1 SPE DISKS – 47 mm diameter, manufactured with octadecyl (C18) sorbent phase (Fisher Cat. No. 14-386-2, or equivalent).

6.9.2 SPE DISK EXTRACTION GLASSWARE – Funnel, PTFE-coated support screen, PTFE gasket, base, and clamp used to support SPE disks and contain samples during extraction. May be purchased as a set (Fisher Cat. No. K971100-0047, or equivalent) or separately.

6.9.3 VACUUM EXTRACTION MANIFOLD – Designed to accommodate extraction glassware and disks (Varian Cat. No. 1214-6001, or equivalent).

6.9.4 GRADUATED COLLECTION TUBES – 50-mL conical glass tubes with beaded rim (Kimax #45165-50) or 40-mL glass tubes with 24-410 screw cap (Kimax #45200-40) suitable for collection of eluent from the solid phase disks.

6.10 LABORATORY OR ASPIRATOR VACUUM SYSTEM – Sufficient capacity to maintain a vacuum of approximately 15 to 25 inches of mercury.

6.11 EXTRACT CONCENTRATION SYSTEM – Extracts are concentrated by blowdown with nitrogen using a water bath set at 40 °C (N-Evap, Model 11155, Organomation Associates, Inc., or equivalent).

6.12 LIQUID CHROMATOGRAPHY ELECTROSPRAY IONIZATION TANDEM MASS SPECTROMETRY SYSTEM (LC-ESI-MS/MS)

6.12.1 LC SYSTEM – The LC system must provide consistent sample injection volumes and be capable of performing binary linear gradients at a constant flow rate.

6.12.2 ANALYTICAL COLUMN – The method was developed using a Waters Xterra® MS C18 2.1 x 150 mm, 3.5 µm d_p column (Waters Part No. 186000408). Any column

capable of tolerating basic conditions (mobile phase pH > 9.5), provides adequate resolution, peak shape, capacity, accuracy and precision (Sect. 9), and does not result in suppression or enhancement of analyte responses may be used.

6.12.3 ELECTROSPRAY IONIZATION-TANDEM MASS SPECTROMETER (ESI-MS/MS) – The mass spectrometer must be capable of rapid switching between negative ion and positive ion electrospray ionization modes. The system must be capable of performing MS/MS to produce unique product ions (Sect. 3.19) for the method analytes within specified retention time segments. At least 10 – 15 scans across the chromatographic peak are needed to ensure adequate precision.

6.12.4 MS/MS DATA SYSTEM – An interfaced data system is required to acquire, store and output MS data. The computer software must have the capability of processing stored data by recognizing a chromatographic peak within a given retention time window. The software must allow integration of the abundance of any specific ion between specified time or scan number limits. The software must be able to construct a linear regression or quadratic calibration curve and calculate analyte concentrations using the internal standard technique.

7. REAGENTS AND STANDARDS

7.1 GASES, REAGENTS AND SOLVENTS – Reagent grade or better chemicals must be used. Unless otherwise indicated, all reagents will conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (ACS), where such specifications are available. Other grades may be used if the reagent is demonstrated to be free of analytes and interferences and all requirements of the IDC (Sect. 9.2) are met when using these reagents.

7.1.1 ACETONITRILE (CH_3CN, CASRN 75-05-8) – (HPLC-grade, Honeywell Burdick & Jackson Brand®, Catalog No. 015 or equivalent).

7.1.2 AMMONIUM HYDROXIDE - (NH_4OH, CASRN 1336-21-6) – Mobile phase modifier (29% by weight, Fisher Cat. No. S93120A, or equivalent).

7.1.3 COLLISION GAS - High purity compressed gas (e.g., nitrogen or argon) used in the collision cell of the mass spectrometer. The specific type of gas, purity and pressure requirements will depend on the instrument manufacturer's specifications.

7.1.4 DESOLVATION GAS - High purity compressed gas (e.g., nitrogen or zero-air) used for desolvation in the mass spectrometer. The specific type of gas, purity and pressure requirements will depend on the instrument manufacturers' specifications.

7.1.5 METHANOL – (CH_3OH, CASRN 67-56-1) – (LC/MS grade, Fisher Optima®, Fisher Cat. No. A456, or equivalent).

7.1.6 REAGENT WATER – Purified water that does not contain any measurable quantities of any method analytes or interfering compounds greater than 1/3 the MRL for each method analyte of interst.

7.1.7 (2-MERCAPTOPYRIDINE-1-OXIDE, SODIUM SALT, "SODIUM OMADINE", (C_5H_4NOSNa, CASRN 3811-73-2) – > 96% (Sigma Cat. No. H3261, or equivalent). Used to inhibit microbial growth in dechlorinated water samples.

7.1.8 SODIUM THIOSULFATE ($Na_2S_2O_3$, CASRN 7772-98-7) – Certified, anhydrous (Fisher Cat. No. S446, or equivalent). Added to remove free chlorine in chlorinated finished waters.

7.2 STANDARD SOLUTIONS – When a compound's purity is assayed to be 96 percent or greater, the weight can be used without correction to calculate the concentration of the stock standard. The solution concentrations listed in this section were used to develop this method and are included only as examples. Guidance on the storage stability of Primary Dilution Standards and calibration standards is provided in the applicable sections below. Although estimated stability times for standard solutions are given, laboratories should use standard QC practices to determine when standards need to be replaced.

7.2.1 INTERNAL STANDARDS – This method uses four isotopically enriched internal standards listed in the table below. Although alternate internal standards may be used, the analyst must ensure that the QC requirements defined in Section 9.3.4 are met.

Internal Standard	CASRN[a]	Neat Materials Catalog No.	Solution Standards , Cat. No.
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
$^{13}C_2$-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

[a] CASRN = Chemical Abstract Registry Number.

7.2.1.1 INTERNAL STANDARD STOCK STANDARDS (ISSS) (500 µg/mL, except as noted) – Prepare α-hydroxyestradiol-d_2 (estriol-d_2) stock standard solution by weighing 5 mg of the solid material into a tared 10-mL volumetric flask and diluting to volume with methanol. The remaining internal standards can be purchased as 100 µg/mL solutions.

7.2.1.2 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 can be prepared in acetonitrile. The IS PDS is stable for about six months provided it is stored at a temperature $\leq$ 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 in the 1-mL extracts. 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	Conc. of 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

7.2.2 SURROGATE ANALYTES – Two isotopically labeled surrogates were evaluated during method development and are listed in the following table. Only one surrogate, however, is required to be evaluated 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. Other alternate surrogate standards may be used provided they meet the QC requirements described in Section 9.3.5.

Surrogate Analyte	CASRN[a]	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

[a] CASRN = Chemical Abstract Registry Number.

7.2.2.1 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.

7.2.2.2 SURROGATE ANALYTE PRIMARY DILUTION STANDARD (SUR PDS) (2.5 – 7.0 µg/mL) – The table below can be used as a guide for preparing the SUR PDS. The SUR PDS can be prepared in methanol and is stable for about six months provided it is stored at a temperature $\leq$ 6 °C. Use 10 µL of SUR PDS to fortify 1-L samples. This will yield 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, µL	Final Volume of SUR PDS, mL	Final Conc. of SUR PDS (µg/mL)
Ethynylestradiol-d_4	1000	70	10	7.0
Bisphenol A-d_{16}	1000	25	10	2.5

7.2.3 METHOD ANALYTE STANDARD SOLUTIONS

7.2.3.1 ANALYTE STOCK STANDARD SOLUTION (1000 µg/mL) – Obtain the analytes listed in the table in Section 1.1 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.

NOTE: The androgens, testosterone and androstenedione, can be purchased as 1 mg/mL drug standards.

7.2.3.2 ANALYTE PRIMARY DILUTION SOLUTION (Analyte PDS) (1.0 – 3.5 µg/mL) – Prepare the Analyte PDS by diluting the Analyte Stock Standard solutions into 50% methanol in reagent water. An example preparation of the Analyte PDS that was used to collect data presented in Section 17 is provided in the table below. 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 analytes.

Analyte Stock	Stock Concentration (µg/mL)	Stock Volume (µL)	Final Volume (mL 50% MeOH)	Analyte PDS Concentration (µg/mL)
16α-Hydroxyestradiol (Estriol)	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

7.2.4 CALIBRATION (CAL) STANDARDS – Prepare at least five calibration standards over the concentration range of interest by diluting aliquots of Analyte PDS into 50% methanol in reagent water. The lowest calibration standard must be at or below the MRL. Using the IS PDS and SUR PDS, add a constant amount of each internal standard and surrogate to each calibration standard. The concentration of the surrogate should match the concentration in the sample extracts, assuming 100% recovery through the extraction process. The CAL standards may also be used as CCCs (Sect. 9.3.2). If the extracts are filtered (Sect. 11.4), it is recommended that the CAL standards also be filtered using 0.2 µm syringe filters. During method development, the CAL standards were shown to be stable for at least two weeks when stored at a temperature $\leq 6\ ^{o}C$.

8. SAMPLE COLLECTION, PRESERVATION, AND STORAGE

8.1 SAMPLE BOTTLE PREPARATION

8.1.1 SAMPLE CONTAINERS – One-liter amber glass bottles with PTFE-lined screw caps and sufficient capacity to allow subsequent preparation of all required sample and QC aliquots.

NOTE: Smaller sample volumes (e.g., 500-mL) can be collected if the laboratory demonstrates acceptable performance in meeting the required MRLs (Sect. 9.2.4) using the smaller sample volume. The amount of added preservatives and surrogate/analyte fortification levels should be adjusted accordingly.

8.1.2 ADDITION OF PRESERVATIVES – Preservation reagents, listed in the table 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

8.2 SAMPLE COLLECTION – Grab samples must be collected in accordance with conventional sampling practices.[5] Fill sample bottles taking care not to flush out the preservatives. Because the method analytes are not volatile, it is not necessary to ensure that the sample bottles are completely headspace-free.

8.2.1 SAMPLING FROM A TAP – When sampling from a cold water tap, remove the aerator, open the tap, and allow the system to flush until the water temperature has stabilized (approximately five minutes). Invert the bottles several times to mix the sample with the preservation reagents.

8.3 SAMPLE SHIPMENT AND STORAGE – 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, samples must be stored at or below 6 °C and protected from light until analysis. Samples must not be frozen.

8.4 SAMPLE HOLDING TIMES – Results of the sample storage stability study (Table 9) indicated that all compounds listed in the method have adequate stability for 28 days when collected, preserved, shipped and stored as described in Sections 8.1 – 8.3. Therefore, samples should be extracted as soon as possible, but must be extracted within 28 days. Extracts must be stored at 0 °C or less and analyzed within 28 days after extraction. The extract storage stability study data are presented in Table 10.

9. QUALITY CONTROL

9.1 QC requirements include the IDC (Sect. 9.2) and ongoing QC requirements (Sect. 9.3). This section describes each QC parameter, its required frequency, and the performance criteria that must be met in order to satisfy EPA quality objectives. The QC criteria discussed in the following sections are summarized in Section 17, Tables 11 and 12. These QC requirements are considered the minimum acceptable QC criteria. Laboratories are encouraged to institute additional QC practices to meet their specific needs.

9.1.1 METHOD MODIFICATIONS – The analyst is permitted to modify LC columns, LC conditions, internal standards and surrogate standards, MS and MS/MS conditions. Each time such method modifications are made, the analyst must repeat the procedures of the IDC. Modifications to LC conditions should minimize co-elution of method analytes to reduce the probability of suppression/enhancement effects.

NOTE: Ultraperformance liquid chromatography (UPLC) was evaluated concurrently with high performance liquid chromatography (HPLC) during development of this method. The UPLC columns were found to be less stable under the basic mobile phase conditions that were used in the HPLC analyses. Basic conditions will improve the negative electrospray ionization response of the estrogens. The UPLC analyses also exhibited a greater degree of matrix suppression when chlorinated (finished) groundwater and surface water samples were evaluated. This does not preclude the use of UPLC for this method. However, analysts must verify that UPLC analyses can meet the established MRLs (Sect. 9.2.4) and that acceptable recoveries are obtained in real drinking water matrices (Sect. 9.4.2).

9.2 INITIAL DEMONSTRATION OF CAPABILITY (IDC) – The IDC must be successfully performed prior to analyzing any field samples. Prior to conducting the IDC, the analyst must generate an acceptable initial calibration following the procedure outlined in Section 10.2.

9.2.1 DEMONSTRATION OF LOW SYSTEM BACKGROUND – Analyze an LRB. Confirm that the blank is free of contamination as defined in Section 9.3.1.

9.2.1.1 Depending on the source and purity, labeled internal standards may contain a small percentage of the corresponding native analyte. Therefore, the analyst must demonstrate that the internal standards do not contain the unlabeled analytes at a concentration $\geq$ 1/3 of the MRL when added at the appropriate concentration to samples.

9.2.1.2 The system should also be checked for carry-over by analyzing a RW blank immediately following the highest CAL standard. If this RW sample does not meet the criteria outline in Section 9.3.1, then carry-over is present and should be identified and eliminated.

9.2.2 DEMONSTRATION OF PRECISION – Prepare, extract and analyze four to seven replicate LFBs. Fortify these samples near the midrange of the initial calibration curve. The method preservatives must be added to the LFBs as described in Section 8.1.2. The percent relative standard deviation (%RSD) of the results of the replicate analyses must be $\leq$ 20%.

$$\%\,\mathrm{RSD} = \frac{\text{Standard Deviation of Measured Concentrations}}{\text{Average Concentration}} \times 100$$

9.2.3 DEMONSTRATION OF ACCURACY – Using the same set of replicate data generated for Section 9.2.2, calculate the average percent recovery. The average percent recovery for each analyte must be within $\pm$ 30% of the true value.

$$\%\,\mathrm{Recovery} = \frac{\text{Average Measured Concentration}}{\text{Fortified Concentration}} \times 100$$

9.2.4 MINIMUM REPORTING LEVEL (MRL) CONFIRMATION – Establish a target concentration for the MRL based on the intended use of the method. Analyze an initial calibration following the procedures in Section 10. The lowest calibration standard used to establish the initial calibration (as well as the low-level CCC) must be at or below the concentration of the MRL. Establishing the MRL concentration too low may cause repeated failure of ongoing QC requirements. Confirm the MRL following the procedure outlined below.

9.2.4.1 Fortify, extract and analyze seven replicate LFBs at or below the proposed MRL concentration. The LFBs must contain the method preservatives as specified in Section 8.1.2. Calculate the mean (*Mean*) and standard deviation for these replicates. Determine the Half Range for the Prediction Interval of Results (HR_{PIR}) using the equation

$$HR_{PIR} = 3.963S$$

where *S* is the standard deviation and 3.963 is a constant value for seven replicates.[1]

9.2.4.2 Confirm that the Upper and Lower limits for the Prediction Interval of Results ($PIR = Mean \pm HR_{PIR}$) meet the upper and lower recovery limits as shown below.

The Upper PIR Limit must be ≤ 150 percent recovery.

$$\frac{Mean + HR_{PIR}}{Fortified\,Concentration} \times 100 \leq 150\%$$

The Lower PIR Limit must be ≥ 50 percent recovery.

$$\frac{Mean - HR_{PIR}}{Fortified\,Concentration} \times 100 \geq 50\%$$

9.2.4.3 The MRL is validated if both the Upper and Lower PIR Limits meet the criteria described above. If these criteria are not met, the MRL has been set too low and must be confirmed again at a higher concentration.

NOTE: These equations are only valid for seven replicate samples.

9.2.5 QUALITY CONTROL SAMPLE (QCS) – Analyze a mid-level Quality Control Sample (Sect. 9.3.8) to confirm the accuracy of the primary calibration standards.

9.2.6 DETECTION LIMIT DETERMINATION *(optional) – While DL determination is not a specific requirement of this method, it may be required by various regulatory bodies associated with compliance monitoring. It is the responsibility of the laboratory to ascertain whether DL determination is required based upon the intended use of the data.*

Analyses for this procedure should be done over at least three days. Prepare at least seven replicate LFBs at a concentration estimated to be near the DL. This concentration may be estimated by selecting a concentration at two to five times the noise level. The method preservatives must be added to the samples as described in Section 8.1.2. Process the seven replicates through all steps of Section 11. Do not subtract blank values when performing DL calculations.

NOTE: If an MRL confirmation data set meets these requirements, a DL may be calculated from the MRL confirmation data, and no additional analyses are necessary.

Calculate the DL using the following equation:

$$DL = s \times t_{(n-1,1-\alpha = 0.99)}$$

where

$t_{(n-1,1-\alpha = 0.99)}$ = Student's t value for the 99% confidence level with n-1 degrees of freedom (for seven replicate determinations, the Student's t value is 3.143 at a 99% confidence level),

n = number of replicates, and

s = standard deviation of replicate analyses.

9.3 ONGOING QC REQUIREMENTS – This section describes the ongoing QC elements that must be included when processing and analyzing field samples.

9.3.1 LABORATORY REAGENT BLANK (LRB) – Analyze an LRB (Sect. 3.12) with each extraction batch (Sect. 3.5). The LRB must contain the method preservatives and the surrogate analytes at the same concentration used to fortify field samples. Background from method analytes or contaminants that interfere with the measurement of method analytes must be $\leq$ 1/3 of the MRL. If method analytes are detected in the LRB at concentrations greater than this level, then all data for the problem analyte(s) must be considered invalid for all samples in the extraction batch. Subtracting blank values from sample results is not permitted.

NOTE: Although quantitative data below the MRL may not be accurate enough for data reporting, such data are useful in determining the magnitude of background interference. Therefore, blank contamination levels may be estimated by extrapolation when the concentration is below the MRL.

9.3.2 CONTINUING CALIBRATION CHECK (CCC) – Analyze CCC standards at the beginning of each analysis batch (Sect. 3.1), after every ten field samples, and at the end of the analysis batch. See Section 10.3 for concentration requirements and acceptance criteria for CCCs.

9.3.3 LABORATORY FORTIFIED BLANK – An LFB (Sect. 3.9) is required with each extraction batch (Sect. 3.5). The concentration of the LFB must be rotated between low, medium, and high concentrations from batch to batch. The low concentration LFB must

be as near as practical to, but no more than two times the MRL. Similarly, the high concentration LFB should be near the high end of the calibration range established during the initial calibration (Sect. 10.2). Results of the low-level LFB analyses must be within ± 50% of the true value for each analyte. Results of the medium and high-level LFB analyses must be within ± 30% of the true value for each analyte. If the LFB results do not meet these criteria, then all data for the problem analyte(s) must be considered invalid for all samples in the extraction batch.

9.3.4 INTERNAL STANDARDS (IS) – The analyst must monitor the peak areas of the internal standards in all injections of the analysis batch (Sect. 3.1). The internal standard responses (as indicated by peak areas) for any chromatographic run must not deviate by more than ± 50% from the average areas measured during the initial calibration for the internal standards. If the IS areas in a chromatographic run do not meet these criteria, inject a second aliquot from the same autosampler vial.

9.3.4.1 If the re-injected aliquot produces an acceptable IS response, report results for that aliquot.

9.3.4.2 If the re-injected aliquot fails the IS criterion, the analyst should check the calibration by injecting the most recent CAL standard that passed. If the CAL standard fails the IS criterion, recalibration is in order as per Section 10.2. If the CAL standard is acceptable, report results from the re-injected aliquot, but annotate as "suspect/IS recovery". Alternatively, collect a new sample and reanalyze.

9.3.5 SURROGATE RECOVERY – The surrogate standard is fortified into all field samples, LRB, LFB, LFSMs, LFSMDs, and FDs prior to extraction. It is also added to the CAL standards. Calculate the percent recovery (%R) for each surrogate using the following equation:

$$\%R = \left(\frac{A}{B}\right) \times 100$$

where
A = calculated surrogate concentration for the QC or field sample, and
B = fortified concentration of the surrogate.

9.3.5.1 Surrogate recovery must be in the range of 70 to 130%. When a surrogate fails to meet this criterion, check 1) calculations to locate possible errors, 2) standard solutions for degradation, 3) contamination, and 4) instrument performance. Correct the problem and reanalyze the extract.

9.3.5.2 If the repeat analysis meets the surrogate recovery criterion, report only data for the reanalyzed extract.

9.3.5.3 If the extract reanalysis fails the 70 to 130% recovery criterion after corrective action, check the calibration by injecting the last CAL standard that passed. If the CAL

standard fails the criteria of Section 10.3, recalibrate as described in Section 10.2. If the CAL standard is acceptable, extraction of the sample should be repeated provided a sample is available and still within the holding time. If the re-extracted sample also fails the recovery criterion, report all data for that sample as "suspect/surrogate recovery." Alternatively, collect a new sample and re-analyze.

9.3.6 LABORATORY FORTIFIED SAMPLE MATRIX (LFSM) – Within each extraction batch (Sect. 3.5), analyze a minimum of one LFSM (Sect. 3.10) for every 20 samples. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot and subtracted from the measured values in the LFSM. If various sample matrices are analyzed regularly, for example, drinking water processed from ground water and surface water sources, performance data should be collected for each source.

9.3.6.1 Prepare the LFSM by fortifying a Field Duplicate with an appropriate amount of the Analyte PDS (Sect. 7.2.3.2). Generally, select a spiking concentration that is greater than or equal to the native concentration for most analytes. Selecting a duplicate aliquot of a sample that has already been analyzed aids in the selection of an appropriate spiking level. If this is not possible, use historical data when selecting a fortifying concentration.

9.3.6.2 Calculate the percent recovery (%R) using the equation:

$$\%R = \frac{(A - B)}{C} \times 100$$

where
A = measured concentration in the fortified sample,
B = measured concentration in the unfortified sample, and
C = fortification concentration.

9.3.6.3 Recoveries for samples fortified at concentrations at or near the MRL (within a factor of two times the MRL concentration) must be within $\pm$ 50% of the true value. Recoveries for samples fortified at all other concentrations must be within $\pm$ 30% of the true value. If the accuracy for any analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control in the CCCs and in the LFB, the recovery is judged matrix biased. Report the result for the corresponding analyte in the unfortified sample as "suspect/matrix."

NOTE: In order to obtain meaningful percent recovery results, correct the measured values in the LFSM and LFSMD for the native levels in the unfortified samples, even if the native values are less than the MRL. This situation and the LRB are the only permitted uses of analyte results below the MRL.

9.3.7 FIELD DUPLICATE OR LABORATORY FORTIFIED SAMPLE MATRIX DUPLICATE (FD or LFSMD) – Within each extraction batch, analyze a minimum of

one Field Duplicate or one Laboratory Fortified Sample Matrix Duplicate. If the method analytes are not routinely observed in field samples, analyze an LFSMD rather than an FD.

9.3.7.1 Calculate the relative percent difference (RPD) for duplicate measurements (FD_1 and FD_2) using the equation:

$$RPD = \frac{|FD_1 - FD_2|}{(FD_1 + FD_2)/2} \times 100$$

9.3.7.2 RPDs for Field Duplicates shoud be ≤ 30% for each analyte. Greater variability may be observed when Field Duplicates have analyte concentrations that are at or near the MRL (within a factor of two times the MRL concentration). At these concentrations, Field Duplicates must have RPDs that are ≤ 50%. If the RPD of an analyte falls outside the designated range, and the laboratory performance for the analyte is shown to be in control in the CCC and in the LFB, the precision is judged matrix influenced. Report the result for the corresponding analyte in the unfortified sample as "suspect/matrix."

9.3.7.3 If an LFSMD is analyzed instead of a Field Duplicate, calculate the RPD for the LFSM and LFSMD using the equation:

$$RPD = \frac{|LFSM - LFSMD|}{(LFSM + LFSMD)/2} \times 100$$

9.3.7.4 RPDs for duplicate LFSMs should be ≤ 30% for each analyte. Greater variability may be observed when the matrix is fortified at analyte concentrations at or near the MRL (within a factor of two times the MRL concentration). LFSMs at these concentrations must have RPDs that are ≤ 50%. If the RPD of an analyte falls outside the designated range, and the laboratory performance for the analyte is shown to be in control in the CCC and in the LFB, the precision is judged matrix influenced. Report the result for the corresponding analyte in the unfortified sample as "suspect/matrix."

9.3.8 QUALITY CONTROL SAMPLE (QCS) – As part of the IDC (Sect. 9.2), each time a new Analyte PDS (Sect. 7.2.3.2) is prepared, and at least quarterly, analyze a QCS sample from a source different from the source of the CAL standards. If a second vendor is not available, then a different lot of the standard should be used. Fortify the QCS near the midpoint of the calibration range. The acceptance criteria for the QCS are the same as the mid- and high-level CCCs (Sect. 10.3). If the accuracy for any analyte fails the recovery criterion, prepare fresh standard dilutions and repeat the QCS evaluation.

9.4 METHOD MODIFICATION QC REQUIREMENTS – The analyst is permitted to modify the separation technique, LC column, mobile phase composition, LC conditions and MS conditions.

9.4.1 Each time method modifications are made, the analyst must repeat the procedures of the IDC (Sect. 9.2) and verify that all QC criteria can be met in ongoing QC samples (Sect. 9.3).

9.4.2 The analyst is also required to evaluate and document method performance for the proposed modifications in real matrices that span the range of waters that the laboratory analyzes. This additional step is required because modifications that perform acceptably in the IDC, which is conducted in reagent water, could fail ongoing method QC requirements in real matrices. This is particularly important for methods subject to matrix effects, such as LC/MS-based methods. For example, a laboratory may routinely analyze drinking water from municipal treatment plants that process ground water, surface water, or a blend of surface and ground water. In this case, the method modification requirement could be accomplished by assessing precision and accuracy (Sects. 9.2.2 and 9.2.3) in a surface water with moderate to high total organic carbon (e.g., 2 mg/L or greater) and a hard ground water (e.g., 250 mg/L as calcium carbonate ($CaCO_3$) equivalent, or greater).

9.4.3 The results of Sections 9.4.1 and 9.4.2 must be appropriately documented by the analyst and independently assessed by the laboratory's QA officer prior to analyzing field samples. When implementing method modifications, it is the responsibility of the laboratory to closely review the results of ongoing QC, and in particular, the results associated with the LFSM (Sect. 9.3.6), FD (Sect. 9.3.7), CCCs (Sect. 10.3), and the internal standard area counts (Sect. 9.3.4). If repeated failures are noted, the modification must be abandoned.

10. CALIBRATION AND STANDARDIZATION

Demonstration and documentation of acceptable MS calibration and initial analyte calibration are required before performing the IDC (Sect. 9.2) and prior to analyzing field samples. Verification of the initial calibration should be repeated each time a major instrument modification or maintenance is performed.

10.1 LC-ESI-MS/MS CALIBRATION AND OPTIMIZATION

10.1.1 MASS CALIBRATION – Calibrate the mass spectrometer with the calibration compounds and procedures specified by the manufacturer.

10.1.2 OPTIMIZING MS PARAMETERS – Each LC-ESI-MS/MS system will have different optimal conditions, which are influenced by the source geometry and system design. Due to the differences in design, follow the recommendations of the instrument manufacturer when tuning the instrument. During the development of this method, instrumental parameters were optimized for the precursor and product ions listed in Section 17, Table 3. Product ions other than those listed may be selected; however, the analyst is cautioned to avoid using ions with lower mass and/or common ions that may not provide sufficient discrimination between the analytes of interest and co-eluting interferences.

10.1.2.1 Optimize the response of the precursor ion (Sect. 3.17) for each analyte by infusing approximately 0.5 – 1.0 µg/mL of each analyte directly into the mass spectrometer as recommended by the instrument manufacturer. Vary the MS parameters (source voltages, source and desolvation temperatures, gas flows, etc.) until optimal analyte responses are obtained. The target analytes may have different optimal instrument parameters, thus requiring some compromise on the final operating conditions. See Section 17, Table 2 for the ESI-MS/MS conditions used in method development.

10.1.2.2 Optimize the response of the product ion (Sect. 3.19) for each analyte by infusing approximately 0.5 – 1.0 µg/mL of each analyte directly into the mass spectrometer as recommended by the instrument manufacturer. Vary the MS/MS parameters (collision gas pressure, collision energy, etc.) until optimal product ion responses are determined.

10.1.3 LIQUID CHROMATOGRAPHY INSTRUMENT CONDITIONS – Establish LC operating parameters that optimize resolution. Suggested LC operating conditions are described in Section 17, Table 1. Conditions different from those listed (e.g., LC columns and mobile phase compositions) may be used if the QC criteria in Sections 9.2 and 9.3 are met and chromatographic separation of the method analytes is achieved.

NOTE: Chromatographic separation as defined does not include the isotopically enriched internal standards and surrogates, which are mass separated. Co-elution of the internal standards with their analogous method analytes helps mitigate matrix suppression and/or enhancement effects.

10.1.4 ESTABLISH LC-ESI-MS/MS RETENTION TIMES AND MRM SEGMENTS – Inject a mid- to high-level calibration standard under optimized LC-ESI-MS/MS conditions to obtain the retention times of each method analyte. Divide the chromatogram into segments that contain one or more chromatographic peaks. For maximum sensitivity in subsequent MS/MS analyses, minimize the number of MRM (Sect. 3.16) transitions that are simultaneously monitored within each segment.

10.2 INITIAL CALIBRATION - During method development, daily calibrations were performed; however, it is permissible to verify the calibration with daily CCCs. Calibration must be performed using peak areas and the internal standard technique. Calibration using peak heights or external standard calibration is not permitted.

10.2.1 CALIBRATION STANDARDS – Prepare a set of at least five calibration standards as described in Section 7.2.4. The analyte concentrations in the lowest calibration standard must be at or below the MRL. Field samples must be quantified using a calibration curve that spans the same concentration range used to collect the IDC data (Sect. 9.2), i.e., analysts are not permitted to use a restricted calibration range to meet the IDC criteria and then use a larger dynamic range during analysis of field samples.

10.2.2 CALIBRATION – Calibrate the LC-ESI-MS/MS and fit the calibration points with either a linear regression or quadratic regression (response vs. concentration). Weighting may

be used. Forcing the calibration curve through the origin is not recommended. The MS/MS instruments used during method development were calibrated using weighted (1/x) quadratic curves. Internal standard assignments appropriate for each method analyte and surrogate analyte are presented in Table 3. The MRM transitions for the internal standards are provided in Table 4.

10.2.3 CALIBRATION ACCEPTANCE CRITERIA – Validate the initial calibration by calculating the concentration of each analyte as an unknown against its regression equation. For calibration levels that are ≤ MRL, the result for each analyte should be within ± 50% of the true value. All other calibration points must calculate to be within ± 30% of their true value. If these criteria cannot be met, the analyst will have difficulty meeting ongoing QC criteria. In this case, corrective action should be taken to reanalyze the calibration standards or restrict the range of calibration.

10.3 CONTINUING CALIBRATION CHECKS (CCCs) – Analyze a CCC to verify the initial calibration at the beginning of each analysis batch, after every tenth field sample, and at the end of each analysis batch. The beginning CCC for each analysis batch must be at or below the MRL. This CCC verifies instrument sensitivity prior to the analysis of samples. Subsequent CCCs should alternate between a medium and high concentration CAL standard.

10.3.1 Inject an aliquot of the appropriate concentration CAL standard and analyze with the same conditions used during the initial calibration.

10.3.2 Verify that the absolute areas of the quantitation ions of each of the internal standards have not changed by more than ± 50% from the average areas measured during the initial calibration. If this limit is exceeded, corrective action is necessary (Sect. 10.4).

10.3.3 The calculated concentration of the surrogate analytes must be within ± 30% of the true value. If the surrogate analytes fail this criterion, corrective action is necessary (Sect. 10.4).

10.3.4 Calculate the concentration of each method analyte in the CCC. Each analyte fortified at a level ≤ MRL must calculate to be within ± 50% of the true value. The calculated concentration of the method analytes in CCCs fortified at all other levels must be within ± 30%. If these limits are exceeded, then all data for the failed analytes must be considered invalid. Any field samples analyzed since the last acceptable CCC that are still within holding time must be reanalyzed after an acceptable calibration has been restored.

10.4 CORRECTIVE ACTION – Failure to meet CCC QC performance criteria requires corrective action. Acceptable method performance may be restored simply by flushing the column at the highest eluent concentration in the gradient. Following this and other minor remedial action, check the calibration with a mid-level CCC and a CCC at the MRL, or recalibrate according to Section 10.2. If internal standard and calibration failures persist, maintenance may be required, such as servicing the ESI-MS/MS system or replacing the LC column.

These later measures constitute major maintenance, and the analyst must return to the initial calibration step (Sect. 10.2).

11. PROCEDURE

This section describes the procedures for sample preparation and analysis. Important aspects of this analytical procedure include proper sample collection and storage (Sect. 8), ensuring that the instrument is properly calibrated (Sect. 10), and that all required QC elements are included (Sect. 9).

11.1 SAMPLE PREPARATION

11.1.1 Samples are dechlorinated, preserved, collected and stored as described in Section 8. All field and QC samples must contain the dechlorinating and preservation agents listed in Section 8.1.2, including the LRB. Before extraction, mark the level of the sample on the outside of the sample bottle for later sample volume determination (Sect. 11.5). If using weight to determine volume, weigh the full sample bottle before extraction.

11.1.2 Add an aliquot of the SUR PDS (Sect. 7.2.2.2) to each sample, cap the sample, and invert to mix. During method devlopment, a 10-µL aliquot of SUR PDS was added to 1-L samples for final concentrations of 25 ng/L bisphenol A-d_{16} and 70 ng/L ethynylestradiol-d_4.

11.1.3 Fortify LFBs, LFSMs, or LFSMDs, with an appropriate volume of Analyte PDS (Sect. 7.2.3.2). Cap and invert each sample several times to mix.

11.2 DISK SPE PROCEDURE

11.2.1 Assemble the extraction glassware onto the vacuum manifold, placing disks on a support screen between the funnel and base.

11.2.2 DISK CLEANING – Add a 10-mL aliquot of methanol and draw through the disk until dry. Add another 5-mL aliquot of methanol and draw through the disk until dry.

11.2.3 DISK CONDITIONING – The conditioning step is critical for recovery of analytes and can have a marked effect on method precision and accuracy. **Once the conditioning has begun, do not allow the disk to dry until the last portion of the sample is drawn through the disk**. If the disk goes dry during the conditioning phase, the conditioning must be repeated.

11.2.3.1 CONDITIONING WITH METHANOL – Add approximately 10 mL of methanol to each disk. Pull about 1 mL of solvent through the disk and turn off the vacuum temporarily; let the disk soak for about one minute. Draw most of the remaining solvent through the disk, but leave a thin layer of methanol on the surface of the disk. The disk must not be allowed to go dry from this point until the end of the sample extraction.

11.2.3.2 CONDITIONING WITH WATER – Add 10 mL of reagent water to each disk and draw through, leaving a thin layer of liquid on the surface of the disk. Follow this with another 10-mL aliquot of reagent water. Draw the water through each disk, again being careful to keep the water level above the disk surface. Turn off the vacuum.

11.2.4 SAMPLE EXTRACTION – Add the sample to the extraction reservoir containing the conditioned disk and turn on the vacuum (approximately 10 to 15 in. Hg). Do not let the disk go dry before the entire sample volume is extracted. When the sample has been drawn through the disk, add a 10-mL aliquot of 15% methanol to the sample container and wash the disk with the rinsate from the container. Pull air through the disk by maintaining full vacuum for 10 – 15 minutes. After drying, turn off and release the vacuum.

11.2.5 DISK ELUTION – Detach the glassware base from the manifold without disassembling the funnel from the base. Insert collection tubes into the manifold to catch the extracts as they are eluted from the disk. The collection tube must fit around the drip tip of the base to ensure collection of all the eluent. Reattach the base to the manifold. Add 5 mL of methanol to the disk and, with vacuum, pull enough methanol into the disk to soak the sorbent. Allow the disk to soak for about one minute. Using vacuum, pull the remaining methanol slowly through the disk into the collection tube. Elute with additional 2 x 5-mL aliquots of methanol. Detach glassware from manifold and remove collection tube.

11.3 EXTRACT CONCENTRATION – Concentrate the extract to approximately dryness under a gentle stream of nitrogen in a warm water bath (~45 ^{o}C). Rinse the collection tube with 500 µL of 50% methanol, and transfer the rinsate to a 1-mL volumetric. Add IS PDS solution, and adjust to the 1-mL volume with 50% methanol. During method development, a 5-µL aliquot of the IS PDS (Sect. 7.2.1.2) was added to each extract.

11.4 FILTERING EXTRACTS – It is highly recommended that all samples be filtered prior to analysis. Finished drinking water matrices will yield extracts that may contain particulates. If filtering is incorporated as part of the sample preparation, the first lot of syringe filters must be included in the procedure when conducting the IDC (Sect. 9.2) to ensure that they do not introduce interferences or retain any of the method analytes. Verification of subsequent lots of syringe filters can be accomplished by examining CAL standards. Filter aliquots of at least two prepared CAL standards. Compare the filtered samples to the unfiltered CAL standards. 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.

11.5 SAMPLE VOLUME OR WEIGHT DETERMINATION – Use a graduated cylinder to measure the volume of water required to fill the original sample bottle to the mark made prior to extraction. Determine the volume of each sample to the nearest 10 mL for use in the final calculations of analyte concentration. If using weight to determine volume, reweigh the empty sample bottle to the nearest 10 g. From the weight of the original sample bottle measured in Section 11.1.1, subtract the empty bottle weight. Assume a sample density of 1.0 g/mL.

11.6 SAMPLE ANALYSIS

11.6.1 Establish LC-ESI-MS/MS operating conditions equivalent to those summarized in Tables 1-4 of Section 17 as per the guidance in Section 10.1. Column choice and instrument parameters should be optimized prior to initiation of the IDC (Sect. 9.2).

11.6.2 Establish a valid initial calibration following the procedures in Section 10.2 or confirm that the calibration is still valid by analyzing a CCC (Sect. 10.3). If establishing an initial calibration for the first time, complete the IDC as described in Section 9.2 prior to analyzing field samples.

11.6.3 Analyze field and QC samples at appropriate frequencies in the analysis batch as described in Section 11.7.

11.7 THE ANALYSIS BATCH – An analysis batch is a sequence of samples, analyzed within a 24-hour period, of no more than 20 field samples and includes all required QC samples (LRB, CCCs, LFB, LFSM and LFSMD (or FD)). The required QC samples are not included in counting the maximum field sample total of 20. LC-MS/MS conditions for the analysis batch must be the same as those used during calibration.

11.7.1 After a valid calibration is established, begin every analysis batch by analyzing an initial low-level CCC at or below the MRL. This initial CCC must be within ± 50% of the true value for each method and surrogate analyte and must pass the IS area criterion (Sect. 10.3.2). The initial CCC confirms that the calibration is still valid. Failure to meet the QC criteria may indicate that recalibration is required prior to analyzing samples. After the initial CCC, continue the analysis batch by analyzing an LRB, followed by field and QC samples at appropriate frequencies (Section 9.3). Analyze a mid- or high-level CCC after every ten field samples and at the end of each analysis batch. Do not count QC samples (LRBs, LFBs, FDs, LFSMs, LFSMDs) when calculating the required frequency of CCCs.

11.7.2 A final CCC completes the analysis batch. The acquisition start time of the final CCC must be within 24 hours of the acquisition start time of the initial low-level CCC at the beginning of the analysis batch. More than one analysis batch within a 24-hour period is permitted.

12. DATA ANALYSIS AND CALCULATIONS

12.1 Establish an appropriate retention time window for each analyte to identify them in QC and field sample chromatograms. Base this assignment on measurements of actual retention time variation for each compound in standard solutions over the course of time. The suggested variation is plus or minus three times the standard deviation of the retention time for each compound for a series of injections. The injections from the initial calibration and from the IDC (Sect. 9.2) may be used to calculate the retention time window. However, the experience of the analyst should weigh heavily on the determination of an appropriate range.

12.2 At the conclusion of data acquisition, use the same software settings established during the calibration procedure to identify peaks of interest in the predetermined retention time windows. Confirm the identify of each analyte by comparison of its retention time with that of the corresponding analyte peak in an initial calibration standard or CCC.

12.3 Calculate analyte concentrations using the multipoint calibration established in Section 10.2. Report only those values that fall between the MRL and the highest calibration standard.

12.4 Calculations must use all available digits of precision, but final reported concentrations should be rounded to an appropriate number of significant figures (one digit of uncertainty), typically two, and not more than three significant figures.

12.5 Prior to reporting the data, the chromatograms must be reviewed for any incorrect peak identifications or improper integration. The laboratory is responsible for ensuring that QC requirements have been met and that any appropriate qualifier is assigned.

12.6 EXCEEDING THE CALIBRATION RANGE – The analyst must not extrapolate beyond the established calibration range. If an analyte result exceeds the range of the initial calibration curve, the extract may be diluted using 50% methanol containing the appropriate amount of internal standard added to match the original level. Re-inject the diluted sample. Incorporate the dilution factor into final concentration calculations. The resulting data must be annotated as a dilution, and the reported MRLs must reflect the dilution factor. Acceptable surrogate performance must be determined from the undiluted sample extract.

13. METHOD PERFORMANCE

13.1 PRECISION, ACCURACY, AND DETECTION LIMITS – Tables for these data are presented in Section 17. LCMRLs and DLs for each method analyte are presented in Table 5. Precision and accuracy are presented for four water matrices: reagent water (Table 6); chlorinated (finished) groundwater (Table 7); moderate TOC chlorinated surface water (Table 8); and, the aqueous holding time study in chlorinated surface water (a separate source from the moderate TOC chlorinated surface water) (Table 9).

13.2 SECOND LABORATORY DEMONSTRATION – The performance of this method was demonstrated in multi-lab studies using triple quadrupole, ion trap, and "hybrid" triple quadrupole/ion trap mass spectrometers. The authors wish to acknowledge the work of MWH Laboratories (Monrovia, CA), Southern Nevada Water Authority (Henderson, NV), and Suffolk County Water Authority (Hauppauge, NY) for assisting in the review of this method and participation in the second laboratory demonstration.

13.3 ANALYTE STABILITY STUDY - Chlorinated surface water samples, fortified with method analytes at 5 – 10 ng/L, were preserved as required in Section 8 and stored over a 28-day period. The accuracy and precision of six replicate analyses, conducted on days 0, 7, 14, 21 and 28, are presented in Section 17, Table 9.

13.4 EXTRACT STORAGE STABILITY – Extract storage stability studies were conducted on extracts obtained from a chlorinated surface water fortified with the method analytes. The precision and accuracy of six replicates conducted on days 0, 7, 14, 21, and 28, are presented in Section 17, Table 10.

14. POLLUTION PREVENTION

This method utilizes SPE to extract analytes from water. It requires the use of very small volumes of organic solvent and very small quantities of pure analytes, thereby minimizing the potential hazards to both the analyst and environment as compared to the use of large volumes of organic solvents in conventional liquid-liquid extractions. For information about pollution prevention that may be applicable to laboratory operations, consult "Less is Better: Laboratory Chemical Management for Waste Reduction" available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C., 20036.

15. WASTE MANAGEMENT

The analytical procedures described in this method generate relatively small amounts of waste since only small amounts of reagents and solvents are used. The matrix of concern is finished drinking water. However, the Agency requires that laboratory waste management practices be conducted consistent with all applicable rules and regulations, and that laboratories protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. In addition, compliance is required with any sewage discharge permits and regulations, particularly the hazardous waste identification rules and land disposal restrictions.

16. REFERENCES

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17. TABLES, DIAGRAMS, FLOWCHARTS, AND VALIDATION DATA

TABLE 1. HPLC CONDITIONS

HPLC			
Column: Waters Xterra® MS C18, 2.1 x 150 mm, 3.5 µm d_p			
Column Temperature: 35 °C			
Column Flow Rate: 0.200 mL/min.			
Injection Volume: 50 µL			
Gradient:			
Time (min.)	**%RW**	**%MeOH**	**%NH_4OH[a] (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

[a]Preparation of 0.2% (v/v) ammonium hydroxide: Filter 1000 mL reagent water using 0.22 µm PVDF disk filter (Sect. 6.7). Add 8 mL 29% ammonium hydroxide (Sect. 7.1.2) and mix well (pH 10 – 10.5). The set-up described in the table entails quarternary mixing; if this option is not available, an alternative approach is to incorporate 0.02% ammonium hydroxide in the RW and MeOH eluents.
As the ammonia solutions age, analyte responses may drop – this is an indication that fresh mobile phase needs to be prepared.

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-d_{16}	9.31	ESI-	241.1[a]	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-d_4	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

[a]The bisphenol A-d_{16} readily exchanges two deuterium atoms in solution. As such, the m/z observed in MS analysis is based on bisphenol A-d_{14}

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. LC-ESI-MS/MS LOWEST CONCENTRATION MINIMUM REPORTING LEVELS (LCMRLs) and DETECTION LIMITS (DLs)

Analyte	DL Fortified Concentration (ng/L)[a]	DL (ng/L)[b]	LCMRL (ng/L)[c]
Estriol	1.10	0.24	0.28
Estrone	1.05	0.19	4.0
17β-Estradiol	1.30	0.39	0.32
17α-Ethynylestradiol	1.75	0.33	1.3
Androstenedione	0.50	0.20	0.37
Testosterone	0.50	0.04	0.062
Equilin	1.25	2.94	3.0

[a]Fortification level used to determine DLs
[b]Detection limits were obtained as described in Section 9.2.6
[c]LCMRLs were calculated according to the procedure in reference 1 with the following modification: Instead of evaluating seven replicates at four concentration levels, LCMRLs are now obtained by analyzing four replicates at seven concentration levels.

TABLE 6. LC-ESI-MS/MS PRECISION AND ACCURACY IN FORTIFIED REAGENT WATER (n=5)

Analyte	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD
Estriol	11	83.5	6.3
Estrone	10.5	86.5	1.2
17β-Estradiol	13	90.6	6.0
17α-Ethynylestradiol	17.5	90.1	3.1
Androstenedione	10	87.2	18
Testosterone	10	93.5	3.3
Equilin	12.5	80.4	5.1
Ethynylestradiol-d_4	67	98.1	2.2
Bisphenol A-d_{16}	24	84.5	8.5

TABLE 7. LC-ESI-MS/MS PRECISION AND ACCURACY IN FORTIFIED CHLORINATED GROUND WATER[a] (n=5)

Analyte	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD
Estriol	11	89.5	5.8
Estrone	10.5	96.5	3.0
17β-Estradiol	13	93.6	2.9
17α-Ethynylestradiol	17.5	105	3.5
Androstenedione	10	94.6	12
Testosterone	10	88.3	2.4
Equilin	12.5	99.9	3.1
Ethynylestradiol-d_4	67	93.0	3.4
Bisphenol A-d_{16}	24	93.2	1.9

[a] Ground water physical parameters: total hardness = 334 milligrams/liter (mg/L) (as $CaCO_3$); free chlorine = 0.60 mg/L; total chlorine = 0.79 mg/L.

TABLE 8. LC-ESI-MS/MS PRECISION AND ACCURACY IN FORTIFIED MODERATE TOC CHLORINATED SURFACE WATER[a] (n=5)

Analyte	Fortified Concentration (ng/L)	Avg. %Recovery	%RSD
Estriol	11	89.3	3.3
Estrone	10.5	86.8	4.8
17β-Estradiol	13	88.1	5.9
17α-Ethynylestradiol	17.5	99.6	6.1
Androstenedione	10	102	3.7
Testosterone	10	86.2	3.1
Equilin	12.5	92.4	11
Ethynylestradiol-d_4	67	94.5	3.6
Bisphenol A-d_{16}	24	93.2	13

[a] Surface water physical parameters: total hardness = 113 milligrams/liter (mg/L) (as $CaCO_3$); free chlorine = 1.35 mg/L; total chlorine = 1.56 mg/L.

TABLE 9. AQUEOUS SAMPLE HOLDING TIME DATA FOR SAMPLES FROM CHLORINATED SURFACE WATER[a], FORTIFIED WITH METHOD ANALYTES AND PRESERVED AND STORED ACCORDING TO METHOD SECTION 8 (n = 6)

Analyte	Fortified Conc. (ng/L)	Day 0		Day 7		Day 14		Day 21		Day 28	
		Avg. %Rec	%RSD	Avg. %Rec	%RSD	Avg. %Rec	%RSD	Avg. %Rec	%RSD	Avg. %Rec	%RSD
Estriol	11	85.5	4.3	83.4	4.0	86.7	3.5	86.9	3.5	86.1	3.8
Estrone	10.5	84.1	3.6	82.8	5.3	89.1	5.8	91.9	6.4	84.3	3.1
17β-Estradiol	13	91.8	2.9	85.3	5.3	92.3	4.8	88.1	4.0	87.3	2.4
17α-Ethynylestradiol	17.5	97.6	3.9	90.4	4.0	97.4	4.9	90.4	3.2	85.1	3.3
Androstenedione	10	104	3.8	103	3.4	93.5	5.9	115	7.1	112	4.6
Testosterone	10	93.0	6.0	88.9	2.5	85.9	3.2	91.7	1.5	95.9	2.3
Equilin	12.5	103	13	92.6	4.1	85.7	14	91.7	11	86.3	5.0
Ethynylestradiol-d_4	67	85.9	5.7	86.2	4.5	90.6	5.5	88.8	3.6	85.0	7.1
Bisphenol A-d_{16}	24	85.8	6.3	87.7	4.7	85.6	8.7	127	7.3	94.7	4.9

[a] Surface water physical parameters: total hardness = 134 milligrams/liter (mg/L) (as $CaCO_3$); free chlorine = 1.05 mg/L; total chlorine = 1.25 mg/L.

TABLE 10. EXTRACT HOLDING TIME DATA FOR SAMPLES FROM CHLORINATED SURFACE WATER FORTIFIED WITH METHOD ANALYTES AND PRESERVED AND STORED ACCORDING TO METHOD SECTION 8 (n = 6)

Analyte	Fortified Conc. (ng/L)	Day 0		Day 7		Day 14		Day 21		Day 28	
		Avg. %Rec	%RSD	Avg. %Rec	%RSD	Avg. %Rec	%RSD	Avg. %Rec	%RSD	Avg. %Rec	%RSD
Estriol	11	85.5	4.3	82.3	5.6	84.0	6.9	82.3	4.4	86.1	5.2
Estrone	10.5	84.1	3.6	85.6	4.9	83.4	7.9	88.8	3.8	84.2	3.5
17β-Estradiol	13	91.8	2.9	86.0	8.9	89.3	5.5	88.6	5.6	89.6	4.0
17α-Ethynylestradiol	17.5	97.6	3.9	91.1	6.8	86.6	6.7	90.4	3.0	88.4	4.8
Androstenedione	10	104	3.8	104	6.0	96.0	8.9	119	8.2	111	3.4
Testosterone	10	93.0	6.0	89.8	4.2	90.4	2.9	96.2	4.9	96.6	4.1
Equilin	12.5	103	13	90.1	4.8	79.5	9.3	91.0	4.1	88.7	4.4
Ethynylestradiol-d_4	67	85.9	5.7	89.4	6.7	88.6	5.9	86.4	2.1	87.8	4.6
Bisphenol A-d_{16}	24	85.8	6.3	89.5	3.7	87.6	15	131	3.9	99.8	3.9

TABLE 11. INITIAL DEMONSTRATION OF CAPABILITY (IDC) QUALITY CONTROL REQUIREMENTS

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
Section 9.2.1	Demonstration of low system background	Analyze a Laboratory Reagent Blank (LRB) prior to any other IDC steps.	Demonstrate that all method analytes are < 1/3 of the Minimum Reporting Level (MRL) and that possible interferences from reagents and glassware do not prevent the identification and quantitation of method analytes.
Section 9.2.2	Demonstration of precision	Prepare, extract, and analyze 4 - 7 replicate Laboratory Fortified Blanks (LFBs) fortified near the midrange concentration.	Percent relative standard deviation must be ≤ 20%.
Section 9.2.3	Demonstration of accuracy	Calculate average percent recovery for replicates used in Section 9.2.2.	Average percent recovery within ± 30% of the true value.
Section 9.2.4	MRL confirmation	Fortify, extract, and analyze 7 replicate LFBs at the proposed MRL concentration. Confirm that the Upper Prediction Interval of Results (PIR) and Lower PIR (Sect. 9.2.4.1 and Sect. 9.2.4.2) meet the recovery criteria.	Upper PIR ≤ 150% Lower PIR ≥ 50%
Section 9.2.5	Quality Control Sample (QCS)	Analyze mid-level QCS.	Results must be within ± 30% of the true value.

TABLE 12. ONGOING QUALITY CONTROL REQUIREMENTS

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
Section 10.2	Initial calibration	Use the internal standard calibration technique to generate a linear or quadratic calibration curve. Use at least five calibration concentrations. Validate the calibration curve as described in Section 10.2.3.	When each calibration standard is calculated as an unknown using the regression equation, the lowest level standard must be within ± 50% of the true value. All other points must be within ± 30% of the true value.
Section 9.3.1	Laboratory Reagent Blank (LRB)	Analyze one LRB with each extraction batch.	Demonstrate that all method analytes are ≤ 1/3 the Minimum Reporting Level (MRL), and that possible interference from reagents and glassware do not prevent identification and quantitation of method analytes.
Section 9.3.3	Laboratory Fortified Blank (LFB)	Extract and analyze one LFB with each extraction batch.	For LFBs fortified at concentrations ≤ 2 x MRL, the result must be within ± 50% of the true value. At concentrations > 2 x MRL, the result must be ± 30% of the true value.
Section 10.3	Continuing Calibration Check (CCC)	Verify initial calibration by analyzing a low-level CCC at the beginning of each analysis batch. Subsequent CCCs are required after every 10 field samples, and at the end of the analysis batch.	The lowest level CCC must be within ± 50% of the true value. All other points must be within ± 30% of the true value. Surrogate analytes must be ± 30% of the true value. Internal standards must be ± 50% of the average peak areas in the initial calibration. Results for field samples that are not bracketed by acceptable CCCs are invalid.
Section 9.3.4	Internal standard (IS)	Isotopically labeled internal standards are added to all standards and samples.	Peak area counts for each IS must be within ± 50% of the average peak areas in the initial calibration.
Section 9.3.5	Surrogate analyte	Fortify the surrogate analytes into all samples prior to extraction.	Surrogate recovery must be in the range of 70 to 130%.
Section 9.3.6	Laboratory Fortified Sample Matrix (LFSM)	Analyze one LFSM per extraction batch. Fortify the LFSM with method analytes at a concentration greater than the native concentrations.	For LFSMs fortified at concentrations ≤ 2 x MRL, the calculated recovery must be within ± 50% of the true value. At concentrations greater than the 2 x MRL, the recovery must be ± 30% of the true value.
Section 9.3.7	Laboratory Fortified Sample Matrix Duplicate (LFSMD) or Field Duplicate (FD)	Analyze at least one LFSMD or FD with each extraction batch.	For LFSMDs or FDs, the calculated relative percent difference must be ≤ 30%. (≤ 50% if concentration ≤ 2 x MRL.)
Section 9.3.8	Quality Control Sample (QCS)	Analyze mid-level QCS at least quarterly.	Results must be ± 30% of the true value.

FIGURE 1. EXAMPLE CHROMATOGRAM OF LC-ESI-MS/MS TRANSITIONS FOR METHOD 539 ANALYTES.

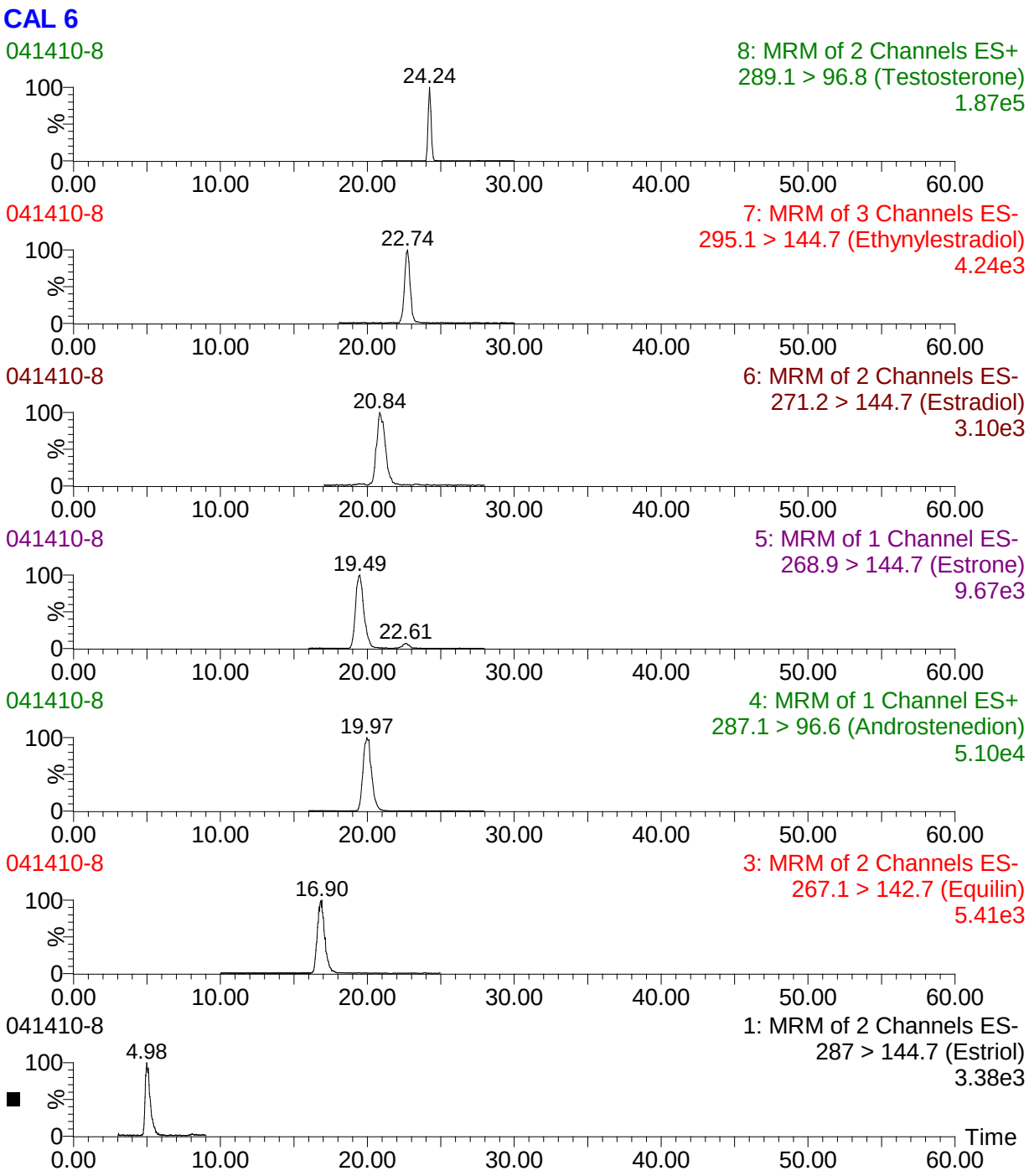

FIGURE 2. EXAMPLE CHROMATOGRAM OF LC-ESI-MS/MS TRANSITIONS FOR METHOD 539 SURROGATES AND INTERNAL STANDARDS.