

Quantitation of 17 β -Estradiol and Estrone by LC-MS/MS



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Introduction

- Measurement of serum estrogens plays a decisive role in assessment of hypogonadism, oligomenorrhea, assessing ovarian status, and in anti-estrogen therapy in cancer.
- Clinical measurement of 17 β -estradiol (E2) and estrone is currently performed by immunoassay and LC-MS/MS. Immunoassay methods lack the selectivity and sensitivity to measure low levels of estrogens. LC-MS/MS methods afford greater sensitivity and accuracy, and are increasingly being adopted in the diagnostic setting.
- Accurate calibrators are required for accuracy and standardization of test results. We present product design and method validation for certification of estrogen reference materials in a surrogate matrix.

Product Design and Certification

- Five calibrator levels in SigMatrix Ultra™ (2% rHSA in PBS): 1000 pg/mL, 500 pg/mL, 100 pg/mL, 20 pg/mL and 10 pg/mL
- Gravimetrically prepared from Certified Reference Materials, E-060 & E-075 (1 mg/mL in acetonitrile)
- Certification based on gravimetric preparation and LC-MS/MS assignment of concentration.

Method Description

- A sensitive and precise LC-MS/MS assay for measuring 17 β -Estradiol and Estrone in serum and surrogate matrix based on CDC Primary Reference Method.¹
- Range: <10 to 1000 pg/mL.
- No derivatization - negative ion mode electrospray

Value Assignment Curves (VACs):

- Catalog products (CRMs E-060, E-073, E-075 & E-108) were used to prepare native and labeled intermediate stocks in acetonitrile
- Intermediate stocks were diluted to two value assignment curves (VACs, high and low range in MeOH/water, 0.1% TFA)

Sample preparation:



Figure 1: Extraction protocol

- Samples at -20°C are thawed to room temperature.
- Double liquid-liquid extraction is optimized for recovery and reduction of matrix interferences.
- Extract reconstitution sets concentration of estrogens in sample to the mid-point of the VAC.

LC-MS/MS Method

- Adapted from literature¹ and modified for use on a SCIEX5500 Qtrap coupled to a Shimadzu Nexera UPLC system.
- Flow rate: 0.5 mL/min.
- Mobile Phase A: 0.3 mM ammonium fluoride in water.
- Mobile Phase B: Methanol.
- Gradient: Initial MPB 10% ramp to MPB 52% in 11 minutes, hold at 52% for 1.75 minutes. Ramp to MPB 90%, hold for 3 mins, return to MPB 10%.
- Column: Phenyl Hexyl, 2.6 μ , 4.6 x 50 mm.
- Estrone 269 \rightarrow 145 ; Estrone ¹³C₃ 272 \rightarrow 148.
- Estradiol 271 \rightarrow 145 ; Estradiol ¹³C₃ 274 \rightarrow 148.

¹ Julianne Cook Botelho, Ashley Ribera, Hans C. Cooper, and Hubert W. Vesper. Anal. Chem., 2016, 88 (22), pp 11123-11129

Method Validation

- Precision and Accuracy was determined across three days.
- Controls: BCR578 and BCR577 higher order controls for 17 β -Estradiol. In house Quality Controls containing 17 β -Estradiol and Estrone used as controls for both 17 β -Estradiol and Estrone.
- Specificity, Robustness, Recovery, Matrix Factors and Extract Stability were also established during validation.

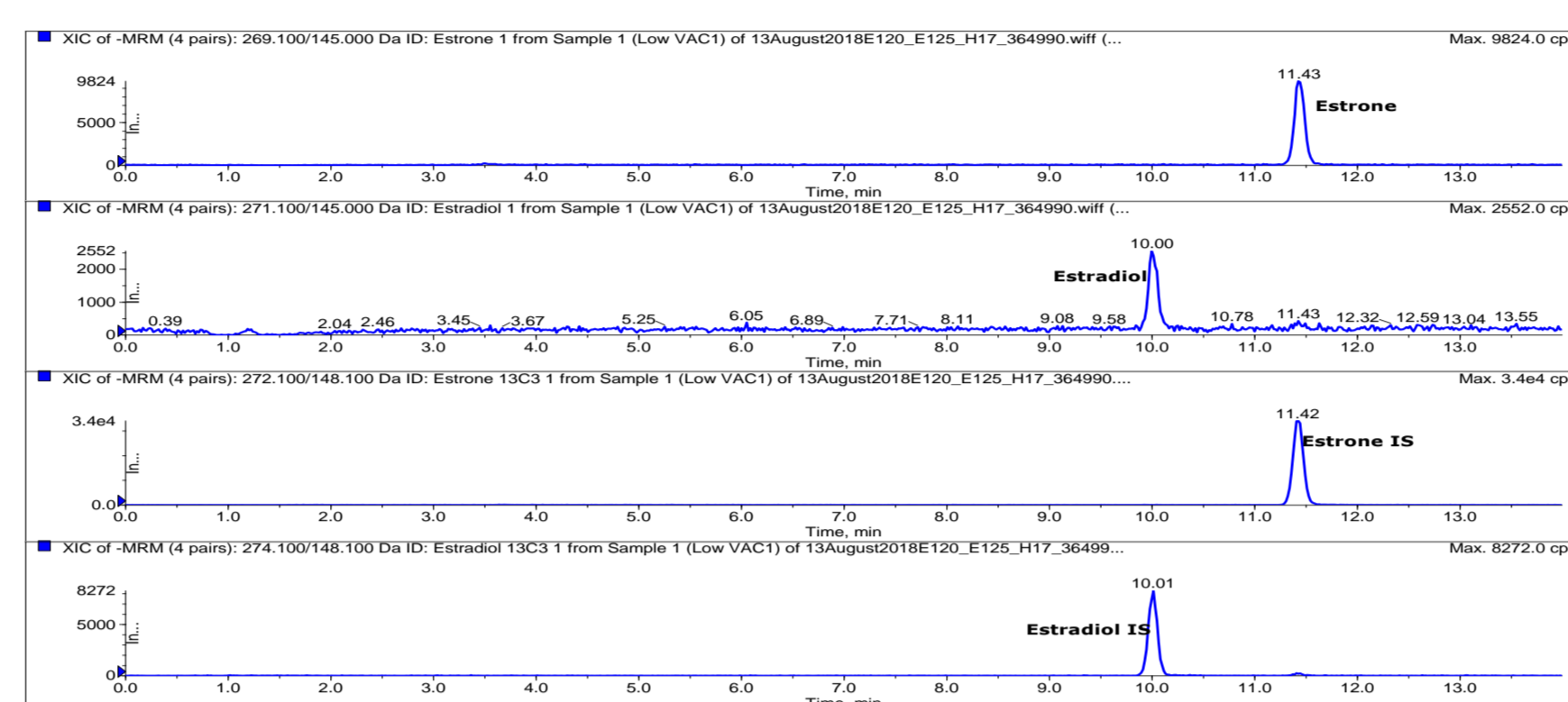


Figure 2: Example Chromatogram for Estrogens

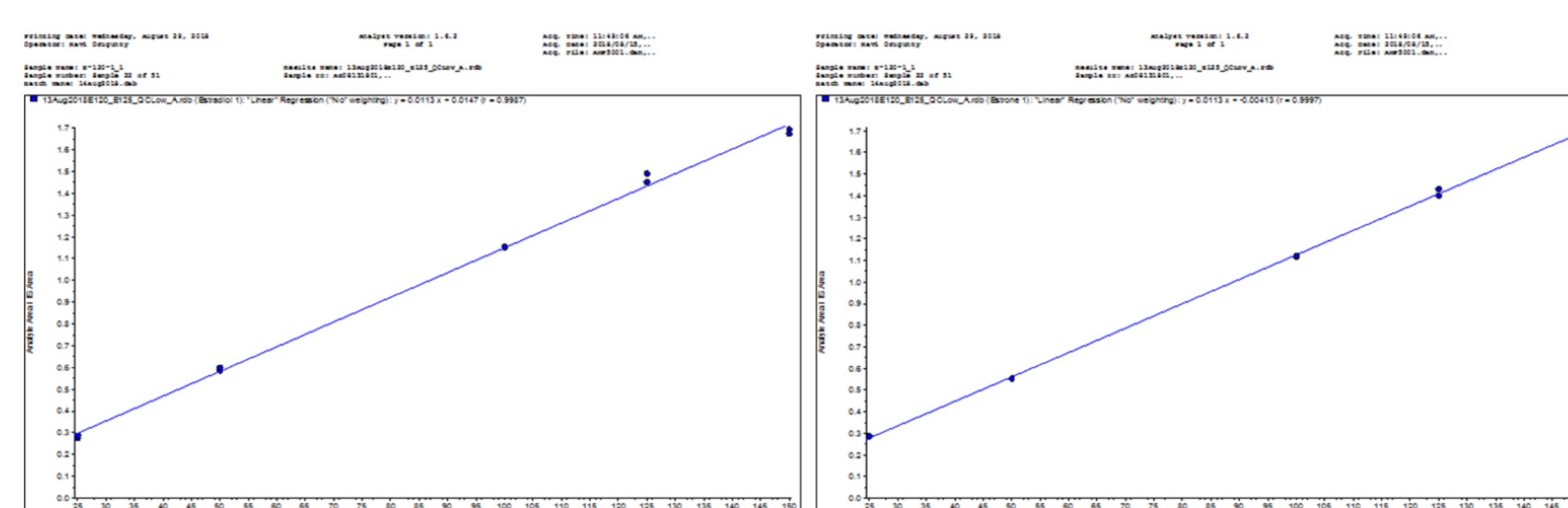


Figure 3: Value Assignment Curve for Estrogens

Method Validation Results

- Data from method validation met all acceptance criteria for precision, accuracy and linearity.
- Table 1 shows precision and accuracy data for the controls. BCR577, BCR578 report certified values for 17 β -Estradiol only.
- Table 2 shows performance of the individual product levels in the LC-MS/MS assay.

Table 1: BCR577, BCR578 and In House QC Data

Product	Project	17 β -Estradiol (pg/mL)				Estrone (pg/mL)			
		CoA value/Prep Value	Mean Assay Conc.	% Diff.	% RSD	CoA value/Prep Value	Mean Assay Conc.	% Diff.	% RSD
BCR577	Val Day 1	187.94	179.84	-4.31	2.28	189.73	187.64	-1.10	1.23
	Val Day 2	187.94	183.95	-2.12	3.09	189.73	181.51	-4.52	2.40
	Val Day 3*	187.94	179.49	-4.50	2.54	189.73	189.93	0.11	1.07
In House QC Low	Val Day 1	190.12	181.09	-3.64	1.37%	189.73	190.65	0.48	2.04
	Val Day 2	190.12	194.59	2.42	4.05	189.73	187.64	-1.10	1.23
	Val Day 3*	190.12	200.34	5.44	2.48	189.73	187.64	-1.10	1.23
BCR578	Val Day 1	364.99	340.00	-6.85	1.17	368.3	356.34	-3.25	1.11
	Val Day 2	364.99	365.08	0.02	1.7	368.3	356.34	-3.25	1.11
	Val Day 3*	364.99	362.88	-1.28	2.28	368.3	356.34	-3.25	1.11
In House QC High	Val Day 1	367.60	367.80	0.05	1.84	368.3	357.10	-3.04	1.32
	Val Day 2	367.60	382.09	3.94	0.88	368.3	356.34	-3.25	1.11
	Val Day 3*	367.60	370.92	0.90	2.69%	368.3	356.53	-3.20	0.14%

Table 2: Certified Reference Material Data

Product	Project	17 β -Estradiol (pg/mL)				Estrone (pg/mL)			
		Prep. Conc.	Mean Assay Conc.	% Diff.	% RSD	Prep. Conc.	Mean Assay Conc.	% Diff.	% RSD
E-120	Val Day 1	10.01	11.16	11.51	2.26	10.00	11.21	12.11	1.13
	Val Day 2	10.01	11.43	14.23	1.29	10.00	11.14	11.41	0.95
	Val Day 3*	10.01	11.31	13.03	1.96	10.00	11.28	12.82	0.21
E-121	Val Day 1	20.02	21.30	5.52	1.41	19.99	21.88	9.46	0.49
	Val Day 2	20.02	21.86	9.20	3.83	19.99	21.83	9.19	1.01
	Val Day 3*	20.02	21.61	7.92	1.96	19.99	22.02	10.13	0.38
E-122	Val Day 1	100.1	107.68	7.57	1.22	99.95	103.60	3.65	3.18
	Val Day 2	100.1	105.12	5.01	3.23	99.95	107.58	7.63	0.98
	Val Day 3*	100.1	105.42	5.32	2.01	99.95	106.04	6.09	2.01
E-123	Val Day 1	500.5	502.40	0.38	0.61	499.7	527.82	5.63	0.21
	Val Day 2	500.5	504.55	0.81	0.19	499.7	526.21	5.31	0.48
	Val Day 3*	500.5	510.75	2.05	0.86	499.7	523.23	4.71	1.21
E-124	Val Day 1	1001.0	997.86	-0.31	1.00	999.5	1050.6	5.11	0.51
	Val Day 2	1001.0	985.84	-1.51	1.31	999.5	1066.3	6.69	0.56
	Val Day 3*	1001.0	1019.0	1.79	0.50	999.5	1063.4	6.39	0.91
Interday			1000.9	-0.01	1.67		1060.1	6.06	0.79

Accelerated Stability Studies

- Accelerated stability studies were performed on products; E-120, E-122 and E-124 at freezer (-20°C), refrigerator (4-8°C), ambient (20°C) and elevated (30°C) temperatures.
- Samples were held at each temperature for 3 days, one week, two weeks, four weeks and eight weeks.
- All samples were analyzed along with unstressed control samples.

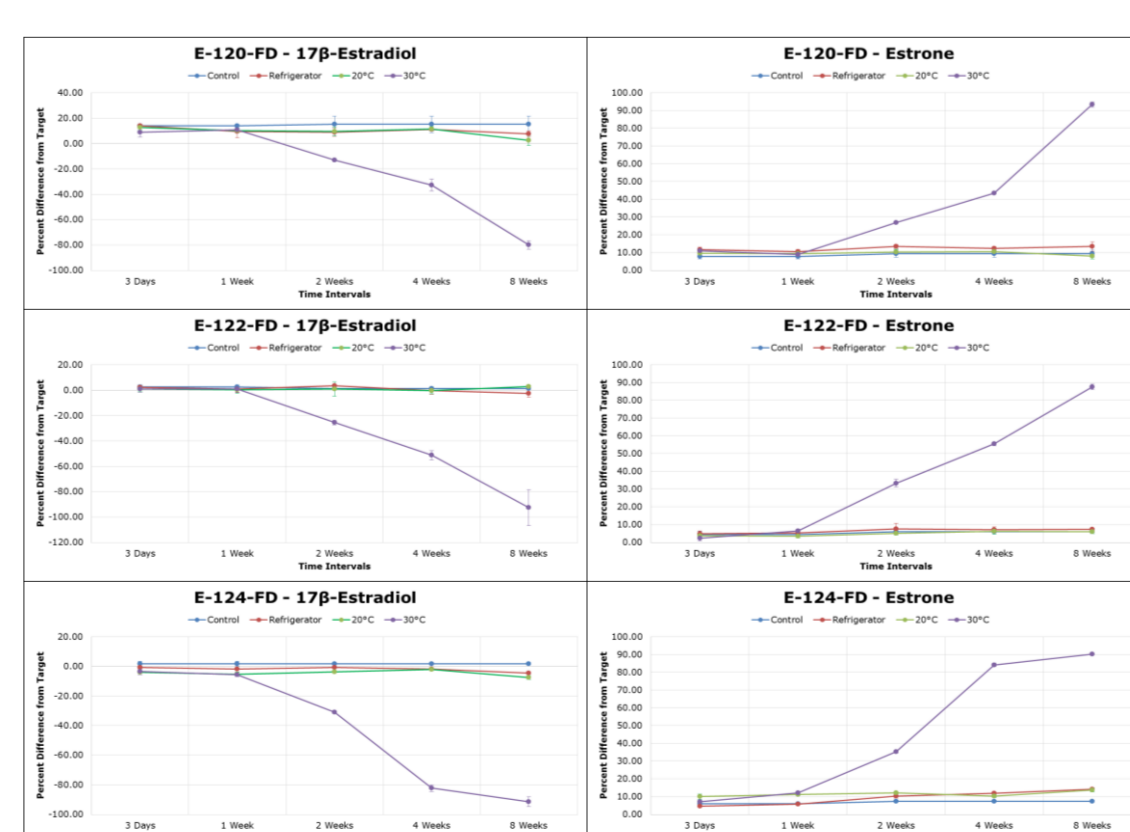


Figure 4: Temperature Stress Studies E-120, E-122 and E-124

- The products are stable when stored at 20°C or below.
- Data shows that at 30°C substantial amounts of 17 β -Estradiol are converted to Estrone.

Performance of Estrogen CRMs in Matrix

- The purpose of this study was to assess performance of the Estrogen CRMs for quantitation of estrogens in real serum samples.
- A method for quantitation of estrogens in serum/matrix samples was developed and validated.
- The range for estrogens in this method is 10-1000 pg/mL using the Estrogen CRMs in Sigmatrix Ultra™ (E-500 through E-504) as calibrators (Figure 5).
- BCR576, BCR577 and serum samples obtained from the Centers for Disease Control (CDC) were used to assess performance of the Estrogen CRMs.
- Results for 17 β -Estradiol are comparable and consistent with the values provided in the Certificates of Analysis for the serum samples (Table 3).
- This study indicates that the Estrogen CRMs are suitable for quantitation of 17 β -Estradiol in serum samples and confirms the accuracy of the value assignment method AMP5001 results.

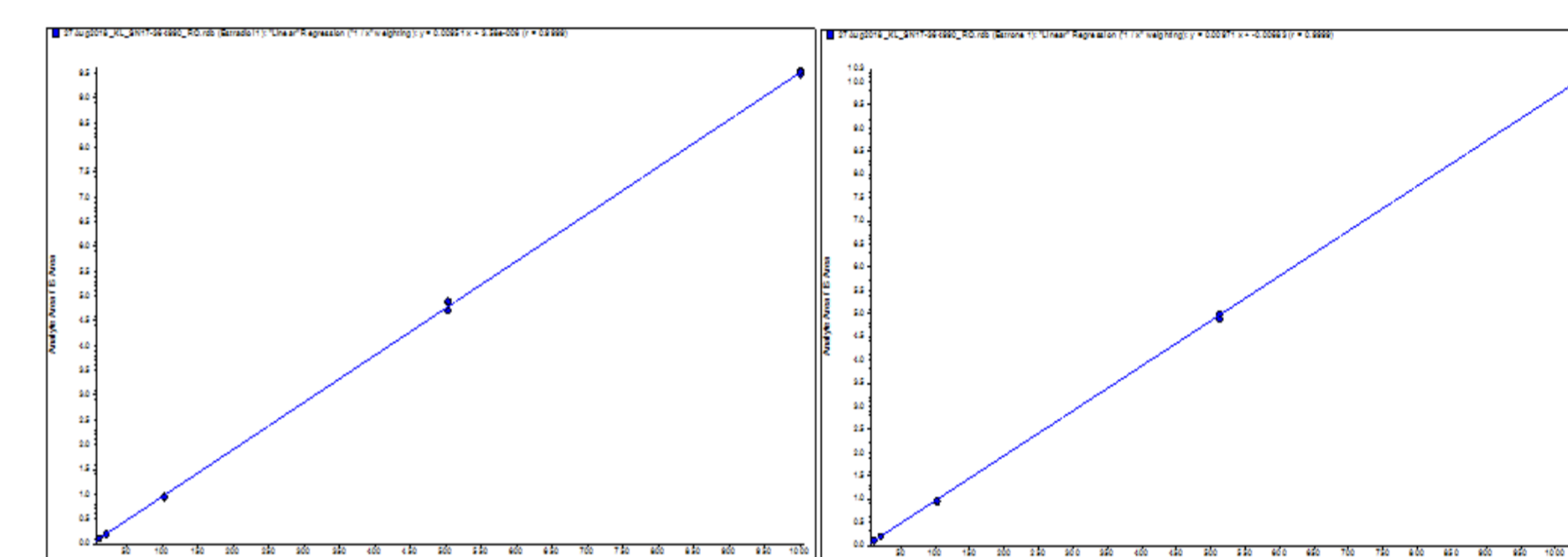
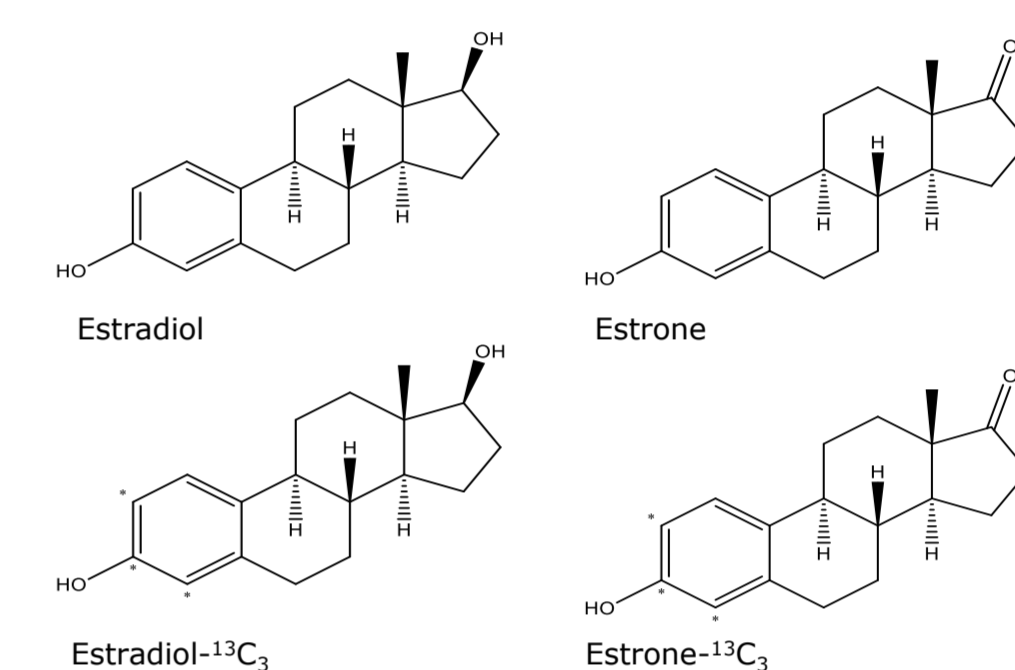


Figure 5: Performance of CRMs in matrix as calibrators in LCMS assay

Table 3: CRM Product Performance Data

	CDC samples			
	17 β -Estradiol		Estrone	
	Low	High	Low	High
Mean (pg/mL)	39.72 \pm 1.00	154.19 \pm 2.93	44.59 \pm 0.29*	85.00 \pm 1.01*
%RSD	2.53	1.90	0.67	1.19
CDC Value (%CV)	40.80 (1.5%)	169.7 (0.8%)	NA	NA
% Diff. to CDC Value	-2.67	-9.14	NA	NA
	BCR-Samples			
	17 β -Estradiol		Estrone	
	BCR576	BCR577	BCR576	BCR577
Mean (pg/mL)	29.45 \pm 0.65	174.76 \pm 2.62	39.70 \pm 0.57	48.77 \pm 0.78
%RSD	2.22	1.50	1.44	1.59
CoA Value	30	188	NA	NA
% difference to CoA	-1.84	-7.04	NA	NA

Conclusions

- Developed matrix based Estrogen Certified Reference Materials (CRMs) for Estrogens.
- Manufactured and certified under ISO17034 and ISO/IEC17025 and stability of product was established.
- Developed and validated LC-MS/MS method to support product certification.
- Sigmatrix Ultra, a surrogate matrix, is a good alternative to stripped serum for calibrator preparation.
- Performance of the CRMs was established by comparison to serum controls.