APPLICATION NOTE

Liquid Chromatography/ Mass Spectrometry

Authors:

Jingcun Wu

Feng Qin

PerkinElmer, Inc.
Woodbridge, Ontario, Canada

High Sensitivity Analysis of Estrogens in Water and Urine Without Derivatization by Direct Injection UHPLC/MS/MS

Introduction

Estrogens are important hormones that are associated with the female reproductive system, and for maintenance of female secondary sexual characteristics.[1] However, numerous studies have found that the concentrations of endogenous estrogens and their metabolites in urine are related to the development and growth of breast cancer and tumors. Further, an increased risk of breast cancer has been reported in women with high urinary estrogen levels, as well as in those exposed to increased estrogens over time as a result of hormone replacement therapy.[2-17]

Studies have shown that the risk factors for breast cancer development in women may be linked to their diets, and that women who eat high fat diets have higher urinary estrogen levels and a higher incidence of cancer, when compared to women with low fat diets.[7-9] Soy consumption can decrease urinary excretion of estrogens, and may have cancer-preventive effects by decreasing estrogen synthesis and altering endogenous estrogen metabolism.[10-11]

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Although various analytical methods have been developed for the determination of estrogens and their metabolites in urine, the most widely applied technique is LC/MS/MS, owing to its high sensitivity and selectivity. To improve analyte ionization efficiency and enhance the method's sensitivity, derivatization of analytes during sample preparation has been used in many LC/MS/MS methods.[4, 12-13] However, derivatization not only increases the sample preparation time and cost, but also introduces variations in the method, which are not suitable for routine clinical laboratories. Thus, it is imperative to develop a rapid and sensitive LC/MS/MS method for analysis of estrogens in urine without derivatization.

Endocrine disrupting compounds (EDCs) are chemicals that can interfere directly with the endocrine system, either by interaction with hormone receptors or by alteration of hormone synthesis or metabolism, and cause adverse effects on the growth and reproduction of animals. Of these EDCs, endogenous estrogens, such as estrone (E1), 17β-estradiol (E2), and estriol (E3) are of great concern, as they can enter the environment through the discharge of treated and raw sewage, and can induce adverse health effects to wildlife organisms, even at sub-nanogram to nanogram per liter levels. Recent evidence has shown that estrogens at low ng/L levels are the main cause for the feminization of male fish in aquatic environments impacted by sewage.[18-20]

Naturally occurring estrogens (E1, E2 and E3) are regularly and consistently released into the environment by the urine and excrement of humans and animals. They have been detected in surface and waste waters at ng/L concentration levels, and trace amounts may be found in drinking water, even after extensive removal treatments. Owing to the very low detection limits required and the complexity of sample matrix, complicated and time-consuming extraction and purification processes, such as liquid-liquid extraction, solid phase extraction (SPE) and on-line SPE are typically performed before final determination by LC/MS/MS, or other methods.[21-24] US EPA Method 539 describes a process for the determination of hormones in drinking water utilizing conventional SPE procedures.[24]

The aim of this study is to develop a fast and sensitive LC/MS/MS method for the analysis of estrogens in urine and water samples, without derivatization and SPE.

Experimental

Hardware/Software

The chromatographic separation was conducted by a PerkinElmer QSight® LX50 ultra-high-performance liquid chromatography (UHPLC) system. MS detection was achieved utilizing a PerkinElmer QSight 420 triple quadrupole mass spectrometer, with electrospray ionization (ESI) source under negative ionization conditions. All instrument control, data acquisition and data processing were performed using the Simplicity™ 3Q Software.

Method

Standard and Chemicals

The following standards were obtained from Sigma-Aldrich: estrone (E1), 17β-estradiol (E2), and estriol (E3); and ammonium fluoride (purity > 99.9%). LC/MS grade methanol (MeOH), acetonitrile (ACN) and water were obtained from MilliporeSigma. The chemical structures of the three estrogens were illustrated in a previous application note.[25]

A variety of drinking water and surface water samples were analyzed in this study: bottled drinking water purchased from a local store (Woodbridge, Ontario), tap water obtained from two different cities in Ontario (Toronto and Kitchener), rain water collected from Kitchener, Ontario, river water samples from the Humber River, Toronto, Canada, and lake water samples from Lake Ontario, Canada. Human urine samples were collected and donated by two healthy volunteers (one female, and one male).

Standard Preparation

Individual primary standard stock solutions (1 mg/mL) were prepared by weighing accurately 10 mg of each standard, and subsequently dissolving each in 10 mL of methanol. A mixed standard stock solution containing 10 µg/mL of each analyte was prepared in methanol by dilution of the primary standard solutions. Several working solutions with various low concentrations (10, 100, 1000, and 10,000 ng/L) were prepared by diluting the mixed stock solution with a 50:50 methanol/water diluent. Calibration standards with concentrations of 0, 1, 2.5, 5, 10, 50, 100, 300, 500, 1000 and 2000 ng/L were prepared by diluting the corresponding working solutions with a 30:70 methanol/water diluent.

Sample Preparation

The various water samples were prepared by extraction with methanol (with water/methanol ratio of 7:3) and vertex mixing for one minute, with analysis performed directly thereafter, without further treatment. The two urine samples were first extracted by methanol (urine/methanol ratio of 7:3), and then chilled in a refrigerator for 30 minutes to promote protein precipitation. The supernatants were then analyzed directly, without further treatment.

Quality Control Sample Preparation

Avoiding contamination of the samples during sample collection, sample preparation and sample analysis are all crucial for reliable sample analysis. To test for possible interference or contamination from reagents and glassware, or from the sample preparation processes itself, a laboratory reagent blank (LRB) was prepared. The values of LRB should be close to zero, or at least less than the limit of quantification (LOQ) of the method. If the LRB value is above the LOQ, an investigation into the source of contamination must be carried out. An LRB sample was prepared for this study by following the same procedures as for a water sample preparation, using the 30:70 methanol/LC/MS grade water as the sample matrix.

To identify possible analyte loss or contamination during sample preparation, a laboratory fortified blank (LFB) sample was prepared by following the same water sample preparation procedures, using a 30:70 methanol/water solution as the sample matrix, spiked with a known amount of analyte solution. During method validation, LFB samples were prepared by spiking the analyte at three different concentration levels (5, 10, and 100 ng/L, respectively).

To evaluate sample matrix effects and analyte recovery from the lake water sample matrix, a laboratory fortified matrix sample

(LFM) was prepared by following the same water sample preparation procedures, using a lake water sample spiked with a known amount of analyte. The percent recovery is calculated by comparing the difference of the spiked (LFM sample) and non-spiked sample results with the expected (spiked) value. During method validation, the LFM samples were prepared at three different analyte concentrations (5, 10, and 100 ng/L, respectively).

LC Conditions and MS Parameters

The LC method and MS source parameters are shown in Table 1. A C18 column (Bownlee, SPP C18, 50 x 3 mm, 2.7 µm) was used to separate estrogens and any interfering components. The applied LC gradient program is shown in Table 2. MS source parameters including gas flows, temperature and probe position settings, were optimized for maximum sensitivity. Compound-dependent parameters such as collision energies (CE), entrance voltages (EV), and lens voltages (CCL2) were optimized, and are shown in Table 3.

Results and Discussion

Method Development and Optimization

For mass detection of estrogens, both positive and negative electrospray ionization (ESI) modes were evaluated initially. The results showed that all analytes in the study demonstrated better sensitivity and signal to noise ratio under negative ionization mode. Similar results were also obtained in previous studies.[26-27] Therefore, negative ESI detection was used in this study.

According to the regulatory guidance on analytical method validation, at least two MS/MS transitions should be used in a method.[28] In this study, three MS/MS transitions were evaluated. Tables 1 and 3 present the optimized parameters for the ion source and compounds.

To improve analyte ionization efficiency and to enhance the method's sensitivity, other studies have utilized ammonium hydroxide with concentrations from 0.01% to 0.1% in mobile phases.[26-27] However, an LC column that can work robustly at the high pH range is required. In other recent studies, ammonium fluoride has been increasingly used as a mobile phase additive to enhance ionization of some analytes, including steroid-like molecules.[29] Compared to ammonium hydroxide, the advantages of using ammonium fluoride include better analyte responses, and no need for a high pH tolerant LC column. In this work, the effect of ammonium fluoride concentrations (such as 0.05, 0.10, 0.15, 0.20, 0.30, and 0.40 mM) on the estrogen response in aqueous mobile phase A was studied. As shown in Figure 1, all analytes showed the highest responses at around 0.20 mM, therefore, 0.2 mM of ammonium fluoride was used in mobile phase A.

Table 1. LC Method and MS Source Conditions.

LC Conditions	
Analytical Column	Bownlee, SPP C18, 50 x 3 mm, 2.7 µm (PN: N9308408)
Mobile Phase A	0.2 mM ammonium fluoride in water
Mobile Phase B	LC/MS grade methanol
Mobile Phase Gradient	See Table 2
Flow Rate	0.6 mL/min
Column Oven Temperature	30 °C
Auto Sampler Temperature	10 °C
Injection Volume	50 µL
Needle Wash 1	30% methanol in water
Needle Wash 2	Methanol
MS Source Conditions	
ESI Voltage (Negative)	-4800 V
Drying Gas	110
Nebulizer Gas	400
Source Temperature	400 °C
HSID Temperature	310 °C
Detection Mode	Multiple reaction monitoring (MRM)

Table 2. LC Gradient Program.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	70	30
8.0	0	100
9.0	0	100
9.1	70	30
12.0	70	30

Table 3. Retention Time (RT), Optimized MRM Transitions and Parameters.

Analyte	RT (min)	MRM Transition	CE	EV	CCL2
Estrone (E1)	4.84	269.3/145.1	50	-77	112
		269.3/143.1	76	-77	112
		269.3/159.1	48	-77	112
Estradiol (E2)	4.85	271.4/145.1	52	-65	150
		271.4/143.1	76	-65	150
		271.4/183.2	50	-65	150
Estriol (E3)	2.90	287.1/171.1	47	-65	160
		287.1/145.1	54	-65	160
		287.1/143.1	69	-65	160

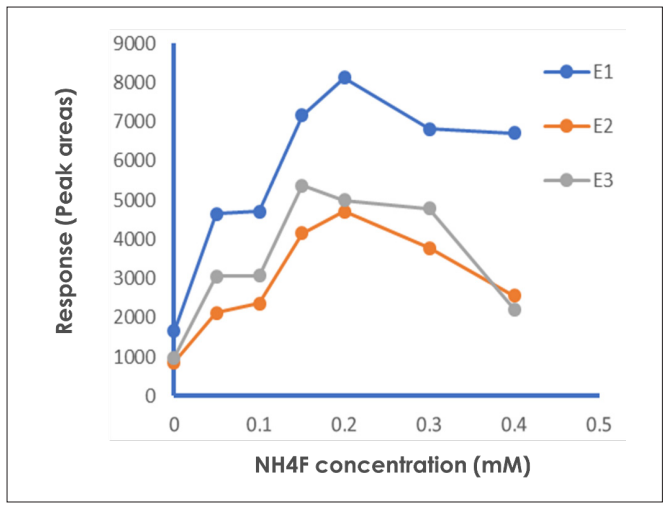

Figure 1. The effects of ammonium fluoride concentration in mobile phase (MP) A on analytes responses.

Methanol and acetonitrile were also compared in terms of sensitivity and separation of the target analytes when used as organic mobile phase B. As shown in Figure 2, higher responses were obtained for all three analytes with methanol as mobile phase, while better separation of E1 and E2 were achieved using acetonitrile, as illustrated in Figure 3. As E1 and E2 can be separated and determined by mass spectrometer, and the sensitivity is more important for this method, methanol was selected as mobile phase B. In addition, methanol is typically less expensive than acetonitrile, making it a more economical choice, especially for a high throughput routine testing laboratory analyzing many samples.

Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Method linearity was studied by external calibration method. Excellent linearity was obtained from 1 to 2000 ng/L (ppt) for each analyte, with regression coefficients (R^2) greater than 0.99, as shown in Figure 4. The limit of detection (LOD) and limit of quantification (LOQ) were estimated based on the signal to noise ratio (S/N ≥ 3 for LOD and S/N ≥ 10 for LOQ) of the analyte's quantifier ion. The estimated LOD for E1 is 0.2 ng/L, and 0.5 ng/L for both E2 and E3. The estimated LOQs are 0.5 ng/L for E1, and 1.0 ng/L for E2 and E3.

Contamination, Analyte Recovery, Sample Matrix Effects and Carryover Effect

Small contamination peaks were found during the initial LRB study for analytes E2 and E3, likely a result of the glassware, including the autosampler vials. After thoroughly cleaning the glassware with acetone, LC/MS grade methanol and water, the contamination peaks were eliminated from the LRB sample. As shown in Table 4, good recoveries were obtained for the LFB samples, indicating no analyte loss or contamination during sample preparations.

Instrument repeatability or precision was assessed based on replicate analyses of a low-level standard (5 ng/L, 7 replicates). The precision was then calculated based on the coefficient of variation (RSD%) of the results. The RSDs for the standard were calculated at 4.6% for E1, 12.8% for E2, and 5.7% for E3.

For water sample analysis, method accuracy was evaluated by the recovery of a known amount of analyte spiked into a lake water sample (LFM samples). As shown in Table 4, the recovery of analytes from the spiked samples were between 75% and 115%, except for E2 in sample LFM1, demonstrating good overall accuracy of the method. The lower recovery for E2 at low concentration levels may be caused by an ion suppression matrix effect. Therefore, it is recommended to include stable isotope labeled internal standards in the method to compensate for the sample matrix effects, and to improve the accuracy of the method.

The carryover effect was investigated by injecting the highest concentration calibration standard (2000 ng/L, in this study), followed by a blank water sample injection. The results demonstrated that the carryover effect is less than the LOQ of the method.

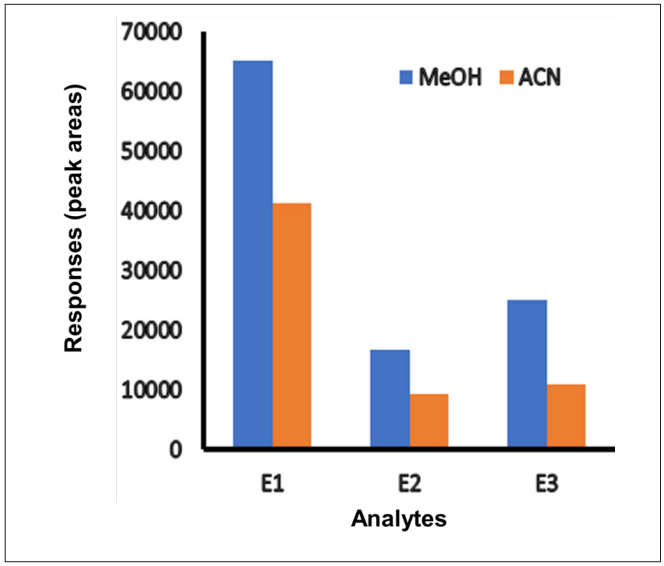

Figure 2. The effects of methanol (MeOH) and acetonitrile (ACN) as mobile phase (MP) B on analytes responses.

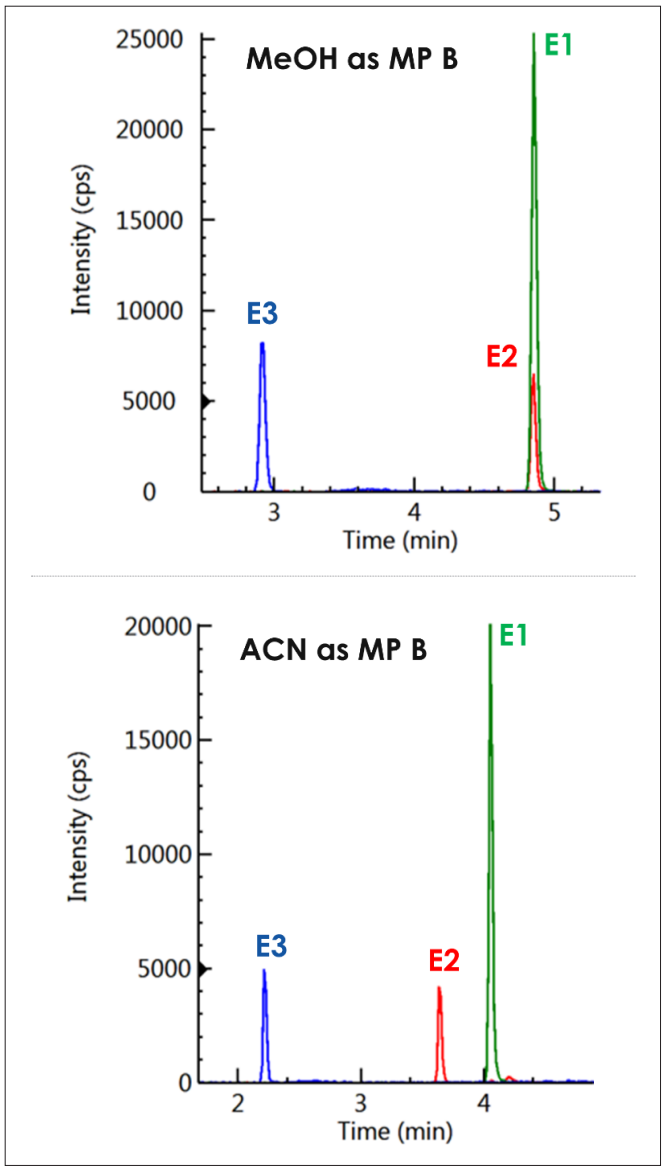

Figure 3. MRM Chromatograms of E1, E2 and E3 obtained using methanol (MeOH) and acetonitrile (ACN) as mobile phase (MP) B.

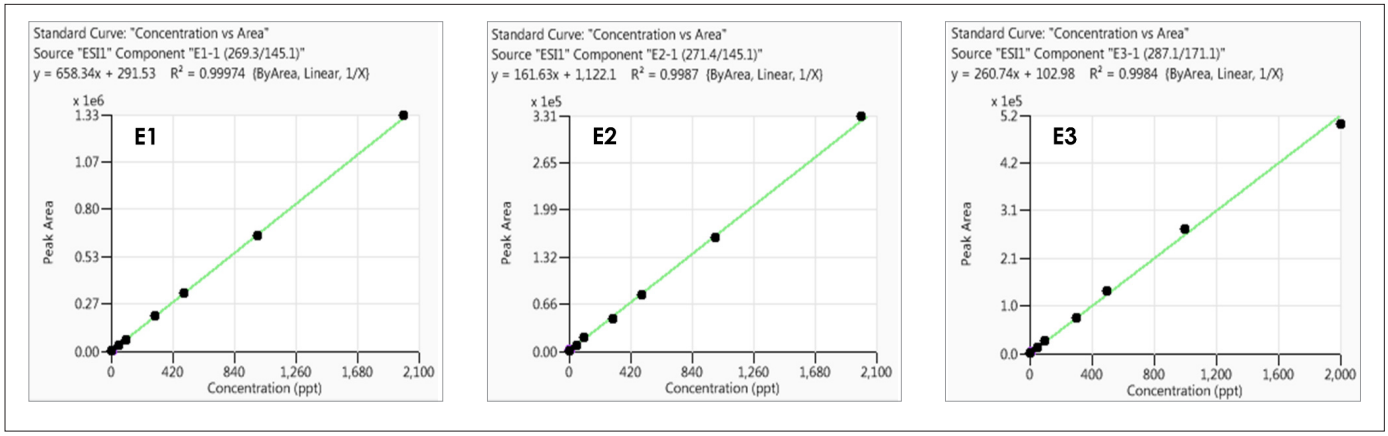

Figure 4. Calibration curves for E1, E2, and E3 with concentrations from 1 to 2000 ng/L (ppt).

Table 4. The Spiked E1, E2 E3 amounts in QC Samples and the Recovery Results.

Sample ID	Spiked (ng/L)			Measured (ng/L)			Recovery (%)		
	E1	E2	E3	E1	E2	E3	E1	E2	E3
LRB	0	0	0	0	0	0			
LFB1	5	5	5	4.9	5.4	4.9	97.5	107	98.2
LFB2	10	10	10	10.1	9.1	8.7	101	91.5	87.1
LFB3	100	100	100	91.9	97.7	94.8	91.9	97.7	94.8
LFM1	5	5	5	5.2	2.3	5.4	104	45.1	109
LFM2	10	10	10	11.5	7.5	9.2	115	75.0	92.0
LFM3	100	100	100	101	94.4	106	101	94.4	106

Sample Analysis

The developed LC/MS/MS method was applied for the analysis of estrogens in fourteen water samples including drinking water, rain water, river water and lake water samples. For the five drinking water samples (including a bottled water sample and four tap water samples collected from different sources), as well as the rain water sample collected from Kitchener Ontario, no analytes were found above the LOQ of the method. However, 7.7 ng/L of E2 was detected in one of the river water samples collected from the Humber River (Toronto, Ontario).

Eight lake water samples from four different lakeshore areas of Lake Ontario in Toronto were collected and analyzed. Four of the lake samples were collected prior to a heavy rain storm, and the remaining four samples were collected immediately following the rain storm. The analysis of the four samples collected before the storm resulted in no target analytes detected. However, as shown in Table 5, trace amounts of E1 were detected from each of the samples collected immediately after the storm, when the municipal health office of Toronto issued a warning for high *E. Coli* levels in waters around the lakeshore areas of Lake Ontario. These results indicate that before entering wastewater treatment plants, human and animal waste may have been flushed into the lake by the heavy rain fall. In addition, high levels of *E. Coli* in water can help transform conjugated estrogens to their free form analytes, which can be detected by the method.

The identity of the analytes in these samples can be confirmed by comparing the analyte retention time and the ion ratios of the qualifier ion against the quantifier ion in the samples with those in the reference standards. As shown in Figure 5, the ion ratios of the qualifier ion against the quantifier ion in the S2 lake sample for E1, and the river sample for E2, are consistent with those obtained from reference standards (0.35 for E1 and 0.56 for E2), confirming the existence of the analytes in these water samples.

As endogenous estrogens are mainly excreted as glucuronide conjugates and small amounts of sulfate conjugates, and the content of free estrogens in urine is typically less than 10% (at low ng/L level), most of the previous urine sample analysis studies have been focused on measuring the total amount of estrogens after hydrolysis by either an enzymatic or acidic method.[12-13] A few studies were also reported for the analysis of conjugated estrogens in urine after SPE clean-up and enrichment,[15-16] but no report was found for direct determination of free form estrogens in urine samples, possibly due to the limited sensitivity of these methods, even with SPE.

For the first time, a simple dilute-and-shoot method has been developed in this study for the analysis of free estrogens in urine, without sample hydrolysis, derivatization and SPE treatments. As shown in Table 5, significantly higher levels of E1 were determined from a female urine sample as compared to that of a male sample, and a small amount of E2 was also found in the female urine. E3 values were not analyzed (NA) in either urine sample, owing to interfering peaks on the second transitions. As shown in Figure 6, the ion ratios of the qualifier ion against the quantifier ion in the female urine sample for both E1 and E2 are consistent with those obtained from reference standards (0.35 for E1 and 0.56 for E2), confirming the identity of the analytes in the sample.

These results from the analysis of all studied matrices clearly demonstrates the superior sensitivity of the QSight 420 triple quadrupole mass spectrometer.

Table 5. Estrogen results obtained from various tested samples in ng/L (ppt).

Analyte	River S	Lake S1	Lake S2	Lake S3	Lake S4	Urine-F	Urine-M
E1	<LOQ	1.2	2.6	1.1	1.4	37.4	1.2
E2	7.7	<LOQ	<LOQ	<LOQ	<LOQ	1.3	<LOQ
E3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	NA	NA

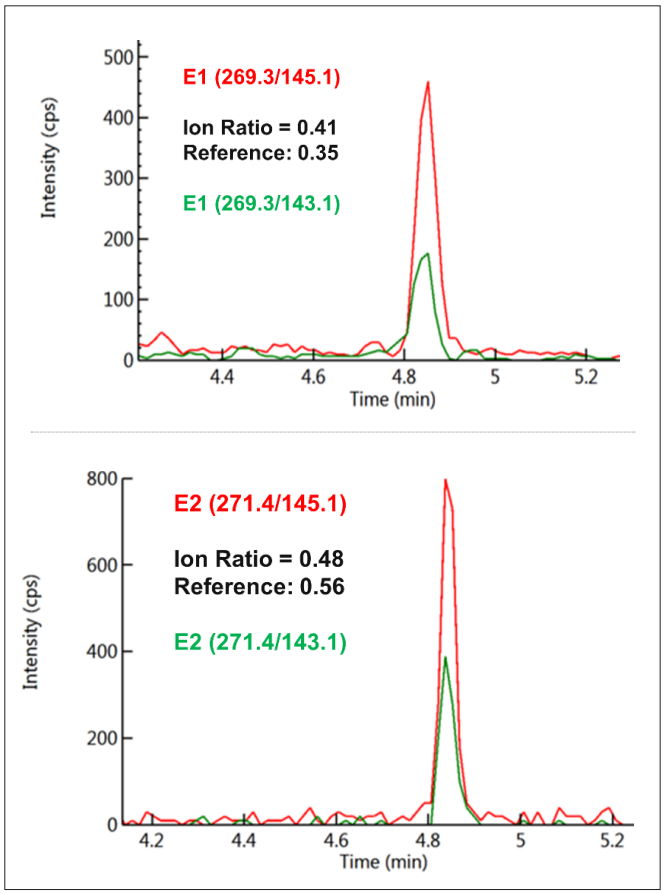

Figure 5. Chromatograms of E1 in Lake S2 water sample and E2 in River water sample (Red - quantifier ion pair; and green, qualifier ion pair).

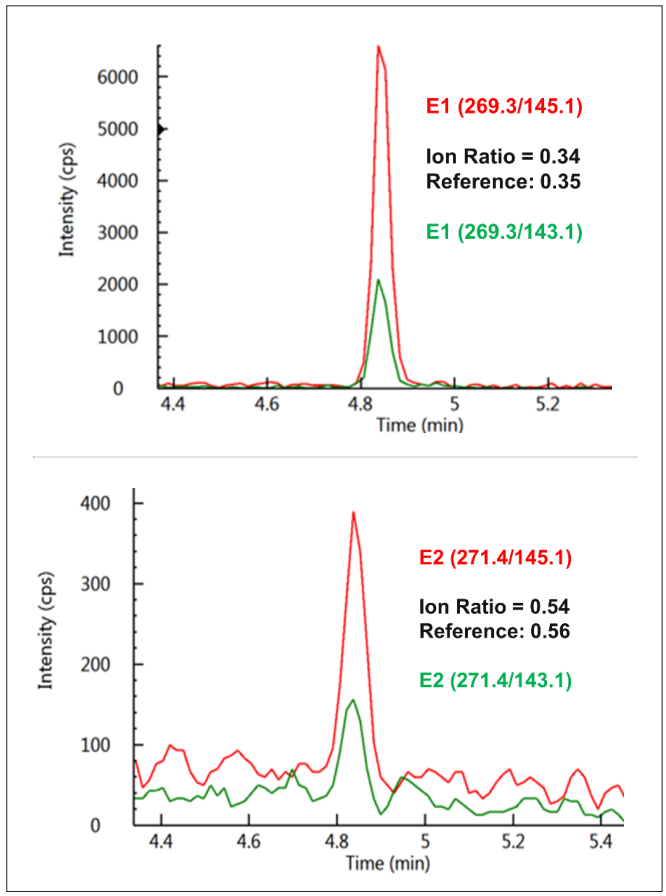

Figure 6. Chromatograms of E1 and E2 in a female urine sample (Red - quantifier ion pair; and green, qualifier ion pair).

Conclusions

A rapid and sensitive LC/MS/MS method has been presented for the analysis of three endogenous estrogens in water and urine samples at low ng/L (ppt) levels by coupling a PerkinElmer QSight LX-50 UHPLC system to a QSight 420 triple quadrupole mass spectrometer. In addition to its high sensitivity, the method shows good linearity, precision and accuracy, without the need for derivatization and concentration with SPE.

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PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
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