

Liquid Chromatography/  
Mass Spectrometry

## Authors:

Jingcun Wu

Saba Hariri

Feng Qin

PerkinElmer, Inc.  
Woodbridge, Ontario, Canada

Keqiang (Peter) Hang

BioPharma Services Inc.  
Toronto, Canada

Chengyuan Cai

PerkinElmer, Inc.  
Shanghai, China

## Analysis of Low Part Per Trillion Levels of Estrogens in Serum without Derivatization Using QSight 420 UHPLC/ MS/MS System

### Introduction

Estrogens are steroid hormones derived from cholesterol. They are crucial for the development of female secondary sex characteristics and reproductive functions, and are also important for many other non-sex-specific processes, including growth, skeleton maintenance,

cardioprotective, and neuroprotective functions. The order of potency of the three major estrogens within the female and male bodies are:  $17\beta$ -estradiol (E2), estrone (E1), and estriol (E3).<sup>1</sup>

Estrogens play an important role in human breast carcinogenesis and are of clinical importance, thus, there have been growing demands for highly selective, sensitive and accurate methods that can measure estrogens at low concentration levels (< 20 pg/mL) in clinical and research laboratories. Currently, the most widely used techniques are automated immunoassays, owing to the fast sample throughput and minimal sample preparation required. However, these techniques suffer from poor accuracy and precision at low concentration levels, and are susceptible to interferences from non-specific antibody interactions with estrogen metabolites and other exogenous metabolites.

Recently, there has been increasing interest in developing LC/MS/MS methods for the analysis of estrogens in serum. Owing to their low polarity, one of the challenges for MS analysis of steroid analytes is the difficulty experienced in ionizing these analytes. To improve analyte ionization efficiency and enhance the method's sensitivity, derivatization of analytes has been used in many LC/MS/MS methods during sample preparation.<sup>2-5</sup> However, derivatization not only increases the sample preparation time and cost, but also introduces variations into the method, making derivatization not suitable for routine clinical laboratories. The objective of this study was to develop a highly sensitive method for the analysis of estrogens in serum, without derivatization, using the QSight® 420 LC/MS/MS system.

## Experimental

### Hardware/Software

Chromatographic separation was conducted using the PerkinElmer QSight LX50 ultra-high-performance liquid chromatography (UHPLC) system, with subsequent mass detection achieved using the 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 Simplicity™ 3Q Software.

### Method

#### Standard and Chemicals

Standards for estrone (E1), 17 $\beta$ -estradiol (E2), estriol (E3), ammonium fluoride (purity > 99.9%) and methyl tert-butyl ether (MTBE) were obtained from Sigma-Aldrich. LC/MS grade methanol (MeOH) and water were obtained from MilliporeSigma. Figure 1 shows the chemical structures of the three estrogens.

#### Standard Preparation

Individual primary standard stock solutions (1mg/mL) were prepared by weighing accurately 10 mg of each standard, and dissolving each in 10 mL of methanol, respectively. A mixed standard stock solution (containing each analyte at 10 $\mu$ g/mL) was prepared in methanol by dilution of the primary standard solutions. Several working standard solutions with various low concentrations (10, 100, 1,000, and 10,000 pg/mL) were prepared by diluting the mixed stock solution with a 50:50 methanol/water diluent. Two sets of calibration standards (with concentrations: 0, 1, 2, 5, 10,

40, 100, and 400 pg/mL) were prepared by diluting corresponding working solutions with an appropriate diluent. One set of calibration standards were prepared using a 30:70 methanol/water diluent; another set of calibration standards were prepared using charcoal stripped serum as the diluent, with serum sample preparation procedures shown below for cleanup and concentration.

#### Serum Sample Preparation

A 400  $\mu$ L serum sample, or spiked serum sample, was extracted utilizing a liquid-liquid extraction procedure using 1 mL of MTBE, followed by vortex mixing for 2 minutes. The sample was then centrifuged at 9,000 rpm for 10 minutes, after which the supernatant (organic phase) was transferred into a clean 1.5 mL microcentrifuge tube, dried under nitrogen gas, and finally reconstituted using 100  $\mu$ L of 30:70 methanol/water diluent.

#### Quality Control Sample Preparation

Avoiding contamination of the samples during sample collection, sample preparation and sample analysis are all crucial for ensuring reliable sample analysis. To test possible interference or contamination from reagents and glassware, and from the sample preparation processes, a laboratory reagent blank (LRB) was prepared. The values of LRB should be close to zero, or at least less than the LOQ of the method. Otherwise, an investigation into the source of contamination must be carried out. An LRB sample was prepared by following the same procedures as described for a serum sample preparation, using the 30:70 methanol/water diluent as sample.

To study possible analyte loss or contamination during sample preparations, a laboratory fortified blank (LFB) sample was prepared. An LFB sample can be prepared by following the same serum sample preparation procedures, using the 30:70 methanol/water diluent spiked with a known amount of analyte solution. During method validation, LFB samples were prepared by spiking the analyte in three different concentration levels (5, 10, and 100 pg/mL, respectively), and three replicates of the LFB samples at each level were prepared.

To check possible interference or contamination from the charcoal stripped serum, a stripped serum blank (SSB) was prepared by following the same procedures as for a serum sample preparation.

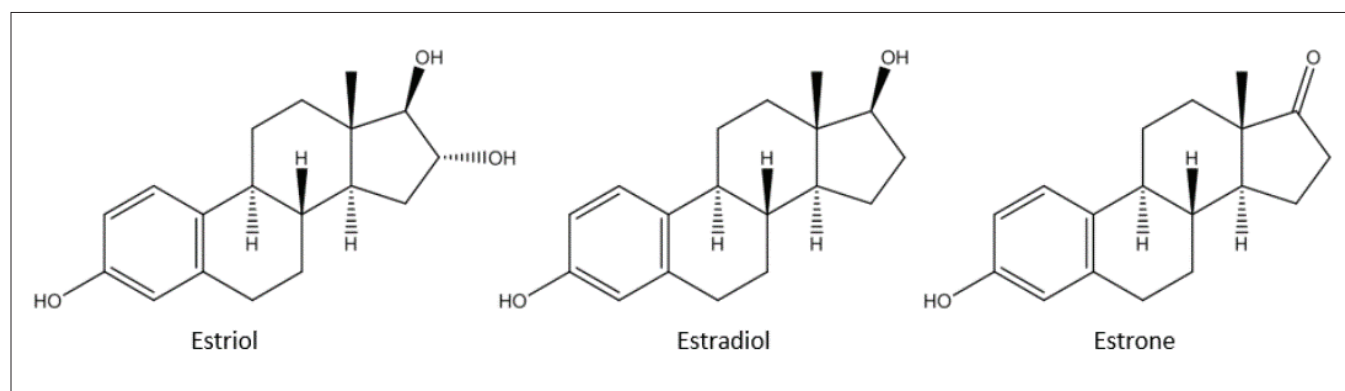


Figure 1. Chemical structures of the three estrogens analyzed in this study.

To evaluate sample matrix effects and analyte recovery from the serum sample matrix, a laboratory fortified matrix sample (LFM) was prepared by following the same serum sample preparation procedures, using a charcoal stripped serum 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 serum sample results to the expected (spiked) value. During method validation, the LFM samples were prepared at three different analyte concentrations (5, 40, and 100 pg/mL, respectively).

### LC Conditions and MS Parameters

The LC method and MS source parameters are shown in Table 1. A C18 column (Brownlee, SPP C18, 50 x 3 mm, 2.7  $\mu$ m) 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 the 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 demonstrated better sensitivity and signal to noise ratio under negative ionization mode. Similar results were also obtained in previous studies.<sup>6-7</sup> In one study,<sup>7</sup> different ionization techniques, such as ESI, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) were compared for analysis of estradiol in serum. The study indicated that positive APCI and APPI modes showed higher background noise, although the limits of quantification (LOQs) of E2 assays using positive APCI and APPI were comparable with LOQs of assays using negative ESI. Therefore, negative ESI detection was used in this study.

Table 1. LC Method and MS Source Conditions.

LC Conditions	
Analytical Column	Brownlee, SPP C18, 50 x 3mm, 2.7 $\mu$ m (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	25 $\mu$ L
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

According to the regulatory guidance on analytical method validation, at least two MS/MS transitions should be used in an LC/MS/MS method.<sup>8-9</sup> In this study, three MS/MS transitions were evaluated. Tables 1 and 3 list the optimized parameters for the ion source and compounds.

To improve analyte ionization efficiency and enhance the method's sensitivity, ammonium hydroxide with concentrations from 0.01% to 0.05% were used in mobile phases.<sup>5-7</sup> However, an LC column that can work robustly at the high pH range is required. Recently, ammonium fluoride has been increasingly used as a mobile phase additive to enhance ionization of some analytes, including steroid-like molecules and estrogens.<sup>10-13</sup> 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 study, the effect of ammonium fluoride concentrations (such as 0.05, 0.10, 0.15, 0.20, 0.30, and 0.40 mM) on estrogen responses in mobile phase A was studied, and it was found that all analytes gave the highest peaks at 0.20 mM and therefore, 0.2 mM of ammonium fluoride was used in mobile phase A.

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

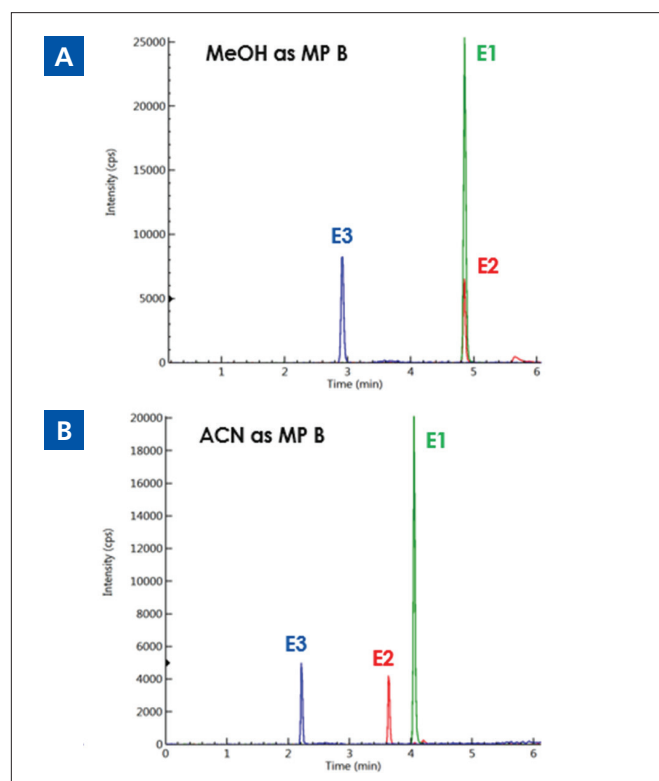


Figure 2. MRM Chromatograms of E1, E2 and E3 obtained using methanol (MeOH) and acetonitrile (ACN) as mobile phase (MP) B, respectively.

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

Method linearity was studied by external calibration method. For the calibration standards that were prepared in neat solutions (refer to experimental section for details), excellent linearity was obtained from 5 to 400 pg/mL (ppt) for each analyte with regression coefficients ( $R^2$ ) greater than 0.99 (data not shown, but available upon request).

To reduce sample matrix effects, matrix matched calibrations were also prepared by spiking analytes into charcoal stripped serum samples from 5 to 400 ng/L (ppt), and again good linearity ( $R^2 \geq 0.99$ ) was achieved for each analyte, as shown in Figure 3. The limit of detection (LOD) and limit of quantification (LOQ) were estimated based on the signal to noise ratio ( $S/N \geq 3$  for LOD and  $S/N \geq 10$  for LOQ) of the analyte's quantifier ion. The estimated LODs are 0.5 pg/mL for E1 and 2 pg/mL for E2 and E3, respectively, and the estimated LOQs are 1 pg/mL for E1 and 5 pg/mL for E2 and E3, respectively.

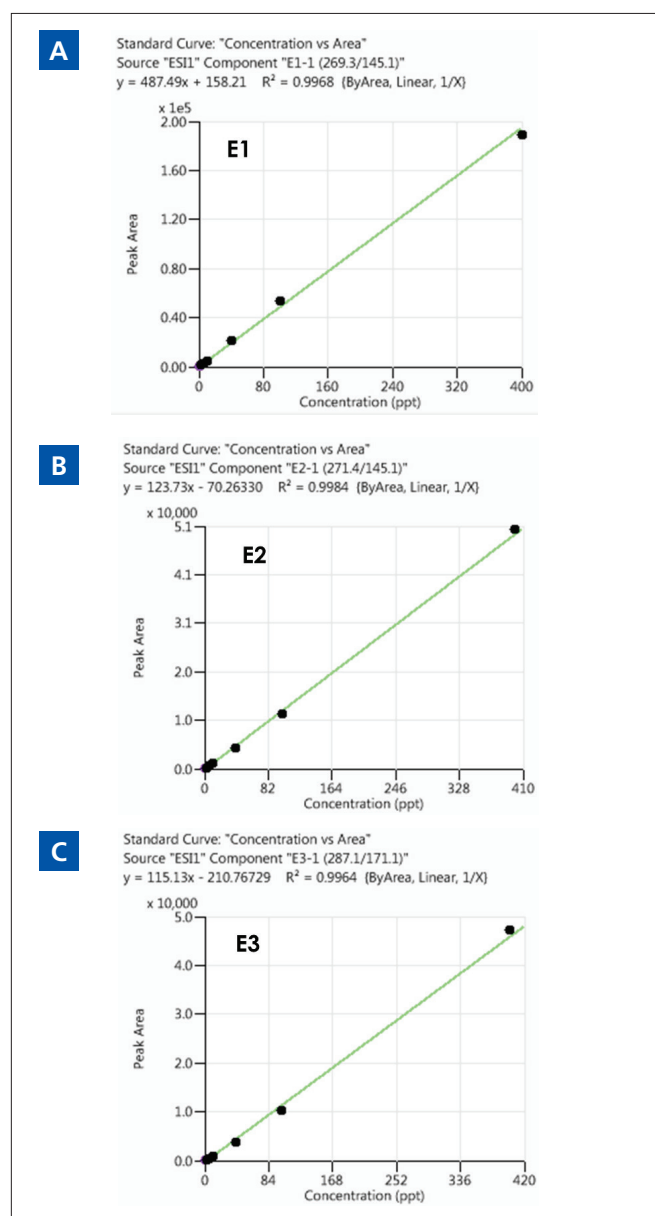


Figure 3. Calibration curves for E1, E2, and E3 obtained from standards prepared by spiking analytes in charcoal stripped serum sample with concentrations from 5 to 400 pg/mL (ppt).

### Contamination, Analyte Recovery and Carryover Effect

Contamination peaks were found for analytes E2 and E3, with the root cause determined to be glassware, including autosampler vials, during initial study with the Laboratory Reagent Blank (LRB). After thoroughly cleaning all glassware with acetone, LC/MS grade methanol, and water, these contamination peaks were finally eliminated from the LRB sample. As shown in Table 4, good recoveries were obtained for LFB samples, indicating no analyte loss or contamination during sample preparations. No interference or contamination peaks were found from the charcoal stripped serum bank (SSB) sample, as shown in Figure 4.

Instrument repeatability or precision was assessed based on replicate analyses of a low-level standard (5 pg/mL, seven

replicates). The precision was then calculated based on the coefficient of variation (RSD%) of the results. The calculated RSDs for the low-level standard were 4.6% for E1, 12.8% for E2, and 5.7% for E3.

Method accuracy assesses how close the experimental value is to the expected value. Method accuracy was evaluated by the recovery of a known amount of analyte spiked into a serum sample (LFM samples). As shown in Table 4, the recoveries of analytes from the spiked samples were between 77.8% and 111%, demonstrating good accuracy of the method. Figure 4 illustrates the overlapped chromatograms of analytes in a stripped serum blank (SSB) and a low concentration spiked serum sample (LFM1).

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

Sample ID	Spiked (pg/mL)			Measured (pg/mL)			Recovery (%)		
	E1	E2	E3	E1	E2	E3	E1	E2	E3
LRB	0	0	0	0	0	0			
LFB1	5	5	5	4.8	5.2	4.5	96.0	104	90.0
LFB2	10	10	10	9.2	8.7	10.4	92.0	87.0	104
LFB3	100	100	100	100	113	96.3	100	113	96.3
SSB	0	0	0	0	0	0			
LFM1	5	5	5	5.2	4.9	5.2	104	97.5	105
LFM2	40	40	40	44.2	37.3	34.1	111	93.4	85.3
LFM3	100	100	100	102	81.6	77.8	102	81.6	77.8

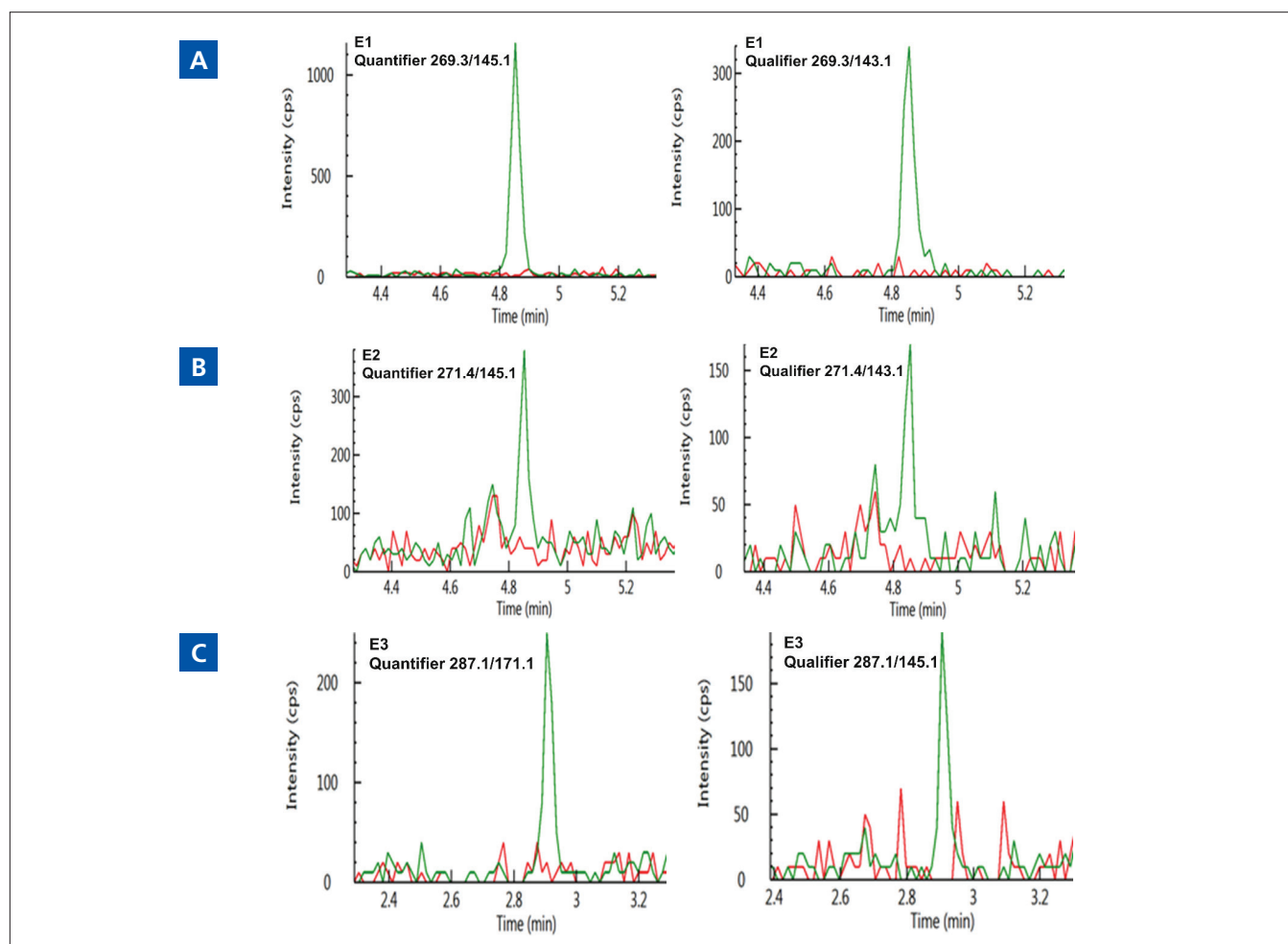


Figure 4. Chromatograms of SSB sample (in red color, prepared using charcoal stripped serum) and LFM1 sample (in green color, prepared using charcoal stripped serum spiked with 5 pg/mL of analytes).

The carryover effect was investigated by injecting the highest concentration calibration standard (400 pg/mL, in this case), followed by a blank serum (SSB) injection. The results demonstrate that the carryover effect is minimum, or at least less than the LOQ of the method.

### Sample Analysis and Sample Matrix Effects

Sample matrix effects are one of the main challenges in LC/MS/MS method development and applications, especially for complex biological sample matrices. In this study, significant serum sample matrix effects, mainly ion suppressions, were observed. To overcome matrix effects, it is strongly recommended that the analytical process include stable isotope labeled internal standards in the method. In addition, more extensive sample clean-up steps, such as solid supported liquid extraction (SLE) and solid phase extraction (SPE), may be needed to remove interfering components<sup>11,14-15</sup>, and/or a longer LC column may be used to separate analyte peaks from interfering components in samples.

### Conclusions

A rapid and sensitive LC/MS/MS method has been developed for the analysis of three naturally occurring estrogens in human serum samples at low pg/mL (ppt) levels by coupling an LX50 UHPLC system to a QSight 420 triple quadrupole mass spectrometer. In addition to its high sensitivity, the method showed good linearity and precision. The method has been validated using stripped serum samples with good accuracy.

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