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Detection of a Low-level Estrogen Panel in Human Serum Without Derivatization Using EVOLUTE® EXPRESS ABN Prior to LC/MS-MS Analysis

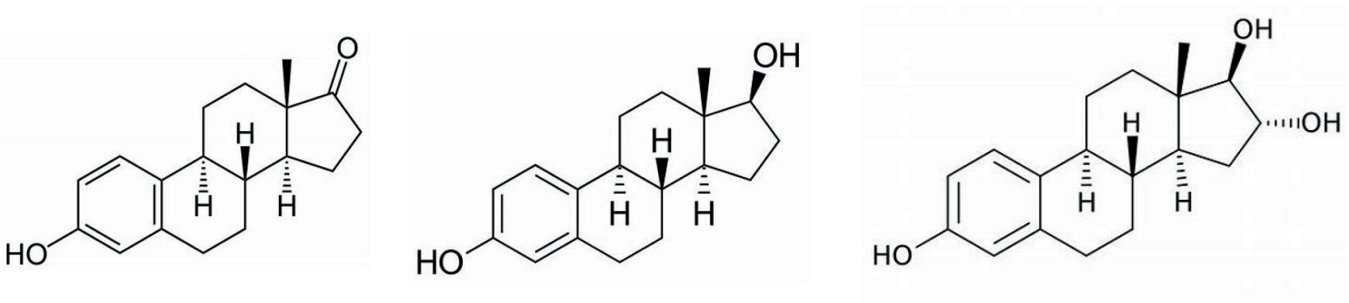

Figure 1. Structures of Estrone(E1), Estradiol(E2), and Estriol(E3).

Introduction

This application note describes the extraction of a panel of estrogenic hormones from human serum using EVOLUTE® EXPRESS ABN solid phase extraction plates prior to LC/MS analysis. The simple sample preparation procedure delivers clean extracts and analyte recoveries greater than 90% with RSDs lower than 5% for all analytes. Linearity of greater than 0.99 is achieved for Estrone (E1) and Estradiol (E2) from 5–2000 pg/mL, and 50–20000 pg/mL for Estriol (E3). No derivatization is required, and detection limits are enhanced using a fluorinated mobile phase.

EVOLUTE EXPRESS products dramatically improve flow characteristics and enhance sample preparation productivity.

Analytes

Estrone, Estradiol, and Estriol

Internal Standards

Estrone-[2,3,4-$^{13}C_3$]

17ß-Estradiol-[2,3,4-$^{13}C_3$]

Sample Preparation Procedure

Format

EVOLUTE® EXPRESS ABN 30 mg plate (P/N 600-0030-PX01).

Sample Pre-Treatment

Spike serum (500 µL) with 20 µL of ISTD in methanol (2 ng/mL) and 2% formic acid (aq) (100 µL).

Condition

Condition wells with methanol (500 µL).

Equilibration

Equilibrate wells with 2% formic acid (aq) (500 µL).

Sample Loading

Load pre-treated serum mix (500 µL total volume).

Wash 1

Elute interferences with 2% formic acid (aq) (500 µL).

Wash 2

Elute interferences with H_2O:MeOH (60:40, v/v, 500 µL)

Elution

Elute analytes with ethyl acetate (500 µL).

Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage ®SPE Dry at 40 °C, 20 to 40 L/min for approximately 20 minutes.

Reconstitute evaporated samples with mobile phase A:B (60:40, v/v, 100 µL) and mix thoroughly.

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UHPLC Conditions

Instrument
Agilent 1260 Infinity II LC System

Column
Raptor Biphenyl 2.7um 50 X 3.0 mm (Restek part # 9309A5E)

Flow Rate
0.5 mL/min

Column Temperature
40 °C

Injection Volume
20 µL

Table 1. HPLC Gradient.

Time (min.)	%A	%B
0	60	40
3.9	10	90
4.0	2	98
4.5	2	98
5.0	60	40

Mass Spectrometry Conditions

Instrument
Agilent 6490 triple quadrupole mass spectrometer with ion funnel technology

Ionization Mode
Agilent Jet Stream Neg.

Gas Temperature
200 °C

Drying Gas
15 L/min

Nebulizer Gas (Nitrogen)
35 psi

Sheath Gas (Nitrogen)
400 °C

Capillary (V)
-3000

VCharging
-2000

Delta EMV
500

Ion Funnel Parameters
Pos. HRH 150 Neg. HRH 150

Pos. LRF 100 Neg. LRF 100

Table 2. MS conditions and retention times for target analytes in positive and negative mode.

Cpd Name	ISTD	MRM Transition	Collision Energy (CE)	RT	Polarity	Cell ACC (V)
Estriol	No	287.2>171,145	41,43	2.5	-	2
Estradiol	No	271.4>182.8,144.9	53,43	3.8	-	2
Estradiol $^{13}C_3$	Yes	274.3>148	49	3.8	-	2
Estrone	No	269.3>145,143.1	43,70	4.4	-	2
Estrone $^{13}C_3$	Yes	272.3>148	45	4.4	-	2

Results and Discussion

Linearity was investigated using diluted synthetic serum spiked between 5–2000 pg/mL for Estrone and Estradiol, and 50–20000 pg/mL for Estriol. Good linearity was observed for all three analytes, typically delivering r^2 values greater than 0.999 Table 3 details linearity performance and associated LOQ for each analyte using ethyl acetate as elution solvent.

Table 3. Analyte calibration curve r^2 and LOQ performance.

Analyte	r^2 EtOAc	LLOQ (pg/mL) EtOAc	Range (pg/mL)
Estrone	0.999	5	5-2000
Estradiol	0.999	5	5-2000
Estriol	0.999	50	50-20000

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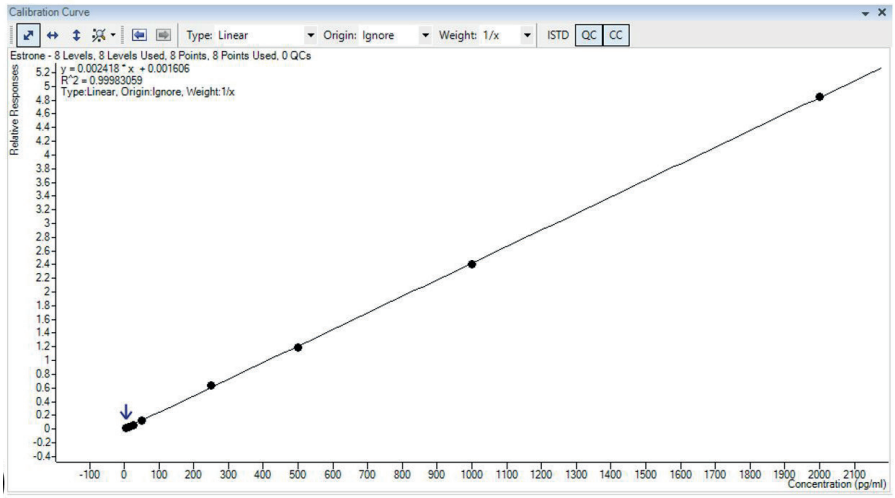
a)



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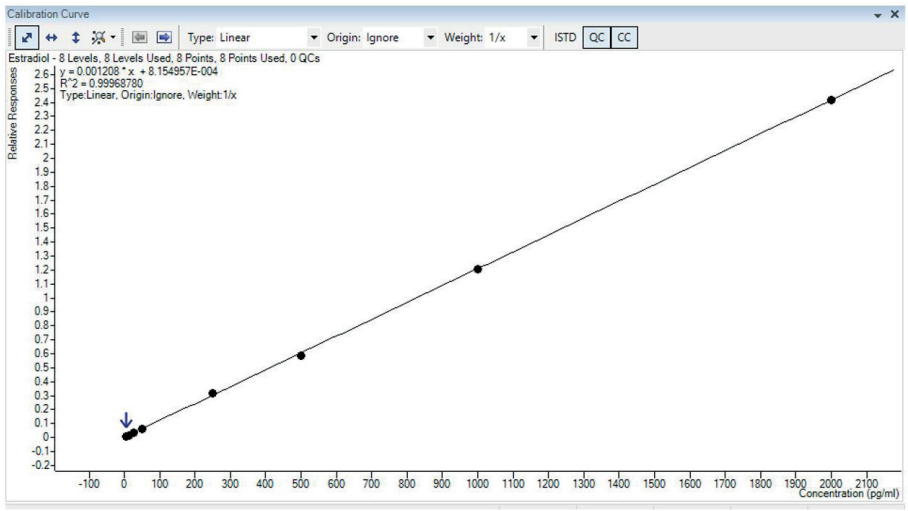

c)

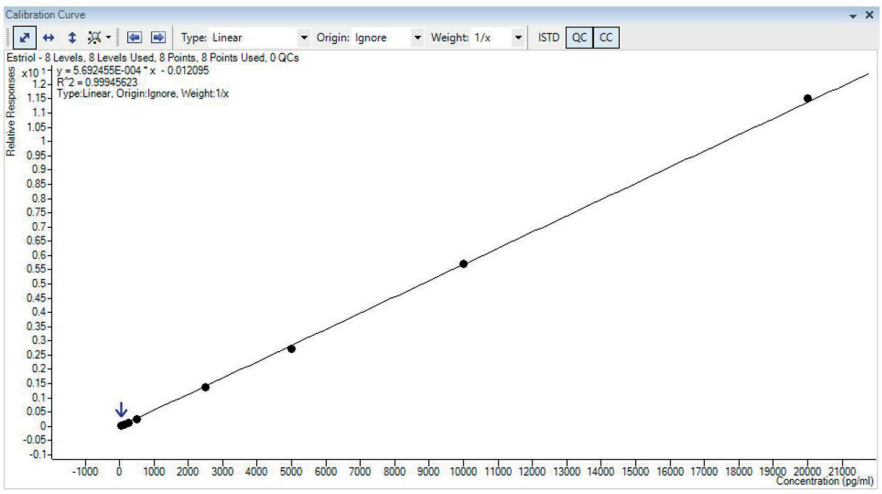

Figure 2. Calibration curves for Estrone (a), Estradiol (b), Estriol (c).

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Peak Shape

Figure 3 shows the estrone (E1) and estradiol (E2) chromatograms at the LOQ of 5 pg/mL in serum, and the estriol (E3) chromatogram at the LOQ of 50 pg/mL. The three major estrogens estrone (E1), estradiol (E2), and estriol (E3) are difficult to ionize in the mass spectrometer source under normal conditions. Traditionally, they are extracted using liquid-liquid extraction followed by derivatization (dansylation) to be detected in positive mode. However, this traditional method involves many steps and intensive labor. When using EVOLUTE® EXPRESS ABN 30 mg plates for extraction we were able to extract these analytes and detect them in negative mode with the help of 0.2 mM ammonium fluoride additive in the mobile phase. The optimized extraction method was verified with 5-day accuracy and precision runs to collect the data shown in table 4.

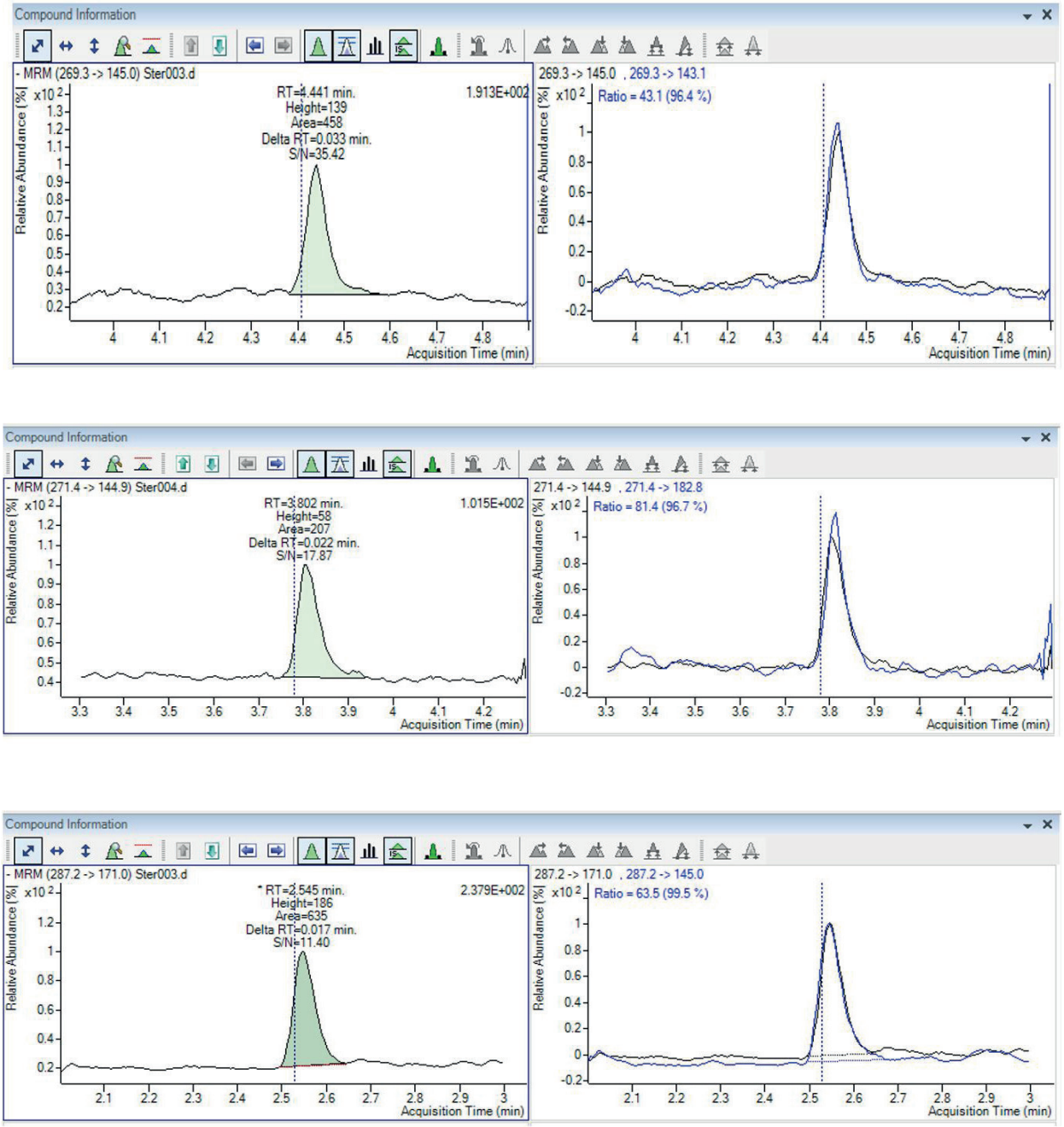


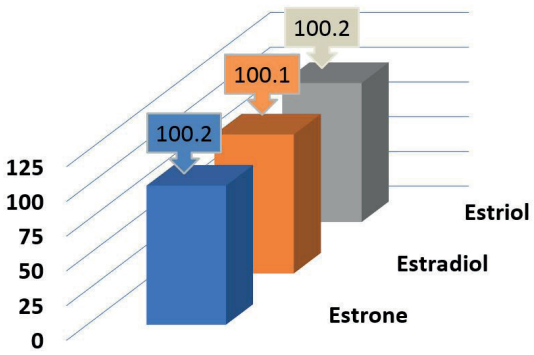
Figure 3. LOQ Peak Shape @ 5 pg/mL for E1 & E2 and 50 pg/mL for E3.

Table 4. Showing Inter - and Intraday Accuracy and Precision (n=5).

Analyte	LOQ (pg/mL)	Calibration Range (pg/mL)	Spiked QC Conc. (pg/mL)	Interday % Accuracy (n=5)	Interday % Precision (n=5)	Intraday % Accuracy (n=5)	Intraday % Precision (n=5)
			10	94.5	4.86	92.8	8.77
Estrone (E1)	5	5–2000	100	95.9	6.24	102.5	8.61
			400	93.2	5.64	96.2	7.31
			10	101.1	4.57	97.8	8.74
Estradiol (E2)	5	5-2000	100	100.5	2.62	104.1	5.64
			400	96.3	5.34	102.3	6.29
			100	99.3	7.25	103.2	4.01
Estriol (E3)	50	50-20000	1000	96.2	3.67	95.8	9.91
			4000	97.3	8.91	98.6	7.62

Recovery data for both elution solvent systems is shown below in Figure 4. EVOLUTE® EXPRESS ABN plates deliver clean extracts and analyte recoveries more than 90% with corresponding RSDs below 10%.

Average % Recovery for Estrogens after Extraction with EVOLUTE® EXPRESS ABN.



Average % RSD for Estrogens after Extraction with EVOLUTE® EXPRESS ABN.

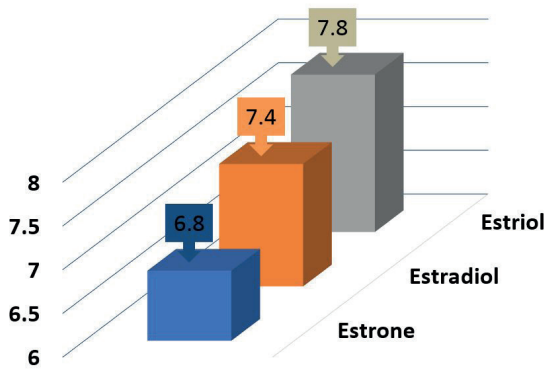


Figure 4. Average % Recovery and % RSD for all three analytes.

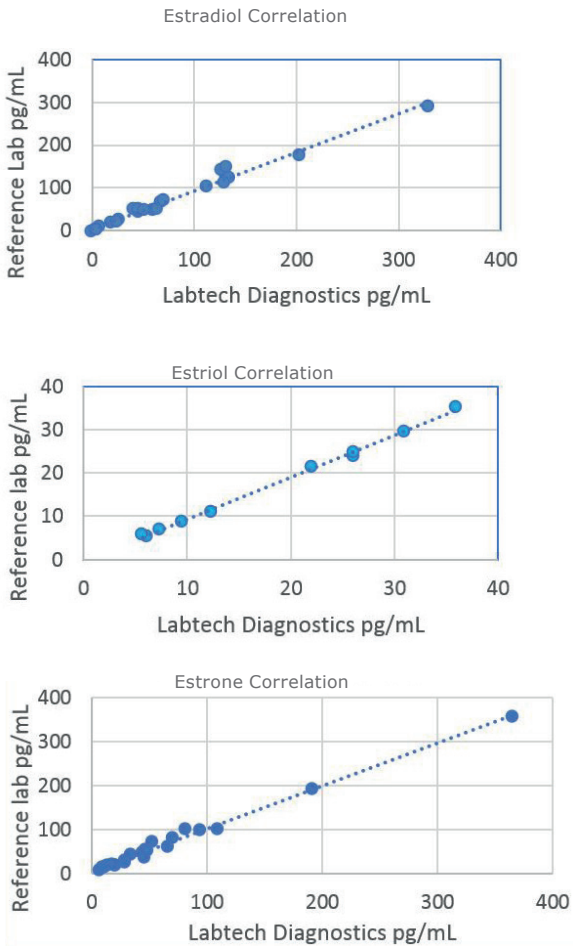


Figure 5. Correlation data for all analytes.

Inter-Laboratory Correlation

Comparing the LC-MS/MS results from two different laboratories showed excellent agreement between these 2 methodologies for the three estrogens (r=0.82–0.91; n=20). Importantly, absolute concentrations also agreed; the median levels were almost identical (±10% difference) according to the two techniques for E1, E2, and E3.

Additional information

- Ammonium fluoride in the mobile phase increased sensitivity in negative ion mode.
- Other strategies for increasing sensitivity:
  - Increase matrix volumes above 500 µL and use 60 mg extraction bed.
  - When pretreating the sample with IS and 2 % formic acid let sit for 5 min to enhance binding to the matrix before proceeding with extraction.
  - Decrease reconstitution solvent volume below 100 µL.
  - Increase injection volumes above 20 µL.

Chemicals and Reagents

- Methanol (LC-MS grade), ammonium fluoride, water (LC-MS grade), formic acid (LC-MS grade) and ethyl acetate (anhydrous, 99.8%) were purchased from Sigma Aldrich (St. Louis, MO).
- All analyte standards and deuterated internal standards were purchased from Cerilliant (Round Rock, TX).
- Mobile phase A 0.2 mM ammonium fluoride in LC/MS Water (aq) was prepared by adding 78 mg of Ammonium fluoride in one Liter of water.
- Mobile phase B 0.2 mM Ammonium Fluoride in LC/MS Methanol (or) was prepared by adding 78 mg of ammonium fluoride in one liter of methanol.

Conclusion

Estrogens in human serum can be quantified accurately and precisely with this LC/MS-MS method that employs SPE using EVOLUTE® EXPRESS ABN 30 mg without derivatization steps. The method can analyze 20 samples per hour, providing a very efficient high-throughput analysis. The achieved sensitivity allows the required lower limits of quantification for all three estrogens to be achieved. Method performance parameters agree with current bioanalytical guidelines.

Ordering Information

Part Number	Description	Quantity
600-0030-PX01	EVOLUTE® EXPRESS ABN 30 mg Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
SD-9600-DHS-EU	Biotage® SPE Dry Sample Concentrator System 220/240 V	1
SD-9600-DHS-NA	Biotage® SPE Dry Sample Concentrator System 100/120 V	1

Acknowledgements

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