Analysis of 7 hormones in drinking water following EPA Method 539

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Introduction

This technical note describes the analysis of hormones in drinking water, following the United States EPA Method 539. Using the SCIEX QTRAP 6500+ system, the UCMR3 minimum reporting levels (MRLs) were demonstrated by performing the initial demonstration of capability (IDC).[1] Confirmed MRLs ranged from 0.1 to 4 ng/L, dependent on the analyte UCMR3 MRL targets. IDC experiments included the assessment of method blanks (<1/3 MRL), the determination of accuracy (70%–130%) and precision (%CV <20%) in laboratory fortified blanks (LFBs) and the confirmation of the MRL (prediction interval of results [PIR] limits 50%-150%). Sample preparation used only 250 mL of water, reducing sample preparation time and potentially reducing shipping and storage costs.

Hormones in drinking water can potentially disrupt endocrine signaling in humans and have been detected in sources of drinking water.[2,3] The US EPA Method 539 is a solid-phase extraction (SPE)-based method that can achieve sub- to low-ng/L detection limits in sample for 7 hormones of concern (Figures 1 and 2).

Key benefits of analysis following EPA Method 539 using the QTRAP 6500+ system

- **MRL confirmation at UCMR3 reporting levels:** Analysis of 7 replicate samples showed PIR limits between 50% and 150% for all analytes, confirming MRLs ranging from 0.1 to 4 ng/L
- **Accuracy and precision of LFB samples surpassed IDC criteria:** LFB samples (n=4) spiked at 5 ng/L were within the EPA Method 539 requirements of ±30% and <20% for accuracy and precision, respectively
- **Low ng/L sensitivity for calibration standards using the QTRAP 6500+ system:** In-vial limits of quantitation (LOQs) for the solvent-based calibration standards ranged from 10 to 100 ng/L
- **Rapid chromatographic gradient:** The gradient runtime was reduced to 10 minutes, while maintaining good chromatographic separation and analyte peak shape

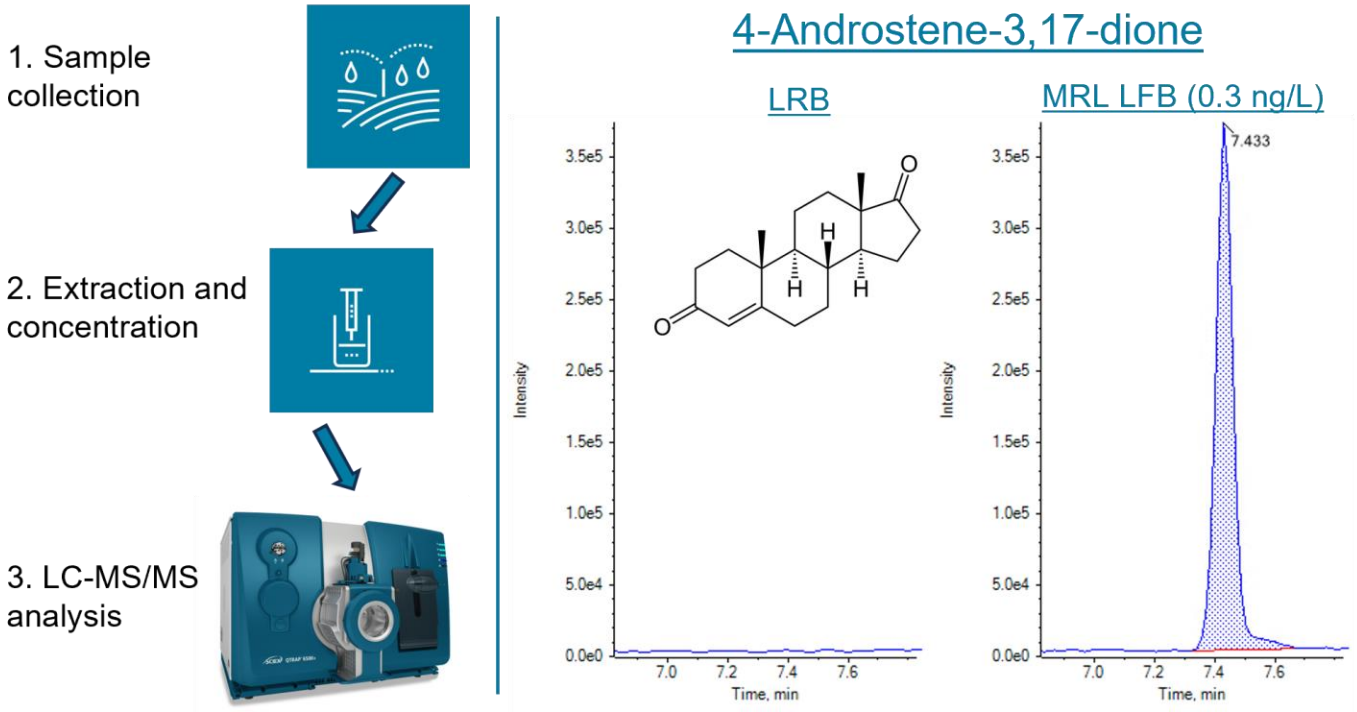

Figure 1. Extracted ion chromatograms (XIC) of androstenedione in LRB and LFB samples at the UCMR3 MRL (0.3 ng/L).

Methods

Standard preparation: Standards were purchased from Vivan Life Sciences, Artis Biotech, Pharmaffiliates Analytics & Synthetics and Minerva Scientific Solutions. All standards were purchased as neat powder, except for androstenedione, which was in solution. Intermediate stock solutions of 1 mg/mL were prepared in methanol and used to prepare the calibration standards in 50:50 (v/v), methanol/water, at concentrations ranging from 10 to 10000 pg/mL. The internal standards used included estriol-D2, estradiol-D4 and testosterone-D5.

IDC experiments: IDC experiments followed the procedures described in section 9.2 of the EPA Method 539 and are briefly described below. Spiking levels were chosen to represent the UCMR3 MRL concentrations.

Initial demonstration of low system background: As outlined in section 9.2.1 of the EPA Method 539, the laboratory reagent blank (LRB, n=1) was 250 mL of blank laboratory water that was spiked with the surrogate standard and carried through the sample preparation procedure.

Initial demonstration of precision and accuracy: As outlined in sections 9.2.2 and 9.2.3 of the EPA Method 539, the LFB samples (n=4) were 250 mL of blank laboratory water spiked with hormones at 5 ng/L. As permitted by section 8.1.1, the sample volume was reduced to 250 mL and the solvent rinsing and washing volumes were adjusted accordingly. The final reconstitution volume was 250 µL. Sample preparation details are thoroughly outlined in section 11.

Briefly, the water samples were dechlorinated and preserved. The samples were then fortified with the surrogate standard (17α-ethynylestradiol-D4, 70 ng/L) and target analytes. The samples were then passed through an Empore solid phase extraction disk (C18, 47 mm). The analytes were eluted using methanol and the extract was reduced to dryness under a gentle stream of nitrogen gas and reconstituted with 250 µL of 50:50 (v/v), methanol/water after adding the internal standards.

MRL confirmation (Section 9.2.4) at the UCMR3 levels: To confirm the MRL, 7 LFB samples were spiked at the UCMR3 level. Sample preparation methods matched those used for the initial demonstration of precision and accuracy. Packaged water was purchased locally, spiked at the UCMR3 MRL levels, extracted and analyzed (laboratory fortified sample matrix samples, LFSM) in the same manner as the LRB and LFB samples.

Table 1. LC gradient conditions used for the analysis of hormones in drinking water following EPA Method 539.

Time	Flow rate (mL/min)	%A	%B
0	*0.400*	*98*	*2*
4.0	*0.400*	*50*	*50*
7.0	*0.400*	*40*	*60*
7.1	*0.400*	*5*	*95*
9.0	*0.400*	*5*	*95*
9.1	*0.400*	*98*	*2.0*
10	*0.400*	*98*	*2.0*

Chromatography: Chromatographic separation was achieved using the Phenomenex Kinetex C8 column (2.6 µm, 100 Å, 100 x 2.1 mm, P/N: 00D-4497-AN). Mobile phase A was 0.1mM ammonium fluoride in water and mobile phase B was methanol. Samples were analyzed using the gradient conditions presented in Table 1. The mobile phase flow rate was 0.400 mL/min. The injection volume was 10 µL and the column oven was set to 40°C.

***Mass spectrometry*:** Samples were analyzed using the QTRAP 6500+ system with the IonDrive Turbo V ion source and electrospray ionization electrode. Multiple reaction monitoring (MRM), source and gas parameters were optimized by infusion. Optimized conditions are presented in Tables 2 and 3. The entrance potential (EP) was set to 10/-10 V for all transitions. Data were acquired using MRM mode with polarity switching (pause time = 5 ms, settling time = 15 ms).

***Data processing*:** Data were processed using SCIEX OS software, version 2.2. For the calibration curves, the linear regression algorithm was used with 1/x weighing. Analyte responses were normalized to the corresponding internal standard response given in Table 3.

Table 2. Source and gas parameters used for the analysis of hormones in drinking using the QTRAP 6500+ system

Parameter	Value
Polarity	*Positive/negative*
Curtain Gas	*30 psi*
CAD Gas	*High*
Ion spray voltage	*3500 V*
Temperature	*450°C*
GS1	*80 psi*
GS2	*70 psi*

Table 3. Compound-specific MRM parameters and internal standard assignment for the analysis of hormones in drinking water using the SCIEX 6500+ system.

Compound	Internal standard	Polarity	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP (V)
16α-Hydroxyestradiol (estriol)_1	Estriol-D2	Negative	286.9	171	-110	-47	-19
16α-Hydroxyestradiol (estriol)_2	Estriol-D2	Negative	286.9	145	-110	-52	-14
17β-Estradiol_1	Estradiol-D4	Negative	270.9	145	-119	-51	-17
17β-Estradiol_2	Estradiol-D4	Negative	270.9	143	-119	-65	-15
Equilin_1	Estradiol-D4	Negative	266.9	143.1	-115	-41	-14
Equilin_2	Estradiol-D4	Negative	266.9	223	-115	-45	-13
Estrone_1	Estradiol-D4	Negative	268.9	145.2	-125	-48	-8
Estrone_2	Estradiol-D4	Negative	268.9	143.1	-125	-66	-15
17α-Ethynylestradiol_1	Estradiol-D4	Negative	294.9	145.1	-141	-51	-13
17α-Ethynylestradiol_2	Estradiol-D4	Negative	294.9	143	-141	-65	-16
Testosterone_1	Testosterone-D5	Positive	289.2	109.1	76	31	12
Testosterone_2	Testosterone-D5	Positive	289.2	97.1	76	29	12
4-Androstene-3,17-dione_1	Testosterone-D5	Positive	287.2	97.1	97	29	13
4-Androstene-3,17-dione_2	Testosterone-D5	Positive	287.2	109.1	97	36	10
17α-Ethinylestradiol-D4 (SUR)	n/a	Negative	298.9	145.2	-130	-68	-12
Testosterone-D5 (IS)	n/a	Positive	294.2	100.1	50	29	15
Estriol-D2 (IS)	n/a	Negative	288.9	173.2	-170	-48	-19
Estradiol-D4 (IS)	n/a	Negative	274.9	147.1	-175	-72	-10

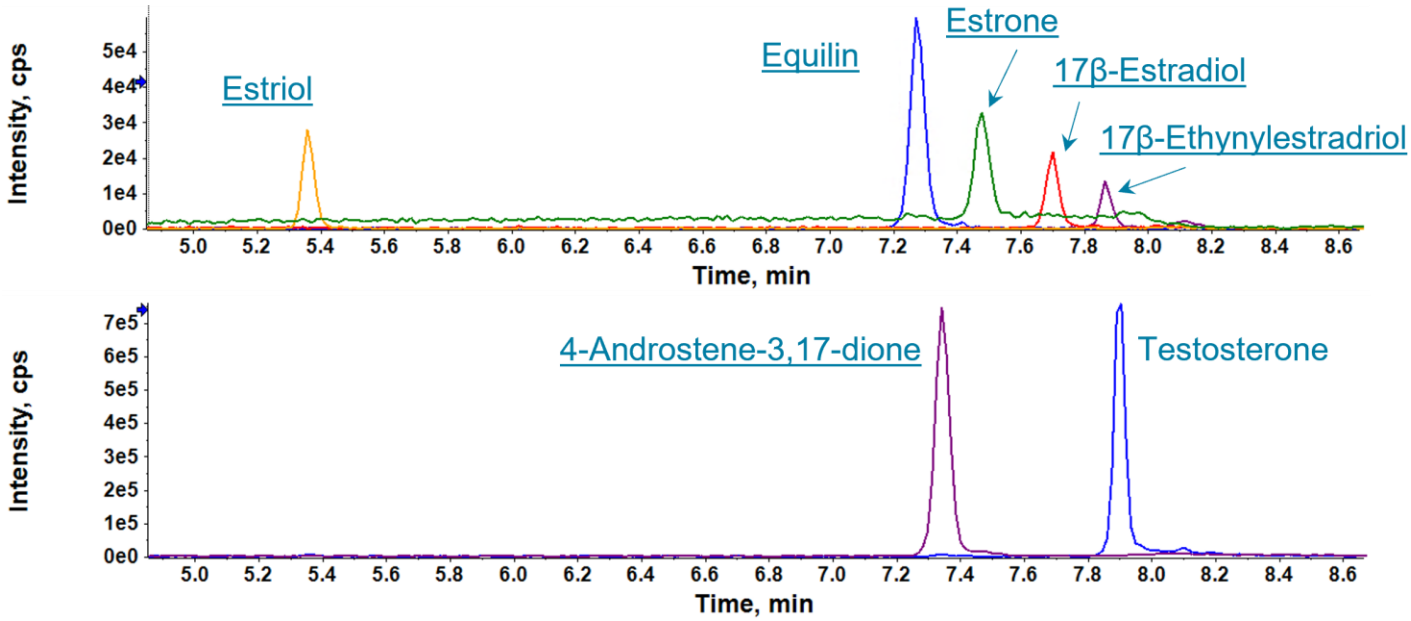

Figure 2. XICs of the 7 hormones analyzed during EPA Method 539. The top and bottom panels show XICs acquired using MRM in negative and positive mode, respectively.

Table 4. LOQ, %CV of LOQ, mean accuracy of LOQ, linearity range and regression coefficient for the 7 hormones analyzed in the solvent-based calibration standards (n=3).

Compound	LOQ (ng/L)	%CV	Mean accuracy (%)	Linearity range (ng/L)	Regression coefficient (r)
4-Androstene-3,17-dione	10	1.0	120	10–10,000	0.999
Testosterone	10	6.9	105	10–10,000	0.999
17α-Ethynylestradiol	100	4.8	104	100–10,000	0.999
Equilin	100	5.8	116	100–10,000	0.999
17β-Estradiol	100	13	103	100–10,000	0.999
Estrone	50	3.6	106	50–10,000	0.999
Estriol	50	1.5	102	50–10,000	0.999

Chromatographic separation of isomer pairs

LC column stationary phase and mobile phase composition were selected to ensure the chromatographic separation of 3 hormone isotope pairs. These included testosterone/4-andro-3,17-dione, estrone/17β-estradiol and equilin/estrone. Chromatographic separation of these pairs ensured that there was no interference from the M+2 precursor ion.

Sensitivity, accuracy, precision and linearity of solvent calibration standards

Instrument performance for the 7 hormone analytes on the QTRAP 6500+ system was evaluated through triplicate injections of the solvent-based calibration standards (Table 4). The in-vial LOQ concentrations ranged from 10 to 100 ng/L. Estimated in-sample LOQs ranged from 0.01 to 0.1 ng/L, considering the 1000-fold SPE concentration factor. Good accuracy (102-120%) and precision (<7% except for 17β-estradiol, 13%) were achieved at the LOQ level. These results demonstrate the high sensitivity of the QTRAP 6500+ system for hormone analysis. Further, linearity was shown across 2-3 orders of magnitude with r-values >0.999.

IDC experiments

Section 9.2 of the EPA Method 539 outlines the procedures to demonstrate laboratory capability.[1] Experiments include the demonstration of low system background (9.2.1), demonstration of precision (9.2.2), demonstration of accuracy (9.2.3) and MRL confirmation (9.2.4). Experiments were performed to achieve these criteria, demonstrating the method performance.

Demonstration of low system background. Extracted LRBs showed minimal background levels, demonstrating negligible contamination from sample preparation and instrumental analysis (Figures 1 and 3). The LRB sample consisted of laboratory water spiked with the surrogate standard and was processed through the SPE sample preparation procedure and analyzed.

Demonstration of precision and accuracy. LFB samples were spiked at 5 ng/L (n=4), representing the mid-point of the calibration curve, and processed through the sample preparation and analysis procedure. Mean accuracy ranged between 75% and 86%. The mean precision ranged between 1.5% and 6.4% (Table 5). These observed values met the standard acceptable criteria of accuracy within ±30% of the nominal value and %CV <20%.

Table 5. Precision and average accuracy of IDC experiments at 5 ng/L (n=4).

Compound	Mean accuracy (%)	Mean precision (%CV)
16α-Hydroxyestradiol (estriol)	*75*	*3.4*
17β-Estradiol	*85*	*1.5*
Equilin	*85*	*4.2*
Estrone	*84*	*2.8*
17α-Ethynylestradiol	*86*	*2.0*
Testosterone	*85*	*4.2*
4-Androstene-3,17-dione	*84*	*6.4*

MRL confirmation at UCMR3 reporting level concentrations. Confirmation of the MRL concentration was performed by spiking LFBs (n=7) at the UCMR3 reporting limits and processing the samples through the extraction and instrumental analysis methods (see Table 6 for the UCMR3 target reporting limit concentrations). MRLs were considered verified if the calculated PIR limits were between 50% and 150%. The PIR limits are a measure of method accuracy and precision.

Chromatograms for the LRBs and MRL LFBs are shown in Figures 1 and 3. Mean recoveries ranged from 77% to 101% with %CV ranging from 5.5% to 10% (Table 6). The mean measured recovery percentage and standard deviation were used to calcuate the PIR using the following equation:

$$PIR\ (upper\ or\ lower) = \frac{Mean \pm\ HR_{PIR}}{Fortified\ concentration} \times 100\%$$

Where HR_{PIR} represents the half range for the PIR and was calculated as,

$$HR_{PIR} = 3.963s$$

HR_{PIR} = Half range for the PIR
s = The standard deviation of replicate analyses
3.963 = Constant value for 7 replicates

Considering all 7 hormone analytes, the lower PIR values were between 53% and 78% and the upper PIR values were between 96% and 138%. Therefore, the MRLs were verified at sub- to low-ng/L levels, achieving the URMR3 MRLs for the analysis of hormones in drinking water. These results demonstrate the ability of the QTRAP 6500+ system to analyze hormones according to EPA Method 539 with sensitivity, accuracy and precision.

To demonstrate the method applicability in real-world water samples, 2 different packaged drinking water samples were purchased from a local store and spiked at the UCMR3 MRL concentration. These are representative of the LFSM and were processed and instrumentally analyzed in the same manner as the MRL LFB samples. The 2 samples were extracted individually but instrumentally analyzed 7 times to obtain statistics for accuracy and precision (duplicate #1 was injected 3 times and duplicate #2 was injected 4 times). The mean accuracy was between 78% and 102% and mean precision (%CV) was between 1.7% and 5.5%.

Table 6. MRL spikes in LFB and LFSM samples at the UCMR3 MRL concentration. LFB samples consisted of 7 individual replicate samples that were injected once each. LFSM samples were comprised of 2 different packaged water samples that were injected a total of 7 times.

		LFB MRL samples (n=7)				LFSM MRL samples (n=2)	
Analyte	UCMR3 concentration (ng/L)	Mean recovery (%)	Mean precision (%CV)	Lower PIR	Upper PIR	Mean recovery (%)	Mean precision (%CV)
Testosterone	*0.1*	*89*	10	53	126	*78*	*3.0*
4-Androstene-3,17-dione	*0.3*	*100*	5.5	78	121	*83*	*1.7*
17β-Estradiol	*0.4*	*80*	9.2	53	110	*82*	*2.6*
16α-Hydroxyestradiol (Estriol)	*0.8*	*77*	*6.1*	*59*	96	*78*	*5.5*
17α-Ethynylestradiol	*0.9*	*101*	9.1	65	138	*85*	*3.7*
Estrone	*2.0*	*96*	8.6	64	129	*97*	*1.7*
Equilin	*4.0*	*100*	8.9	65	135	*102*	*2.3*

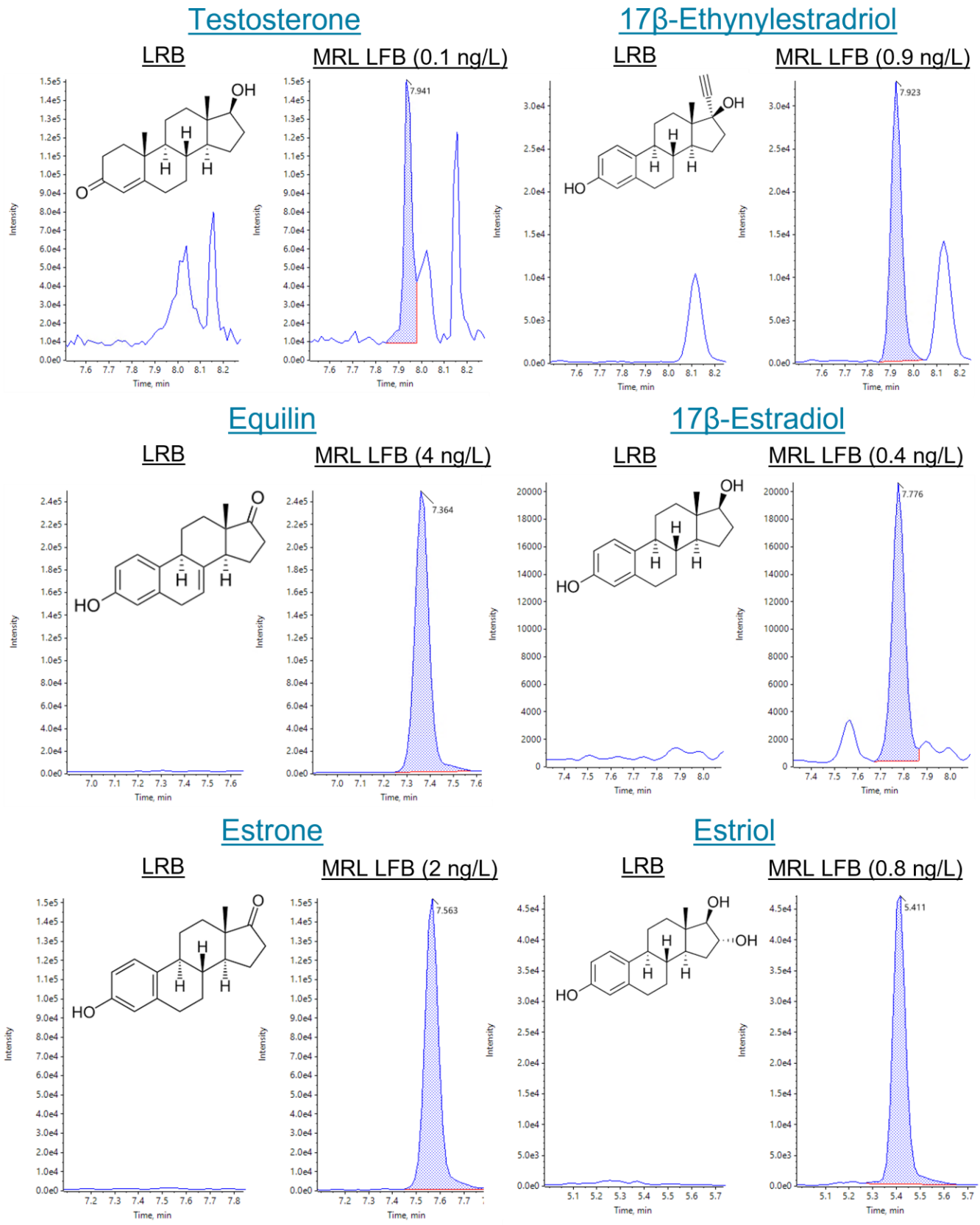

Figure 3. XICs of LRB (left) and LFB (right) at the UCMR3 MRL concentration. Chromatograms are shown for testosterone, 17β-ethynylestradiol, equilin, 17β-estradiol, estrone and estriol.

Conclusions

This technical note demonstrated the successful analysis of hormones in drinking water using the QTRAP 6500+ system, following the EPA Method 539.

- EPA Method 539 IDC experiments necessary to demonstrate analytical proficiency, including low LRB background, LFB accuracy and precision and MRL verification, were performed
- MRL confirmation was attained at the UCMR3 reporting levels ranging from 0.1 to 4 ng/L
- LFB spikes (5 ng/L) met the acceptable IDC criteria of ±30% and <20% for accuracy and precision, respectively
- Low-ng/L sensitivity of the QTRAP 6500+ system for hormone analysis with solvent-based calibration standard LOQs ranging from 10 to 100 ng/L
- Fast 10 min runtime yielded good analyte separation and peak shape

References

1. 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). United States Environmental Protection Agency, Cincinnati, OH, November 2010. EPA Document No. 815-B-10-001.
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3. Benotti, MJ; Trenholm, RA; Vanderford, BJ; Holady, JC; Stanford, BD; Snyder, SA. (2008) Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. Environ. Sci. Technol. 43: 597-603.

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