Improved Analysis of Trace Hormones in Drinking Water by LC/MS/MS (EPA 539) using the Agilent 6460 Triple Quadrupole LC/MS

Application Note

Environmental

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Abstract

A modification to the chromatographic conditions of EPA method 539 has been developed that reduces the total run time by more than a factor of three, while providing complete baseline resolution of all seven hormones and DLs that meet the EPA requirements. Response is 7 to 11 times more sensitive in negative mode compared to the use of ammonium hydroxide, and 1.7 to 2.4 times more sensitive in positive mode. The performance of the Agilent 6460 Triple Quadrupole LC/MS System can be used to determine where problems may be occurring, if labs have trouble in method implementation.

Agilent Technologies

Introduction

Chemicals are being discovered in water that previously had not been detected, or are being detected at levels that may be significantly different than expected [1]. These are often referred to as contaminants of emerging concern (CECs) because the risk to human health and the environment associated with their presence, frequency of occurrence, or source may not be known. As a result, the EPA has instituted Unregulated Contaminant Monitoring Rules (UCMRs) to assess the potential threat of CECs in the water supply. The latest, UCMR3, requires monitoring for 30 contaminants from 2013 to 2015. States, laboratories, and public water systems will participate in assessment monitoring, a screening survey, and prescreen testing [2].

The screening survey, which uses analytical method technologies not commonly used by drinking water laboratories, is done for List 2 Contaminants, which are seven hormones. The EPA has developed Method 539 for the monitoring of these hormones. This application note describes modifications to Method 539, that provide full baseline resolution of all target hormones and detection limits (DLs) that meet or exceed EPA DLs. The LC analysis is performed in less than one-third of the time, and positive-negative ion switching on the Agilent 6460 Triple Quadrupole LC/MS System during a single injection also reduces overall run time. The modified method uses ammonium fluoride in place of ammonium hydroxide in the aqueous mobile phase, premixed acetonitrile/methanol solvents in the organic mobile phase, and an Agilent Poroshell 120 Phenyl-Hexyl Column with backpressure less than 250 bar, and is, therefore, suitable for use on all HPLC pumps.

Experimental

Reagents and Standards

All target hormones and ammonium fluoride were supplied by Sigma-Aldrich Canada Ltd (Oakville, Ontario). Surrogates and internal standards were supplied by Cambridge Isotope Laboratories (Andover, MD). Reagent water, methanol, and acetonitrile (all HPLC grade) were supplied by Caledon Laboratories (Georgetown, Ontario).

Testosterone and androstenedione were supplied as 1 mg/mL solutions in dimethoxyethane. All other target hormones were prepared from neat materials by dissolving an accurately weighed amount in methanol. Stock solutions were sonicated for 5 minutes to ensure dissolution before subsequent dilutions. Primary dilution standards (PDS) and calibration standards were prepared as described in EPA Method 539, with all final solutions in 50% methanol in reagent water.

Instruments

This method was developed on an Agilent 1290 Infinity LC System with a 1260 Autosampler, running an Agilent Poroshell 120 Phenyl-Hexyl Column (p/n 695975-312). The LC system was coupled to an Agilent 6460 Triple Quadrupole LC/MS System. Table 1 lists the instrument conditions.

Table 1. LC and MS Run Conditions

LC conditions			
Column	Agilent Poroshell 120 Phenyl-Hexyl Column 3.0 × 100 mm, 2.7 µm (p/n 695975-312)		
Column temperature	15 °C		
Injection volume	50 µL		
Mobile phase	A = 1 mM ammonium fluoride B = 35% acetonitrile + 65% methanol		
Flow rate	0.3 mL/min		
Gradient	Time (min)	% A	% B
	0	100	10
	0.5	90	10
	12.5	0	100
Post time	2.5 minutes		
Total run time	15 minutes		
MS conditions			
Ionization mode	ESI with pos/neg switching		
Drying gas temperature	300 °C		
Drying gas flow	4 L/min		
Nebulizer pressure	40 psig		
Sheath gas temperature	375 °C		
Sheath gas flow	11 L/min		
Capillary voltage	Pos 4000 V, Neg 4500 V		
Nozzle voltage	Pos 500 V, Neg 1500 V		

Sample Preparation

Laboratory fortified blanks were prepared by first adding 2-mercaptopyridine-1-oxide, sodium salt (65 mg/L final concentration), and sodium thiosulfate (80 mg/L) as preservatives to 1 L of reagent water, as well as 17α-ethynylestradiol-d_4 added as surrogate (70 ng/L final concentration). Fortification (IDC) levels for the target hormones ranged from 0.05 to 2 µg/L, as shown in Table 5. Each 1 L sample was extracted using solid phase extraction (SPE) on a C18 disk (p/n 12145004), as described in EPA Method 539, eluted with methanol, followed by concentration under a gentle stream of nitrogen to near dryness, and brought to 1 mL with 50% methanol before analysis.

Analysis Parameters

Tables 2 and 3 show the 6460 Triple Quadrupole LC/MS System multiple reaction monitoring (MRM) analysis parameters.

Table 2. MRM Analysis Parameters for the Target Compounds

Compound name	Precursor ion	Product ion	Fragmentor voltage (V)	Collision energy (V)	Polarity
17α-Ethynylestradiol	295.2	145.0	139	36	Negative
Testosterone	289.2	109.1	116	24	Positive
Testosterone	289.2	97.1	116	20	Positive
Estriol	287.2	171.2	159	36	Negative
Estriol	287.2	145.0	159	44	Negative
Androstenedione	287.2	109.1	107	24	Positive
Androstenedione	287.2	97.1	107	20	Positive
17β-Estradiol	271.2	183.2	171	40	Negative
17β-Estradiol	271.2	145.1	171	44	Negative
Estrone	269.1	145.0	136	40	Negative
Estrone	269.1	143.2	136	56	Negative
Equilin	267.1	265.1	136	20	Negative
Equilin	267.1	143.1	136	40	Negative

Table 3. MRM Analysis Parameters for the Internal Standards and Surrogates

Compound name	Precursor ion	Product ion	Fragmentor voltage (V)	Collision energy (V)	Polarity
17α-Ethynylestradiol-$^{13}C_2$	297.2	144.9	148	36	Negative
17α-Ethynylestradiol-$^{13}C_2$	297.2	143.1	148	52	Negative
Testosterone-d_3	292.2	109.1	116	24	Positive
Testosterone-d_3	292.2	97.1	116	20	Positive
Estriol-d_4	291.2	173.1	145	44	Negative
Estriol-d_4	291.2	147.2	145	48	Negative
17β-Estradiol-$^{13}C_6$	277.2	186.2	168	44	Negative
17β-Estradiol-$^{13}C_6$	277.2	145.1	168	48	Negative
17α-Ethynylestradiol-d_4	299.2	147.0	121	44	Negative
17α-Ethynylestradiol-d_4	299.2	145.2	121	56	Negative

Results and Discussion

EPA Method 539 Performance

EPA Method 539 is an electrospray LC/MS/MS method for the analysis of hormones in finished drinking water, and it uses analysis by internal standardization, based on peak areas [3].

The EPA detection limits (DLs) range from 0.04 to 2.9 ng/L, and laboratories must demonstrate a Minimum Reporting Limit (MRL) for each target analyte with a Lower Prediction Interval of Results (PIR) ≥ 50% and an Upper PIR Limit ≤ 150%. The method allows flexibility in LC columns, LC conditions, and MS conditions, as long as method performance is not affected, and the analytes should be adequately resolved.

Improving the Chromatography

However, Method 539 resolves only five of the seven target hormones, with DLs ranging from 0.04 to 2.94 ng/L and a run time of 50 minutes. A chromatographic approach has been developed that provides improved resolution of the hormones, and in much less time [4,5]. This approach uses ammonium fluoride (NH_4F) in place of ammonium hydroxide in mobile phase A, at a pH of 6.2 rather than 9.3. The lower pH reduces the wear on the HPLC system, improves column lifetime, and allows the use of columns other than high pH-tolerant columns such as Extend-C18. Figure 1 shows a typical separation of the seven target hormones using this chromatographic configuration, illustrating baseline resolution of all compounds, as well as bisphenol-A-d_{16}, in less than 16 minutes.

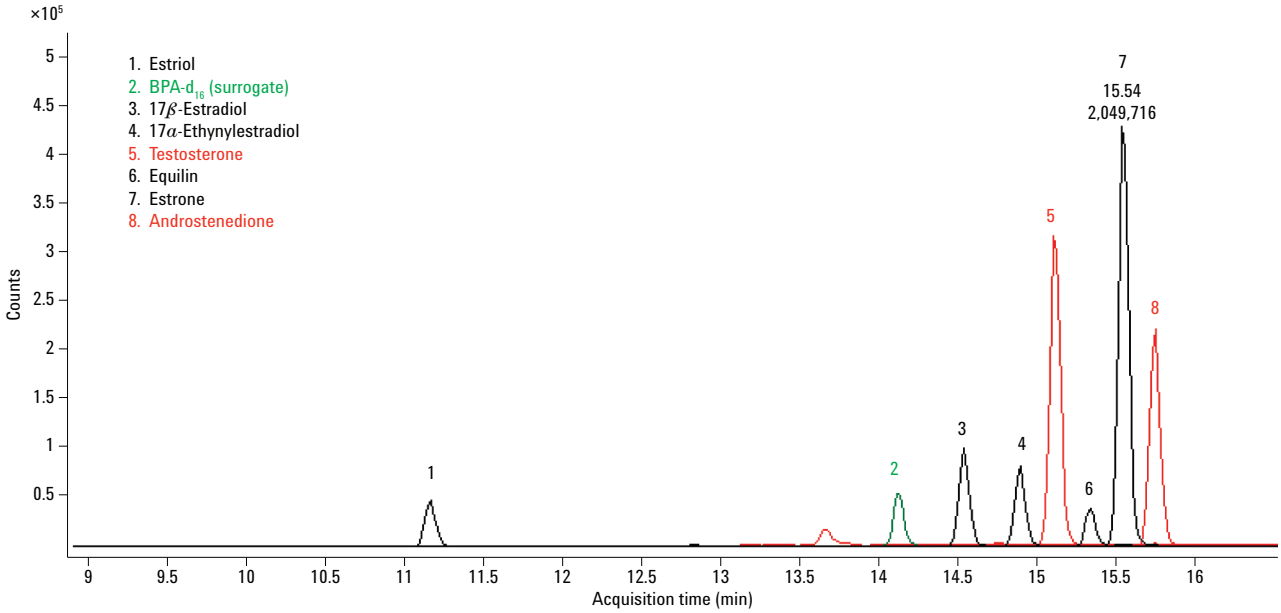


Figure 1. Chromatogram showing baseline separation of the seven target hormones for EPA Method 539, using the 1 mM NH_4F mobile phase A.

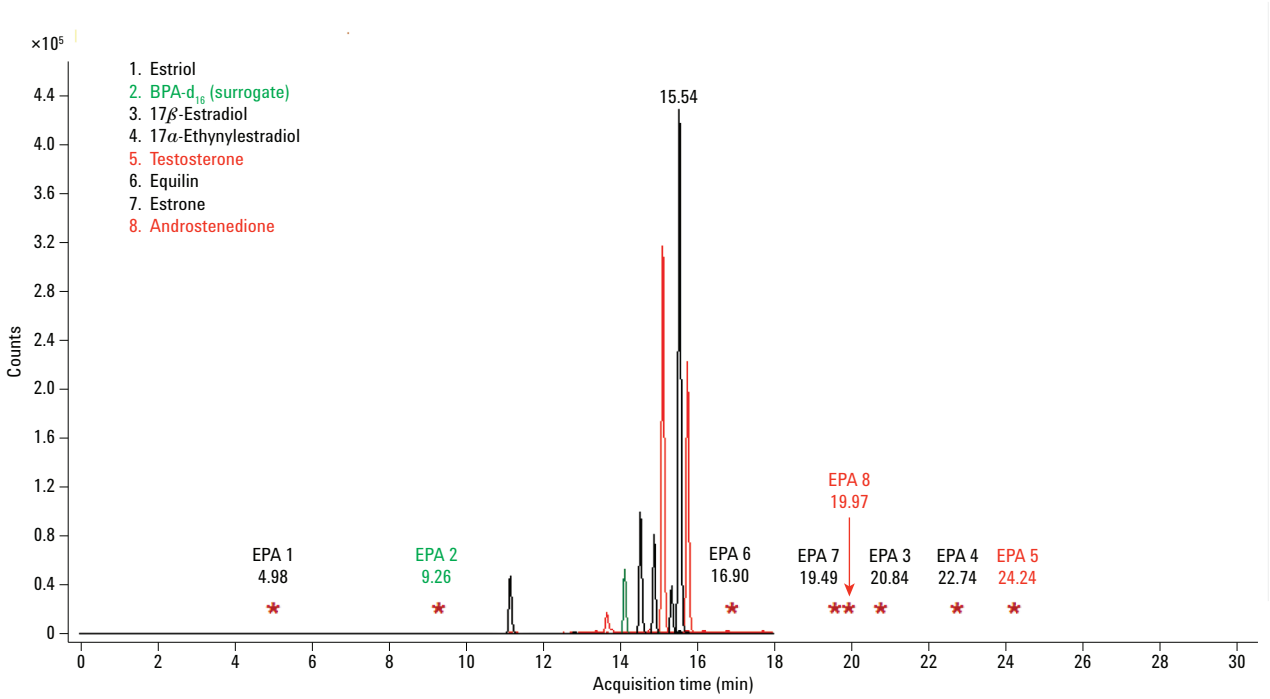
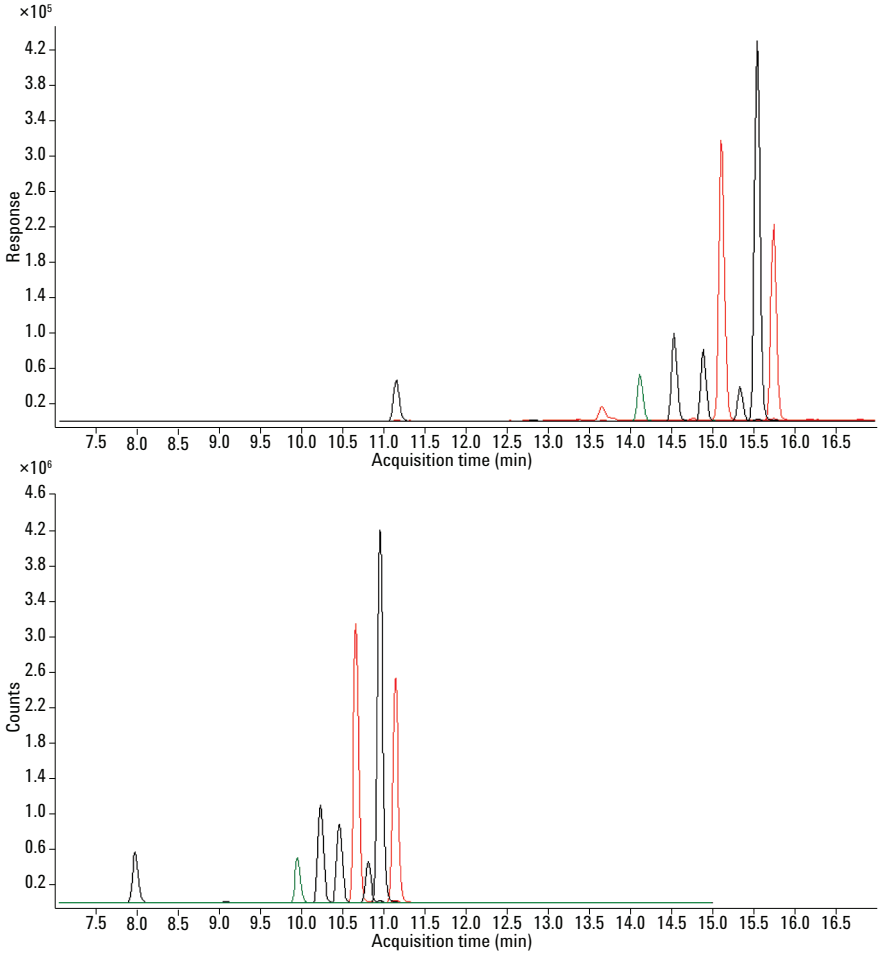


Figure 2. A comparison of the chromatographic separation provided by the NH_4F buffer system, versus the Method 539 NH_4OH system. Asterisks indicate the retention times from Method 539.

In contrast, the EPA chromatographic approach provides only partial resolution of the seven hormones in 25 minutes (Figure 2).

Changing the mobile phase gradient from 5% B/minute to 7.5% B/minute cut the separation time in half, without any appreciable loss of resolution (Figure 3). The end result is that the total run time of 15 minutes required for the modified method is less than one-third that required by the EPA Method 539 chromatographic approach, including column equilibration. This chromatographic configuration was used for all of the method validation studies.



Initial gradient conditions

1. Hold of 1.0 minute
2. Gradient = 5% B/min

Time	% B	Flow
0	10	0.3
1	10	0.3
19	100	0.3

Faster Gradient for Faster Separation

1. Reduce hold to 0.5 minutes
2. Increase gradient to 7.5% B/min

Time	% B	Flow
0	10	0.3
0.5	10	0.3
12.5	100	0.3

Figure 3. Reducing the time required for the chromatographic separation using the NH_4F buffer system by 50%, using a more rapid % B gradient. Black trace = negative ionization; green = negative-ion surrogate; red =positive ionization.

A further benefit of the mobile phase in this method is a significant increase in sensitivity. In negative mode, responses increased between 6.8 and 11.3 times, while in positive mode the increase was approximately doubled, with an increase of 1.7 times for androstenedione and 2.4 times for testosterone (Figure 4).

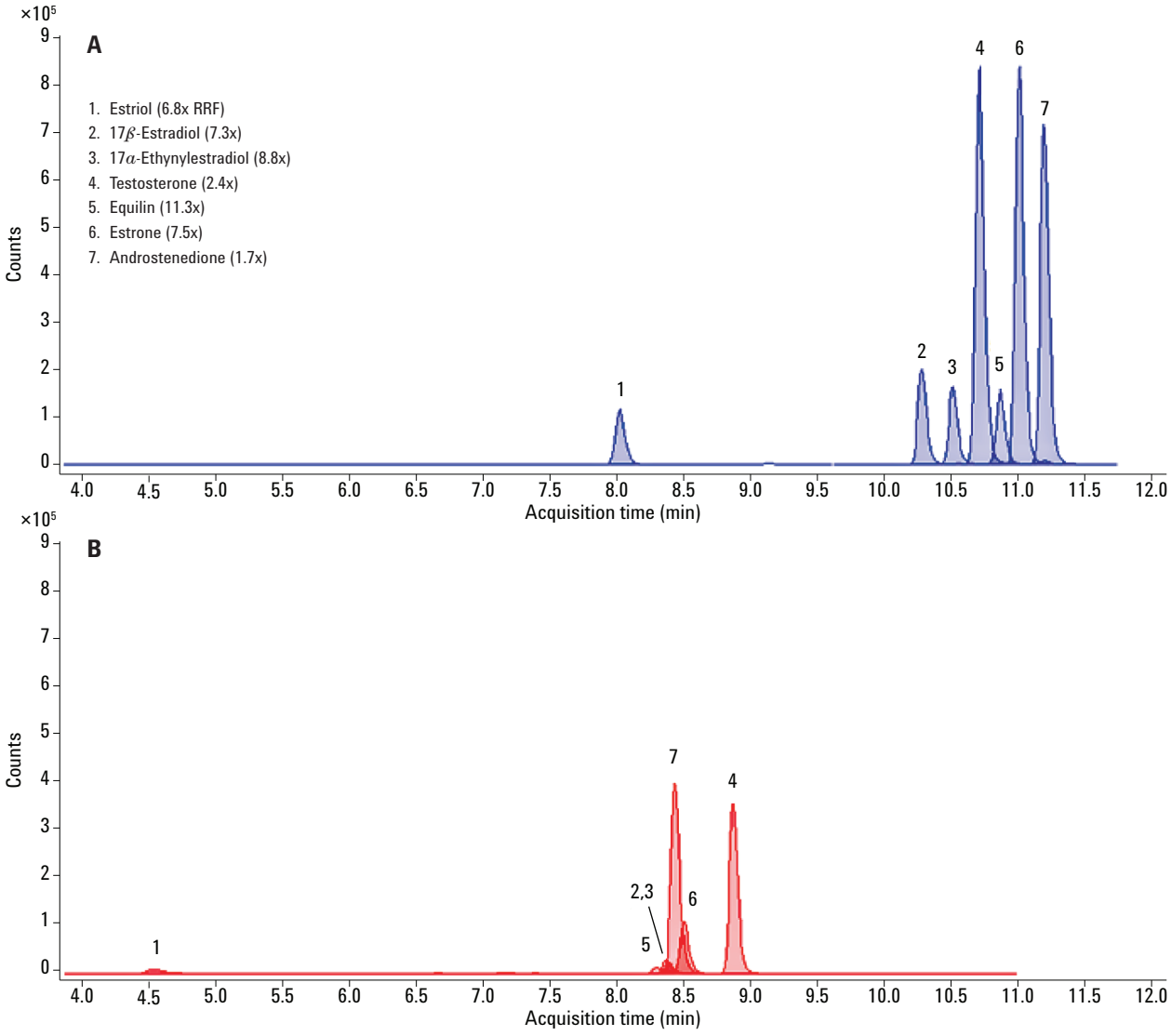


Figure 4. Response improvement using ammonium fluoride as the mobile phase modifier (A) instead of ammonium hydroxide (B).

Validating the Modified Method

To show that the Triple Quadrupole LC/MS System was suitable to perform this method, a set of seven laboratory fortified blanks (LFBs) was taken through EPA Method 539 as written, using the modified chromatographic approach and MS/MS analysis using pos-neg switching and dynamic MRM. During implementation of a method, laboratories may discover that some analytes show poor recoveries in all samples, while some samples may show poor recoveries for all analytes. Results presented in this batch show examples of both of these problems. The excellent reproducibility of the 6460 Triple Quadrupole LC/MS System allows determination of where the problems originate. Concentrations for the LFBs that were chosen were similar to those used in the EPA method as a starting point for this work.

As required by the Initial Demonstration of Capability (IDC) in EPA Method 539 (Section 9), calibration curves were first constructed for all hormones as shown in Table 4, to cover the concentration range that would be used in the laboratory fortified blanks (LFBs). Correlation coefficients (R^2) were ≥ 0.999, as shown for estrone (Figure 5). The precision and accuracy of the method were determined for all seven hormones, using seven LFBs (Table 5). One compound (androstenedione) demonstrated poor accuracy throughout the batch, and LFB 6 gave poor recoveries for all seven compounds.

Table 4. Calibrator Concentrations Used for the Seven Hormones (ng/mL)

Compound	Cal L1	Cal L2	Cal L3	Cal L4	Cal L5
Estriol	0.20	0.40	1.0	2.0	4.0
17β-Estradiol	0.25	0.50	1.25	2.5	5.0
17α-Ethynylestradiol	0.35	0.70	1.75	3.5	7.0
Equilin	0.1	0.20	0.50	1.0	2.0
Estrone	0.2	0.40	1.0	2.0	4.0
Testosterone	0.005	0.01	0.025	0.05	0.10
Androstenedione	0.005	0.01	0.025	0.05	0.10

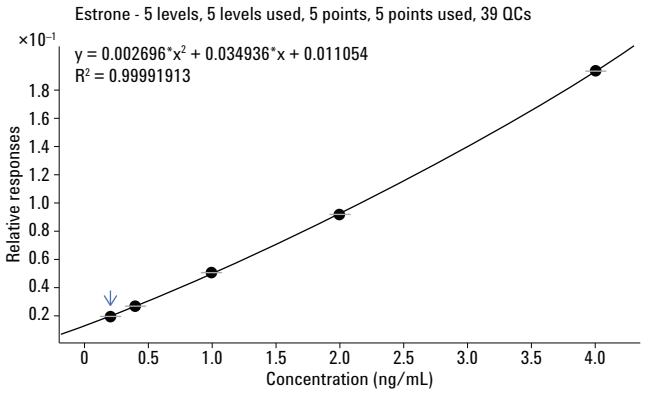


Figure 5. Calibration curve for estrone illustration an R^2 value of 1.000, quadratic fit.

Table 5. IDC Using Seven LFBs

	Estriol	17β-Estradiol	17α-Ethynylestradiol	Testosterone	Equilin	Estrone	Androstenedione	17α-Ethynylestradiol-d_4*
EPA Spike Level (ng/mL)	1.10	1.30	1.75	0.500	1.25	1.05	**0.500**	
IDC Spike Level (ng/mL)	1.00	1.00	2.00	0.050	1.00	1.00	**0.050**	70.0
LFB 1	0.99	0.95	1.81	0.051	1.25	1.13	**0.033**	74.5
LFB 2	0.92	0.90	1.79	0.047	1.18	0.99	**0.028**	70.6
LFB 3	1.04	0.96	1.95	0.055	1.28	1.16	**0.035**	81.0
LFB 4	0.90	0.81	1.79	0.043	1.11	0.97	**0.030**	57.0
LFB 5	0.87	0.89	1.76	0.046	1.19	0.97	**0.032**	58.5
LFB 6	**0.76**	**0.73**	**1.37**	**0.036**	**0.97**	**0.82**	**0.026**	**48.4**
LFB 7	0.90	0.82	1.84	0.045	1.23	0.96	**0.032**	69.1
Mean	0.91	0.87	1.76	0.046	1.17	1.00	**0.031**	65.6
Standard deviation	0.09	0.08	0.18	0.006	0.11	0.11	**0.003**	11.4
Avg % Rec (accuracy)	91.1%	86.7%	87.8%	92.0%	117.3%	100.1%	**62.1%**	93.7%
%RSD (precision)	9.8%	9.8%	10.4%	13.1%	9.0%	11.4%	**10.4%**	17.3%

*Surrogate

In method development, one is faced with determining whether the two errant results could be due to instrument errors, or analyst errors in sample handling (extraction and cleanup), or LFB and calibrator preparations. The true cause was addressed by assessing instrument repeatability, to indicate the source of greatest variability in the results.

Instrument Repeatability

The maximum standard deviation (S) allowed by EPA Method 539 is 12.6%, when the recovery is 100%. This follows from the equation for the Half Range for the Predicted Interval of Results (HR_{PIR}), as described in Section 9.2.4.1 of the EPA method.

$HR_{PIR} = 3.963 * S$

Since the HR_{PIR} is ± 50%, S must be no more than 50%/3.963 (that is, 12.6%), and S gets smaller as recoveries deviate from 100%. For example, if average recovery was only 80%, the maximum allowable standard deviation would then be (80%–50%)/3.963, equaling 7.6%. Therefore, it is crucial that the instrument delivers low RSDs in order to meet this strict requirement, and provides as much room as possible for deviations in the rest of the method.

Eight replicate injections were made from two calibrators and two fortified LFB extracts (including LFB 6 that showed low recoveries for all compounds). These replicates were intended to show whether the initial result was due to random injection error, or whether the initial result was representative of the sample as processed through the entire

%RSD Summary	Estriol	17β-Estradiol	17α-Ethynylestradiol	Testosterone	Equilin	Estrone	Androstenedione	17α-Ethynylestradiol-d_4
Cal L3	2.7%	1.8%	5.9%	1.5%	2.8%	3.5%	2.9%	0.9%
Cal L4	1.4%	2.0%	1.6%	1.5%	2.4%	1.2%	0.5%	1.2%
LFB 6	1.6%	3.0%	3.3%	2.3%	1.9%	1.7%	2.1%	0.9%
LFB 7	2.0%	6.2%	4.6%	4.6%	4.9%	4.6%	2.7%	0.7%

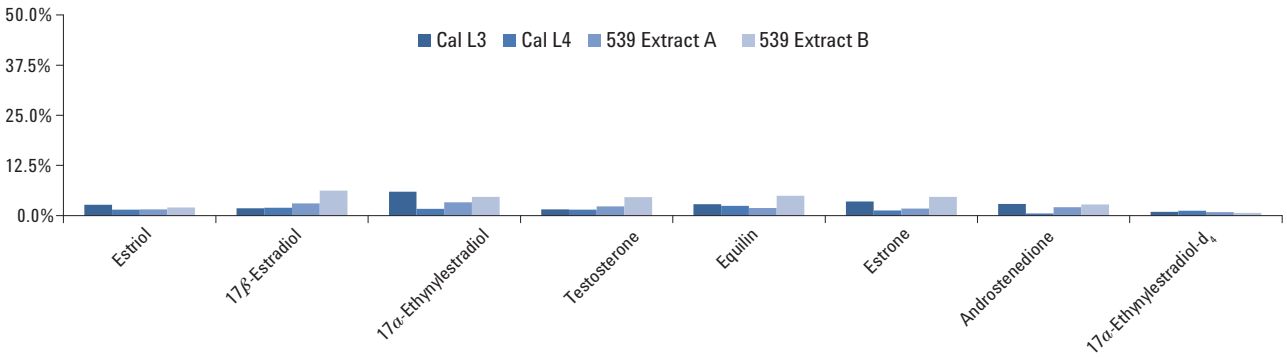


Figure 6. RSD summary for eight injections each of two calibrators and two LFBs, illustrating that the source of the error observed in the IDC was not due to instrument performance, which is well within Method 539 requirements.

method. The average %RSD obtained across the calibrator targets was 2.3%, and the average %RSD across the LFB targets, including LFB 6, was 3.3% (Figure 6).

These low %RSD values indicate that the error observed in the IDC was due to method materials or sample handling by the analyst, and not instrument performance. It can be argued that the low recoveries for LFB 6 were due to a spiking error, but the low recovery of the surrogate at 69.1% is in line with the average recovery of the targets (74.1%). Since the surrogate is spiked independently of the target compounds at the beginning of the method, a more likely explanation is that the low recovery is related to sample handling. Figure 7 illustrates just how tightly the replicate chromatograms fit with each other.

Androstenedione showed low recoveries for all fortified samples, with an average of 62.1%. This is an unexpected result, as testosterone recoveries averaged 92.0%. Subsequent androstenedione spikes of blanks that were taken through the entire method showed responses that matched standards at the same level, so it appears that there was a mismatch between the intended spiking level and the preparation of the standards. Instrument precision was still within the requirements of the EPA method, but poor apparent accuracy due to the incorrect spiking amount caused the accuracy parameter to fail.

Accuracy, Precision and Detection Limit (DL)

Given the proven instrument repeatability, the IDC results were analyzed to determine if they met the accuracy, precision, and DL requirements for Method 539. The method requires precision (%RSD) of ≤ 20% and accuracy of ± 30% of the true value. Table 6 shows a comparison of this dataset to that of the EPA method, and that the modified method meets the Method 539 requirements for precision and accuracy, in spite of the sample handling errors with LFB 6. Androstenedione failed due to a spiking error. Accuracy ranged from 86.7 to 117.3%, while precision ranged from 9.0 to 13.1%. Detection limits for estrone

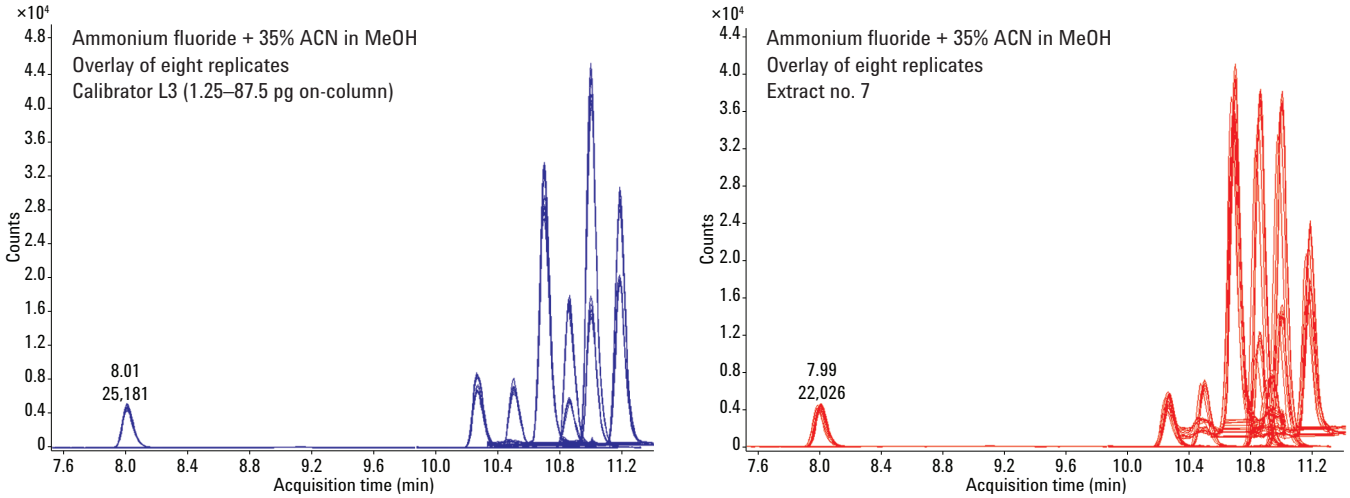


Figure 7. Overlay of extracted ion chromatograms (EICs) for eight replicates each of calibrator L3 and LFB7, illustrating the excellent instrument repeatability. Both target and qualifier MRMs are shown.

Table 6. Accuracy, Precision, and Detection Limit (DL)

Compound	IDC Accuracy[a] (%)	EPA Accuracy (%)	IDC Precision[a] (%)	EPA Precision (%)	IDC DL[a] (ng/L)	EPA DL (ng/L)
Estriol	91.1	83.5	9.8	6.3	0.21	0.24
17β-Estradiol	86.7	90.6	9.8	6.0	0.22	0.39
17α-Ethynylestradiol	87.8	90.1	10.4	3.1	0.23	0.33
Testosterone	92.0	93.5	13.1	3.3	0.014	0.040
Equilin	117.3	80.4	9.0	5.1	0.20	2.94
Estrone	100.1	86.5	11.4	1.2	0.29	0.19
Androstenedione	62.1	87.2	10.4	18.0	0.008	0.200

[a] n = 6 (LFB-6 removed); t-stat = 3.365 at 99% confidence level

were slightly higher than EPA's DLs for this batch, at 0.29 ng/L compared to 0.19 ng/L in the method. DLs for the remaining compounds were lower than those achieved by EPA, and up to 15 times lower in the case of equilin.

Because the spiking levels used for this batch were comparable to those used by the EPA method, it is not surprising that the calculated DLs were also comparable. However, lower DLs could conceivably be achieved by repeating the work at lower fortification levels. The responses of all compounds were significantly higher using mobile phase with ammonium fluoride as the modifier instead of ammonium hydroxide, offering the possibility of either lowering DLs or reducing the volume of water to be extracted.

Minimum Reporting Level (MRL) Confirmation

Method 539 requires MRL confirmation by fortifying, extracting, and analyzing seven replicate LFBs at or below the proposed MRL concentration. The mean and standard deviation are calculated for these replicates, and the HR_{PIR} is determined by using the equation 3.963 (for seven replicates) times the standard deviation. The Upper and Lower limits for the Prediction Interval of Results (PIR = Mean ± HR_{PIR}) must meet the requirements shown below:

Upper PIR Limit (Mean + HR_{PIR}) ≤150% Recovery

Lower PIR Limit (Mean – HR_{PIR}) ≥ 50% Recovery

In this case, only six replicates were used (LFB-6 removed), so the value to determine the HR_{PIR} became 4.336 rather than 3.963. Table 7 gives the results of the MRL confirmation, which shows that all of the analytes passed the PIR test except for androstenedione. This indicates that the MRL was set too low for androstenedione. However, this result could also be due to analyst error involving an incorrect calculation of the spike amount relative to the standard, or incorrect preparation of the calibrators, since this is the only compound with a low recovery (62.1% across seven LFBs). Good instrument precision indicates that the problem of low accuracy is related to sample or calibrator preparation.

Table 7. MRL Confirmation Results

Compound	Lower PIR limit (%)	Upper PIR limit (%)	Confirmation result
Estriol	66.1	121.3	Pass
17β-Estradiol	60.6	117.4	Pass
17α-Ethynylestradiol	76.2	106.0	Pass
Testosterone	58.5	132.4	Pass
Equilin	94.6	146.8	Pass
Estrone	65.2	141.0	Pass
Androstenedione	42.2	85.4	Fail

Conclusion

This modification to Method 539, using a different mobile phase and the Agilent 6460 Triple Quadrupole LC/MS System, provides detection limits that meet or, in some cases, greatly exceed EPA levels. Complete chromatographic resolution of target hormones can be achieved in less than 16 minutes, due to the use of the unique mobile phase A and Agilent Poroshell column technology, which also reduces hardware requirements. Instrument precision ranged from 0.5–6.2% RSD in calibrators and sample extracts. Therefore, uncertainty in accuracy and precision as measures of the complete method performance are more dependent upon the abilities of the analyst, and not the instrumentation.

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