Appendix VII

Analytical Chemistry Support Report

SUMMARY REPORT OF THE ANALYTICAL CHEMISTRY SUPPORT PROVIDED BY THE NCTR DIVISION OF BIOCHEMICAL TOXICOLOGY – CHEMISTRY SUPPORT GROUP FOR NTP EXPERIMENT 2190

Prepared by F. Michelle McLellen
And Matthew S. Bryant, Ph.D.
Division of Biochemical Toxicology
National Center for Toxicological Research

For

K. Barry Delclos, Ph.D.
Principal Investigator for NCTR Experiment 2190
National Center for Toxicological Research

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APPENDICES:

1. Test Articles

The test article was bisphenol A (synonyms: 2, 2-bis(4-hydroxyphenyl)propane; 4, 4'-isopropylidenediphenol or BPA, CAS # 80-05-7) and was supplied by TCI America (Portland, OR) as Lot #6052012, with a purity assessment on the Certificate of Analysis by Battelle, Inc. (Columbus, OH) of 99.9%. The inventory and chain of custody documentation for this compound were maintained by the Diet Preparation group. This lot of BPA was purchased by NTP in 2009 and was characterized for identity and purity by proton NMR and high performance liquid chromatography with photodiode array detection (HPLC-PDA) at NCTR prior to the start of the sub-chronic BPA toxicity study (NCTR E2176.01). Battelle Laboratories later extensively characterized this same lot of BPA and reported the analysis to be consistent with the manufacturer's stated purity of 99.9% (Bulk Chemical Limited Analysis Report: Bisphenol A, Battelle Project No. G005430-DSU, October 29, 2010).

The reference estrogen control test article (Ethinyl estradiol, E4876, CAS #57-63-6) was supplied by Sigma-Aldrich Corporation (St. Louis, MO) as lot number 071M1492V and had a stated purity of > 98%. A sample of this test article was evaluated by HPLC with PDA and electrospray mass spectrometry and found to contain a single peak that contained fragment ions consistent with and matching a reference sample of ethynyl estradiol from Steraloids (See Appendix 15).

For the present study, Battelle air milled the same lot of BPA (used for NCTR E2176.01) prior to shipment to NCTR and provided a current statement of purity to NCTR. The purity assessment was conducted at NCTR at intervals during the study and after study completion, using the technique of HPLC-PDA (spectral purity). Data from these purity checks of the BPA test article and EE2 reference standard are shown in Tables 1 and 2.

For the spectral purity analysis, a sample of the test article was subjected to HPLC separation at least in triplicate using chromatography developed for dose certification work (Appendix 5). The PDA was scanned from 200 to 400 nm. A single major peak was obtained at the expected HPLC retention time for BPA and showed 99-100% purity (Table 1). EE2 was examined in the same manner and also showed purity above 99% (Table 2).

Table 1. Spectral Purity of BPA by HPLC-PDA*

Analysis Date	Sampling Date	SCR #	Lot #	Purity (% of Peak Area)	Average Purity (%)
07/23/2012	07/22/2012	2190 99 00011	6052012 (Bucket #1)	99.23	99.25
				99.25	
				99.20	
				99.30	
02/14/2013	02/07/2013	2190 99 00070	6052012 (Bucket #1)	99.43	99.49
				99.56	
				99.48	
02/28/2014	02/21/2014	2190 99 00265	6052012 (Bucket #1)	99.97	99.99
				99.99+	
				99.99+	
01/21/2015	01/21/2015	2190 99 00328	6052012 (Bucket #1)	100	100
				100	
				100	

*BPA dissolved in methanol at a concentration of 0.5 or 1 mg/ml. Injections (20ul) onto Novapak C18 5um 3.9x150mm column; PDA at 210 to 600 nm,

Table 2. Spectral Purity of EE2 by HPLC-PDA*

Analysis Date	Sampling Date	SCR #	Lot #	Purity (% of Peak Area)	Average Purity (%)
05/11/2012	05/12/2012	Not applicable	071M1492V	98.47	98.59
				98.81	
				98.80	
				98.29	
02/12/2013	02/012/2013	Not applicable	071M1492V	99.96	99.72
				99.35	
				99.59	
				99.98	
01/21/2015	01/21/2015	Not applicable	071M1492V	100	100
				100	
				100	

*EE2 dissolved in methanol at a concentration of 0.5 or 1 mg/ml. Injections (20ul) onto Novapak C18 5um 3.9x150mm column; PDA at 210 to 800 nm or 210 to 600 nm,

Stable isotope-labeled BPA (d6-BPA, Catalog D-2476, Lot X195P1, 99.4% atom%) was obtained from CDN Isotopes (Pointe-Claire, Quebec) for evaluating internal doses at low BPA dose levels (2.5, 25, and 250 ug /kg body weight/day). Compound purity was verified by liquid chromatography with ultraviolet detection and full scan liquid chromatography-electrospray mass spectrometry in the Division of Biochemical Toxicology, NCTR. The dose vehicle excipient carboxymethylcellulose (sodium salt; Catalog C5013, Lot 041M0105V) was obtained from Sigma-Aldrich (St. Louis, MO).

2. Dose Certification of BPA Dose Preparations

On a regular basis during the study, dose certification analyses were performed for samples of BPA in 0.3% Carboxymethylcellulose prepared and supplied by Diet Preparation. Samples were prepared for analysis by DBT/CHEM SOP 547 or 548 (Appendices 4 and 5) and were assayed for BPA content by HPLC-UV or submitted to the DBT Mass Spectrometry laboratory for HPLC-MS analysis (NCTR MSL 17; Appendix 7). Corrections were made for actual sample weights taken for analysis. Samples were assayed at least in triplicate and concentration results reported in µg/ml and % of target.

Table 3. Dose certification of BPA concentrations in 0.5% CMC formulations

SCR Date	SCR#	Dose Level (µg/kg)	Target Dose Conc (µg/ml)	Mean (µg/ml)	% CV	% of Target
8/8/2012	2190 99 00018	0	0	<LOQ*	-	-
8/8/2012	2190 99 00013	2.5	0.5	0.493	1.7	99.3
8/8/2012	2190 99 00014	25	5	5.22	4.6	104
8/8/2012	2190 99 00015	250	50	50.6	0.9	101
8/9/2012	2190 99 00019	2,500	500	457	5.5	91.5
8/9/2012	2190 99 00020	25,000	5,000	4670	6.8	93.4
8/9/2012	2190 99 00031	250,000	50,000	47300	1.2	94.7
10/3/2012	2190 99 00041	0	0	<LOQ*	-	-
10/3/2012	2190 99 00042	2.5	0.5	0.491	2.8	98.1
10/3/2012	2190 99 00043	25	5	4.52	0.3	90.4
10/3/2012	2190 99 00044	250	50	46.9	1.0	93.9
10/16/2012	2190 99 00047	2,500	500	488	0.9	97.6
10/16/2012	2190 99 00048	25,000	5,000	5,024	1.2	100.5
10/16/2012	2191 99 00032 (was 49)**	250,000	50,000	45,600	1.1	91.2
12/4/2012	2190 99 00050	0	0	<LOQ*	-	-
12/4/2012	2190 99 00051	2.5	0.5	0.481	0.4	96.2
12/4/2012	2190 99 00052	25	5	4.87	2	97.5
12/4/2012	2190 99 00053	250	50	54.5	0.4	109
12/4/2012	2190 99 00056	2,500	500	512	1.4	102
12/4/2012	2190 99 00057	25,000	5,000	5,102	3.2	102
2/4/2013	2190 99 00071	0	0	<LOQ*	-	-
2/4/2013	2190 99 00063	2.5	0.5	0.481	0.4	96.2
2/4/2013	2190 99 00064	25	5	4.56	0.004	91.2
2/4/2013	2190 99 00065	250	50	48.5	0.03	96.9

Table 3. Dose certification of BPA concentrations in 0.5% CMC formulations						
2/4/2013	2190 99 00066	2500	500	477	3.5	95.5
2/4/2013	2190 99 00067	25,000	5,000	4619	0.02	92.3
4/9/2013	2190 99 00085	0	0	<LOQ*	-	-
4/9/2013	2190 99 00086	2.5	0.5	0.477	4.5	95.4
4/9/2013	2190 99 00087	25	5	4.76	2.4	95.2
4/9/2013	2190 99 00088	250	50	51.6	0.5	103
4/9/2013	2190 99 00089	2,500	500	498	2.9	99.5
4/9/2013	2190 99 00090	25,000	5,000	5,138	3.1	103
5/10/2013	2190 99 00096	0	0	<LOQ*	-	-
5/20/2013	2190 99 00099	2.5	0.5	0.454	7.5	90.8
5/20/2013	2190 99 00100	25	5	4.93	7.3	98.6
5/20/2013	2190 99 00161	250	50	52.3	3.3	105
5/21/2013	2190 99 00162	2,500	500	515	4.3	103
5/21/2013	2190 99 00163	25,000	5,000	5,219	1.3	104
8/6/2013	2190 99 00173	0	0	<LOQ*	-	-
8/6/2013	2190 99 00176	2.5	0.5	0.468	3	93.6
8/6/2013	2190 99 00177	25	5	5.37	2	107
8/6/2013	2190 99 00178	250	50	49.5	1.1	99.1
8/6/2013	2190 99 00181	2,500	500	478	2.9	95.7
8/6/2013	2190 99 00182	25,000	5,000	4,652	0.5	93
10/23/2013	2190 99 00186	0	0	<LOQ*	-	-
10/23/2013	2190 99 00191	2.5	0.5	0.451	2.2	90.1
10/23/2013	2190 99 00192	25	5	4.95	7.2	99
10/23/2013	2190 99 00193	250	50	52.3	5.8	105
10/23/2013	2190 99 00187	2,500	500	513	11	103
10/23/2013	2190 99 00188	25,000	5,000	5,206	8.6	104
12/13/2013	2190 99 00256	0	0	<LOQ*	-	-
12/11/2013	2190 99 00199	2.5	0.5	0.494	1.8	98.4
12/11/2013	2190 99 00200	25	5	4.64	0.37	92.7
12/11/2013	2190 99 00253	250	50	48.4	4	96.8
12/11/2013	2190 99 00254	2,500	500	492	3.7	98.5
12/11/2013	2190 99 00255	25,000	5,000	4870	4	97.4
1/27/2014	2190 99 00257	0	0	<LOQ*	-	-
2/11/2014	2190 99 00260	2.5	0.5	0.521	0.7	106
2/11/2014	2190 99 00261	25	5	5.16	2.10	103.2
2/11/2014	2190 99 00262	250	50	47.49	3.6	95.0
2/11/2014	2190 99 00263	2,500	500	483.14	0.8	96.6
2/11/2014	2190 99 00264	25,000	5,000	4948	2.7	99.0
4/9/2014	2190 99 00269	0	0	<LOQ*	-	-
4/9/2014	2190 99 00272	2.5	0.5	0.469	2	92
4/9/2014	2190 99 00273	25	5	5.17	0.7	103
4/9/2014	2190 99 00274	250	50	50.5	2.6	101
4/9/2014	2190 99 00270	2,500	500	473	4.3	94.5
4/9/2014	2190 99 00280	25,000	5,000	5260	6.6	105
05/29/2014	2190 99 00281	0	0	<LOQ*	-	-
6/4/2014	2190 99 00284	2.5	0.500	0.488	0.8	96.9
6/4/2014	2190 99 00285	25	5.00	4.67	1.1	93.5
6/4/2014	2190 99 00286	250	50.0	50.5	0.3	101
6/4/2014	2190 99 00282	2,500	500	467	2.3	93.3
6/4/2014	2190 99 00283	25,000	5,000	4988	3.9	99.8

Table 3. Dose certification of BPA concentrations in 0.5% CMC formulations						
7/9/2014	2190 99 00292	0	0	<LOQ*	-	-
8/8/2014	2190 99 00294***	0	0.00	0.00*	-	-
8/11/2014	2190 99 00295	2.5	0.500	0.470	2.6	93.6
8/11/2014	2190 99 00296	25	5.00	4.63	1.6	92.6
8/11/2014	2190 99 00297	250	50.0	50.9	2.5	101.9
8/11/2014	2190 99 00298	2,500	500	528	3.7	105.6
8/11/2014	2190 99 00299	25,000	5,000	5082	4.1	101.6
9/23/2014	2190 99 00304	0	0	<LOQ*	-	-
10/7/2014	2190 99 00307	2.5	0.500	0.470	2.6	93.6
10/7/2014	2190 99 00308	25	5.00	4.73	2.5	94.7
10/7/2014	2190 99 00309	250	50.0	47.6	3.8	95.1
10/7/2014	2190 99 00310	2,500	500	499	0.8	99.9
10/7/2014	2190 99 00311	25,000	5,000	4732	1.9	94.6
12/8/2014	2190 99 00320	0	0.00	0.00	-	-
12/8/2014	2190 99 00321	2.5	0.500	0.467	3.6	92.5
12/8/2014	2190 99 00322	25	5.00	4.75	2.5	95.0
12/8/2014	2190 99 00323	250	50	47.1	0.5	94.1
12/8/2014	2190 99 00324	2,500	500	465	3.7	93.1
12/8/2014	2190 99 00325	25,000	5,000	4625	1.8	92.5

***<**LOQ = less than limit of quantitation (no peak detected or was well below lowest analytical standard)
********The highest dose of 50,000 ug/ml was used only for the NTP study E02191 and was conducted concurrently to the E02190 study. All other dose preparations were shared between the two studies and were designated with identifications for the E02190 study. All data associated with E02191 is included in this report for E02190.
***A trace amount of signal was observed in the BPA channel for one of the sample replicates, but was not observed in the other three.

Table 4. Summary Statistics for BPA Dose Certification Results.					
Dose Level (µg/kg)	Target Dose Conc (µg/ml)	Mean Measured Value (µg/ml)	% CV	Minimum Result(µg/ml)	Maximum Result(µg/ml)
0	0	0.000	__	0.000	0.000
2.5	0.5	0.478	3.7	0.451	0.521
25	5	4.86	5.4	4.52	5.37
250	50	49.9	4.5	47.1	54.5
2500	500	490	4.3	457	528
25000	5000	4942	4.7	4619	5260
250000	50000	46450	2.6	45600	47300

3. Dose Verification of BPA Dose Preparations

On a regular basis during the study, dose verification analyses were performed for samples of BPA in 0.3% Carboxymethylcellulose that were obtained from the animal rooms at the end of their use. Samples were prepared for analysis by DBT/CHEM SOP 547 or 548 (Appendices 4 and 5) and were assayed for BPA content by HPLC-UV or submitted to the DBT Mass Spectrometry laboratory for HPLC-MS analysis (NCTR MSL 17; Appendix 7). Corrections were made for actual sample weights taken for analysis. Samples were assayed at least in triplicate and concentration results reported in µg/ml and % of target. Dose verification results for BPA solutions are contained in Table 5 and summarized in Table 6.

Table 5. Dose verification of BPA concentrations in 0.3% CMC formulations

SCR Date	SCR#	Dose Level (µg/kg)	Target Dose Conc (µg/ml)	Mean (N=3) Result (µg/ml)	% CV	% of Target
9/10/2012	2190 99 00133	2.5	0.5	0.503	2.7	101
9/24/2012	2190 99 00138	25	5	4.96	1.2	99.2
9/10/2012	2190 99 00134	250	50	52.3	0.8	105
9/26/2012	2190 99 00141	250	50	53.4	2.1	107
9/24/2012	2190 99 00139	2,500	500	496	1	99.2
9/24/2012	2190 99 00140	25,000	5000	5029	2.2	101
9/26/2012	2191 99 00011	250,000	50000	47700	1.5	95.5
3/26/2013	2190 99 00150	0	0	<LOQ*	-	-
3/26/2013	2190 99 00151	2.5	0.500	0.448	4.7	89.5
3/26/2013	2190 99 00152	25	5	4.68	0.6	93.7
3/26/2013	2190 99 00153	250	50	49.05	2.1	98.1
3/26/2013	2190 99 00154	2,500	500	504.41	1.9	100.9
3/26/2013	2190 99 00155	25,000	5,000	4698.13	1.2	94.0
10/28/2013	2190 99 00208	0	0	<LOQ*	-	-
10/28/2013	2190 99 00209	2.5	0.5	0.463	4	92.6
10/28/2013	2190 99 00210	25	5	5.28	0.7	106
10/28/2013	2190 99 00211	250	50	50.65	0.6	101.3
10/28/2013	2190 99 00212	2,500	500	488	0.1	97.6
10/28/2013	2190 99 00213	25,000	5,000	5,334	0.8	107
3/31/2014	2190 99 00224	0	0	0.002	200	-
3/31/2014	2190 99 00225	2.5	0.5	0.489	4.3	97.8
3/31/2014	2190 99 00226	25	5	4.74	1.5	94.9
3/31/2014	2190 99 00227	250	50	51.5	1.7	103
3/31/2014	2190 99 00228	2,500	500	462	1.8	92.3
3/31/2014	2190 99 00229	25,000	5,000	5,140	5	103
9/18/2014	2190 99 00247	0	0	0.002	173	-
9/18/2014	2190 99 00246	2.5	0.5	0.446	1.4	89.2
9/22/2014	2190 99 00248	25	5	4.55	1.1	91
9/22/2014	2190 99 00249	250	50	51.3	0.5	103
9/22/2014	2190 99 00250	2,500	500	499	0.1	99.9

Table 5. Dose verification of BPA concentrations in 0.3% CMC formulations						
9/22/2014	2190 99 00351	25,000	5,000	4,560	0.9	91.2
1/14/2015	2190 99 00356	0	0	<LOQ*	-	-
1/14/2015	2190 99 00357	2.5	0.5	0.5	1	100
1/14/2015	2190 99 00358	25	5	4.71	1.4	94.2
1/14/2015	2190 99 00359	250	50	47.4	1.3	94.7
1/14/2015	2190 99 00360	2,500	500	482	2.6	96.3
1/14/2015	2190 99 00361	25,000	5,000	5,246	0.3	105

***<**LOQ = less than limit of quantitation (no peak detected or was well below lowest analytical standard)

Table 6. Summary Statistics for BPA Dose Verification Results:					
Dose Level (µg/kg)	Target Dose Conc (µg/ml)	Mean Measured Value (µg/ml)	% CV	Minimum Result(µg/ml)	Maximum Result(µg/ml)
0	0	0.000	-	0.000	0.000
2.5	0.5	0.475	5.4	0.446	0.503
25	5	4.82	5.4	4.55	5.28
250	50	50.8	4.0	47.4	53.4
2,500	500	489	3.1	462	504
25,000	5,000	5001	6.2	4560	5334
250,000	50,000	47700	-	47700	47700

4. Dose Certification, Verification and Accuracy of EE2 Dose Preparations

On a regular basis during the study, dose certification analyses were performed for samples of EE2 in 0.3% carboxymethylcellulose prepared and supplied by Diet Preparation from stock solutions provided by DBT/Chemistry. Samples were prepared for analysis by DBT/CHEM SOP 547 or 548 (Appendices 4 and 5) and were assayed for EE2 concentration by HPLC/MS/MS analysis by the DBT Mass Spectrometry laboratory (NCTR MSL 16; Appendix 6). Corrections were made for actual sample weights taken for analysis. Samples were assayed at least in triplicate and concentration results reported in µg/ml and % of target. Dose certification results for the two EE2 solutions are summarized in Tables 7A and 8A.

On a regular basis during the study, dose verification analyses were also performed for samples of EE2 in 0.3% Carboxymethylcellulose that were obtained from the animal rooms at the end of their use. These results are summarized in Tables 7B and 8B.

In addition to extensive dose accuracy studies conducted for E02176, similar dose accuracy analyses were conducted for the current study (7/7/2012) to document that EE2 doses delivered by gavage pump were achieving the target levels in both adult animals and pups. Samples were also collected utilizing positive displacement pipette for comparative purposes. Samples were prepared for analysis using DBT/CHEM SOP 548 (Appendix 5) and assayed for EE2 concentration by HPLC/MS/MS by the DBT Mass Spectrometry Laboratory (NCTR MSL 16: Appendix 6). The results of this work (Table 8C) confirmed the accuracy of the delivery pumps.

Table 7A. Dose Certification of EE2 (0.01 µg/ml) in 0.3% CMC

Expiration Date of Solution	SCR #	EE2 Concentration (µg/ml)	% CV
9/11/2012	2190 99 000 17	0.01000	4.4
11/22/2012	2190 99 000 45	0.00907	2.1
1/23/2013	2190 99 00054	0.00980	7.7
3/26/2013	2190 99 00068	0.00970	0.7
5/28/2013	2190 99 00091	0.00954	7.9
7/9/2013	2190 99 00097	0.00932	4.8
9/24/2013	2190 99 00179	0.01034	2.0
12/11/2013	2190 99 00189	0.00989	3.7
1/29/2014	2190 99 00197	0.01021	5.1
4/1/2014	2190 99 00258	0.01009	3.8
5/28/2014	2190 99 00275	0.00901	4.3
7/24/2014	2190 99 00287	0.00912	2.4
9/30/2014	2190 99 00301	0.01005	5.2
10/7/2014	2190 99 00317	0.00996	5.7
1/27/2015	2190 99 00326	0.00928	4.6
	Average =	**0.00969**	**4.5**
	Minimum =	**0.00901**	
	Maximum =	**0.01034**	

Table 7B. Dose Verification of EE2 (0.01 µg/ml) in 0.3% CMC

Expiration Date of Solution	SCR #	Mean (N=3) EE2 Concentration (µg/ml)	% CV
09/10/2012	2190 99 00135	0.00996	6.5
03/26/2013	2190 99 00156	0.00869	6.0
10/28/2013	2190 99 00214	0.00979	3.1

Table 7B. Dose Verification of EE2 (0.01 µg/ml) in 0.3% CMC			
03/31/2014	2190 99 00230	0.00937	2.1
09/15/2014	2190 99 00244	0.00895	1.4
01/14/2015	2190 99 00362	0.00933	7.4

Table 8A. Dose Certification of EE2 (0.10 µg/ml) in 0.3% CMC			
Expiration Date of Solution	**SCR #**	**EE2 Concentration (µg/ml)**	**% CV**
9/11/2012	2190 99 00016	0.10600	1.7
11/22/2012	2190 99 00046	0.09558	2.3
1/23/2013	2190 99 00055	0.09560	2.1
3/26/2013	2190 99 00069	0.09380	2.2
5/28/2013	2190 99 00092	0.09130	4.1
7/9/2013	2190 99 00098	0.09480	5.4
9/24/2013	2190 99 00180	0.09770	6.4
12/11/2013	2190 99 00190	0.09850	1.7
1/29/2014	2190 99 00198	0.09773	3.7
4/1/2014	2190 99 00259	0.09414	9.1
5/28/2014	2190 99 00276	0.09692	1.7
7/24/2014	2190 99 00288	0.09842	1.5
9/30/2014	2190 99 00302	0.10787	4.9
12/5/2014	2190 99 00319	0.09540	6.4
1/27/2015	2190 99 00327	0.10178	2.1
	Average =	**0.09770**	**4.6**
	Minimum =	0.0913	
	Maximum =	0.10787	

Table 8B. Dose Verification of EE2 (0.10 µg/ml) in 0.3% CMC			
Expiration Date of Solution	**SCR #**	**Mean (N=3) EE2 Concentration (µg/ml)**	**% CV**
09/10/2012	2190 99 00136	0.100	2.6
03/26/2013	2190 99 00157	0.09340	3.9
10/28/2013	2190 99 00215	0.09547	2.3
03/31/2014	2190 99 00231	0.08524	7.4
09/15/2014	2190 99 00245	0.09079	5.4
01/14/2015	2190 99 00363	0.10175	5.0

Table 8C. Analysis of Dose Accuracy Samples for EE2 (0.01 µg/ml)				
SCR #	**Sample ID**	**Measured EE2 Conc (µg/ml)**	**% of Target**	**Mean (N=3) % of Target**
2190 99 00117	Adult (Pipet)	0.0100	100	99.2
2190 99 00118		0.0098	97.8	
2190 99 00119		0.0100	99.9	
2190 99 00120	Adult (Pump)	0.0086	86.4	87.2
2190 99 00121		0.0089	88.7	
2190 99 00122		0.0086	86.4	
2190 99 00123	Pup (Pipet)	0.0095	95.3	97.0
2190 99 00124		0.0094	94.3	
2190 99 00125		0.0101	101	
2190 99 00126	Pup (Pump)	0.0087	87.0	93.7
2190 99 00127		0.0084	83.9	
2190 99 00128		0.0110	110	

5. Stability and Homogeneity of BPA and EE2 Dosing Solutions

The stability of BPA and EE2 dose solutions at room temperature was evaluated to confirm the frequency of preparation during the study. A low and high dose concentration was tested for each test article. The initial solutions were prepared June 5, August 8 and August 9, 2012 by Diet Preparation and are designated as Day 1. The solution was stored at room temperature in Diet Preparation and aliquots were delivered periodically to DBT/Chemistry for quantitative analysis to determine stability. Samples were prepared for analysis by DBT/CHEM SOP 547 or 548 (See Appendices 4 and 5) and submitted to the DBT Mass Spectrometry laboratory for HPLC-MS/MS analysis (NCTR MSL 16 and 17;Appendices 6 and 7). Corrections were made for actual sample weights taken for analysis. Samples were assayed at least in triplicate and concentration results reported in µg/ml and % of target. The stability study indicates that EE2 and BPA are stable in 0.3% CMC for up to 50 days after preparation at the concentration range utilized for the experiment.

Homogeneity analyses of select BPA and EE2 dose formulations was conducted for concentrations not already covered by an earlier BPA study (E02176). After preparation of the BPA suspension (5,000 µg/ml) via stirring overnight in stainless steel mixing vessel, nine independent samples were taken for analysis using a positive displacement pipet. Comparison of the measured concentrations of BPA in these samples (Table 12B) indicate that the suspension was homogeneous.

Similar analyses were conducted for the EE2 solutions utilized in this study. After preparation of the EE2 solutions (0.01 and 0.10 µg/ml in 0.3% CMC), three independent samples were taken from the top,

middle and bottom of the solution. Comparison of the mean values for the top, middle and bottom indicate that these solutions were homogeneous (Table 12C).

Table 9. Stability of Low Dose (0.01 µg/ml) EE2 Solution in 0.3% CMC

SCR Number	Days of Storage	Overall Mean (N=9 or 10) EE2 Conc. (µg/ml)*	Mean Result (% of Target)
2190 99 00001	1	0.0106	106
2190 99 00002	8	0.0103	103
2190 99 00005	15	0.0094	93.7
2190 99 00006	22	0.0098	97.6
2190 99 00008	29	0.0092	92.1
2190 99 00009	36	0.0090	90.2
2190 99 00010	43	0.0108	108
2190 99 00012	50	0.0092	91.6

*Concentrations corrected for actual sample weights

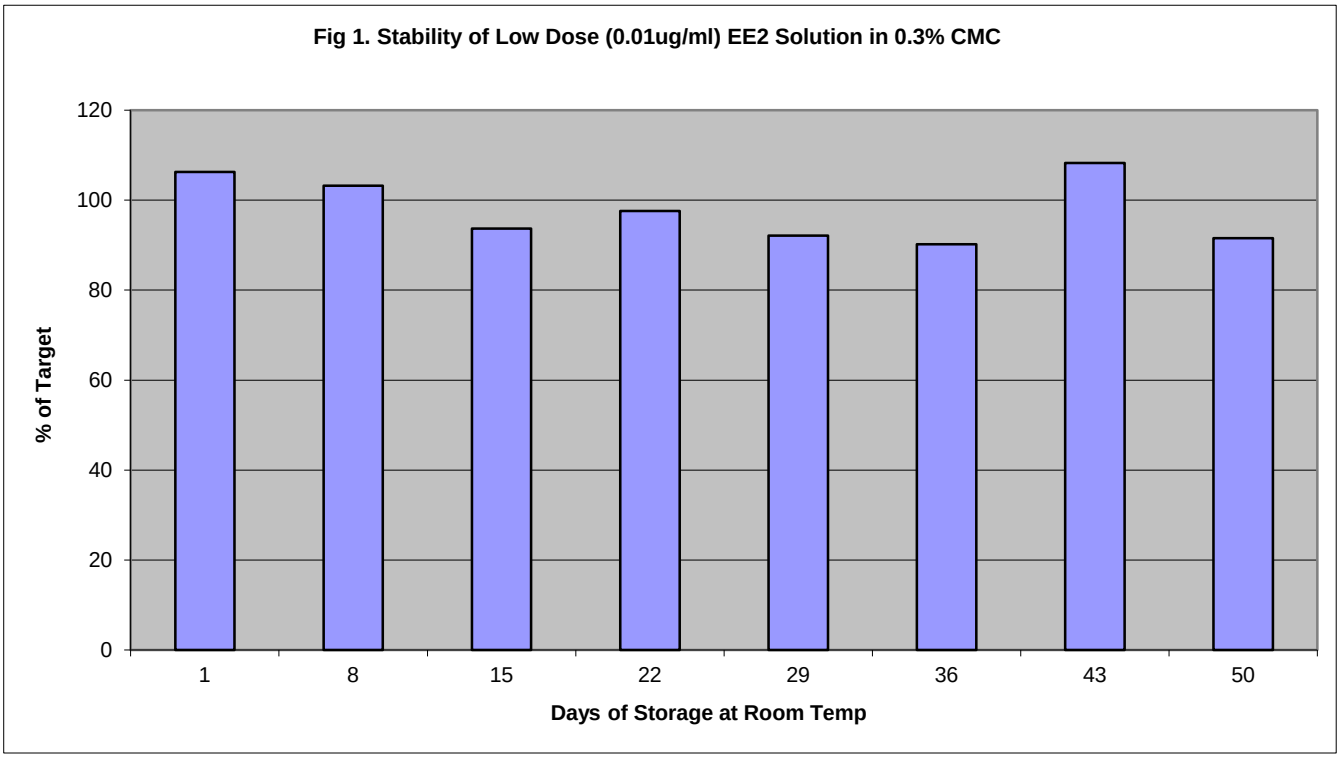


Table 10. Stability of High Dose (0.1 µg/ml) EE2 Solution in 0.3% CMC			
SCR Number	**Days of Storage**	**Overall Mean (N=9 or 10) EE2 Conc. (µg/ml)***	**Mean Result (% of Target)**
2190 99 00016	1	0.106	106.0
2190 99 00024	15	0.107	106.9
2190 99 00028	29	0.106	105.6
2190 99 00031	36	0.0988	98.8
2190 99 00034	43	0.0982	98.2
2190 99 00039	50	0.102	101.8

*Concentrations corrected for actual sample weights

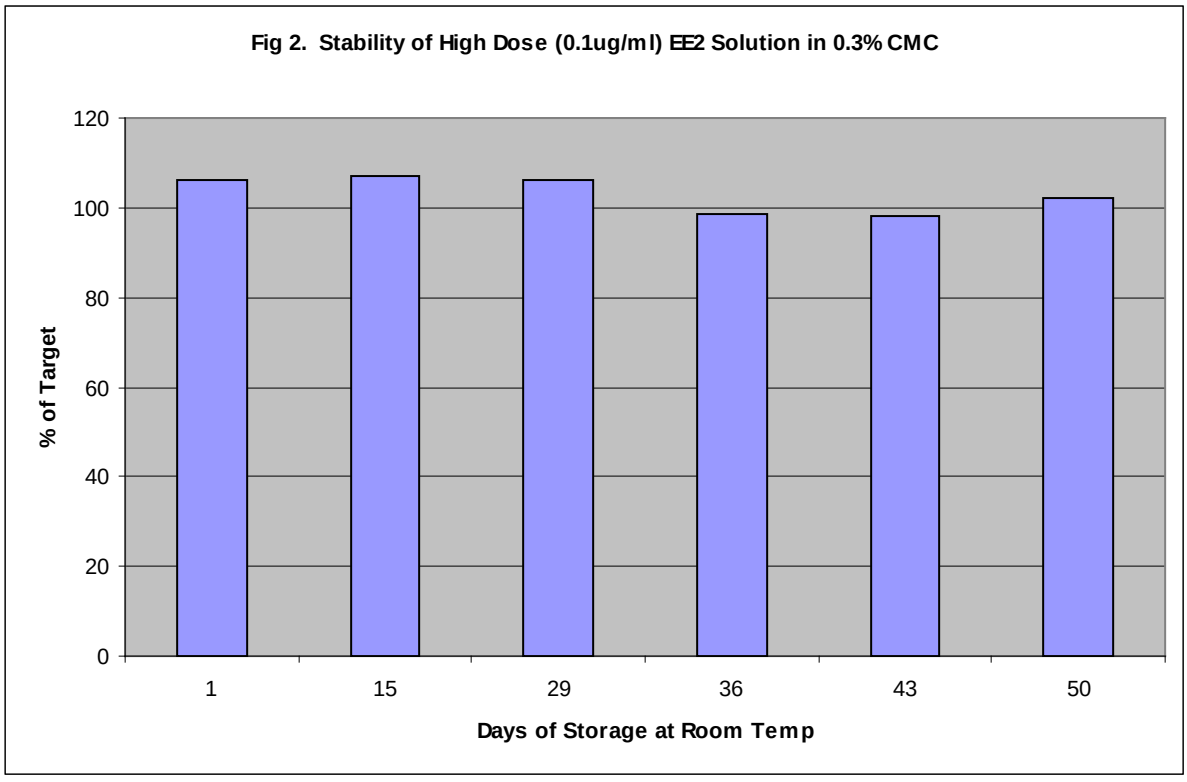


Table 11. Stability of Low Dose (0.5 µg/ml) BPA Solution in 0.3% CMC			
SCR Number	**Days of Storage**	**Overall Mean (N=9) BPA Conc. (µg/ml)***	**Mean Result (% of Target)**
2190 99 00013	1	0.493	99.3
2190 99 00023	15	0.479	95.8
2190 99 00027	29	0.540	107.8
2190 99 00030	36	0.506	101.3
2190 99 00033	43	0.523	104.6
2190 99 00038	50	0.505	101

*Concentrations corrected for actual sample weights

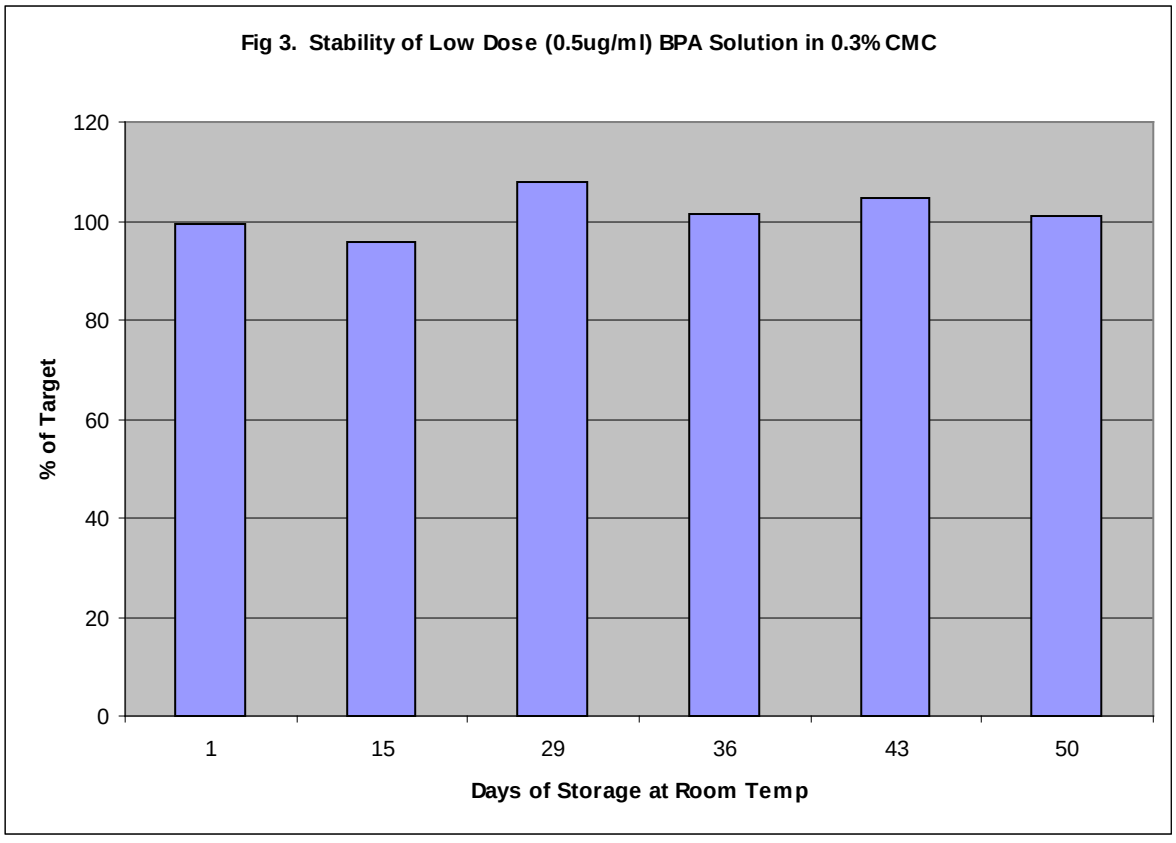


Table 12. Stability of High Dose (5000 µg/ml) BPA Solution in 0.3% CMC			
SCR Number	**Days of Storage**	**Overall Mean (N=3) BPA Conc. (µg/ml)***	**Mean Result (% of Target)**
2190 99 00020	1	4670	93.4
2190 99 00026	15	4716 (N=9)	94.3
2190 99 00029	29	5208	104
2190 99 00032	36	5414	108
2190 99 00035	43	5173	103
2190 99 00040	50	5120	102.4

*Concentrations corrected for actual sample weights

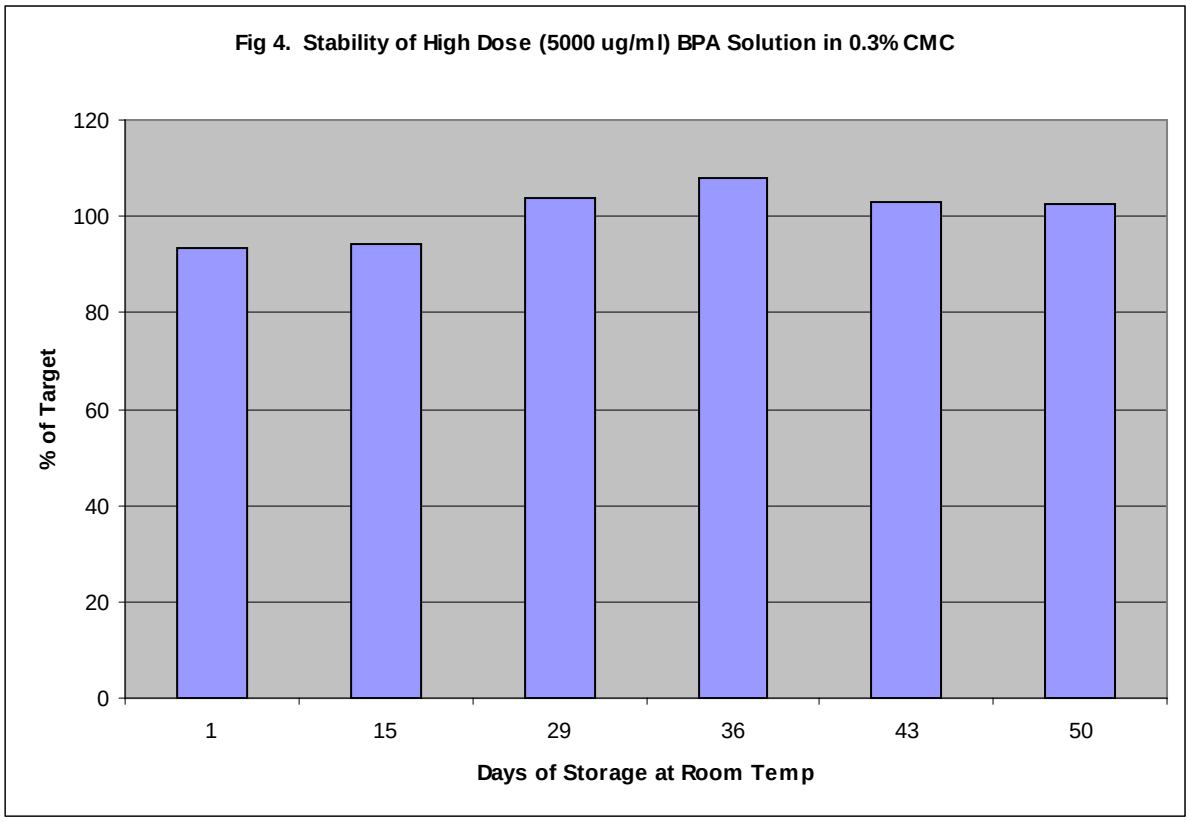


Table 12B. Homogeneity Analysis of High Dose (5,000 µg/ml) BPA Preparation in 0.3% CMC			
SCR Number	Sample Number	Measured BPA Conc. (µg/ml)*	% of Target
2190 99 00022	1	4615	92.3
	2	4537	90.7
	3	4518	90.4
	4	4632	92.6
	5	4549	91.0
	6	4736	94.7
	7	4621	92.4
	8	4665	93.3
	9	4894	97.9
	Mean (N=9)	4641	92.8
	SD(%CV)	117 (2.5)	

*Concentrations corrected for actual sample weights

Table 12C. Homogeneity Analysis of EE2 Solutions in 0.3% CMC			
SCR Number	Sample ID	Mean (N=3) EE2 Conc. (µg/ml)*	% of Target
2190 99 00017	0.01 µg/ml Target Conc. Bottom	0.0103	103
	0.01 µg/ml Target Conc. Middle	0.01004	100.4
	0.01 µg/ml Target Conc. Top	0.00948	94.8
	Mean (%CV) for N=9 Samples	0.00994 (4.2)	99.5
2190 99 00016	0.10 µg/ml Target Conc. Bottom	0.108	108
	0.10 µg/ml Target Conc. Middle	0.106	106
	0.10 µg/ml Target Conc. Top	0.103**	103
	Mean (%CV) for N=8 Samples	0.106 (2.4)	106

*Concentrations corrected for actual sample weights
**N=2 after sample with poor chromatography excluded

6. Assessment of Background BPA levels in 0.3% CMC Solutions

Additional analyses of control 0.3% CMC solutions prepared by Diet Preparation were also conducted. These stock solutions were utilized by Diet Preparation to formulate the dosing solutions at specified concentrations. Samples were prepared for analysis by DBT/CHEM SOP 547 or 548 (Appendices 4 and 5) and submitted to the DBT Mass Spectrometry laboratory for HPLC-MS analysis (NCTR MSL 17; Appendix 7). Corrections were made for actual sample weights taken for analysis. Samples were assayed at least in triplicate and concentration results reported in µg/ml and % of target.

Only one sample (from batch prepared Jan 31, 2013) showed a detectable BPA concentration above the analytical background.

Table 13. Background BPA levels in 0.3% CMC Solutions

SCR#	Solution Preparation Date	Mean (N=3 or 4) Result (µg/ml)	% CV
2190 99 00072	9/4/2012	0	-
2190 99 00073	9/17/2012	0	-
2190 99 00074	10/1/2012	0	-
2190 99 00077	11/25/2012	0	-
2190 99 00078	12/7/2012	0	-
2190 99 00079	12/24/2012	0	-
2190 99 00080	1/18/2013	0	-
2190 99 00081	1/31/2013	0.106	4.5
2190 99 00082	2/25/2013	0	-
2190 99 00083	3/7/2013	0	-
2190 99 00084	3/20/2013	0	-
2190 99 00095	4/30/2013	0	-
2190 99 00096	5/10/2013	0	-
2190 99 00164	5/30/2013	0	-
2190 99 00171	6/20/2013	0	-
2190 99 00172	7/11/2013	0	-
2190 99 00173	8/2/2013	0	-
2190 99 00183	8/20/2013	0	-
2190 99 00184	9/5/2013	0	-
2190 99 00185	9/24/2013	0	-
2190 99 00194	11/13/2013	0	-

Table 13. Background BPA levels in 0.3% CMC Solutions			
2190 99 00196	11/29/2013	0	-
2190 99 00256	12/12 2013	0	-
2190 99 00257	1/27/2014	0	-
2190 99 00300	2/25/2014	0	-
2190 99 00269	3/31/2014	0	-
2190 99 00279	4/16/2014	0	-
2190 99 00281	5/29/14	0	-
2190 99 00292	7/9/14	0	-
2190 99 00294	8/7/14	0	-
2190 99 00304	9/22/14	0	-
2190 99 00320	11/20/14	0	-

7. Assessment of Background BPA Levels in Materials Utilized for the Study

Various materials utilized in the conduct of the study were evaluated for the presence of background levels of the test article BPA to ensure that they would not have an impact on the results of the study. These materials included the drinking water, cages, bedding and feed.

A. Rodent Water

The drinking water utilized for the rodents in the study was periodically assayed for background BPA. These samples were prepared for MS analysis per DBT/CHEM SOP 322 (Appendix 1) and submitted to the Mass Spectrometry Laboratory for LC-ESI-MRM by SOP NCTR MSL-19 (Appendix 9). Duplicate analyses were conducted for each of the four water bottle samples. The mean values (ppb BPA) obtained for each bottle are reported in Table 14. All samples showed BPA levels that were close to or less than the limit of quantitation or limit of blank for the assay.

Table 14. Background BPA levels in rodent drinking water		
SCR Date	**SCR# & Identification**	**BPA Result (ppb)***
7/3/2012	2190 99 00129 (Bottle #1)	< LOQ
	2190 99 00130 (Bottle #2)	< LOQ
	2190 99 00131 (Bottle #3)	< LOQ
	2190 99 00132 (Bottle #4)	< LOQ
12/11/2012	2190 99 00142 (Bottle #1)	0.22
	2190 99 00143 (Bottle #2)	0.005
	2190 99 00144 (Bottle #3)	< LoB
	2190 99 00145 (Bottle #4)	0.39
3/11/2013	2190 99 00146 (Bottle #1)	<LoB
	2190 99 00147 (Bottle #2)	<LoB
	2190 99 00148 (Bottle #3)	< LoB
	2190 99 00149 (Bottle #4)	0.039
6/11/2013	2190 99 00159 (Bottle #1)	< LoB
	2190 99 00160 (Bottle #2)	< LoB
	2190 99 00201 (Bottle #3)	<LoB
	2190 99 00202 (Bottle #4)	<LoB
9/24/2013	2190 99 00204 (Bottle #1)	< LoB
	2190 99 00205 (Bottle #2)	< LoB
	2190 99 00206 (Bottle #3)	< LoB
	2190 99 00207 (Bottle #4)	< LoB
12/24/2013	2190 99 00216 (Bottle #1)	< LoB
	2190 99 00217 (Bottle #2)	< LoB
	2190 99 00218 (Bottle #3)	< LoB
	2190 99 00219 (Bottle #4)	< LoB
3/24/2014	2190 99 00220 (Bottle #1)	< LoB
	2190 99 00221 (Bottle #2)	0.30
	2190 99 00222 (Bottle #3)	0.10
	2190 99 00223 (Bottle #4)	0.03
5/27/2014	2190 99 00232 (Bottle #1)	< LoB
	2190 99 00233 (Bottle #2)	< LoB
	2190 99 00234 (Bottle #3)	< LoB
	2190 99 00235 (Bottle #4)	< LoB
9/8/2014	2190 99 00240** (Bottle #1)	< LoB
	2190 99 00241 (Bottle #2)	< LoB
	2190 99 00242 (Bottle #3)	< LoB
	2190 99 00243 (Bottle #4)	< LoB
12/15/2014	2190 99 00352 (Bottle #1)	< LoB
	2190 99 00353 (Bottle #2)	< LoB
	2190 99 00354 (Bottle #3)	< LoB
	2190 99 00355 (Bottle #4)	< LoB

*LOQ = limit of quantitation. LoB = limit of blank

**One analytical sample discarded due to laboratory contamination

B. Rodent Cages

The animal cages utilized for the housing of animals in the study were examined for the presence of background BPA levels. Cages were extracted and prepared for MS analysis according to procedures described in DBT/CHEM SOP 322.04 (Appendix 1) and submitted to the Mass Spectrometry Laboratory for LC-ESI-MRM by SOP MSL-19.04 (Appendix 9). Three new cages purchased for this study, plus three old cages from a previous study were examined in this analysis. None of the cages (old or new) showed a quantifiable BPA concentration above the analytical background of the assay (Table 15).

Table 15. Background BPA levels in Rodent Cages

Sample Identification	**Average BPA Results (ng/g or ppb)**		
	Cages Extract (N=2)*	Reagent Blank for Extract (N=1)	Net Result (Cage result minus reagent blank)
SCR 0022 77 07324 (Old Cages)	0.091	0.077	< LOB**
SCR 0022 77 07323 (New Cages)	0.140	0.077	<LOB**

*One cage in each group was used for recovery determination (spiked with d0 and d6-BPA)
**The observed BPA levels for both cage extracts did not exceed the 'limit of blank', which was estimated for this matrix to be about 0.15 ppb, or twice the value determined for the reagent blank.

C. Hardwood Chip Bedding

The hardwood chip bedding utilized for housing of animals in this study was examined for background BPA levels. Composite samples (N=3 or more) for each lot of bedding were prepared from a sampling of individual bags received for each lot of bedding. Samples were prepared and assayed in duplicate using DBT/CHEM SOP 326.00 (Appendix 2) and submitted to the Mass Spectrometry Laboratory for MS analysis using SOP MSL 20.03 or 34.01 (Appendices 10 and 12).

All lots of bedding showed BPA levels that were less than or close to the analytical limits of the BPA assay (Table 16).

Table 16. Background BPA levels in Hardwood Chip Bedding				
SCR # and Delivery Date* **	**BPA Result (ppb)**	**Average BPA (ppb)**	**SD**	**%CV**
0022 77 07335 25 June 2012	< LOQ* (N=2)	< LOQ	-	-
0022 77 07336 25 June 2012	< LOQ* (N=2)	< LOQ	-	-
0022 77 07337 25 June 2012	< LOQ* (N=2)	< LOQ	-	-
0022 77 07338 25 June 2012	< LOQ* (N=2)	< LOQ	-	-
0022 77 07339 25 June 2012	< LOQ* (N=2)	< LOQ	-	-
0022 77 07461 7 March 2013	< LOQ*	< LOQ	-	-
0022 77 07462 7 March 2013	< LOQ*			
0022 77 07463 7 March 2013	< LOQ*			
0022 77 07532 08 Aug 2013	< LOQ*	< LOQ	-	-
0022 77 07533 08 Aug 2013	< LOQ*			
0022 77 07534 08 Aug 2013	< LOQ*			
0022 77 07575 26 Nov 2013	0.9	0.9	0.2	17
0022 77 07576 26 Nov 2013	1.2			
0022 77 07577 26 Nov 2013	0.5			
0022 77 07630 2 April 2014	<LoB**	0.6	0.5	91
0022 77 07631 2 April 2014	0.7			
0022 77 07632 2 April 2014	1.0			
0022 77 07678 27 June 2014	0.80	0.78	0.22	28
0022 77 07679 27 June 2014	0.56			
0022 77 07680 27 June 2014	0.98			

Table 16. Background BPA levels in Hardwood Chip Bedding				
0022 77 07726 15 Sept 2014	0.15	0.07	0.13	176
0022 77 07727 15 Sept 2014	0.07			
0022 77 07728 15 Sept 2014	0.00			

*LOQ = Limit of quantitation
**Values have been corrected via subtraction of the experimentally determined limit of blank (LoB) from N=3 determinations.
***Date bedding sample prepared and submitted for analysis

D. Cellulose Bedding

A portion of the animals were housed on cellulose bedding. Composite samples (N=2) from each lot of this bedding material was examined for background BPA levels. Samples were prepared and assayed using MS Lab SOP MSL 44.00 (Sample Preparation; Appendix 14) and MSL 43.00 (Determination by LC/MS/MS; Appendix 13). The analytical methodology involves extraction of bedding samples using acetonitrile which contains d6-BPA as internal standard, concentrating the extract and analysis using HPLC-ESI-MRM. An experimentally determined limit of blank (LoB) based on N=3 reagent samples was subtracted from N=3 bedding samples processed in the same manner. Spiked bedding samples were also utilized to verify performance of the assay.

The average value for all the cellulose bedding samples examined during the study was about 0.12 ppb (%CV = 6.4), a value close to the limit of blank for the analytical assay.

Table 17. Background BPA levels in Cellulose Bedding					
SCR #	Delivery Date*	BPA Result (ppb)	Average (ppb)	SD	% CV
022 77 07582	12/12/2013	<LoB	<LoB	-	-
022 77 07583	12/12/2013	<LoB			
022 77 07610	02/10/2014	<LoB	<LoB	-	-
022 77 07611	02/10/2014	<LoB			

Table 17. Background BPA levels in Cellulose Bedding					
022 77 07622	3/14/2014	0.08	0.08	0.00	0.9
022 77 07623	3/14/2014	0.08			
022 77 07640	4/23/2014	0.12	0.13	0.01	9.3
022 77 07641	4/23/2014	0.14			
0022 77 07671	6/13/2014	0.128	0.128	0.00	0.07
0022 77 07672	6/13/2014	0.129			
0022 77 07698	8/1/2014	0.103	0.101	0.002	2.1
0022 77 07699	8/1/2014	0.100			
0022 77 07713	8/19/2014	0.107	0.121	0.02	16.1
0022 77 07714	8/19/2014	0.135			
0022 77 07737	9/22/2014	0.124	0.119	0.007	5.9
0022 77 07738	9/22/2014	0.114			
0022 77 07776	12/04/2014	0.102	0.108	0.008	7.6
0022 77 07777	12/04/2014	0.113			

*Date bedding sample prepared and submitted for analysis

E. 5K96 Diet

The manufacturer of the 5K95 diet (Purina) provided reports from an analytical laboratory on various nutrients and contaminants. These are included in a separate appendix for the study. Each lot of 5K96 diet received and utilized to feed animals in the study were also examined for background BPA levels. A single composite sample for each lot of diet was prepared from a sampling of individual bags received for each lot. Samples were prepared using DBT/CHEM SOP 326 (Appendix 2) and submitted to the Mass Spectrometry Laboratory for MS analysis

using SOP MSL-20 (Appendix 10). An experimentally determined limit of blank (LoB) based on N=3 reagent samples was utilized for this assay. Feed samples were likewise prepared (N=3) and are reported after subtraction of the LoB. The analytical methodology involves extraction of feed samples using acetonitrile, followed by solid phase extraction cleanup and analysis by the Mass Spectrometry Laboratory using HPLC-ESI-MRM utilizing d6-BPA as internal standard.

The average background level of BPA observed above the analytical background in diet samples for the study was about 1 ng/g or ppb.

Table 18. Background BPA levels in 5K96 Diet

Report Date*	SCR #	Limit of Blank (LoB)	Average BPA Results (ng/g or ppb)
6/8/2012	2191-99-00001	0.601	1.207
6/26/2012	2190-99-00003	0.961	1.65
10/5/2012	2190-99-00036	10.1	< LoB**
1/11/2013	2190-99-00058	2.4	0.74
2/4/2013	2190-99-00060	0.61	0.51
5/6/2013	2190-99-00093	2.42	1.43
8/21/2013	2190-99-00174	10.2	3.03
12/13/2013	2190-99-00251	1.82	0.96
3/20/2014	2190-99-00267	1.48	0.78
7/1/2014	2190-99-00290	3.27	1.31
10/15/2014	2109-99-00305	4.19	2.49

Animal Diet	Average BPA (ng/g or ppb)	-	1.28

*Date results reported to study director.
**Values have been corrected via subtraction of the experimentally determined limit of blank (LoB) from N=3 determinations. For this assay, one of the replicates was significantly higher than the other two. Nonetheless, the mean value for the LoB was 8.67 ppb.

8. Assessment of Background Isoflavone Levels in 5K95 Diet

A low isoflavone diet was selected for use in this study. Background levels of four common xenoestrogens (genistein, daidzein, coumestrol and zearalenone) were nonetheless assessed in each lot of 5K96 diet received and utilized for feeding of animals in the study. The analytical methodology involves extraction of diet samples using acetonitrile, followed by solid phase extraction cleanup and analysis by the Mass Spectrometry Laboratory using HPLC-ESI-MRM utilizing d6-BPA as internal standard. An experimentally determined limit of blank (LoB) based

on N=3 reagent samples was utilized for this assay. The LoB is determined as the mean blank value plus 1.645 times the SD of the blank, according to D. Armbruster and T. Pry (2008) Clin. Biochem. Rev, Vol 29, S49 - 52. Feed samples were likewise prepared (N=3) and are reported after subtraction of the LoB. The methods used were NCTR/DBT CHEM SOP No. 525 (Appendix 3) and MSL-30.01 (Appendix 11) and reported in ppm. A calculation error resulted in incorrect results being reported to the Study Director during the course of the study. These values represent the verified results (Table 19).

Table 19. Background Isoflavone levels in 5K96 Diet

5K95 Diet lot #	SCR #*	Concentration (µg/g or ppm)				
		Genistein*	Daidzein*	Coumestrol	Zearalenone	Totals
12FEB15RTD1	2191 99 00001	0.46	0.23	0	0	0.69
12MAY02RTD1	2190 99 00003	1	0.46	0	0	1.46
12JUL23RTD1	2190 99 00036	0.26	0.41	0	0	0.67
12NOV29RTD1	2190 99 00058	0.13	0.07	0	0	0.2
12DEC10RTD1	2190 99 00060	0.3	0.17	0	0	0.47
13MAR01RTD1	2190 99 00093	0.32	0.25	0.05	0.01	0.63
13JUL01RTD1	2190 99 00174	6.27	6.51	0	0	12.78
13SEP19RTD1	2190 99 00251	3.04	2.78	0.08	0	5.9
14JAN08RTD1	2190 99 00267	3.28	2.54	0	0	5.82
14APR30RTD1	2190 99 00290	3.13	3.83	0	0.05	7.01
14AUG08RTD1	2190 99 00305	1.54	0.97	0	0	2.51

*Acid hydrolysis utilized to convert genistin and daidzin to aglycone analogs (total aglycones reported)

Table 20. Summary of Background Isoflavone levels in 5K96 Diet			
Isoflavones	**Mean Result (µg/g or ppm)**	**Minimum (µg/g or ppm)**	**Maximum (µg/g or ppm)**
Genistein	1.79	0.13	6.27
Daidzein	1.66	0.07	6.51
Coumestrol	0.01	< LOQ	0.08
Zearalenone	0.005	< LOQ	0.05

Standard Operating Procedure for Sample Preparation of Animal Diet, Bedding, Carboxlymethyl cellulose (CMC) and CMC Solutions, Polysulfone Cage extracts, or Water for Determination of Bisphenol A by LC/MS

1. PURPOSE

This Standard Operating Procedure (SOP) describes the method for preparation of samples for analysis of Bisphenol A (BPA) by LC/MS. BPA in water or extracts is concentrated using a Waters Oasis HLB solid phase extraction (SPE) method. BPA is quantified by LC/MS utilizing d_6-BPA as the internal standard.

2. SCOPE

This procedure applies to the analyses of background levels of BPA in all Polysulfone animal cages, bedding, diet, CMC or CMC solutions, and water utilized at the NCTR and is for compliance with FDA Good Laboratory Practice (GLP) as specified by 21 CFR 58.

3. CHEMICALS

3.1. Acetonitrile, (Acros, 26827-0040, HPLC grade)
3.2. Ammonium Hydroxide 30% (Fisher Sci., A669S-500)
3.3. Bisphenol A (Sigma-Aldrich, St. Louis, MO,)
3.4. Bisphenol A-d_6 (CDN Isotopes, Bisphenol-A-d_6, dimethyl-d_6,)
3.5. Deionized Water, ultra-purified (Barnstead NANOPure, 18.2 MΩ cm^{-1})
3.6. Methanol (Fisher Sci. , E127-4, HPLC grade)
3.7. Methyl Tertiary Butyl Ether (J.T. Baker, HPLC grade)
3.8. Nitrogen, purified GC grade
3.9. Sodium Thiosulfate, $Na_2S_2O_3$, (Sigma-Aldrich, 217263)

4. EQUIPMENT

4.1. Agilent GC vials, amber 1.5ml with Teflon lined caps
4.2. Analytical balance (4 or 5-place) with NIST traceable weights
4.3. Culture tubes, Kimble, 160ml with Teflon lined cap
4.4. Gas-tight syringes, Hamilton, 100 & 500µl
4.5. Graduated cylinders, 50 to 1000ml sizes, Pyrex®(or equivalent)
4.6. LC/MS vials, XPERTEK®, 0.5ml, with Teflon lined caps (PJ Colbert# 954019 & 958850B)
4.7. Nitrogen sample evaporation gas manifold apparatus
4.8. Pasteur 9 cm glass transfer pipettes (2 ml with latex rubber bulbs)
4.9. Pipettes, volumetric (Class A)
4.10. Pipettes, disposable glass sterile serological, 10ml (Kimble Glass Inc.)
4.11. SPE extraction vacuum manifold
4.12. SPE columns, Waters Oasis HLB, 5cc, 200mg (Waters Part# 186000683)
4.13. Syringeless filter system, 0.2 µm, (Whatman Mini-UniPrep™)
4.14. Volumetric flasks, various sized, glass class A

5. PROCEDURES

Note: Prior to weighing operations, the analytical balance is calibrated and the calibration is documented with NIST traceable weights in the weight range of intended operation.

5.1 Stock Solutions

5.1.1 Aldrich BPA 10.00 mg/ml stock solution: Weigh exactly 0.100g BPA on an analytical balance into a borosilicate glass scintillation vial. Add 10 ml MeOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. Prepare stock standard at 6 month intervals or when necessary. Additional standard concentrations of BPA are obtained by serial dilutions.

5.1.2 Ammonium hydroxide solution, 0.05M: Add 6ml of 30% NH_4OH to 994ml of deionized water and mix.

5.1.3 CDN Isotopes Bisphenol-A-d_6 10.00 mg/ml stock solution: Weigh exactly 0.100g BPA on an analytical balance into a borosilicate glass scintillation vial. Add 10 ml MeOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. After dissolved, dispense 1ml aliquots into 1.5ml amber GC vials and store at -20°C until needed. Prepare stock solution as needed. Other standard concentration of d_6-BPA are obtained by serial dilution.

5.1.4 Sodium Thiosulfate 0.1M solution: Weigh 1.58g of $Na_2S_2O_3$ into a 100ml Class A volumetric flask. Dilute to volume with deionized water.

5.1.5 External Standards of d_6-BPA & d_0-BPA at 10ng/ml or 100ng/ml: Add 50µl of a combined standard of d_6-BPA & d_0-BPA, each at a concentration of either 1.00 µg/ml or 0.100 µg/ml, to 450µl of deionized water contained in a 1.5ml GC vial. Cap, mix and supply with appropriate samples for each set of samples submitted for LC/MS analysis.

5.2 Sample Preparation

Note: One or more aliquots are processed from each submitted sample to provide adequate analytical precision (i.e. a single sample is assayed in triplicate, while multiple samples from the same source may be assayed once or in duplicate).

5.2.1 Water Treatment

Prior to Oasis SPE, add 5ml 0.1M Sodium Thiosulfate to a 200ml volume of water. Resulting sample contains ~400ppm $Na_2S_2O_3$. Allow water sample to react for 30 minutes before proceeding to next step. Label six 50ml graduated cylinders 1-6. Add 50ml of 0.05M NH_4OH to cylinders 1-3 to make three blanks. Add 50ml of water sample to cylinders 3-6. Add 50µl of 0.100ng/µl d_6-BPA (5.0ng) to all six. Apply Oasis SPE method as indicated in 5.2.6.

5.2.2 Bedding Extraction

Weigh three 20g samples into 160 ml culture tubes. The bedding samples are spiked with 200µl of 1.00 ng/ul d_6-BPA (200 ng d_6-BPA).To make three blanks add 25ml of Acetonitrile: H2O (2:1) to 50 ml graduated cylinders spike with 200 µl of 1.0ng/µl d_6-BPA (200ng of d_6) . Add 100 ml Acetonitrile: H20 (2:1) to each sample to extract. Place on flat-bed reciprocal shaker at low speed for 1 hour. Centrifuge at low speed (less than 500 RMPs) for 30 minutes. Remove 25 ml liquid extract from each culture tube and add to a 50ml graduated cylinder. Then add 25 ml 0.05M NH4OH (Ammonium Hydroxide) to make 50 ml. Oasis SPE method is applied as indicated in 5.2.6.

5.2.3 5K96 Diet Extraction

Three 20g diet samples are weighed into separate 160ml culture tubes. The diet samples are fortified with 200ng of d_6-BPA. Make three blanks by adding 25ml of Acetonitrile: H2O (2:1) to a 50 ml graduated cylinder spike with 200 µl of 1.0ng/µl d_6-BPA (200ng of d_6). Each sample is extracted with 100ml of ACN: H2O (2:1) for 1 hr on a flat-bed reciprocal shaker at low speed. Samples are centrifuged at less than 500 rpm for 30 min. 25ml of each diet extract (5.0 g-eq) is transferred to 50ml graduated cylinders. Each sample volume is adjusted to 50ml with 0.05M NH_4OH, and then the Oasis SPE method is applied as indicated in 5.2.6.

5.2.4 Polysulfone Cage Extraction:

Three Polysulfone cages are extracted for 64 hours at room temperature on a flat horizontal surface, each with 250ml of 0.05M NH_4OH. Cover the cages with aluminum foil to minimize loss of NH_4OH. After 64 hours contact time, collect from each cage the 0.05M NH4OH in a 250ml graduated cylinder, adjust the volume up to 250ml with additional 0.05M NH_4OH and mix. Take 50 ml of each cage sample and add 50µl of 0.100 ng/µl D6-BPA (5.0 ng). Add 50 ml of 0.05 M NH_4OH to three additional cylinders to make three blanks and add 50 µl of 0.100 ng/µl D6-BPA (5.0 ng). Apply Oasis SPE method as indicated in 5.2.6.

5.2.5 CMC and Solution Extraction:

5.2.5.1 Powder CMC samples are dissolved in DI H2O to make a 1.0 % w/w sample.
5.2.5.2 Weigh three 10 g samples (record actual wt.).
5.2.5.3 Weigh three equivalent control DI H2O samples.
5.2.5.4 Add 50 µl of 0.100 ng/µl D6-BPA (5.0 ng) to each.
5.2.5.5 Liquid: liquid extract each sample with MTBE (2X25ml & 1X10ml) and combine the extracts into separate 125 ml flat-bottomed flasks.
5.2.5.6 Roto-evaporate the MTBE extracts to dryness and dissolve the residues in 25 ml 0.05M NH4OH. Condition SPE columns as in 5.2.6.1 and 5.2.6.2.
5.2.5.7 Transfer the 25ml sample residues to Waters Oasis HLB columns followed by rinsing the sample flasks with 2 X 12 ml 0.05M NH4OH and continue Oasis HLB SPE Method beginning at 5.2.6.4.

5.2.6 Oasis HLB SPE Method

5.2.6.1 Oasis glass columns labeled and attached to a vacuum manifold with waste container in manifold
5.2.6.2 To condition columns add in succession so that the column doesn't go dry
3 ml MTBE (Methyl Tertiary Butyl Ether)
3ml MeOH (Methanol)
3ml H2O
5.2.6.3 Add 50ml samples to columns under vacuum
5.2.6.4 Wash sample cylinders with 3ml 40 % MeOH in H2O and add the column
5.2.6.5 Re-equilibrate column by adding 3 ml H2O to column
5.2.6.6 Wash sample cylinder with 3ml 10% MeOH/2% NH4OH in H2O and add to the column
5.2.6.7 Dry columns under vacuum change waste container to collection tubes with 0.5 ml marked.
5.2.6.8 Elute samples with 6ml of 10% MeOH in MTBE
5.2.6.9 Remove collection tubes and dry samples under nitrogen in an evaporation gas manifold apparatus until volume reduced to less than 0.5 ml
5.2.6.10 Adjust volume to 0.5ml 50% MeOH.
5.2.6.11 Filter samples with the whatman mini-prep 0.2 um syringeless filtration system.

5.3 Label each filter, to coordinate with the sequence list given with the mass spectrometry work request sheet, Form FDA-3710.Submit samples to DBT Mass Spectrometry Support Group for HPLC/MS analysis.

Standard Operating Procedure for Sample Preparation of Animal Diet and Bedding for Determination of Bisphenol A by LC/MS

1. PURPOSE

This Standard Operating Procedure (SOP) describes the method for preparation of samples for analysis of Bisphenol A (BPA) by LC/MS. BPA in extracts is concentrated using a Waters Oasis HLB solid phase extraction (SPE) method. BPA is quantified by LC/MS utilizing d_6-BPA as the internal standard.

2. SCOPE

This procedure applies to the analyses of background levels of BPA in all bedding and diet utilized at the NCTR and is for compliance with FDA Good Laboratory Practice (GLP) as specified by 21 CFR 58.

3. CHEMICALS

3.1. Acetonitrile, (Acros, 26827-0040, HPLC grade)
3.2. Ammonium Hydroxide 30% (Fisher Sci., A669S-500)
3.3. Bisphenol A (Sigma-Aldrich, St. Louis, MO, Lot
3.4. Bisphenol A-d_6 (CDN Isotopes, Bisphenol-A-d_6, dimethyl-d_6,)
3.5. Deionized Water, ultra-purified (Barnstead NANOPure, 18.2 MΩ cm^{-1})
3.6. Methanol (Fisher Sci. , E127-4, HPLC grade)
3.7. Methyl Tertiary Butyl Ether (J.T. Baker, HPLC grade)
3.8. Nitrogen, purified GC grade
3.9. Sodium Thiosulfate, $Na_2S_2O_3$, (Sigma-Aldrich, 217263)

4. EQUIPMENT

4.1. Analytical balance (4 or 5-place) with NIST traceable weights
4.2. Gas-tight syringes, Hamilton, 100,250 and 500µl
4.3. Graduated cylinders, 50 to 1000ml sizes, Pyrex®(or equivalent)
4.4. Nitrogen sample evaporation gas manifold apparatus
4.5. Pasteur 9 cm glass transfer pipettes (2 ml with latex rubber bulbs)
4.6. Pipettes, volumetric (Class A)
4.7. Pipettes, disposable glass sterile serological, 10ml (Kimble Glass Inc.)
4.8. SPE extraction vacuum manifold
4.9. Syringeless filter system, 0.2 µm, (Whatman Mini-UniPrep™)
4.10. Volumetric flasks, various sized, glass class A
4.11. Corning Disposable 50 ml polypropylene flat screw top centrifuge tube(Sigma-Aldrich CLS430291)
4.12. Waters Oasis 6cc Polypropylene HLB Extraction Cartridge (Waters WAT106202)
4.13. 60cc polypropylene Reservoir adapter (Waters 186005587)
4.14. Polyethylene Adapter Cap (Phenomenex AHO-7191)
4.15. Whatman mini-prep 0.2 um Syringeless filtration system

5. PROCEDURES

Note: Prior to weighing operations, the analytical balance is calibrated and the calibration is documented with NIST traceable weights in the weight range of intended operation.

5.1 Stock Solutions

5.1.1 Aldrich BPA 10.00 mg/ml stock solution: Weigh exactly 0.100g BPA on an analytical balance into a borosilicate glass scintillation vial. Add 10 ml MeOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. Prepare stock standard at 6 month intervals or when necessary. Additional standard concentrations of BPA are obtained by serial dilutions.

5.1.2 Ammonium hydroxide solution, 0.05M: Add 6ml of 30% NH_4OH to 994ml of deionized water and mix.

5.1.3 CDN Isotopes Bisphenol-A-d_6 10.00 mg/ml stock solution: Weigh exactly 0.100g BPA on an analytical balance into a borosilicate glass scintillation vial. Add 10 ml MeOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. After dissolved, dispense 1ml aliquots into 1.5ml amber GC vials and store at -20°C until needed. Prepare stock solution as needed. Other standard concentrations of d6-BPA are obtained by serial dilution.

5.1.4 Sodium Thiosulfate 0.1M solution: Weigh 1.58g of $Na_2S_2O_3$ into a 100ml Class A volumetric flask. Dilute to volume with deionized water.

5.2 Sample Preparation

Note: One or more aliquots are processed from each submitted sample to provide adequate analytical precision (i.e. a single sample is assayed in triplicate, while multiple samples form the same source may be assayed once or in duplicate).

5.2.1 Bedding Extraction

Make three blanks by adding 20 ml of Acetonitrile:H_2O (2:1) to three 50 ml centrifuge tubes, and spike each with 200 µl of 1.0 ng/µl d_6-BPA (200 ng of d_6) vortex to mix. Weigh three 5 g samples into 50 ml centrifuge tubes. Add 40 ml Acetonitrile: H20 (2:1) to each sample to extract. Spike the samples with 200 µl of 1.0ng/µl d_6-BPA (200ng of d_6) and vortex to mix. Place all samples and blanks on flat-bed reciprocal shaker at low speed for 1 hour. Centrifuge all at medium speed ~ 2500 (less than 5000 RMPs) for 30 minutes. Add 20 ml of each sample to 50ml graduated cylinder. Pour the blanks into a 50 ml graduated cylinder. Add 30 ml 0.05M NH4OH (Ammonium Hydroxide) to each cylinder, to make 50 ml in each. Oasis SPE method is applied as indicated in 5.2.3.

Note: Bedding extracts should be decanted into a flask and pipetted from there to avoid transferring any bedding.

5.2.2 5K96 Diet Extraction

Make three blanks by adding 20 ml of Acetonitrile:H_2O (2:1) to three 50 ml centrifuge tubes, and spike each with 200 µl of 1.0 ng/µl d_6-BPA (200 ng of d_6) vortex to mix. Weigh three 5g samples into 50 ml centrifuge tubes. Add 40 ml Acetonitrile: H20 (2:1) to each sample to extract. Spike the samples with 200 µl of 1.0ng/µl d_6-BPA (200ng of d_6) vortex to mix. Place all samples and blanks on flat-bed reciprocal shaker at low speed for 1 hour. Centrifuge all at medium speed ~ 2500 (less than 5000 RMPs) for 30 minutes. Add 20 ml of each sample to 50ml graduated cylinder. Pour the blanks into a 50 ml graduated cylinder. Add 30 ml 0.05M NH4OH (Ammonium Hydroxide) to each cylinder, to make 50 ml in each. Oasis SPE method is applied as indicated in 5.2.3.

Note: Diet extracts should be drawn from the clear liquid layer.

5.2.3 Oasis HLB SPE Method

5.2.3.1 Attach labeled Oasis 6cc extraction columns with 60cc water reservoir connected using Phenomenex adapter cap to a vacuum manifold with waste container in manifold

5.2.3.2 To condition columns add in succession:
3 ml MTBE (Methyl Tertiary Butyl Ether)
3ml MeOH (Methanol)
3ml H2O

5.2.3.3 Add 50ml samples to columns under vacuum

5.2.3.4 Wash sample cylinders with 3ml 40 % MeOH in H2O and add the column

5.2.3.5 Re-equilibrate column by adding 3 ml H2O to column

5.2.3.6 Wash sample cylinder with 3ml 10% MeOH/2% NH4OH in H2O and add to the column

5.2.3.7 Dry columns under vacuum change, and waste container to labeled collection tubes with 0.5 ml marked.

5.2.3.8 Elute samples with 6ml of 10% MeOH in MTBE

5.2.3.9 Dry samples under nitrogen until volume reduced to less than 0.5 ml
Adjust volume to 0.5ml 75% MeOH.

5.2.3.10 Filter samples with the whatman mini-prep 0.2 um Syringeless filtration system. Label each filter, to coordinate with the sequence list given with the mass spectrometry work request sheet, Form FDA-3710

5.3 Label each filter, to coordinate with the sequence list given with the mass spectrometry work request sheet, Form FDA-3710.Submit samples to DBT Mass Spectrometry Support Group for HPLC/MS analysis.

5.4 The Limit of Quantitation (LOQ) for Mass Spectrometry BPA is to be calculated for each individual run. The LOQ is less than 250 ppb. Samples that are more than 250 ppb must be repeated for verification.

5.5 To determine the amount of the labeled material it will to be compared to unlabeled material. Each will be sampled 5 times and run by LC-UV. The value of the labeled material will be calculated by the amount in the unlabeled material. The adjusted value will be use.

Standard Operating Procedure
Preparation of Animal Diets for Determination of Background Daidzein & Genistein levels by LC/MS

1. PURPOSE

This Standard Operating Procedure (SOP) describes the procedure utilized for preparation of diet samples for analysis by mass spectrometry of Daidzein and Genistein (Isoflavones).

2. SCOPE

This procedure applies to the analyses of background levels of Isoflavones in all animal diets utilized at the NCTR and is for compliance with FDA Good Laboratory Practice (GLP) as specified by 21 CFR 58.

3. CHEMICALS

3.1. Acetonitrile, MeCN, (Acros, 26827-0040, HPLC grade)
3.2. Ammonium Hydroxide 30% (Fisher Sci., A669S-500)
3.3. Daidzein, (Toronto Research Chemicals, Inc.)
3.4. Deionized Water, ultra-purified (Barnstead NANOPure, 18.2 MΩ cm-1)
3.5. Ethyl acetate (HPLC grade)
3.6. Formic acid, 0.1% in water
3.7. Genistein, (Toronto Research Chemicals, Inc.)
3.8. Methanol (Fisher Sci., E127-4, HPLC grade)
3.9. Hydrochloric acid (JT Baker, 9535-00)

4. EQUIPMENT

4.1. Agilent GC vials, amber 1.5ml with Teflon lined caps
4.2. Analytical balance (4 or 5-place) with NIST traceable weights
4.3. Culture tubes, Kimble, 12 & 50ml with Teflon-lined screw caps
4.4. Eppendorf pipette, 100-1000µl, with tips
4.5. Erlenmeyer glass flasks, 125 ml with 24/40 glass joint
4.6. Graduated cylinders, 25 to 1000ml sizes, Pyrex®(or equivalent)
4.7. Pasteur 9 cm glass transfer pipettes (2 ml with latex rubber bulbs)
4.8. Syringeless filter system, 0.2 µm, (Whatman Mini-UniPrep™)
4.9. Volumetric flasks, various sized, glass class A

5. PROCEDURES

Note: Prior to weighing operations, the analytical balance is calibrated and the calibration is documented with NIST traceable weights in the weight range of intended operation.

5.1 Stock Solutions

5.1.1 Daidzein 10.00 mg/ml stock solution: Weigh exactly 0.100g Daidzein on an analytical balance into a borosilicate glass scintillation vial. Add 10.0 ml MeOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. Prepare stock standard at 6 month intervals or when necessary. Additional standard concentrations of Daidzein are obtained by serial dilutions.

5.1.2 Genistein 10.00 mg/ml stock solution: Weigh exactly 0.100g Genistein on an analytical balance into a borosilicate glass scintillation vial. Add 10.0 ml MeOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. Prepare stock standard at 6 month intervals or when necessary. Additional standard concentrations of Genistein are obtained by serial dilutions.

5.2 Sample Preparation

5.2.1 Animal Diet Extraction

Note: Fortification level described is 5.00µg/g. Various diets may require fortification at 50.0µg/g or even 500µg/g depending on the Isoflavones content of the diets. In these instances, the combined Daidzein and Genistein standard utilized will be at a factor of 10 or 100 higher concentration respectively.

5.2.1.1 For each separate diet sample, weigh four 2.00 ± 0.01g diet samples into separate 125 ml screw-cap Erlenmeyer flasks.
5.2.1.2 Fortify 2 of the above diet aliquots with 1 ml of 10.0 µg/ml combined Daidzein and Genistein standard in MeOH. Fortified samples are at 5.00 µg/g level.
5.2.1.3 Allow fortified samples to stand for 10 min., and then add a Teflon® stirbar to each.
5.2.1.4 Add 20ml 10% HCl in MeOH to each diet sample (19ml to fortified samples) and swirl.
5.2.1.5 Connect a Snyder distillation column to each sample flask.
5.2.1.6 Place each flask on a 5-position stirplate/heater and stir/heat on the lowest possible settings for 6 hours or at room temperature for 48 hours.
5.2.1.7 Remove each flask from stirplate and allow to cool to room temperature.
5.2.1.8 Transfer extracts to a graduated cylinder, then rinse flask with additional MeOH to achieve 20 ml volume.
5.2.1.9 Transfer each 20ml extract to separate 50ml tubes and then centrifuge extracts for 10 min. at 1000 rpm.
5.2.1.10 Withdraw a 5ml aliquot of each methanolic acid extract and add to separate 125ml flat-bottomed flasks with 24/40 ground glass joint.
5.2.1.11 Rotoevaporate contents with ~60°C bath to near dryness and add ~1ml of methanol to each flask and swirl to resuspend contents.
5.2.1.12 Add 4 ml of 0.1% formic acid to each flask and swirl.
5.2.1.13 Transfer the contents of each flask to a 12ml screw-capped tube and then rinse flasks with 4 ml of ethyl acetate & transfer ethyl acetate to 12ml tubes.
5.2.1.14 Invert all tubes 20 times to mix and centrifuge to separate solvents.
5.2.1.15 Transfer the ethyl acetate (top layer) to 125ml flat bottomed flasks.
5.2.1.16 Repeat the liquid-liquid extraction procedure two additional times with ethyl acetate.
5.2.1.17 Rotoevaporate the combined ethyl acetate fractions to near dryness.
5.2.1.18 Transfer residues to 5ml Class A volumetric flasks using ACN.
5.2.1.19 Just prior to submitting samples to quantification, filter each through a 0.2µm filter into a 1.5ml HPLC vial.

5.3 Submit samples to DBT Mass Spectrometry Support Group for HPLC/MS analysis.

Standard Operating Procedure for Sample Preparation of 0.3% Carboxyl methylcellulose (CMC) for Determination of Bisphenol A (BPA) or Ethynylestradiol (EE2) by HPLC-PDA or LC/MS

1. PURPOSE:

This Standard Operating Procedure (SOP) describes the method for preparation of samples for analysis of BPA or EE2. BPA in 0.3% CMC at concentrations from 5.0 to 60,000 µg/ml is diluted with methanol and analyzed by HPLC-PDA. BPA at 0.5 & 1.6 µg/ml or EE2 at 0.1 & 1.0 µg/ml in 0.3% CMC is fortified with BPA-d6 or EE2-d4, extracted with toluene and quantified by LC/MS.

2. SCOPE

This procedure applies to the analyses of BPA or EE2 in 0.3% CMC utilized for animal dosing at the NCTR and is for compliance with FDA Good Laboratory Practice (GLP) as specified by 21 CFR 58.

3. CHEMICALS

3.1. Acetonitrile, MeCN, (Acros, 26827-0040, HPLC grade)
3.2. Bisphenol A (Sigma-Aldrich, St. Louis, MO, Lot # 09128 LD)
3.3. BPA-d6 (bisphenol-A-d6, C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada, D-2476)
3.4. EE2-d4 (17α-ethynylestradiol-2,4,16,16-d4, C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada, D-4319)
3.5. Deionized Water, ultra-purified (Barnstead NANOPure, 18.2 MΩ cm^{-1})
3.6. 2-propanol (J.T. Baker, 9084-03)
3.7. Methanol (Fisher Sci., E127-4, HPLC grade)
3.8. Nitrogen, purified GC grade
3.9. Sodium Lauryl Sulfate, (Fisher Sci., S529-500)
3.10. Toluene (Fisher Scientific, T290-4, HPLC grade)

4. EQUIPMENT

4.1. Analytical balance (4 or 5-place) with NIST traceable weights
4.2. Graduated cylinders, 50 to 1000ml sizes, Pyrex® (or equivalent)
4.3. High-recovery autosampler vials for LC-MS
4.4. Micro centrifuge
4.5. Eppendorf 100-1000 µl micropipette and tips
4.6. Eppendorf 20-200 µl micropipette and tips
4.7. Speed vac concentrator
4.8. TissueLyser II (QIAGEN)
4.9. Pasteur 9 cm glass transfer pipettes (2 ml with latex rubber bulbs)
4.10. Pipettes, volumetric (Class A)
4.11. Volumetric flasks, various sized, glass (Class A)

5. PROCEDURES

Note: Prior to weighing operations, the analytical balance is calibrated and the calibration is documented with NIST traceable weights in the weight range of intended operation.

5.1 Stock Solutions

5.1.1 BPA 10.00 mg/ml stock solution: Weigh 0.1000g BPA on an analytical balance into a borosilicate glass scintillation vial. Add 10 ml EtOH to vial with a class A volumetric pipette, cap vial, and swirl contents until completely dissolved. Prepare stock standard at 6 month intervals or when necessary. Analytical standards of BPA are obtained by serial dilution of Stock into MeOH.

5.1.2 BPA-d6 Stock Solution 100 ng/µl – Weigh exactly 0.00100g of BPA-d6 into a 20 ml glass scintillation vial. Dissolve with 10 ml MeOH using a 10 ml Class A volumetric pipet.

5.1.3 BPA-d6 5 ng/µl solution – Dilute 500 µl of 100 ng/µl BPA-d6 to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.4. EE2-d4 Stock Solution 100 ng/µl - Weigh exactly 0.00100g of EE2-d4 into a 20 ml glass scintillation vial. Dissolve with 10 ml MeOH using a 10 ml Class A volumetric pipet.

5.1.5. EE2-d4 10 ng/µl solution – Dilute 1000 µl of 100 ng/µl EE2-d4 Stock Solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.6. EE2-d4 1 ng/µl solution – Dilute 1000 µl of 10 ng/µl EE2-d4 solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.2 Sample Preparation

5.2.1 BPA Concentrations from 5.0 to 1000,000 µg/ml are submitted as triplicate samples which are each diluted with Methanol as follows:

Sample BPA µg/ml	Dilution
5.00	1:10
16.0	1:10
52.0	1:10
168	1:100
540	1:100
20,000	1:10,000
60,000	1:10,000

5.2.2 Diluted samples are analyzed for BPA content by HPLC-PDA analysis.

5.2.3 **Weigh triplicate 500 µl samples of 0.5 µg/ml BPA in 0.3% CMC (or Control 0.3% CMC) into 1.5 ml micro tubes to the nearest mg.**

5.2.3.1 Add 50 µl of 5 ng/µl BPA-d6 solution to the 1.5 ml micro tube using an Eppendorf micropipette.

5.2.3.2 Vortex 1.5 ml micro tube for about 1 minute.

5.2.3.3 Successively add 50 µl saturated NaCl and 500 µl toluene to the 1.5 ml micro tube using Eppendorf micropipette.

5.2.3.4 Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.

5.2.3.5 Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*

5.2.3.6 Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.

5.2.3.7 Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.

5.2.3.8 Add 100 µl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about 1 minute.

5.2.3.9 Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.4 **Weigh triplicate 313 μl samples of 1.6 μg/ml BPA in 0.3% CMC into 1.5 ml micro tubes to the nearest mg.**

5.2.4.1 Add 100 μl of 5 ng/μl BPA-d6 solution to the 1.5 ml micro tube using an Eppendorf micropipette.
5.2.4.2 Vortex 1.5 ml micro tube for about 1 minute.
5.2.4.3 Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using Eppendorf micropipette.
5.2.4.4 Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.
5.2.4.5 Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*
5.2.4.6 Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.
5.2.4.7 Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.
5.2.4.8 Add 200 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about 1 minute.
5.2.4.9 Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.5 **Weigh triplicate 500 μl samples of 0.1 μg/ml EE2 in 0.3% CMC (or Control 0.3% CMC) into 1.5 ml micro tubes to the nearest mg.**

5.2.5.1 Add 50 μl of 1 ng/μl EE2-d4 solution to the 1.5 ml micro tube using an Eppendorf micropipette.
5.2.5.2 Vortex 1.5 ml micro tube for about 1 minute.
5.2.5.3 Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using Eppendorf micropipette.
5.2.5.4 Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.
5.2.5.5 Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 5 minutes at > 18,000 x g.
5.2.5.6 Carefully transfer the top organic layer into a new 1.5 ml micro tube using Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.
5.2.5.7 Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.
5.2.5.8 Add 100 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about 1 minute.
5.2.5.9 Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.6 **Weigh triplicate 500 μl samples of 1.0 μg/ml EE2 in 0.3% CMC into 1.5 ml micro tubes to the nearest mg.**

5.2.6.1 Add 50 μl of 10 ng/μl EE2-d4 solution to the 1.5 ml micro tube using an Eppendorf micropipette.
5.2.6.2 Vortex 1.5 ml micro tube for about 1 minute.
5.2.6.3 Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using Eppendorf micropipette.
5.2.6.4 Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.
5.2.6.5 Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x g.
5.2.6.6 Carefully transfer the top organic layer into a new 1.5 ml micro tube using Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.
5.2.6.7 Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.
5.2.6.8 Add 100 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about 1 minute.
5.2.6.9 Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

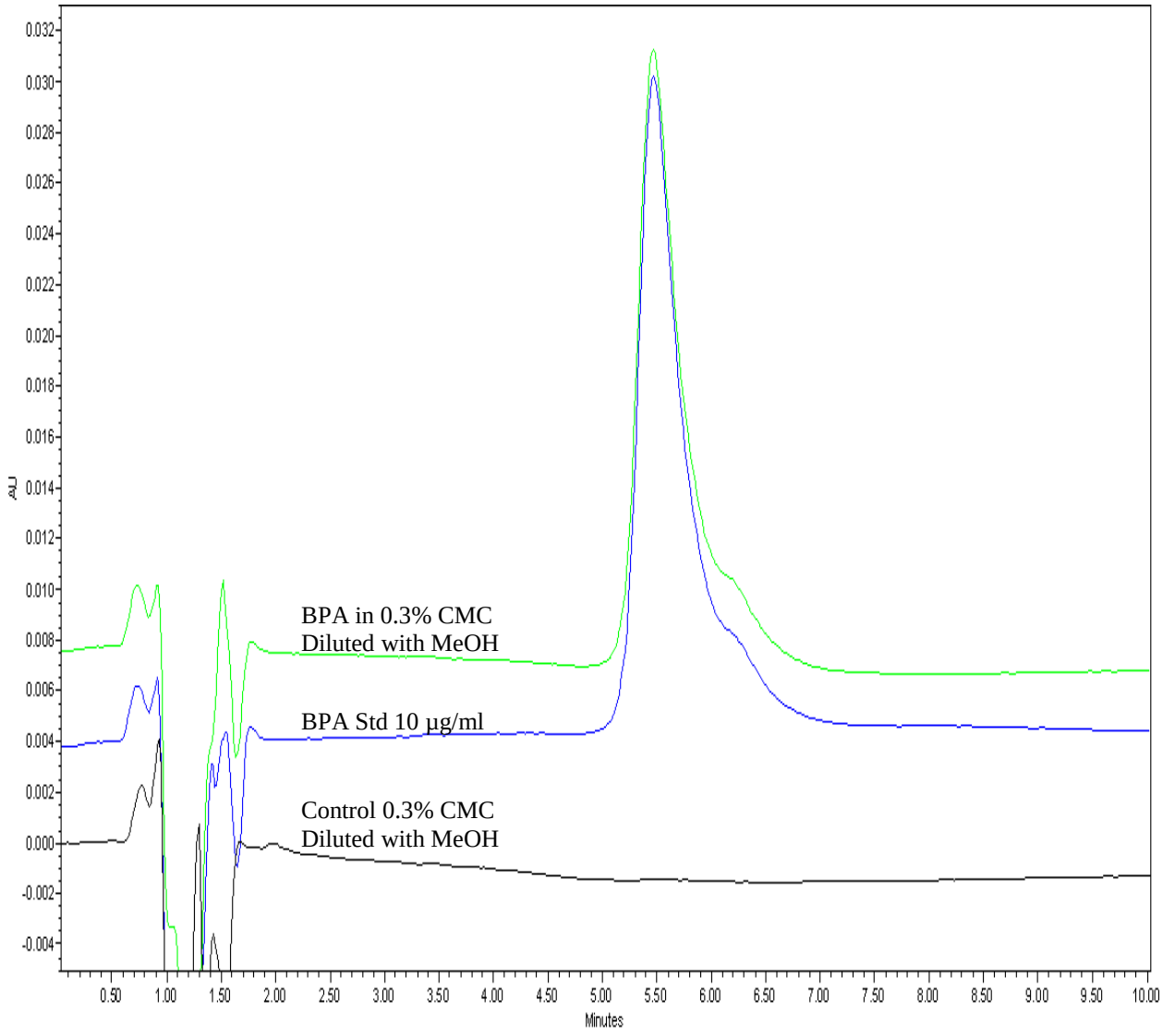
5.3 Transfer samples (5.2.3.9 – 5.2.6.9) to DBT Mass Spectrometry Support personnel for analyses.

6 HPLC-PDA analyses of BPA for Concentration Certification of Dose

6.1 The HPLC analysis of BPA is performed utilizing a Waters Millennium HPLC system equipped with a Waters 996 PDA detector with 227 nm wavelength extracted utilizing a Waters 717plus autosampler. Sample or standard injections volumes are from 10 to 50 µl from 1 ml amber LC vials. A Waters Nova-Pak® 4µm, column (3.9 mm x 150 mm length) is used for the analysis. The mobile phase is 4.5% 2-Propanol: SLS, 75 mM, delivered at a flow of 1 ml/min. The HPLC sample sequence is arranged such that each 3 sample injections are bracketed by injections of BPA quantitation standard.

6.2 The SLS stock is prepared at 150mM by dissolving 216.3g SLS in 5 liters of water. The mobile phase is prepared by combining 500 ml SLS stock with 45 ml 2-propanol and adjusting volume to 1000 ml. After dissolution, the mobile phase is filtered through a 0.45 µm filter.

7 Example Overlaid Chromatograms



Standard Operating Procedure for Sample Preparation of 0.3% Carboxyl methylcellulose Solutions for Determination of Bisphenol A or Ethynylestradiol by HPLC or LC-MS

1. PURPOSE

This Standard Operating Procedure (SOP) describes the method for preparation of samples for extraction of Bisphenol A (BPA) or Ethynylestradiol (EE2) from 0.3% carboxyl methylcellulose (CMC) solutions, for subsequent analysis by HPLC or LC-MS.

2. SCOPE

This procedure applies to the analyses of BPA or EE2 in 0.3% CMC utilized for animal dosing at the NCTR and is for compliance with FDA Good Laboratory Practice (GLP) as specified by 21 CFR 58.

3. CHEMICALS

3.1. BPA-d6 (bisphenol-A-d6, C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada, D-2476)
3.2. EE2-d4 (17α-ethynylestradiol-2,4,16,16-d4, C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada, D-4319)
3.3. Methanol (Fisher Scientific, E127-4, HPLC grade)
3.4. Toluene (Fisher Scientific, T290-4, HPLC grade)
3.5. Deionized Water, ultra-purified (Barnstead NANOPure, 18 MΩ cm-1) or equivalent

4. EQUIPMENT

4.1. 1.5 ml micro tubes
4.2. Glass scintillation vials
4.3. Glass 10 ml Class A volumetric flasks and pipettes
4.4. High-recovery autosampler vials for LC-MS
4.5. Micro centrifuge
4.6. Eppendorf 100-1000 μl micropipette and tips
4.7. Eppendorf 20-200 μl micropipette and tips
4.8. Speed vac concentrator
4.9. TissueLyser II (QIAGEN)
4.10. Vortex

5. PROCEDURES

Note: Prior to weighing operations, the analytical balance is calibrated and the calibration is documented with NIST traceable weights in the weight range of intended operation.

5.1 Standard Solutions Preparation

5.1.1. **BPA-d6 Stock Solution 100 ng/μl** – Weigh exactly 0.00100g of BPA-d6 into a 20 ml glass scintillation vial. Dissolve with 10 ml MeOH using a 10 ml Class A volumetric pipet.

5.1.2. **BPA-d6 5 ng/μl solution** – Dilute 500 μl of 100 ng/μl BPA-d6 to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.3. **BPA-d6 55.6 pg/μl solution** – Dilute 111 μl of 5 ng/μl BPA-d6 solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.4. **EE2-d4 Stock Solution 100 ng/μl** - Weigh exactly 0.00100g of EE2-d4 into a 20 ml glass scintillation vial. Dissolve with 10 ml MeOH using a 10 ml Class A volumetric pipet.

5.1.5. **EE2-d4 10 ng/μl solution** – Dilute 1000 μl of 100 ng/μl EE2-d4 Stock Solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.6. **EE2-d4 1 ng/μl solution** – Dilute 1000 μl of 10 ng/μl EE2-d4 solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.7. **EE2-d4 0.111 ng /μl solution** – Dilute 1110 μl of 1 ng/μl EE2-d4 solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.1.8. **EE2-d4 0.011 ng/μl solution** - Dilute 1000 μl of 0.111 ng/μl EE2-d4 solution to 10 ml with 10% MeOH using a class A 10 ml volumetric flask.

5.2 Sample Preparation

Note: For each dosing group set of accuracy of dose samples, triplicate samples are collected following AC Bionetics SOP and dispensed directly into either 20ml glass scintillation vials (250 mg/kg dose group only) or 1.5 ml borosilicate glass vials. Samples are then processed as follows:

5.2.1 **30 μl and 500 μl samples of 60,000 μg/ml BPA in 0.3% CMC**

Samples are diluted with methanol 1:10,000 and analyzed by HPLC-UV.

5.2.2 **30 μl samples of 0.5 μg/ml BPA in 0.3% CMC**

5.2.2.1. Add 270 μL of 55.6 pg/μl BPA-d6 solution to the 1.5 ml vial using an Eppendorf micropipette.

5.2.2.2. Vortex 1.5 ml glass vial for about 1 minute, then transfer sample to 1.5 ml micro tube.

5.2.2.3. Sucessively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using Eppendorf micropipette.

5.2.2.4. Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.

5.2.2.5. Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*

5.2.2.6. Carefully transfer the top organic layer into a new 1.5 mL micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.

5.2.2.7. Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.

5.2.2.8. Add 50 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about *1* minute.

5.2.2.9. Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.3 **500 μl samples of 0.5 μg/ml BPA in 0.3% CMC**

5.2.3.1 Add 50 μl of 5 ng/μl BPA-d6 solution to the 1.5 ml vial using an Eppendorf micropipette.

5.2.3.2 Vortex 1.5 ml glass vial for about 1 minute then transfer sample to 1.5 ml micro tube.

5.2.3.3 Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using Eppendorf micropipette.

5.2.3.4 Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.

5.2.3.5 Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*

5.2.3.6 Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.

5.2.3.7 Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.

5.2.3.8 Add 100 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about 1 minute.

5.2.3.10 Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.4 **30 μl samples of 1 μg/ml EE2 in 0.3% CMC**

5.2.4.1. Add 270 μL of 0.111 ng/μl EE2-d4 solution to the 1.5 ml micro tube using an Eppendorf micropipette.

5.2.4.2. Vortex 1.5 ml glass vial for about 1 minute, then transfer sample to 1.5 ml micro tube.

5.2.4.3. Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using an Eppendorf micropipette.

5.2.4.4. Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.

5.2.4.5. Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*

5.2.4.6. Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.

5.2.4.7. Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.

5.2.4.8. Add 50 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about *1* minute.

5.2.4.9. Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.5 **500 μl samples of 1 μg/ml EE2 in 0.3% CMC**

5.2.5.1. Add 50 μl of 10 ng/μl EE2-d4 solution to the 1.5 ml glass vial using an Eppendorf micropipette.

5.2.5.2. Vortex 1.5 ml glass vial for about 1 minute, then transfer sample to 1.5 ml micro tube.

5.2.5.3. Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using an Eppendorf micropipette.

5.2.5.4. Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.

5.2.5.5. Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*

5.2.5.6. Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.

5.2.5.7. Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.

5.2.5.8. Add 100 μL MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about *1* minute.

5.2.5.9. Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.6 **30 μl samples of 0.1 μg/ml EE2 in 0.3% CMC**

5.2.6.1. Add 270 μl of 0.011 ng/μl EE2-d4 solution to the 1.5 ml glass vial using an Eppendorf micropipette.

5.2.6.2. Vortex 1.5 ml glass vial for about 1 minute, then transfer sample to 1.5 ml micro tube.

5.2.6.3. Add 500 μl toluene to the 1.5 ml micro tube using an Eppendorf micropipette.

5.2.6.4. Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.

5.2.6.5. Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 min at > 18,000 *x g.*

5.2.6.6. Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.

5.2.6.7. Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.

5.2.6.8. Add 50 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about *1* minute.
5.2.6.9. Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

5.2.7 **500 μl samples of 0.1 μg/ml EE2 in 0.3% CMC**

5.2.7.1. Add 50 μl of 1 ng/μl EE2-d4 solution to the 1.5 ml glass vial using an Eppendorf micropipette.
5.2.7.2. Vortex 1.5 ml glass vial for about 1 minute, then transfer sample to 1.5 ml micro tube.
5.2.7.3. Successively add 50 μl saturated NaCl and 500 μl toluene to the 1.5 ml micro tube using an Eppendorf micropipette.
5.2.7.4. Shake the 1.5 ml micro tubes in a TissueLyser II instrument for 5 minutes at 30Hz.
5.2.7.5. Centrifuge the 1.5 ml micro tubes in a microcentrifuge for 10 minutes at > 18,000 x *g.*
5.2.7.6. Carefully transfer the top organic layer into a new 1.5 ml micro tube using an Eppendorf micropipette. As much as possible of the organic layer should be transferred without transferring any of the bottom aqueous layer.
5.2.7.7. Dry the organic solution in a speedvac (slowly apply vacuum) for 30 minutes.
5.2.7.8. Add 100 μl MeOH: water (1:1, v/v) to the 1.5 ml micro tube and vortex for about *1* minute.
5.2.7.9. Transfer the solution into a high recovery autosampler vial for LC-MS analysis.

6 Transfer samples (5.2.2 – 5.2.7) to DBT Mass Spectrometry Support personnel for analyses.

Analysis of ethinylestradiol in 0.3% CMC solutions

I. PURPOSE

To establish the LC-MS conditions required to analyze solutions of ethinylestradiol (EE2) in 0.3% carboxymethylcelulose (CMC).

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectroscopists/chemists.

IV. EQUIPMENT

Quantum Ultra TSQ Tandem MS (Bar Code 4000219)
Thermo Accela LC system

V. SUPPLIES & REFERENCES

- Methanol (Baker Analyzed HPLC solvent, J.T. Baker Chemical Co.)
- Water (OPTIMA LC/MS grade, Fisher Chemical)
- Gemini C18 2.0 x 50mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the team leader of the Chemistry Support Group.

VII. OPERATIONS

- The samples are loaded in the autosampler as received and run using single ion monitoring in negative electrospray mode as indicated below.
- An external standard at the expected final target sample concentration will be analyzed and results should be at target level +/- 10%.
- The volumes of sample to inject are as follows:

	Ext. std. (EE2-d0&d4)
Sample prepared from 500µL of EE2 solution at 1 µg/mL - 1µL	5 ng/µl
Sample prepared from 500µL of EE2 solution at 0.1 µg/mL - 10µL	0.5 ng/µl
Sample prepared from 500µl of EE2 solution at 0.01 µg/mL - 20µL	0.05 ng/µl
Sample prepared from 30µL of EE2 solution at 1 µg/mL - 10µL	600 pg/µl
Sample prepared from 30µL of EE2 solution at 0.1 µg/mL - 20µL	60 pg/µl

Sample prepared from 30µL of EEs solution at 0.01 µg/mL - 20µL 6pg/µl

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Accela Autosampler
Injection volume: 1-20 µl
Flush volume: 400 µl
Flush/wash source: bottle
Needle height from bottom: 1.5 mm
Wash volume: 1000 µl
Flush speed: 100 µl/sec
Syringe speed: 8 µl/sec
Injection mode: No waste
Column temperature: 40 °C

Accela Pump
Solvent A: water
Solvent B: Baker MeOH
Start settings: Accela AS inj logic
Method finalizing: first line conditions
Min pressure: 0 bar
Max pressure: 1250 bar

Gradient Table

Time (min)	%A	%B	µl/min
0	45	55	500
4	45	55	500

TSQ Quantum Instrument Method

Method Type: Regular Method
MS Run Time (min): 4.00
Segment 1
Duration (min) 4.00
Scan Events 1
Segment 1:
Tune Method C:\Xcalibur\methods\EE2 in CMC.TSQTune
Chrom filter: 10
Q2 Gas Pressure: Not used
Syringe Pump: Off

Scan Events:
1) – SIM Q1MS, Skimmer 22, Micro scans 1

295.100, width 0.5, time 0.350, Q1 0.7, Q3 0.7, Tube 133

2) – SIM Q1MS, Skimmer 22, Micro scans 1

299.090, width 0.5, time 0.350, Q1 0.7, Q3 0.7, Tube 133

IX- CALCULATIONS

As prepared by the Chemistry Support Group, the samples were spiked with an amount of labeled EE2-d4 corresponding to 100% dose accuracy. Solutions of labeled and unlabeled standards were carefully standardized by LC-UV analysis. QC external standard solutions are prepared at each dose level. QC samples are injected in triplicate prior to the analysis of the dosing solutions. The QCs are also injected once after every 6 samples, to ensure instrument stability and reproducibility. The response factors are determined at each dose level.

The average response factor for each day is then used to determine the dose accuracy of the submitted samples. The dose accuracy (% of target) of unlabeled EE2-d0 is determined by dividing the peak area for unlabeled EE2-d0 (*m/z* 295.2) by the peak area for labeled EE2-d4 (*m/z* 299.2) peak, dividing the determined isotopic response factor, and multiplying by 100%.

Example calculation for a sample prepared from 500µL of EE2 solution at 1 µg/mL:

Unlabeled peak area: 6,886,715 Labeled peak area: 6,424,426

Accuracy = [(6,886,715/6,424,426)/1.072] X100% = 99.99%

Analysis of bisphenol A in 0.3% CMC solutions

I. PURPOSE

To establish the LC-MS conditions required to analyze solutions of bisphenol A (BPA) in 0.3% carboxymethylcelulose (CMC).

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectroscopists/chemists.

IV. EQUIPMENT

Quantum Ultra TSQ Tandem MS (Bar Code 4000219)
Thermo Accela LC system

V. SUPPLIES & REFERENCES

- Methanol (Baker Analyzed HPLC solvent, J.T. Baker Chemical Co.)
- Water (OPTIMA LC/MS grade, Fisher Chemical)
- Gemini C18 2.0x50mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the team leader of the Chemistry Support Group.

VII. OPERATIONS

- The samples are loaded in the autosampler as received and run in MRM mode using negative ion mode electrospray as indicated below.
- An external standard at the expected final target sample concentration will be analyzed and results should be at target level +/- 10%.
- The volumes of sample to inject are as follows:

	Ext. std. (BPA-d0&d6)
Sample prepared from 500µL of BPA solution at 0.5 µg/mL - 1µL	2.5 ng/µl
Sample prepared from 30µL of BPA solution at 0.5 µg/mL - 10µL	300 pg/µl

UNCONTROLLED WHEN PRINTED

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Accela Autosampler
Injection volume: 1-10 µl
Flush volume: 400 µl
Flush/wash source: bottle
Needle height from bottom: 1.5 mm
Wash volume: 1000 µl
Flush speed: 100 µl/sec
Syringe speed: 8 µl/sec
Injection mode: No waste
Column temperature: 40 °C

Accela Pump
Solvent A: water
Solvent B: Baker MeOH
Start settings: Accela AS inj logic
Method finalizing: first line conditions
Min pressure: 0 bar
Max pressure: 1250 bar

Gradient Table

Time (min)	%A	%B	µl/min
0	50	50	500
4	50	50	500

TSQ Quantum Instrument Method

Method Type: Regular Method
MS Run Time (min): 4.00
Segment 1
Duration (min) 4.00
Scan Events 4
Segment 1:
Tune Method C:\Xcalibur\methods\BPA dosing solution and water.TSQTune
Chrom filter: 10
Q2 Gas Pressure: 1.5
Syringe Pump: Off

Scan Events:
1) – SRM Skimmer 20, Micro scans 1

227.030 > 133.083, width 0.5, time 0.350, CE 25, Q1 0.7, Q3 0.7, Tube 92

2) – SRM Skimmer 20, Micro scans 1

227.030 > 212.079, width 0.5, time 0.350, CE 21, Q1 0.7, Q3 0.7, Tube 92

3) – SRM Skimmer 20, Micro scans 1

233.050 > 138.144, width 0.5, time 0.350, CE 29, Q1 0.7, Q3 0.7, Tube 81

4) – SRM Skimmer 20, Micro scans 1

233.050 > 215.084, width 0.5, time 0.350, CE 20, Q1 0.7, Q3 0.7, Tube 81

IX- CALCULATIONS

As prepared by the Chemistry Support Group, the samples were spiked with an amount of labeled BPA-d6 corresponding to 100% dose accuracy. Solutions of labeled and unlabeled standards were carefully standardized by LC-UV analysis. QC external standard solutions are prepared at each dose level. QC samples are injected in triplicate prior to the analysis of the dosing solutions. The QCs are also injected once after every 6 samples, to ensure instrument stability and reproducibility. The response factors are determined at each dose level.

The average response factor for each day is then used to determine the dose accuracy of the submitted samples. The dose accuracy (% of target) of unlabeled BPA-d0 is determined by dividing the peak area for unlabeled BPA-d0 (*m/z* 212.079) by the peak area for labeled BPA-d6 (*m/z* 215.084) peak, dividing the determined isotopic response factor, and multiplying by 100%.

Example of calculation for a sample prepared from 500µL of BPA solution at 0.5 µg/mL:

Integration of the unlabeled peak BPA-d0: 125,000
Integration of the labeled peak BPA-d6: 150,000

The d0/d6 ratio for the given analysis is divided by the response factor and then multiplied by 100 to give % accuracy.

Accuracy = ((9,690,193/7,861,796)/1.232)x100 = 100.0%

Determination of the isoflavones, genistein and daidzein, in 5K96 diet samples

I. PURPOSE

To establish the LC-ESI-MRM conditions required to determine genistein (GTN) and daidzein (DDZ) in 5K96 control feed samples submitted to the mass spectrometry lab.

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectroscopists/chemists.

IV. EQUIPMENT

Quantum Ultra TSQ Tandem MS (Bar Code 4000219)
Agilent 1100 HPLC system

V. SUPPLIES & REFERENCES

- Methanol (Baker Analyzed HPLC grade, J.T. Baker Chemical Co.)
- Water (OPTIMA LC/MS grade, Fisher Chemical)
- Acetonitrile (OPTIMA LC/MS grade, Fisher Chemical)
- Gemini C18 2.0x150mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the Chemistry Support Group.

VII. Operations

- The samples are loaded in the autosampler as received and run in MRM negative ion mode electrospray as indicated below.
- An external standard at 0.5 ug/mL GEN and 0.5 ug/mL DDZ will be used as quality control samples.
- 3 ul of quality control standard is injected.

UNCONTROLLED WHEN PRINTED

- Each sample set with an external standard containing GTN and DDZ at 0.5 ug/ml each and duplicate samples for each feed SCR number, with and without spikes at 5 ppm prior to cleanup, is loaded into autosampler as received. All samples are in 60% acetonitrile 40% water and 3 µl of each sample is analyzed by LC-ESI-MRM in triplicate. The external standard is injected continually until peak areas are consistent before analyzing the feed sample set.

Tune page

Spray Voltage	4000V
Sheath Gas Pressure	20 (arbitrary units)
Ion Sweep Gas Pressure	5 (arbitrary units)
Aux Gas Pressure	40 (arbitrary units)
Capillary Temperature	335ºC
Tube Lens Offset	79V

Based on Method: Isoflavone –ESIMRM TMH feed

Agilent1100 MicroAutoSampler
Drawing speed (ul/min): 10.0
Ejecting speed (ul/min): 50.0
Needle draw position offset (mm): 1.0
Injection volume (ul): 3.0
Wash vial: 91
Wash Cycle: 1
Wash Stroke: 20.0
Analysis stop time (min): 0.00
Post run time (min): 0.00
Timed events:
Time Contact Number Contact State
0.00(min)

Agilent1100 Heater
Oven: On
Separate Mode: Off
Post run time (min): 0.00
Time Left Temperature Right Temperature Valve Position
0.00(min) 40.00(C) 0.00(C) 1
18.00(min) 40.00(C) 0.00(C) 1

Agilent1100 Quaternery Pump
Solvent A: H2O 0.1% formic acid
Solvent B: 95% ACN 5%H2O 0.1%formic acid
Solvent C: 100 %Water
Solvent D: 100%MeOH
Minimun pressure limit (bar): 0.0
Maximum pressure limit (bar): 400.0
Post run time (min): 0.00
Gradient program:

Time Flow Rate Composition
0.00(min) 0.20(ml/min) A=0.0% B=0.0% C=40.0% D=60.0%
7.00(min) 0.20(ml/min) A=0.0% B=0.0% C=40.0% D=60.0%
7.50(min) 0.20(ml/min) A=0.0% B=0.0% C=1.0% D=99.0%
12.50(min) 0.20(ml/min) A=0.0% B=0.0% C=1.0% D=99.0%
13.00(min) 0.20(ml/min) A=0.0% B=0.0% C=40.0% D=60.0%
18.00(min) 0.20(ml/min) A=0.0% B=0.0% C=40.0% D=60.0%

TSQ Quantum Instrument Method
Method Type: Regular Method
MS Run Time (min): 7.50
Segment 1
Duration (min) 7.50
Scan Events 4
Segment 1:
Tune Method C:\Xcalibur\methods\Bisphenol A -ESI with ammonia by TMH.TSQTune
Chrom filter: 10
Q2 Gas Pressure: 1.5
Syringe Pump: Off
Scan Events:
1: - c SRM Skimmer Offset 21, Micro Scans 1,
Parent Center Width Time CE Q1 PW Q3 PW Tube Lens
253.000 208.000 0.700 0.200 30 0.70 0.70 Tuned Value
2: - c SRM Skimmer Offset 21, Micro Scans 1,
Parent Center Width Time CE Q1 PW Q3 PW Tube Lens
253.000 223.000 0.700 0.200 30 0.70 0.70 Tuned Value
3: - c SRM Skimmer Offset 21, Micro Scans 1,
Parent Center Width Time CE Q1 PW Q3 PW Tube Lens
269.000 159.000 0.700 0.200 27 0.70 0.70 Tuned Value
4: - c SRM Skimmer Offset 21, Micro Scans 1,
Parent Center Width Time CE Q1 PW Q3 PW Tube Lens
269.000 133.000 0.700 0.200 27 0.70 0.70 Tuned Value
Syringe pump not in use
Divert Valve: in use during run
Divert Time (min) Valve State
0.00 Waste
3.00 Load \ Detector
7.00 Waste
12.50 Load \ Detector

VIII. CALCULATIONS

As prepared by the Chemistry Support Group for each feed, duplicate feed samples that are spiked with unlabeled GEN and DDZ corresponding to 1 ppm each in the feed and duplicate unspiked feed samples are submitted. Determine the peak areas of two transitions for DDZ (*m/z* 253>208 & *m/z* 253>223) and GEN (*m/z* 269>133 & *m/z* 269>159) in each sample analysis. Average the peak area data for the triplicate analyses. The concentrations of unlabeled GEN and DDZ are determined by the

method of standard additions. For each feed, subtract the average area for each transition of the unspiked samples from the average areas for each transition of the spiked feed samples, and determine the area/ppm for each transition. Using these values, calculate the amounts of DDZ and GEN (ppm) in each feed. Values calculated for each transition should be close and should be averaged also. An example for one feed is shown below:

Averaged peak areas for one feed, with and without spikes at 5 ppm:

	DDZ 253>208	DDZ 253>223	GEN 269>133	GEN 269>159
1455000310-1	232,227	256,282	175,299	76,378
1455000310-2	160,773	186,779	219,916	100,149
1455000310 Spike 1	6,428,422	6,735,992	2,150,198	1,032,499
1455000310 Spike 2	4,646,122	4,873,400	4,480,951	2,165,816
The duplicates should replicate fairly well, and they are averaged again below.				
1455000310 Avg	196,500	221,531	197,608	88,264
1455000310 Spike Avg	5,537,272	5,804,696	3,315,574	1,599,157
Subtract unspiked avg values from the avg spiked values to get area/ppm for each transition for each isoflavone target.				
Area/ppm	1,068,154	1,116,633	623,593	302,179
Now calculate the original ppm of each isoflavone for the avg unspiked feed.				
Isoflavone ppm	0.184	0.198	0.317	0.292
Report avg for isoflavones	**0.191 ppm DDZ**		**0.304 ppm GTN**	

IX. DOCUMENTATION

1. Prepare hardcopies of the 4 mass chromatograms for all ion transitions for each sample analysis.
2. Compile the data into report documents according to the analytical technique.
3. Send Reports to the NTP Chemistry Support Team Leader.
4. Store electronic copies of the data files and report files in the DBT-MS Lab/ Data subdirectory for the given Protocol.
5. Store original hardcopies of the Report, Sample sequence list, Work Request with any attachments & Data in the locked file cabinet in 26-B001A.

Determination of Bisphenol A in Water, Cage Leachates, Stopper Extracts and Similar Samples

I. PURPOSE

To determine BPA concentrations in samples submitted to the Mass Spectrometry Laboratory

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectroscopists/chemists.

IV. EQUIPMENT

Quantum Ultra TSQ Tandem MS (Bar Code 4000219)
Thermo Accela LC system

V. SUPPLIES & REFERENCES

- Bisphenol A
- Bisphenol A-d_6
- Methanol (OPTIMA LC/MS grade, Fisher Chemical)
- Water (OPTIMA LC/MS grade, Fisher Chemical)
- Acetonitrile (J.T. Baker, HPLC grade)
- Gemini C18 2.0x50mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the Chemistry Support Group.

VII. OPERATIONS

- The samples are loaded in the autosampler as received and run in MRM negative ion mode electrospray as indicated below.
- External standards at 50 ng/mL BPA-d0/10 ng/mL BPA-d6, 10ng/mL BPA-d0/10ng/mL BPA-d6, and 2.5ng/mL BPA-d0/10ng/mL BPA-d6 will be used as quality control samples.
- 20 ul of each quality control standard is injected.

UNCONTROLLED WHEN PRINTED

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Accela Autosampler
Injection volume: 20 µl
Flush volume: 400 µl
Flush/wash source: bottle
Needle height from bottom: 1.5 mm
Wash volume: 1000 µl
Flush speed: 100 µl/sec
Syringe speed: 8 µl/sec
Injection mode: No waste
Column temperature: 40°C

Accela Pump
Solvent A: water
Solvent B: Baker MeOH
Start settings: Accela AS inj logic
Method finalizing: first line conditions
Min pressure: 0 bar
Max pressure: 1250 bar

Gradient Table

Time (min)	%A	%B	µl/min
0	50	50	500
4	50	50	500

TSQ Quantum Instrument Method

Method Type: Regular Method
MS Run Time (min): 4.00
Segment 1
Duration (min) 4.00
Scan Events 4
Segment 1:
Tune Method C:\Xcalibur\methods\BPA in dosing solution and water.TSQTune
Chrom filter: 10
Q2 Gas Pressure: 1.5
Syringe Pump: Off

Scan Events:
1) – SRM Skimmer 20, Micro scans 1

227.030 > 133.083, width 0.5, time 0.350, CE 25, Q1 0.7, Q3 0.7, Tube 92

2) – SRM Skimmer 20, Micro scans 1

227.030 > 212.079, width 0.5, time 0.350, CE 21, Q1 0.7, Q3 0.7, Tube 92

3) – SRM Skimmer 20, Micro scans 1

233.050 > 138.144, width 0.5, time 0.350, CE 29, Q1 0.7, Q3 0.7, Tube 81

4) – SRM Skimmer 20, Micro scans 1

233.050 > 215.084, width 0.5, time 0.350, CE 20, Q1 0.7, Q3 0.7, Tube 81

IX- CALCULATIONS

The quality control samples will be run repeatedly until the system is equilibrated. **The LLOQ is 2.5 ng/mL.**

The response between the BPA-d0 and BPA-d6 is determined by running a calibration curve from 0-100ng/mL, with the amount of BPA-d6 remaining constant at 10ng/mL in each sample. The slope of the generated curve is then used to determine the amount of BPA-d0 present in all samples. All concentration determinations are performed using the QuanBrowser quantitation program as part of the Xcalibur data acquisition system. All quantitation files are stored electronically on the data acquisition computer. Hard copies are stored in the locked file cabinet in 26-B001A.

A new calibration curve is generated when the following events occur:

- Quality control samples are unexpectedly lower or higher than usual
- Annual performance maintenance
- At the beginning of the protocol

Determination of Bisphenol A in Feed and Bedding

I. PURPOSE

To determine BPA concentrations in samples submitted to the Mass Spectrometry Laboratory

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectroscopists/chemists.

IV. EQUIPMENT

Quantum Ultra TSQ Tandem MS (Bar Code 4000219)
Thermo Accela LC system

V. SUPPLIES & REFERENCES

- Bisphenol A
- Bisphenol A-d_6
- Methanol (Baker Analyzed HPLC solvent, J.T. Baker Chemical Co.)
- Water (OPTIMA LC/MS grade, Fisher Chemical)
- Gemini C18 2.0x150mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the Chemistry Support Group.

VII. OPERATIONS

- The samples are loaded in the autosampler as received and run in MRM negative ion mode electrospray as indicated below.
- External standard at 100 ng/mL BPA-d0/100 ng/mL BPA-d6, 25ng/mL BPA-d0/100ng/mL BPA-d6 and 5ng/mL BPA-d0/100ng/mL BPA-d6 will be used as quality control samples.
- 10 ul of each quality control standard is injected.

UNCONTROLLED WHEN PRINTED

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Accela Autosampler
Injection volume: 10 µl
Flush volume: 400 µl
Flush/wash source: bottle
Needle height from bottom: 1.5 mm
Wash volume: 1000 µl
Flush speed: 100 µl/sec
Syringe speed: 8 µl/sec
Injection mode: No waste
Column temperature: 40°C

Accela Pump
Solvent A: water
Solvent B: Baker MeOH
Start settings: Accela AS inj logic
Method finalizing: first line conditions
Min pressure: 0 bar
Max pressure: 1250 bar

Gradient Table

Time (min)	%A	%B	µl/min
0	40	60	200
1	40	60	200
2	1	99	200
5	1	99	200
6	40	60	200

TSQ Quantum Instrument Method

Method Type: Regular Method
MS Run Time (min): 9.5
Segment 1
Duration (min) 9.5
Scan Events 4
Segment 1:
Tune Method C:\Xcalibur\methods\BPA in feed and bedding Aug2010.TSQTune
Chrom filter: 10
Q2 Gas Pressure: 1.5
Syringe Pump: Off

Scan Events:
1) – SRM Skimmer 20, Micro scans 1

227.030 > 133.064, width 0.5, time 0.350, CE 26, Q1 0.7, Q3 0.7, Tube 114

2) – SRM Skimmer 20, Micro scans 1

227.030 > 212.153, width 0.5, time 0.350, CE 20, Q1 0.7, Q3 0.7, Tube 114

3) – SRM Skimmer 20, Micro scans 1

232.980 > 138.130, width 0.5, time 0.350, CE 27, Q1 0.7, Q3 0.7, Tube 110

4) – SRM Skimmer 20, Micro scans 1

232.980 > 215.084, width 0.5, time 0.350, CE 21, Q1 0.7, Q3 0.7, Tube 110

IX- CALCULATIONS

The quality control samples will be run repeatedly until the system is equilibrated. **The LLOQ for 10µl injected on column is 2.5 ng/mL.**

The response between the BPA-d0 and BPA-d6 is determined by running a calibration curve from 0-250ng/mL, with the amount of BPA-d6 remaining constant at 100ng/mL in each sample. The slope of the generated curve is then used to determine the amount of BPA-d0 present in all samples. All concentration determinations are performed using the QuanBrowser quantitation program as part of the Xcalibur data acquisition system. All quantitation files are stored electronically on the data acquisition computer. Hard copies are stored in the locked file cabinet in 26-B001A.

A new calibration curve is generated when the following events occur:

- Quality control samples are unexpectedly lower or higher than usual
- Annual performance maintenance
- At the beginning of the protocol

Determination of the isoflavones and zearalenone in diet samples

I. PURPOSE

To establish the LC-ESI-MRM conditions required to determine isoflavone and zearalenone levels in feed samples submitted to the mass spectrometry lab.

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectroscopists/chemists.

IV. EQUIPMENT

Quantum Ultra TSQ Tandem MS (Bar Code 4000219)
Thermo Accela LC system

V. SUPPLIES & REFERENCES

- Methanol (Baker Analyzed HPLC grade, J.T. Baker Chemical Co.)
- Water (OPTIMA LC/MS grade, Fisher Chemical)
- Gemini C18 2.0x150mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the Chemistry Support Group.

VII. Operations

- The samples are loaded in the autosampler as received and run in MRM mode using negative mode electrospray as indicated below.
- An external standard at 0.5 ug/mL DDZ, GEN, COU, and ZEA will be used as the quality control sample.
- 3 ul of quality control standard is injected.

UNCONTROLLED WHEN PRINTED

- Each sample set will also contain an external standard containing the isoflavones and zearalenone at 0.5 ug/ml each. Triplicate samples for each feed SCR number, with and without spikes at the appropriate levels prior to cleanup, are loaded into autosampler as received. 3 µl of each sample is analyzed by LC-ESI-MRM. The external standard is injected continually until peak areas are consistent before analyzing the feed sample set.

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Accela Autosampler
Injection volume: 3 µl
Flush volume: 400 µl
Flush/wash source: bottle
Needle height from bottom: 1.5 mm
Wash volume: 1000 µl
Flush speed: 100 µl/sec
Syringe speed: 8 µl/sec
Injection mode: No waste
Column temperature: 40°C

Accela Pump
Solvent A: water
Solvent B: Baker MeOH
Start settings: Accela AS inj logic
Method finalizing: first line conditions
Min pressure: 0 bar
Max pressure: 1250 bar

Gradient Table

Time (min)	%A	%B	µl/min
0	40	60	200
1	40	60	200
6	1	99	200
7	1	99	200
7.5	40	60	200

TSQ Quantum Instrument Method
Method Type: Regular Method
MS Run Time (min): 11.00
Segment 1
Duration (min) 11.00
Scan Events 8
Segment 1:
Tune Method C:\Xcalibur\methods\Isoflavones in feed.TSQTune
Chrom filter: 10

Q2 Gas Pressure: 1.5
Syringe Pump: Off
Scan Events:
1) – SRM Skimmer 22, Micro scans 1

253.010 > 208.053, width 0.5, time 0.2, CE 31, Q1 0.7, Q3 0.7, Tube 145

2) – SRM Skimmer 22, Micro scans 1

253.010 > 223.041, width 0.5, time 0.2, CE 33, Q1 0.7, Q3 0.7, Tube 145

3) – SRM Skimmer 22, Micro scans 1

268.840 > 133.030, width 0.5, time 0.2, CE 32, Q1 0.7, Q3 0.7, Tube 141

4) – SRM Skimmer 22, Micro scans 1

268.840 > 159.03, width 0.5, time 0.2, CE 29, Q1 0.7, Q3 0.7, Tube 141

5) – SRM Skimmer 22, Micro scans 1

266.950 > 211.012, width 0.5, time 0.2, CE 29, Q1 0.7, Q3 0.7, Tube 141

6) – SRM Skimmer 22, Micro scans 1

266.950 > 135.011, width 0.5, time 0.2, CE 30, Q1 0.7, Q3 0.7, Tube 141

7) – SRM Skimmer 22, Micro scans 1

316.810 > 175.088, width 0.5, time 0.2, CE 25, Q1 0.7, Q3 0.7, Tube 129

8) – SRM Skimmer 22, Micro scans 1

316.810 > 273.153, width 0.5, time 0.2, CE 21, Q1 0.7, Q3 0.7, Tube 129

VIII. CALCULATIONS

As prepared by the Chemistry Support Group for each feed, feed samples that are spiked with unlabeled isoflavones and zearalenone corresponding to the appropriate level in the feed and unspiked feed samples are submitted. The peak areas are determined for the main transitions of each compound. The peak area data for the triplicate analyses is averaged. The concentrations of unlabeled isoflavones and zearalenone are determined by the method of standard additions. For each feed, subtract the average area for each transition of the unspiked samples from the average areas for each transition of the spiked feed samples, and determine the area/ppm for each transition. Using these values, calculate the amounts of isoflavones and zearalenone (ppm) in each feed. Values calculated for each transition should be close and should also be averaged. An example for one feed lot is shown below:

Averaged peak areas for one feed, with and without spikes at 75ppm DDZ, 50ppm GEN, 10ppm COU and 0.5ppm ZEA
:

	DDZ	GEN	COU	ZEA
	253>208	269>133	266>239	317>175
Unspiked feed 1	3,050,445	1,565,683	210,099	820

Unspiked feed 2	3,584,231	2,937,727	283,092	<LOQ
Unspiked feed 3	3,898,308	3,990,786	456,786	378
Spiked feed 1	6,968,039	6,164,213	1,258,783	29,032
Spiked feed 2	6,942,915	4,804,566	1,044,606	24,119
Spiked feed 3	6,889,267	5,083,986	1,124,195	19,533

The samples are then averaged by group:

Unspiked Avg	3,510,995	2,831,399	316,659	599
Spiked Avg	6,933,407	5,350,922	1,142,528	24,228

Subtract unspiked avg values from the avg spiked values to get area/ppm for each transition for each isoflavone target.

Area/ppm	45,632.16	50,390.46	82,586.9	47,258

Now calculate the original ppm of each isoflavone for the avg unspiked feedby dividing the unspiked average by the area/ppm:

Isoflavone ppm	76.94	56.19	3.83	0.013

IX. DOCUMENTATION

All peak area integrations are performed using the QuanBrowser quantitation program as part of the Xcalibur data acquisition system. All quantitation files are stored electronically on the data acquisition computer. Hard copies are stored in the locked filed cabinet in 26-B001A.

All further calculations are performed with Excel, using the method shown above.

Determination of Bisphenol A in Feed and Bedding Using the Waters Premiere XE

I. PURPOSE

To determine BPA concentrations in samples submitted to the Mass Spectrometry Laboratory

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectrometrists and chemists.

IV. EQUIPMENT

Waters Premiere XE Triple Quadrupole Mass Spectrometer (VAB 1074)
Waters Acquity UPLC system

V. SUPPLIES & REFERENCES

- Bisphenol A
- Bisphenol A-d_6
- Methanol (Baker Analyzed HPLC solvent)
- Water (OPTIMA LC/MS grade)
- Gemini C18 2.0x150mm 3 µm HPLC column (Phenomenex)

VI. FREQUENCY

Whenever analyses are solicited by the Chemistry Support Group.

VII. OPERATIONS

- The samples are loaded in the autosampler as received and run in MRM negative ion mode electrospray as indicated below.
- External standard at 100 ng/mL BPA-d0/100 ng/mL BPA-d6, 25ng/mL BPA-d0/100ng/mL BPA-d6 and 5ng/mL BPA-d0/100ng/mL BPA-d6 will be used as quality control samples.
- 10 ul of quality control standard is injected.

UNCONTROLLED WHEN PRINTED

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Waters Acquity SDS

Run time: 21.5 min
Solvent A: Optima water
Solvent B: Baker MeOH
Low Pressure Limit: 0psi
High Pressure Limit: 4000psi
Seal Wash: 3.5 min

Gradient Table

Time (min)	Flow rates (µL/min)	%A	%B
Initial	0.200	50	50
3.00	0.200	50	50
13.00	0.200	1	99
18.00	0.200	1	99
18.50	0.200	50	50

Waters Acquity Autosampler

Run time: 1 min
Loop option: Partial loop
Weak wash volume: 700 µL
Strong wash volume: 100 µL
Column temperature: 40 °C
Sample temperature: 18 °C
Sample loop size: 50 µL
Injection volume: 10 µL

Mass Spectrometry Functions

Inter-scan delay: 0.050 sec
Inter-channel delay: 0.005 sec
Span: 0 Da
Ionization mode: ES-
Data type: SIR or MRM data
Function type: MRM of 4 channels

Channel	Reaction	Dwell (sec)	Cone Voltage	Collision Energy
BPA-d0 133	227.04>132.86	0.250	35	26
BPA-d0 212	227.04>212.00	0.250	35	21
BPA-d6 138	233.05>137.88	0.250	35	26
BPA-d6 215	233.05>215.03	0.250	35	21

IX- CALCULATIONS

The quality control samples will be run repeatedly until the system is equilibrated. **The LLOQ for 10µl injected on column is 1.0 ng/mL.**

The response between the BPA-d0 and BPA-d6 is determined by running a calibration curve from 0-250ng/mL, with the amount of BPA-d6 remaining constant in each sample. The slope of the generated curve is then used to determine the amount of BPA-d0 present in all samples. All concentration determinations are performed using the QuanLynx quantitation program as part of the MassLynx data acquisition system. All quantitation files are stored electronically on the data acquisition computer. Hard copies are stored in the locked file cabinet in 26-B001A.

A new calibration curve is generated when the following events occur:

- Quality control samples are unexpectedly lower or higher than usual
- Annual performance maintenance
- At the beginning of the protocol

X-DOCUMENTATION

A copy of the report is sent via email to the requestor. All data files are stored on the data acquisition computer and in the locked file cabinet in 26-B001A as outlined in the file name and data storage SOP. An original signed report is also stored in the locked file cabinet. Upon conclusion of the GLP study, all stored records are boxed and supplied to the Principal Investigator.

National Center for Toxicological Research US Food & Drug Administration, Jefferson, AR Mass Spectrometry Laboratory Standard Operating Procedures	
SOP No.: NCTR MSL-43.00	Approved by Director, Division of Biochemical Toxicology
Supersedes: N/A	Date Approved: 3/10/2014
Page 1 of 3	Effective Date: 3/10/2014

Determination of Bisphenol A in Cellulose Bedding Using the Waters Xevo TQS

I. PURPOSE

To determine BPA concentrations in samples submitted to the Mass Spectrometry Laboratory

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectrometrists and chemists.

IV. EQUIPMENT

Waters Xevo TQS Triple Quadrupole Mass Spectrometer (WAA 1011)
Waters Acquity UPLC system I-class

V. SUPPLIES & REFERENCES

- Bisphenol A
- Bisphenol A-d_6
- Methanol (Baker Analyzed HPLC solvent)
- Water (OPTIMA LC/MS grade, Fisher Scientific)
- BEH C18 1.7µm, 2.1x50mm UPLC column (Waters)

VI. FREQUENCY

Whenever analyses are solicited by the Chemistry Support Group.

VII. OPERATIONS

- The samples are loaded in the autosampler as received and run in MRM negative ion mode electrospray as indicated below.
- External standard at 100 ng/mL BPA-d0/100 ng/mL BPA-d6, 25ng/mL BPA-d0/100ng/mL BPA-d6 and 5ng/mL BPA-d0/100ng/mL BPA-d6 will be used as quality control samples.
- 10 ul of quality control standard is injected.

UNCONTROLLED WHEN PRINTED

Mass Spectrometry Parameters: Optimized as needed for all voltages and gas flow rates.

Waters Acquity SDS

Run time: 3.5 min
Solvent A: Optima water
Solvent B: Baker MeOH
Low Pressure Limit: 0psi
High Pressure Limit: 14500psi
Seal Wash: 1 min

Gradient Table

Time (min)	Flow rates (µL/min)	%A	%B
Initial	0.300	50	50

Waters FTN Acquity Autosampler

Run time: 1 min
Wash solvent: 50/50 MeOH/water
Pre-inject wash: 3 sec
Post inject wash: 7 sec
Purge solvent: 50/50 MeOH/water
Column temp: 40°C
Sample temp: 15°C
Injection volume: 10 µl

Mass Spectrometry Functions

Inter-scan delay: automatic
Inter-channel delay: automatic
Span: 0 Da
Ionization mode: ES-
Data type: SIR or MRM data
Function type: MRM of 4 channels

Channel	Reaction	Dwell (sec)	Cone Voltage	Collision Energy
BPA-d0 133	227.16>133.06	0.05	14	28
BPA-d0 212	227.16>212.04	0.05	14	22
BPA-d6 138	233.22>138.11	0.05	14	24
BPA-d6 215	233.22>215.09	0.05	14	22

IX- CALCULATIONS

The quality control samples will be run repeatedly until the system is equilibrated. **The LLOQ for 10µl of pure standard injected on column is 1 ng/mL.** The LLOQ for the neat standard was determined by injecting the standard 5 times. The average of these injections was 1.002 ng/mL. The percent RSD was 9.56% while the accuracy of the injections was determined to be 100.2%.

The response between the BPA-d0 and BPA-d6 is determined by running a calibration curve from 0-250ng/mL, with the amount of BPA-d6 remaining constant in each sample. The slope of the generated curve is then used to determine the amount of BPA-d0 present in all samples. All concentration determinations are performed using the QuanLynx quantitation program as part of the MassLynx data acquisition system. All quantitation files are stored electronically on the data acquisition computer. Hard copies are stored in the locked file cabinet in 26-B001A.

The spiked bedding samples should contain approximately 12.5 ng/mL BPA-d0 when compared to the non-spiked bedding samples. The control and non-spiked bedding samples are only spiked with the internal standard (BPA-d6) at 100ng/mL. The spiked bedding samples are used to confirm method performance. They also allow for the determination of any signal suppression that may be due to the bedding itself.

A new calibration curve is generated when the following events occur:

- Quality control samples are unexpectedly lower or higher than usual
- Annual performance maintenance
- At the beginning of the protocol

X-DOCUMENTATION

A copy of the report is sent via email to the requestor. All data files are stored on the data acquisition computer and in the locked file cabinet in 26-B001A as outlined in the file name and data storage SOP. An original signed report is also stored in the locked file cabinet. Upon conclusion of the GLP study, all stored records are boxed and supplied to the Principal Investigator.

National Center for Toxicological Research US Food & Drug Administration, Jefferson, AR Mass Spectrometry Laboratory Standard Operating Procedures	
SOP No.: NCTR MSL-44.00	Approved by Director, Division of Biochemical Toxicology
Supersedes: N/A	Date Approved: 3/10/2014
Page 1 of 2	Effective Date: 3/10/2014

Sample Preparation for the Determination of Bisphenol A in Cellulose Bedding by LC/MS/MS

I. PURPOSE

To prepare cellulose bedding samples for the detection of BPA by LC/MS/MS

II. POLICY

Standard operations procedures (SOPs) shall be written for conducting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

III. APPLICABILITY

This SOP applies to these operations performed by all Service Mass Spectrometrists and chemists.

IV. EQUIPMENT

- Analytical balance
- 15mL centrifuge tubes with caps
- Shaking water bath
- Centrifugal evaporator
- 2mL microcentrifuge tubes
- Mass spec certified LC/MS vials with 300 µl insert

V. SUPPLIES & REFERENCES

- Bisphenol A
- Bisphenol A-d_6
- Acetonitrile (OPTIMA LC/MS grade, Fisher)
- Water (OPTIMA LC/MS grade, Fisher)

VI. FREQUENCY

Whenever analyses are solicited by diet prep or the chemistry support group

VII. SAMPLE PREPERATION

- Prepare at least 500mL of acetonitrile containing 10ng/mL BPA-d6 for use as the extraction solvent.
- Add approximately 1.5g of cellulose bedding to each of 6 15mL Falcon tubes. Record the precise weight of the sample as this can be used to correct the final concentration for the weight of the bedding. Three tubes will be used for the bedding extraction while the remaining 3 tubes will be used for spiked bedding samples.

- Add 6mL of acetonitrile containing BPA-d6 to each tube. Also prepare 3 tubes with the extraction solvent only. These will be used as a control sample.
- For the spiked solvent and bedding samples, add 15µl of 500ng/mL BPA-d0. This will allow the solvent and bedding to be spiked at an equivalent of 5ng of BPA per gram of bedding (5ng/g).
- Incubate all tubes for ~3hrs at 50°C in the shaking water bath. Allow the samples to cool to room temperature.
- Remove 1.5mL of each sample to a 2mL microcentrifuge tube. Evaporate to dryness in a centrifugal evaporator at 40°C.
- Reconstitute with 150µl of 50/50 methanol/water for LC/MS/MS. Place in a mass spec certified LC/MS vial with a 300µl insert.
- All samples are analyzed following the procedures outlined in MSL-43.00.

Mass Spectrometry Laboratory
NCTR 870-543-4114

Mass Spectrometry Report

SAMPLE:	EE2 test article; EE2 reference standard, Steraloids Batch G745	REQUESTER:	Matt Bryant
FILES:	2190m726-730	DATE:	25 June 2012
INSTRUMENT:	Quantum Ultra	INLET/MODE:	**HPLC/- ESI-MS/MS**
SCAN:	Q1 90-700/1 sec	OPERATOR:	Kellie A. Woodling
Energy	40 eV	METHODS:	Test article certification Test article certification MSMS 40 eV
Capillary Temp.:	320°C	MOBILE PHASE:	40-100% MeOH in 5 min
Spray Voltage	4.0 kV	LC FLOW RATE:	500 µl/min
Sheath Gas:	40	LC Column:	Gemini C18 column, 2x50mm, 3 µm particle size
Sweep Gas:	0	PDA:	210-700nm, 2nm step
Aux. Gas:	40	MS/MS Conditions:	1.5 mT argon @ 20 eV

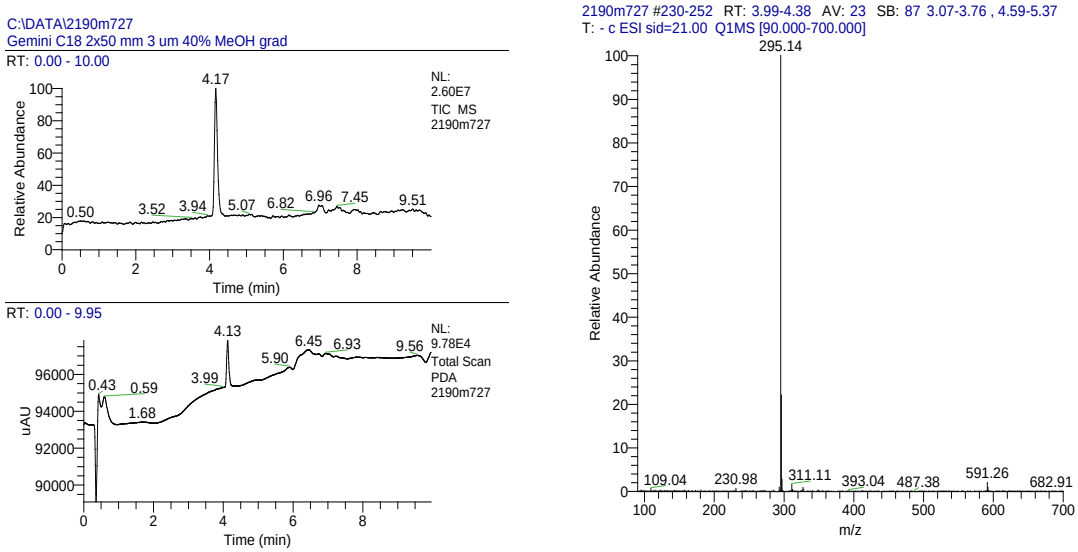
Sample preparation

Dissolved the test articles in 1 mL of acetonitrile. Diluted appropriately in 50/50 methanol/water to afford a concentration of *ca.* 0.1 mg/mL. Injection volumes were 1 µl.

HPLC-PDA / -ESI results

A major UV peak was observed at *ca.* 4.2 min in all samples, with similar UV absorption spectra. A base peak with *m/z* = 295, consistent with the deprotonated ethinylestradiol molecule was observed at that same retention time (Figure 1) in the TIC (Total Ion Chromatogram).

Ethinylestradiol Test Article Lot # 071M1492V



Ethinylestradiol Steraloids Batch G745

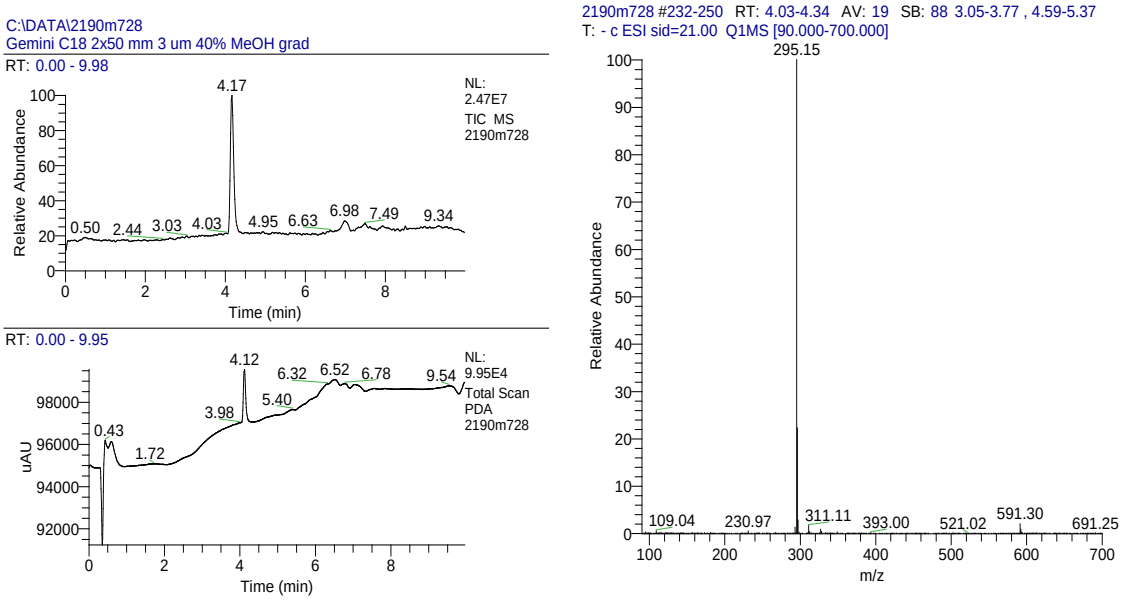


Figure 1

HPLC-PDA / -ESI-MS/MS results

The analyses of the Steraloids ethinylestradiol reference standards (Batch G745) and the test article revealed a deprotonated molecule at *m/z* = 295, consistent with the deprotonated ethinylestradiol molecule and numerous fragment ions. This can be seen in Figure 2.

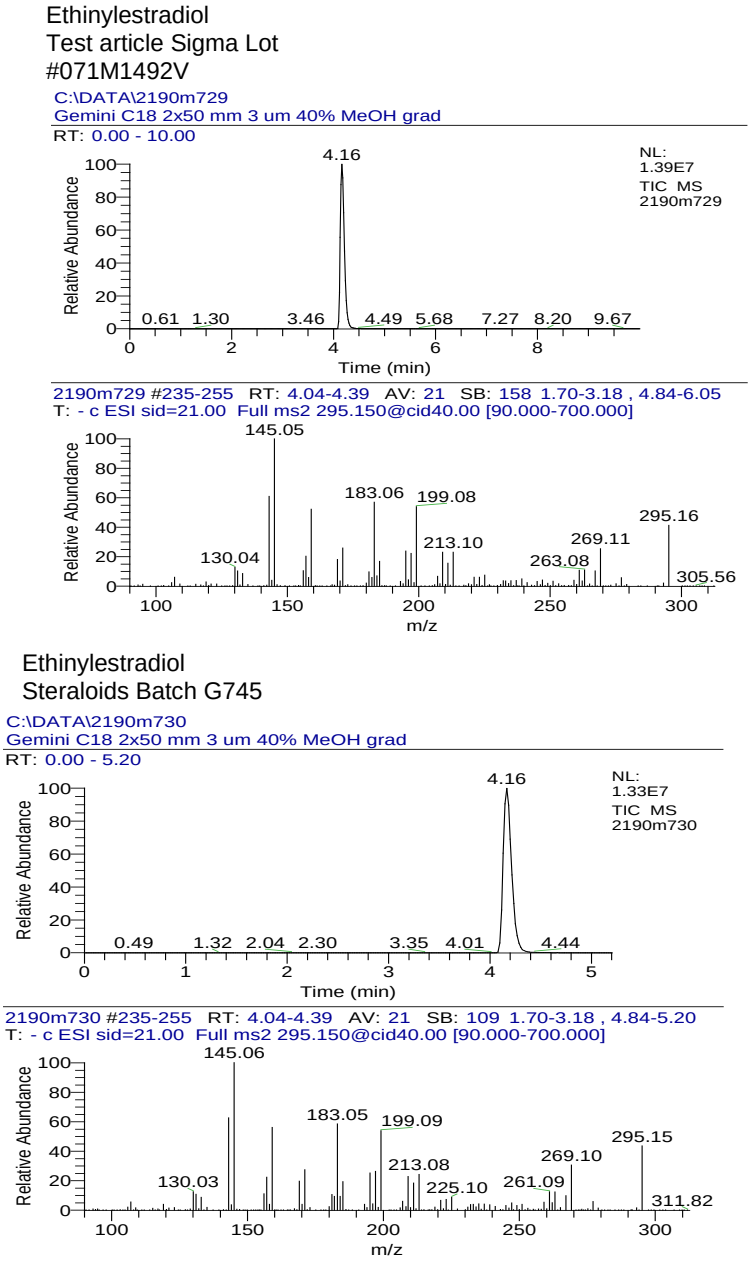


Figure 2.

Conclusion

Considering the coincidence of the chromatographic and spectrometric analytical data of the test compound with the Steraloids reference standard [ethinylestradiol, Batch G745], it is concluded that within the limitations inherent to the technique utilized, the main component of the test compound submitted for analysis is ethinylestradiol.