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Liquid Chromatography Mass Spectrometry

Direct Determination of Trace Hormones in Drinking Water by Large Volume Injection using the LCMS-8050 Triple Quadrupole Mass Spectrometer

David R. Baker, Neil Loftus
Shimadzu Corporation, Manchester, UK

■ Abstract

Endocrine disrupting compounds enter the aquatic environment primarily through the discharge of treated and raw sewage and are detrimental to aquatic organisms even at sub-nanogram per litre levels. In the majority of North American and European cities wastewater treatment plant effluent is indirectly re-used, through discharge into rivers which are also a source of drinking water. Consequently, there is the possibility that trace amounts may enter into drinking water even after special treatment processes. Several hormones (estrone, estriol, 17-β-estradiol, equilin, androstenedione, testosterone and 17-α-ethynylestradiol) are routinely monitored by the US EPA in drinking water as part of the Unregulated Contaminant Monitoring program (UCMR3). In this study, the LCMS-8050 triple quadrupole mass spectrometer was used for the highly selective and sensitive detection of hormones in water to meet the requirements of UCMR3. This direct high volume injection method of analysis avoids the disadvantages associated with extracting samples using SPE as is commonly performed. Ammonium fluoride as an aqueous mobile phase additive was found to significantly improve response for all studied hormones in comparison to ammonium hydroxide. The excellent sensitivity of the final method provided detection limits ranging from 0.005 ng/L (testosterone) to 0.330 ng/L (17-α-ethynylestradiol).

Keywords: Hormones, Steroids, LCMS-8050, Drinking Water, UCMR3, Estrone, Estriol, 17-β-estradiol, Equilin, Androstenedione, Testosterone, 17-α-Ethynylestradiol, Ammonium Fluoride

■ Introduction

There is growing concern over the exposure of fish, wildlife and humans to the aquatic environment contaminated with trace levels of hormones due to their endocrine disruption potential.[1,2] Endocrine disrupting compounds may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological, and immune effects.[3] These compounds include naturally occurring steroid hormones such as estrone (E1), 17-β-estradiol (E2), and estriol (E3) and synthetically prepared ones such as 17-α-ethynylestradiol (EE2).

There are a variety of ways hormones enter the aquatic environment. Primarily this is due to the discharge of treated and untreated sewage water into receiving waters.[4] During wastewater treatment these hormones are susceptible to removal by biodegradation or sorption to sewage sludge, where secondary treatment can reduce concentrations consistently by over 85%,[5] however nanogram per litre concentrations of individual compounds may still be present in effluents.[4] Further routes include runoff into receiving waters from cattle given certain growth promoters, and from sludge and manure applied to agricultural fields.

In the majority of North American and European cities wastewater treatment plant effluent is indirectly re-used, through discharge into rivers which are also a source of drinking water.[6] Consequently, there is the possibility that trace amounts may penetrate into drinking water even after special treatment processes.[2] Several hormones are routinely monitored by the US EPA in drinking water as part of the Unregulated Contaminant Monitoring program (UCMR3).[7] These hormones include estrone, estriol, 17-β-estradiol, equilin, androstenedione, testosterone and 17-α-ethynylestradiol. The European Union has identified a list of priority substances, which includes estradiol and 17-α-ethynylestradiol (Directive 2013/39/EU amending Directives 2000/60/EC and 2008/105/EC).[8] Both regulations require highly sensitive and selective methods with ng/L or pg/L reporting levels. Previously published methods typically use solid phase extraction as a concentration step to achieve the regulatory reporting limits[2]. However, this approach adds an additional expense and complexity.

This technical report describes an optimized approach to the direct analysis of hormones in water regulated by EPA Method 539 and UCMR3. The integration of a high volume injection cycle with a highly robust and sensitive MS/MS detection system has resulted in an effective solution for routine hormone analysis in drinking water without the need for extensive sample preparation using conventional SPE methods.

■ Experimental

Table 1. Acquisition parameters for the analysis of steroids in drinking water using a large volume injection mode.

Liquid chromatography			Mass spectrometry	
UPLC	Nexera LC system		**LC/MS/MS**	LCMS-8050
Analytical column	Shim-pack XR-ODS III column (150 x 2 mm, 2.2 µm particle size).		**Ionisation mode**	Heated electrospray
Column temperature	45°C		**Polarity switching time**	5 ms
Column fitted between the mixer and autosampler	Kinetex XB-C18 column (50 x 2.1, 1.7 µm particle size)		**Pause time**	1 ms
Injection cycle	3 x 400 µL injections (500 µL loop fitted) Total injection volume 1200 µL		**Dwell times**	10-100ms
Flow rate	0.3mL/minute		**Interface temperature**	400°C
Solvent A	0.15mM ammonium fluoride		**Heating block**	400°C
Solvent B	Methanol		**Desolvation line**	200°C
Binary Gradient	**Time (mins)**	**%B**	**Heating gas**	10 L/min
	0	10	**Drying gas**	5 L/min
	0.3	10	**Nebulising gas**	2.8 L/min
	1	45		
	15	100		
	17	100		
	17.1	10		
	22	Stop		
Needle wash	500 µL Methanol / acetonitrile / 2-propanol / Water (1:1:1:1) 0.1 % formic acid			

Table 2. MRM transitions for the target compounds

Compound	Formula	CAS	Retention Time	Polarity	SRM Transitions	Q1 (V)	CE	Q3 (V)	MS1 Res.	MS2 Res.
Estriol	$C_{18}H_{24}O_3$	50-27-1	8.9	Negative	287 > 171	11	36	17	Unit	Unit
					287 > 145	11	42	27	Unit	Unit
Equilin	$C_{18}H_{20}O_2$	474-86-2	12.0	Negative	267 > 143	19	34	27	Unit	Unit
					267 > 223	19	33	24	Unit	Unit
17-α-Ethynylestradiol	$C_{20}H_{24}O_2$	57-63-6	12.1	Negative	295 > 145	11	44	26	Unit	Unit
					295 > 143	11	54	29	Unit	Unit
17-β-Estradiol	$C_{18}H_{24}O_2$	50-28-2	12.1	Negative	271 > 145	10	40	27	Unit	Unit
					271 > 183	10	40	19	Unit	Unit
Estrone	$C_{18}H_{22}O_2$	53-16-7	12.2	Negative	269 > 154	20	38	29	Unit	Unit
					269 > 143	20	55	27	Unit	Unit
Androstenedione	$C_{19}H_{26}O_2$	63-05-8	12.2	Positive	287 > 97	-14	-23	-18	Unit	Unit
					287 > 109	-14	-24	-11	Unit	Unit
Testosterone	$C_{19}H_{28}O_2$	58-22-0	12.7	Positive	289 > 97	-30	-22	-18	Unit	Unit
					289 > 109	-30	-24	-21	Unit	Unit

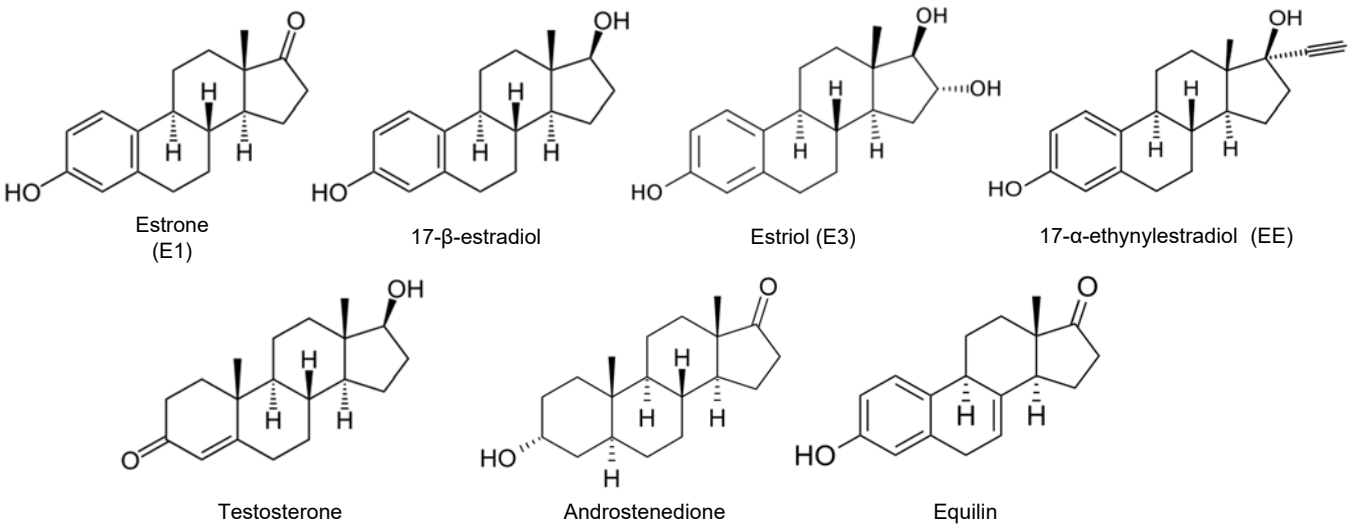

Figure1. Endocrine disruptor structures.

■ Results and Discussion

Method development

Previously published methods for the analysis of endocrine disruptors have used ammonium hydroxide as the mobile modifier and it is the currently recommended approach in EPA method 539.[9]

In this study ammonium fluoride was tested at different concentrations (0.1, 0.2, 0.3 and 0.5mM) in the aqueous phase, with methanol used as the organic phase. Improved response was observed for all hormones using ammonium fluoride, in comparison to ammonium hydroxide, as is shown in Figure 2. The optimum concentration was determined to be 0.15mM which is consistent with the results of others.[10] Ammonium fluoride (approx. pH 6) offers further benefits in comparison to ammonium hydroxide (approx. pH 9.5) as the lower pH means that analytical columns, other than those stable at high pH, can be employed.

Methanol was used as the organic solvent although acetonitrile resulted in a marginal improvement in signal to noise for compounds responding in negative ion but this advantage was countered by a marked reduction in the positive ion response.

As the panel of target compounds resulted in an optimal response in both positive and negative ion detection, a rapid polarity switching method was used in routine analysis without compromising data quality or response (Figure 3).

Key points in enhancing EPA method 539

0.15mM ammonium fluoride generated higher sensitivity compared to ammonium hydroxide. Heated electrospray further enhanced sensitivity and a 5 ms polarity switching optimized the hormone panel detection.

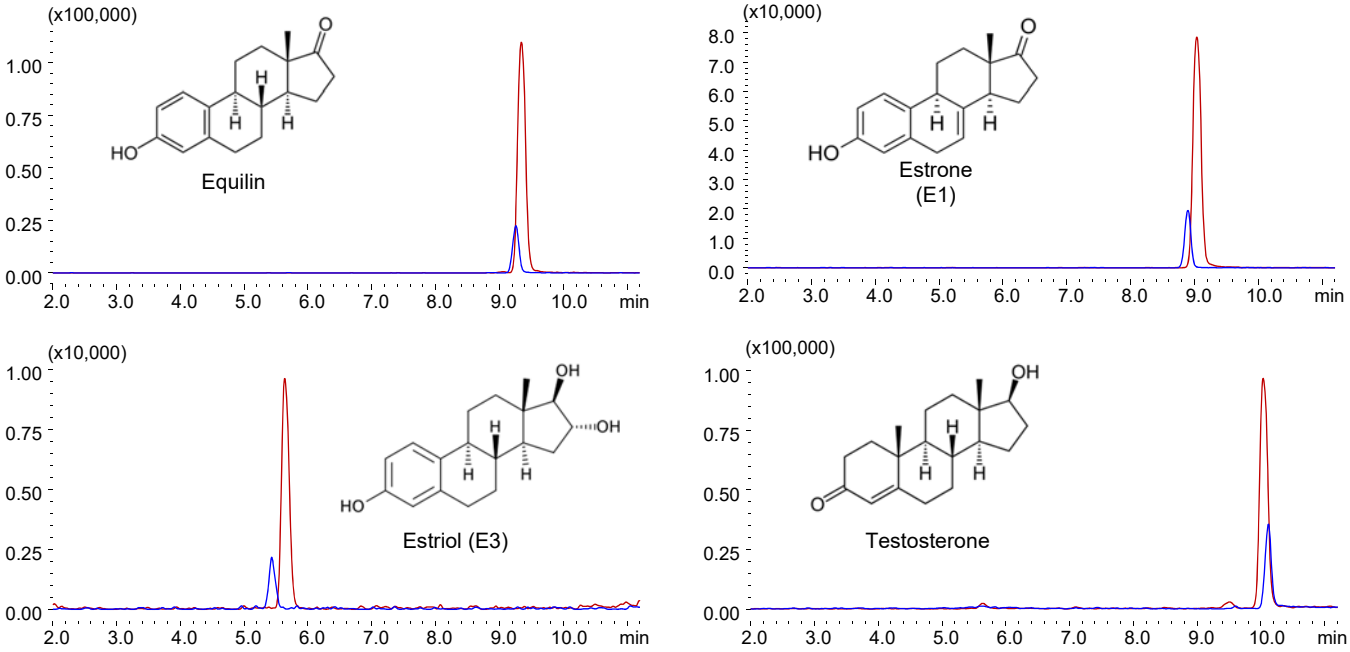

Figure 2. A comparison between the response generated using ammonium hydroxide (blue trace) and ammonium fluoride (red trace). Ammonium fluoride delivers an increase signal to noise for all compounds (for example, equilin x4.0, estrone x4.8, estriol x4.5 and testosterone x2.8).

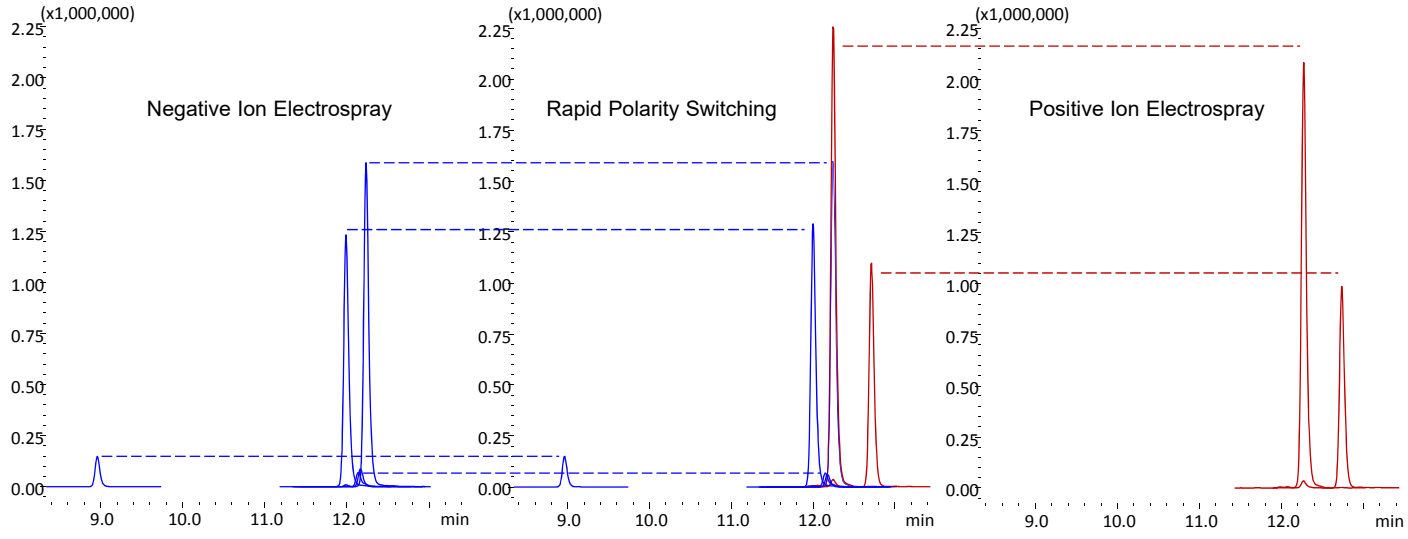

Figure 3. Rapid positive/negative switching using a 5ms switching time results in the highest data quality for all target hormone compounds in a single analysis.

Table 3. Concentration of each compound in the calibration series in drinking water.

Compound	Level 1 (ng/L)	Level 2 (ng/L)	Level 3 (ng/L)	Level 4 (ng/L)	Level 5 (ng/L)	Level 6 (ng/L)	Level 7 (ng/L)	Level 8 (ng/L)
Equilin	2	4	8	20	40	80	200	400
Estrone	1	2	4	10	20	40	100	200
17-α-Ethynylestradiol	0.45	0.9	1.8	4.5	9	18	45	90
Estriol	0.4	0.8	1.6	4	8	16	40	80
17-β-Estradiol	0.2	0.4	0.8	2	4	8	20	40
Androstenedione	0.15	0.3	0.6	1.5	3	6	15	30
Testosterone	0.05	0.1	0.2	0.5	1	2	5	10

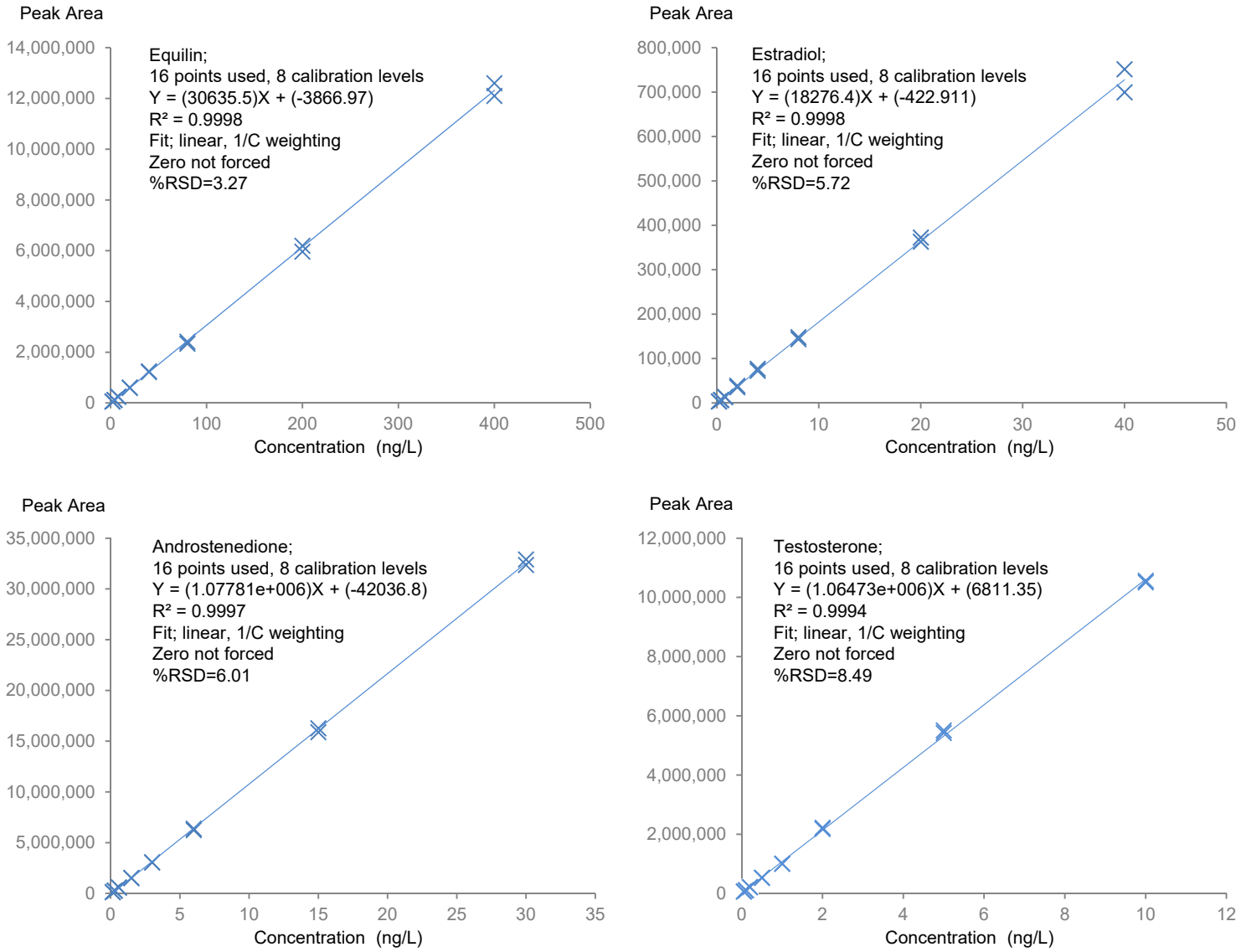

Figure 4. Calibration curves for equilin (2-400ng/L), estradiol (0.2-40ng/L), androstenedione (0.15-30ng/L) and testosterone (0.05-10ng/L) spiked into drinking water.

Linearity was investigated over an eight point calibration curve in drinking water, analysed in duplicate, covering two and a half orders of magnitude. Concentrations of each compound at each level are listed above in Table 3. Peak area repeatability (n=7) was assessed at low (level 2) and high (level 5) concentrations. The robustness study was performed using drinking water spiked at level 5.

Hormone limits of detection were calculated based on the method described by the the EPA Method 539,[9] using a standard deviation of 7 replicates at a concentration value that corresponds to an instrument signal to noise ratio in the range of 2.5 to 5 and a Student's t 99% confidence interval.

$$DL = t\,(n - 1,\ 1 - \alpha = 0.99) \times s.d.$$

Parameter	Description
DL	Detection Limit
t(n-1,1-α=0.99)	Student's t value for the 99% confidence level with n-1 degrees of freedom (t = 3.14 for 7 replicates),
n	number of replicates
s.d	standard deviation of the replicate analyses

■ Method validation

Quantitative Method Validation

In order to test the performance of the developed method, limits of detection, linearity, repeatability (low and high concentrations), and longer term robustness were assessed.

Linearity was assessed from 0.5 x the required reporting level to 100 x times the reporting level. The concentration for each compound in spiked drinking water is listed in Table 3.

Table 4. Detection Limit (DL) is defined as the minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero.

Compound	DL (ng/L)	DL (fg on column)
17-α-Ethynylestradiol	0.330	396
Equilin	0.073	88
17-β-Estradiol	0.052	62
Estriol	0.035	42
Estrone	0.031	38
Androstenedione	0.012	14
Testosterone	0.005	6

All seven hormones achieved excellent correlation coefficients R^2>0.999 using a weighted (1/C) least squares regression analysis. Calibration curves for equilin, estradiol, androstenedione and testosterone are shown in Figure 4. Hormone limits of detection were calculated based on the method described by the EPA Method 539 and are listed in Table 4. Using the developed method on the LCMS-8050 detection limits ranged from 0.0058 ng/L for testosterone to 0.33 ng/L for 17-α-ethynylestradiol. Figure 5 shows the chromatograms at the lowest calibration standard (level 1).

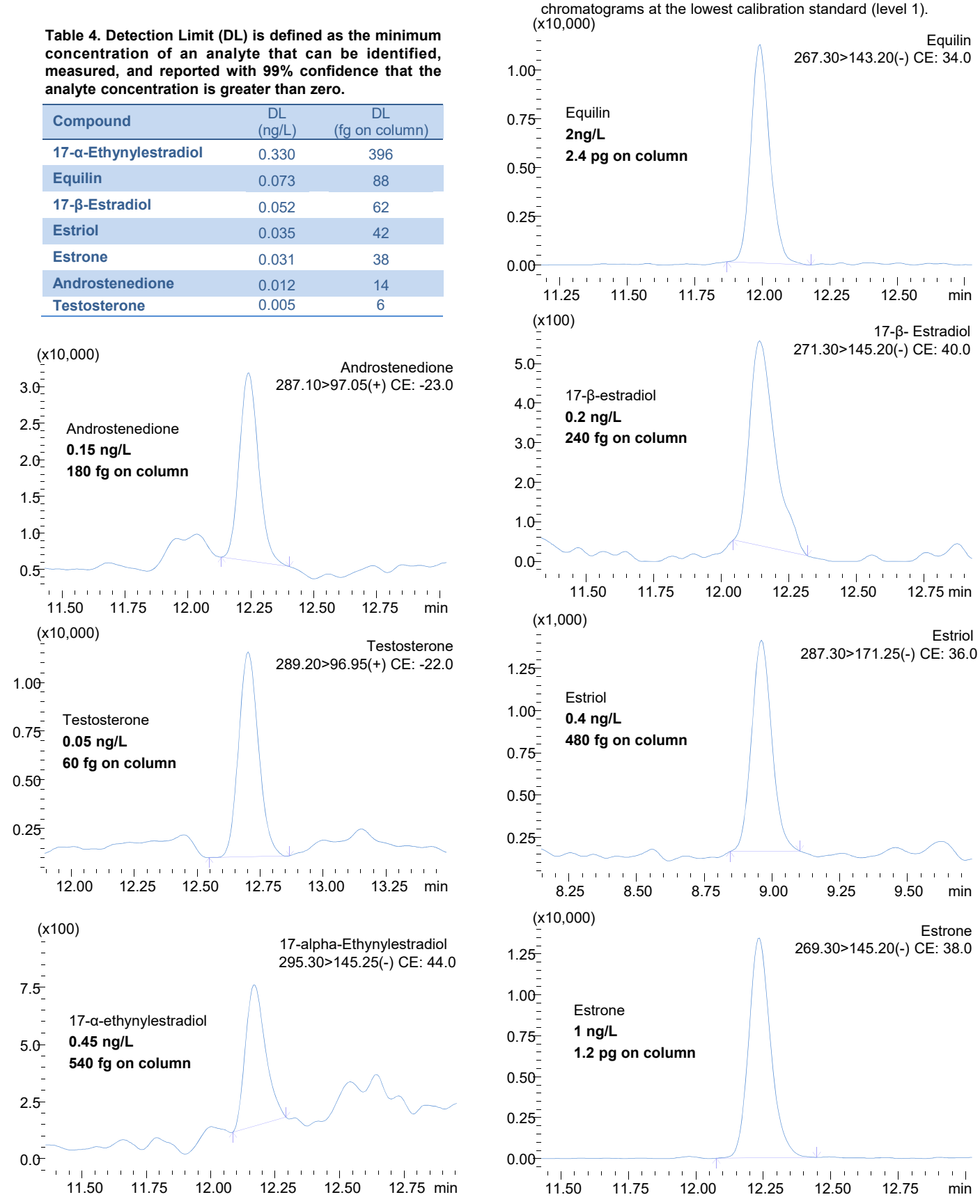

Figure 5. MRM chromatograms of target hormones at the lowest calibration standard (level 1) using an injection volume of 1200uL.

■ Reproducibility

Peak area reproducibility (n=8) was assessed at the reporting level corresponding to 'low concentration' (level 2) and a 'high concentration' (level 5). At the low concentration repeatability was < 4.3 %RSD, with the exception of 17-α-ethynylestradiol (12.2 %RSD). At the high concentration repeatability was < 3.9 %RSD for all compounds. Table 5 lists the repeatability results.

To assess the robustness of system, repeat injections were performed over a 62 hour period using drinking water spiked at level 5.

Results for the three compounds with the lowest peak area are displayed in Figure 6. These results show that even over a much extended time period deviation of less than 5 %RSD was achieved for the three compounds.

Table 5. Peak area repeatability (n=7) at low and high concentrations

Compound	Low (level 2) %RSD	High (level 5) %RSD
17-α-Ethynylestradiol	12.2	2.4
Androstenedione	2.2	2.9
17-β-Estradiol	4.3	3.9
Equilin	3.5	2.7
Estriol	3.4	1.5
Estrone	4.2	1.7
Testosterone	2.8	3.5

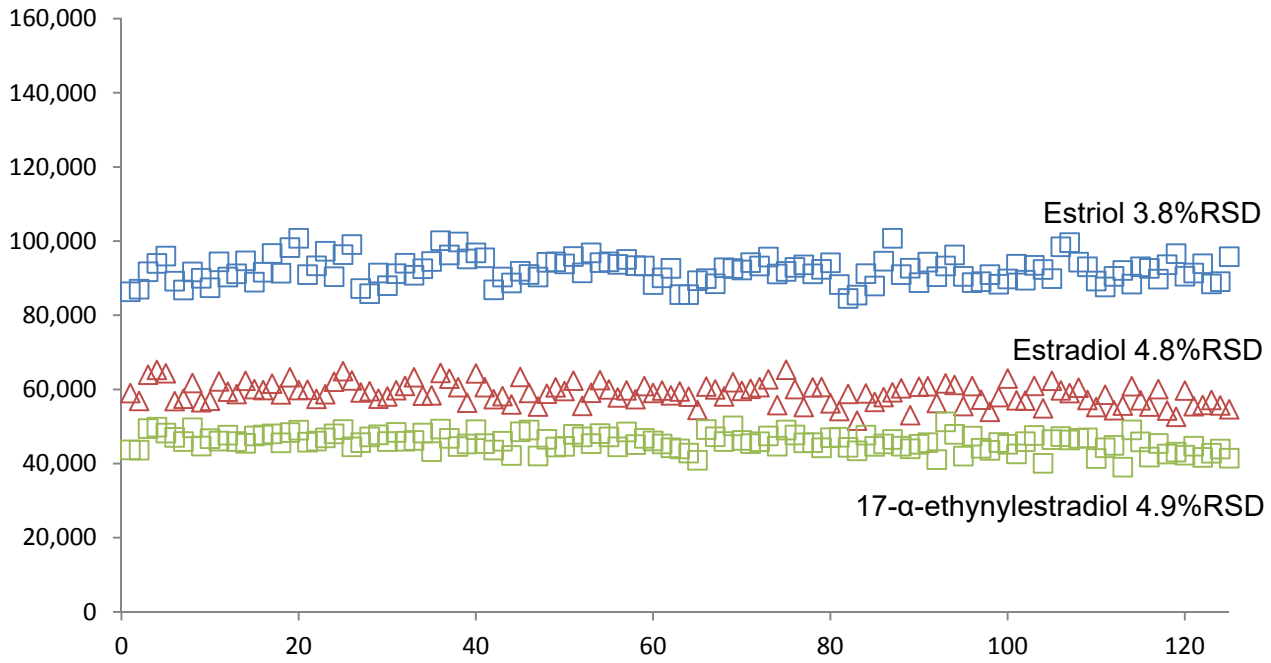

Figure 6. Peak area response for three hormones over 62 hours. The legend displays the %RSD for each compound.

■ Conclusion

A fast, selective and highly sensitive method has been developed for the measurement of hormones in drinking water. By integrating a direct high volume injection cycle with a fully optimised LC/MS/MS method, the LCMS-8050 delivers precise and accurate detection limits regulated by EPA method 539 and is in accordance with UCMR3.

The LCMS-8050 triple quadrupole mass spectrometer method delivered high sensitivity with detection limits ranging from 0.005 ng/L (testosterone) to 0.330 ng/L (17-α-ethynylestradiol). Correlation coefficients for all compounds were greater than 0.999 and peak area repeatability was determined to be typically less than 5%RSD at 'low' (corresponding to the reporting level) and 'high' concentrations.

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