APPENDIX IV

Chemistry Report

Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

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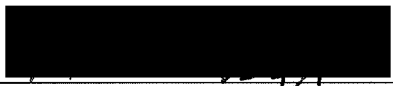
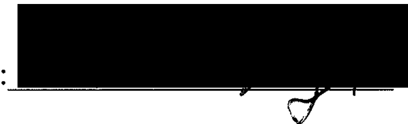
Prepared by:  Date: 31 July, 2015

Approved by:  Date: 31 July, 2015

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ADDENDICES:

Appendix 1: NCTR DBT CHEM SOP No. 549.01

Appendix 2: NCTR DBT CHEM SOP No. 547.02

Appendix 3: Mass Spectrometry Report for Oxybenzone Test Article

Appendix 4: Mass Spectrometry Report for EE2 Test Article

Appendix 5: NMR Report for Oxybenzone Test Article

Appendix 6: NMR Report for EE2 Test Article

1. Receipt, Storage and Handling

The test material oxybenzone (2-hydroxy-4-methoxybenzophenone) was received from Ivy Fine Chemical (Cherry Hill, NJ; Lot # 1F100604). An oxybenzone reference standard (CAS 131-57-7, 98% purity, Lot #S42088) was purchased from Sigma-Aldrich (St. Louis, MO). The test article estrogen (ethinylestradiol; EE2; Lot #071M14392V) was received from Sigma-Aldrich. Reference standards of EE2 were received from Sigma-Aldrich (Lot #028K141188) and from Steraloids (Newport, RI; Batch G745). Standards of the metabolites of oxybenzone were all obtained from Sigma-Aldrich: 2,4-dihydroxybenzophenone, 2,3,4 -trihydroxybenzophenone and 2,2'-dihydroxy-4-methoxybenzophenone. Reserpine, the compound used as an internal standard for the HPLC/MS work, was also obtained from Sigma-Aldrich. Methanol, acetonitrile and formic acid were all HPLC grade or better and obtained from Fisher Scientific (Pittsburgh, PA). All chemicals used for analytical work were stored (light-protected) at room temperature in Building 26, Room A144. The bulk test article was maintained by Diet Preparation and stored in Building 50, Room 161.

2. Characterization and Purity of Oxybenzone and EE2 Test Articles using HPLC-PDA, HPLC-Mass Spectrometry and ^{1}H NMR Spectroscopy

Both the study test article (oxybenzone) and reference estrogen (ethinylestradiol) were characterized using appropriate analytical systems.

(a) HPLC-PDA Analysis

***Oxybenzone**:*

A standard solution of the test article (Ivy Fine Chemical) was prepared in methanol at a concentration of 80 µg/mL. A solution of a reference standard from Sigma-Aldrich was also prepared at 100 µg/mL. Both of these solutions were then subjected to reverse phase HPLC separation using conditions described in SOP No. NCTR DBT CHEM 549.01 (Appendix 1).

The UV spectrum (190 to 400 nm) of each solution was acquired using the Photodiode Array Detector (PDA). One major peak was observed for each solution at identical retention times of 13.15 to 13.16 minutes. The UV spectra for both the test article oxybenzone and the reference standard were equivalent. The chromatographic area of the UV signal corresponding to the oxybenzone peak was then compared to the total area for all components which eluted during the HPLC run (below) and used to estimate the purity of both the test article and the reference standard. Table 1 shows the results for this study. The results indicate that the chromatographic purity of the oxybenzone test article was determined to be greater than 99% by this method.

Table 1: HPLC-PDA Purity of Oxybenzone Test Article and Reference Standard

Oxybenzone Test Article	% Peak Area		Oxybenzone Sigma Ref standard	% Peak Area
Injection 1	99.31		Injection 1	99.48
Injection 2	99.38		Injection 2	99.51
Mean	99.35		Mean	99.49

Ethinylestradiol:

A standard solution of the test article was prepared in methanol at 1 mg/ml. A solution of reference standard was likewise prepared. Both of these solutions were then subjected to reverse phase HPLC separation using conditions described in SOP No. NCTR DBT CHEM 547.02 (Appendix 2).

The UV spectrum (200 to 600 nm) of each solution was acquired using the Photodiode Array Detector. One major peak was observed for each solution at identical retention times of 4.70 to 4.71 minutes. The UV spectra for both the test article and reference standards were also equivalent. The chromatographic area of the UV signal corresponding to the EE2 peak was then compared to the total area for all components which eluted during the HPLC run (below) and used to estimate the purity of both the test article and the reference standard. Table 2 shows the results for this study. The results indicate that the chromatographic purity of the EE2 reference estrogen was determined to be greater than 99% by this method.

Table 2: HPLC-PDA Purity of EE2 Test Article and Reference Standard

EE2 Test Article	% Peak Area		EE2 Sigma Ref standard	% Peak Area
Injection 1	99.92		Injection 1	99.92
Injection 2	99.92		Injection 2	99.89
Injection 3	99.85		Injection 3	99.91
Mean	99.9		Mean	99.9

(b) HPLC-Mass Spectrometry Analysis

Oxybenzone:

Standard solutions of the test article and the reference standard (Sigma Aldrich H36206, Lot S42088) were both prepared in acetonitrile and diluted with methanol/water to approximately 0.1 mg/ml. Injections of each were made onto an HPLC/MS system equipped with a PDA detector. A single UV peak was observed in both samples at about 4.6 minutes, with similar UV spectra. The mass spectra of each of these peaks were identical and consisted of a base peak of m/z 227, which is consistent with the deprotonated oxybenzone molecule. A major fragment ion of m/z 211 was also observed and is consistent with loss of 16 daltons from the base peak. It can be concluded that the major component of the test article is oxybenzone and this matched the reference standard. (See Appendix 3).

Ethinylestradiol:

Standard solutions of the test article (Sigma E4876, Lot 071M1492V) and the reference standard (Steraloids, Batch G745) were both prepared in acetonitrile and diluted with methanol/water to approximately 0.1 mg/ml. Injections of each were made onto an HPLC/MS system equipped with a PDA detector. A single UV peak was observed in both samples at about 4.2 minutes, with similar UV spectra. The mass spectra of each of these peaks were identical and consisted of a base peak of m/z 295, which is consistent with the deprotonated ethinylestradiol molecule. Numerous fragment ions were also observed and these were similar in both the test article sample and the reference standard. It can be concluded that the major component of the test article is ethinylestradiol. (See Appendix 4).

(c) 1H Proton NMR Analysis

Oxybenzone:

The test article and the reference standard were each dissolved in DMSO-d6 and transferred into standard NMR tubes for analysis according to the procedure described in NCTR SOP NMR 04-00 ”Test Article Analysis”. The spectra were referenced to the residual non-deuterated solvent (2.5 ppm). The Proton NMR spectrum of the test article was consistent with the structure of oxybenzone and matched that of the reference standard. Other than the resonances of the residual non-deuterated solvent (2.5 ppm) and water (ca 3.3 ppm), no other significant traces of contaminants were detected, indicating the test article was of high purity. (See Appendix 5).

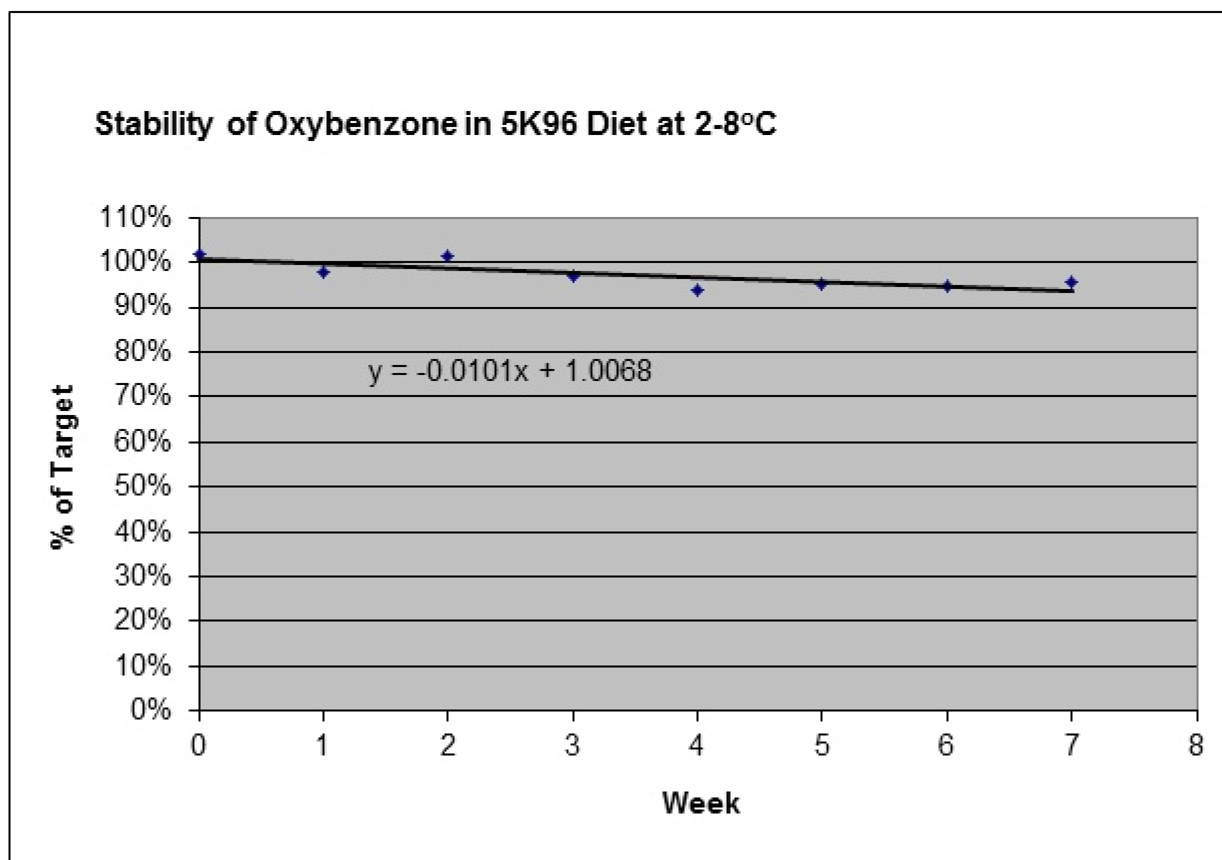
Ethinylestradiol:

The test article and the reference standard were each dissolved in DMSO-d6 and transferred into standard NMR tubes for analysis according to the procedure described in NCTR SOP NMR 04-00 ”Test Article Analysis”. The spectra were referenced to the residual non-deuterated solvent (2.5 ppm). The Proton NMR spectrum of the test article was consistent with the structure of ethinylestradiol and matched that of the reference standard. Other than the resonances of the residual non-deuterated solvent (2.5 ppm) and water (ca 3.3 ppm), no other significant traces of contaminants were detected, indicating the test article was of high purity. (See Appendix 6).

3. Oxybenzone in 5K96 Diet Storage Stability Test

The stability of oxybenzone in 5K96 diet stored in the refrigerator (2 – 8 ºC) in the dark was evaluated. Oxybenzone was mixed at a concentration of 1000 ppm (µg/g) in 5K96 diet (SCR# 2178 98 00002) and provided to DBT/Chemistry on 10/26/10. Diet samples were stored in the refrigerator (covered to prevent exposure to light) and weighed aliquots of diet from the top, middle and bottom were removed at weekly intervals and analyzed for oxybenzone concentration by HPLC-PDA analysis (Appendix 1: NCTR DBT CHEM SOP No. 549). As seen in the following graph, acceptable stability through seven weeks of storage was demonstrated. Note: this data was provided by email communication from P. Siitonen to the Study Director.

Figure 1. Summary of Stability Data for 5K96 Diet (1000 ppm Oxybenzone)



4. Dose Certification (DC) and Homogeneity of Oxybenzone in 5K96 Diet

Oxybenzone was mixed into 5K96 diet by Diet Preparation and aliquots supplied to DBT/Analytical Chemistry for dose certification prior to use in the animal studies. Oxybenzone was thus prepared at various concentrations up to 50,000 ppm (µg/g) in diet and supplied as 3 to 10 g aliquots. For the homogeneity study, samples were collected from the Top, Middle and Bottom from each batch. A sample of control or 0 ppm diet was also supplied with each submission.

Samples were prepared for HPLC-PDA analysis by DBT/Chemistry and analyzed according to the method described in NCTR DBT CHEM SOP No. 549.01 (Appendix 1). Results of HPLC-PDA analysis for oxybenzone in 5K96 diet are reported in µg oxybenzone per g of diet and % of target. Tables 3 to 5 summarize the dose certification results from each of the studies.

Table 3. Dose Certification and Homogeneity of Oxybenzone in 5K96 Diet: Study E02178

SCR Number	Sample Date	Sample ID	Target concentration (µg/g)	Result (µg/g)	Mean ± SD µg/g	% CV	Result (% of Target)
2178 98 00001	10/26/10	-	0	<LOQ*	-	-	-
2178 98 00002		-	1,000	-	1,019 ± 29	2.8	102
2178 98 00003		-	50,000	-	49,900 ± 900	1.7	100
2178 98 00004	10/28/10	-	3,000	-	3,210 ± 120	3.6	107
2178 98 00005			10,000	-	10,400 ± 300	3.0	104
2178 98 00006			25,000	-	23,800 ± 1,100	4.7	95.2
2178 98 00007	07/28/11	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*	-	-	-
		Bottom	0	<LOQ*	-	-	-
2178 98 00008		Top	1,000	991	991 ± 3	0.3	99.1
		Middle	1,000	988			-
		Bottom	1,000	994			-
2178 98 00009		Top	3,000	2,741	2,760 ± 30	0.9	92.1
		Middle	3,000	2,757			
		Bottom	3,000	2,790			
2178 98 00010		Top	10,000	10,078	10,200 ± 90	0.9	102
		Middle	10,000	10,244			
		Bottom	10,000	10,092			
2178 98 00011		Top	25,000	24,058	24,200 ± 300	1.1	97.0
		Middle	25,000	24,140			
		Bottom	25,000	24,546			
2178 98 00012		Top	50,000	47,328	49,800 ± 2,500	5.1	100.0
		Middle	50,000	52,364			
		Bottom	50,000	50,335			
2178 98 00013	08/25/11	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*	-	-	-
		Bottom	0	<LOQ*	-	-	-
2178 98 00014		Top	1,000	933	927 ± 10	1.1	92.7
		Middle	1,000	933			
		Bottom	1,000	916			
2178 98 00015 (See re-sample below)		Top	3,000	2,610**	2,612±103	4.0	87.1
		Middle	3,000	2,510**			
		Bottom	3,000	2,717**			
2178 98 00016		Top	10,000	9,889	9,810±146	1.5	98.1
		Middle	10,000	9,643			
		Bottom	10,000	9,901			
2178 98 00017		Top	25,000	23,476	24,300±830	3.4	97.2
		Middle	25,000	24,319			
		Bottom	25,000	25,135			
2178 98 00018		Top	50,000	50,743	51,200±478	0.9	102
		Middle	50,000	51,699			
		Bottom	50,000	51,236			
2178 98 00015 (Re-Sample)**	08/31/11	Top	3,000	2,841**	2,796±57	2.1	93.2
		Middle	3,000	2,731**			
		Bottom	3,000	2,816**			

* The limit of quantitation is estimated to be 20 µg/g in diet.
** Original analysis was below target. Re-sample on 08/31/2011 and reported

Table 4. Dose Certification and Homogeneity of Oxybenzone in 5K96 Diet: Study E02188

SCR Number	Sample Date	Sample ID	Target concentration in Diet (µg/g)	Result (µg/g)	Mean ±SD µg/g	% CV	Result (% of Target)
2188 99 00004	06/06/12	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*			
		Bottom	0	<LOQ*			
2188 99 00005		Top	3,000	2,984	2,947±124	4.2	98.2
		Middle	3,000	2,808			
		Bottom	3,000	3,048			
2188 99 00006		Top	10,000	10,699	9,911±683	6.9	99.1
		Middle	10,000	9,538			
		Bottom	10,000	9,496			
2188 99 00007		Top	30,000	30,260	30,263±1,284	4.2	101
		Middle	30,000	28,981			
		Bottom	30,000	31,549			
2188 99 00008	07/18/12	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*			
		Bottom	0	<LOQ*			
2188 99 00009		Top	3,000	2,743	2,769±141	5.1	92.3
		Middle	3,000	2,922			
		Bottom	3,000	2,642			
2188 99 00010		Top	10,000	9,963	9,576±357	3.7	95.8
		Middle	10,000	9,260			
		Bottom	10,000	9,506			
2188 99 00011		Top	30,000	30,819	29,360±1,582	5.4	97.9
		Middle	30,000	29,582			
		Bottom	30,000	27,679			
2188 99 00014	08/23/12	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*			
		Bottom	0	<LOQ*			
2188 99 00015		Top	3,000	3,027	2,862±143	5.0	95.4
		Middle	3,000	2,780			
		Bottom	3,000	2,779			
2188 99 00016		Top	10,000	9,706	9,609±119	1.2	96.1
		Middle	10,000	9,645			
		Bottom	10,000	9,475			
2188 99 00017		Top	30,000	26,860	28,639±1,558	5.4	95.5
		Middle	30,000	29,293			
		Bottom	30,000	29,763			

* The limit of quantitation is estimated to be 20 µg/g in diet.

Table 5. Dose Certification and Homogeneity of Oxybenzone in 5K96 Diet: Study E02186

SCR Number	Sample Date	Sample ID	Target concentration (µg/g)	Result (µg/g)	Mean ±SD µg/g	% CV	Result (% of Target)
2186 98 00001	01/22/13	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*	-	-	-
		Bottom	0	<LOQ*	-	-	-
2186 98 00002		Top	3,000	2,938	3,018±73	2.4	101
		Middle	3,000	3,080			
		Bottom	3,000	3,036			
2186 98 00003		Top	10,000	9,966	10,056±148	1.5	101
		Middle	10,000	10,227			
		Bottom	10,000	9,974			
2186 98 00004		Top	30,000	28,749	28,765±464	1.6	96
		Middle	30,000	29,237			
		Bottom	30,000	28,310			
2186 98 00015	03/06/13	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*	-	-	-
		Bottom	0	<LOQ*	-	-	-
2186 98 00016		Top	3,000	3,040	3,059±48	1.6	102
		Middle	3,000	3,114			
		Bottom	3,000	3,024			
2186 98 00017**		Top	10,000	5,650	5,882±246	4.2	59**
		Middle	10,000	6,140			
		Bottom	10,000	5,858			
2186 98 00018		Top	30,000	30,735	28,798±1,689	5.9	96
		Middle	30,000	28,032			
		Bottom	30,000	27,627			
2186 98 00017** (Re-Mixed)		Top	10,000	9,990	10,000±248	2.5	100
		Middle	10,000	9,758			
		Bottom	10,000	10,254			
2186 98 00019	04/01/13	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*	-	-	-
		Bottom	0	<LOQ*	-	-	-
2186 98 00020		Top	3,000	2,924	3,065±144	4.7	102
		Middle	3,000	3,212			
		Bottom	3,000	3,060			
2186 98 00021		Top	10,000	9,826	10,024±238	2.4	100
		Middle	10,000	10,288			
		Bottom	10,000	9,957			
2186 98 00022		Top	30,000	29,915	29,854±92	0.3	100
		Middle	30,000	29,899			
		Bottom	30,000	29,747			
2186 98 00026	05/03/13	Top	0	<LOQ*	-	-	-
		Middle	0	<LOQ*			
		Bottom	0	<LOQ*			
2186 98 00027		Top	3,000	3,007	2,958±77	2.6	98.6
		Middle	3,000	2,998			
		Bottom	3,000	2,869			
2186 98 00028		Top	10,000	9,778	10,029±276	2.8	100
		Middle	10,000	9,985			
		Bottom	10,000	10,325			
2186 98 00029		Top	30,000	30,436	29,692±686	2.3	99.0
		Middle	30,000	29,084			
		Bottom	30,000	29,557			

* The limit of quantitation is estimated to be 20 µg/g in diet.
**Batch failed to meet specifications and was re-mixed. Sample date of 3/7/13 for new batch which met specifications.

5. Analysis of EE2 in 5K96 Feed

A concentrated solution of the reference test article ethinylestradiol (EE2) in ethanol was prepared by DBT/Chemistry and provided to Diet Preparation. This solution was mixed with the 5K96 feed and then the ethanol was removed by heating under vacuum in the blender. Samples of the fortified feed (top, middle, bottom) were then submitted for confirmation of the target concentration of 50 ppb and homogeneity evaluation. Quantitative analysis of the samples was conducted by UPLC-MS/MS using NCTR/DBT MSL SOP No. 33.01. Briefly, samples were spiked with a deuterated EE2 analog as internal standard and then extracted with acetonitrile. The centrifuged extract was then derivatized with dansyl chloride to produce the dansylated products of EE2 and the internal standard, and these were quantified using UPLC-positive ion electrospray tandem mass spectrometry.

As indicated below, the target concentration of 50 ppb was achieved for each of the study samples, and the distribution of EE2 in the feed was found to be homogenous (no appreciable difference in measured EE2 concentration between samples removed from the top, middle or bottom portions of the feed batch).

Table 6. Results of UPLC-Tandem MS Analysis for EE2 in 5K96 Diet: Study E02188

SCR #	Samples	Conc [ppb]	Mean conc.for triplicate [ppb]	StDev	RSD	Conc [ppb]	Accuracy [% of target]
2188 99 00003	Top	50.0 49.5 49.4	49.6	0.3	0.6	**49.0**	**98.0**
	Middle	49.4 49.7 48.3	49.1	0.8	1.5		
	Bottom	47.4 48.2 49.7	48.4	1.2	2.4		
2188 99 00018	Top	48.7 49.0 48.8	48.8	0.2	0.3	**50.1**	**100.3**
	Middle	51.6 51.2 51.3	51.4	0.2	0.4		
	Bottom	50.6 49.9 50.2	50.2	0.3	0.7		

Table 7. Results of UPLC-Tandem MS Analysis for EE2 in 5K96 Diet: Study E02186

SCR #	Samples	Conc [ppb]	Mean conc.for triplicate [ppb]	StDev	RSD	Conc [ppb]	Accuracy [% of target]
2186 98 00006	Top	45.1 46.8 46.5	46.2	0.9	2.0	**46.4**	**92.7**
	Middle	46.8 46.6 45.2	46.2	0.9	1.9		
	Bottom	47.3 46.3 46.6	46.7	0.5	1.1		
2186 98 00023	Top	46.1 47.9 48.4	47.5	1.2	2.6	**47.7**	**95.3**
	Middle	47.4 48.4 47.0	47.5	0.9	1.8		
	Bottom	47.5 49.4 47.0	48.0	1.2	2.6		

6. Analysis of Serum for Oxybenzone and Metabolites: E02178

An analytical method for oxybenzone and three metabolites (2,4-dihydroxybenzophenone, 2,3,4-trihydroxybenzophenone and 2,2'-dihydroxy-4-methoxybenzophenone) was initially developed using HPLC with UV detection (Waters Alliance system) and then enhanced using the Waters Acquity Ultra High Performance Liquid Chromatography (UHPLC) system. The detection limit achieved with this method was down to 1 ug/ml in serum for each of the 4 analytes. Stability studies were conducted on these analytes in serum and found that all were relatively stable in frozen serum, except for one of the metabolites, 2,3,4-trihydroxybenzophenone, which appeared to degrade even in the frozen state. Serum samples from the highest several dose groups from the E02178.01 study were examined and found to contain levels of oxybenzone and metabolites which were significantly below the 1 ug/ml limit of detection. For that reason, a new analytical method was developed using a Sciex 3200 Qtrap HPLC/MS/MS system, which had a detection limit of about 0.005 ug/ml for oxybenzone, 2,4-dihydroxybenzophenone and 2,2'-dihydroxy-4-methoxybenzophenone and about 0.1 ug/ml for 2,3,4-trihydroxybenzophenone . After assaying several serum samples from the study, it was determined that the new method would be adequate for the work.

At least 5-6 samples from dams in each of the treatment groups (0,1000, 3000, 10000, 25000 and 50000 ppm) and collection study days (PND21, GD10, GD15, GD20) were assayed for oxybenzone and the three metabolites. The individual values for each animal for levels of oxybenzone and the three metabolites were reported to the Study Director in an email of Oct 29, 2013 and not duplicated in this report. Nonetheless, the summary graphs from this report are

shown below in Figures 2 and 3. The results indicate that only oxybenzone and one of the metabolites (2,4-dihydroxybenzophenone) were present above the limits of detection of the assay. The stability work would indicate that 2,3,4-trihydroxybenzophenone, if present in the samples at the time of original collection, would likely not have been stable during storage. For both oxybenzone and the 2,4-dihydroxybenzophenone metabolite, serum exposure generally increased with increasing treatment dose. Levels observed in the PND21 group were somewhat higher than those seen in the other three collection days.

Figure 2. Serum Levels of Parent Compound Oxybenzone in Treated Animals: E02178

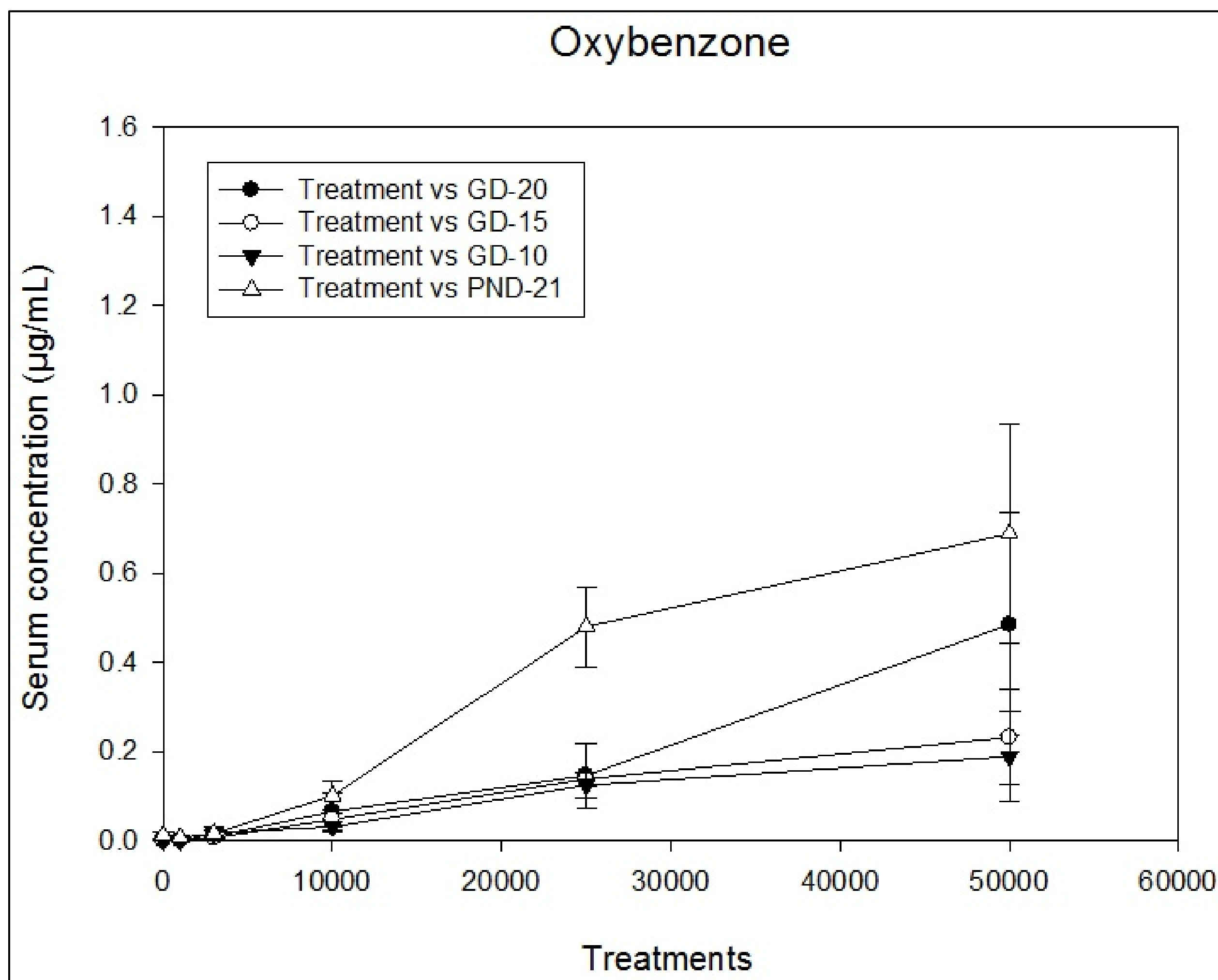
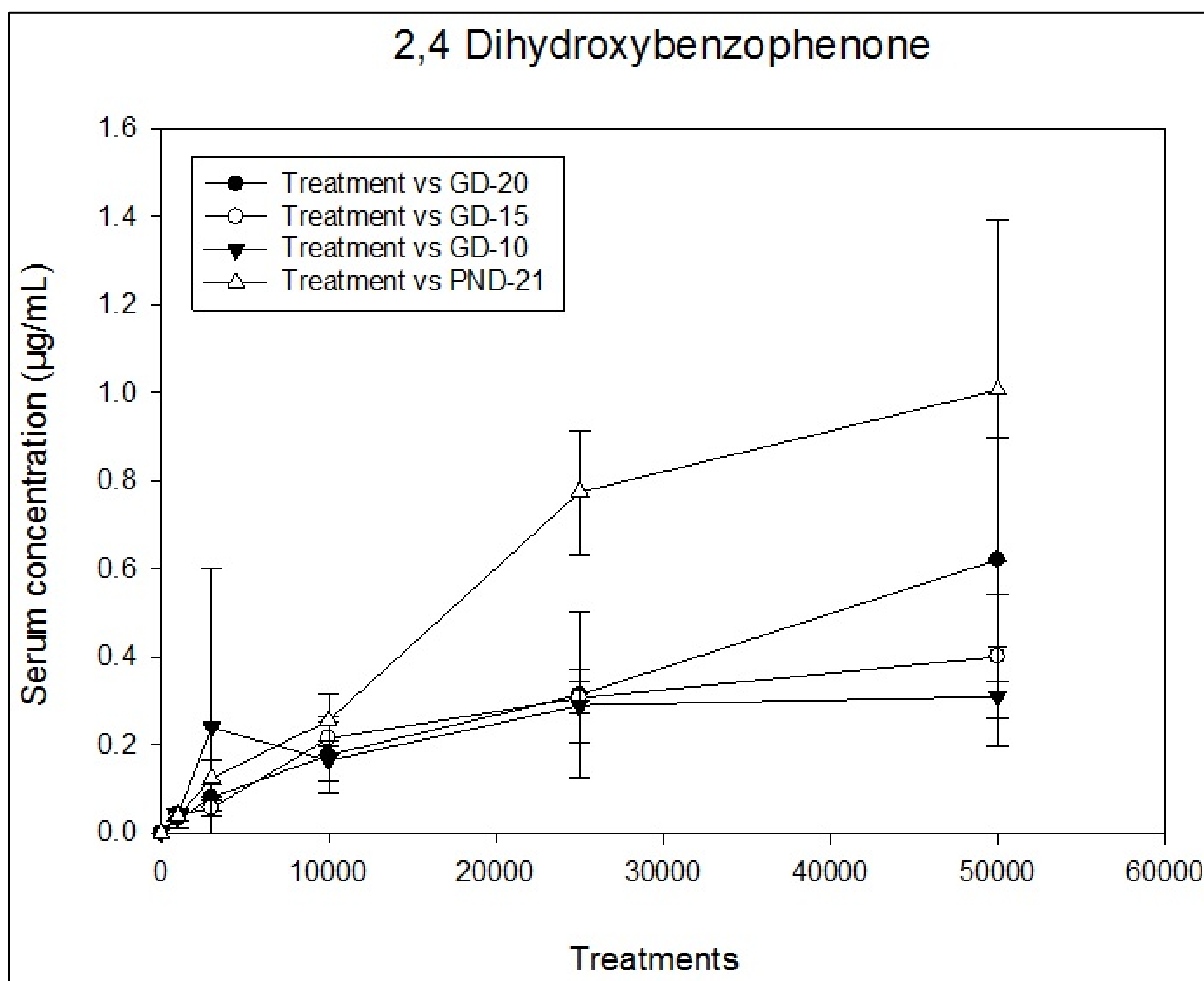


Figure 3. Serum Levels of Metabolite 2,4-Dihydroxybenzophenone in Treated Animals: E02178



7. Analysis of Isoflavones in 5K96 Diet

A special consideration for this study was to ensure that the level of major isoflavones in the 5K96 feed did not exceed a level of about 2 ppm. Analyses were conducted on each delivered batch of 5K96 diet prior to utilization. A validated method was conducted to measure the amount of genistein and daidzein in the diet according to NCTR DBT CHEM SOP No 525.01 or 525.02 The method involves preparation of spiked and unspiked samples of ground diet, followed by addition of 10% hydrochloric acid in methanol and then distillation in a Snyder column at 65-70°C for six hours to hydrolyze the glycoside forms of these compounds. Methanol is then used to extract the analytes, then rotoevaporation to reduce the volume. Ethyl acetate is used to extract and these extracts are then evaporated to dryness. The residue is reconstituted in acetonitrile and the samples are submitted for analysis of the genistein and daidzein by HPLC/MS/MS. As seen in Table 8, the total level of these two isoflavones did not exceed the specified limit of 2 ppm for any of the utilized batches of 5K96 diet and averaged a total of about 0.23 ppm.

Table 8. Results of Analysis of Isoflavones in 5K96 Diet[1]

Sample SCR #	Daidzein (ppm)	Genistein (ppm)	Total (ppm)
01455000332	0.18	0.31	0.49
01455000323	0.13	0.18	0.31
21889900001	0.07	0.33	0.40
21889900012	0.01	0.03	0.04
21889900020	0.01	0.02	0.04
21869900024	0.04	0.05	0.09
Mean (N=6)	0.07	0.15	0.23
Std Dev.	0.07	0.14	0.20

1 Samples were prepared for analysis by HPLC-Tandem MS using NCTR DBT CHEM SOP No. 525.01.

8. Routine Analyses of 5K96 Diet

Analyses were conducted on the 5K96 diet prior to utilization and were conducted per NCTR DBT CHEM SOPs numbers 302.02, 303.03, 304.02, 305.03, 306.02, 307.04, 308.02, 315.03, 320.04 and 321.01. The original copies of these SOPs are maintained by the NCTR GLP Archivist, Deborah Jackson. Trace metals analyses were conducted by Applied Research and Development Laboratories, Inc. Mount Vernon, IL. utilizing prescribed QC measures. Primary data for this routine analytical work is maintained and archived separately from the Chemistry Report for these oxybenzone studies. Values included here are for informational purposes only.

Table 9. Nutrient Composition Determined for 5K96 Diet

Nutrient	Average Result	Number of Lots[1]
Crude protein (% by weight)	20.7	5
Crude Fat (% by weight)	5.54	5
Volatiles (% by weight)	8.34	5
Vitamins		
A (µg/g)	6.72	5
B1 (µg/g)	33.1	5
E (µg/g)	72.2	5
Minerals		
Selenium (µg/g)	0.36	5

1 Analyses are summarized for the following lots of diet: SCR #'s 01455000332, 01455000323, 21889900001, 21889900012 and 21889900020.

Table 10. Contaminant Levels Determined in 5K96 Diet

Contaminant	Average Result (LOQ)	No. of Lots[1] (No. Positive)
Aflatoxin B1 (ng/g)	<LOQ (0.1)	5 (0)[1]
Aflatoxin B2 (ng/g)	<LOQ (0.1)	5 (0)[1]
Aflatoxin G1 (ng/g)	<LOQ (0.1)	5 (0)[1]
Aflatoxin G2 (ng/g)	<LOQ (0.1)	5 (0)[1]
Arsenic (µg/g)	0.08 (0.03)	5 (4)[1]
Cadmium (µg/g)	<LOQ (0.1)	5 (0)[1]
Dieldrin (ng/g)	1.7 (5)	3 (1)[2]
DDT, total (ng/g)	<LOQ (0.1)	3 (0)[2]
Fumonisins, total (ng/g)	136 (20)	5 (5)[1]
Heptachlor (ng/g)	<LOQ (5)	3 (0)[2]
Lead (µg/g)	0.44 (0.20)	5 (4)[1]
Lindane (ng/g)	<LOQ (1)	3 (0)[2]
Malathion (ng/g)	561 (50)	3 (2)[2]
PCBs (ng/g)	42.3 (10)	3 (1)[2]

1 Analyses are summarized for the following lots of diet: SCR #'s 01455000332, 01455000323, 21889900001, 21889900012 and 21889900020.

2 Analyses are summarized for the following lots of diet: SCR #'s 01455000332, 01455000323 and 21889900001.

Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

Appendix 1

NCTR DBT CHEM SOP No. 549.01

National Center for Toxicological Research US Food and Drug Administration, Jefferson, AR Division Of Biochemical Toxicology Chemistry Support Group Laboratory Operating Procedures	
SOP No. NCTR DBT CHEM 549.01 Prepared by: [redacted]	Approved by: DBT/Chemistry Team Leader [redacted]
Supersedes: SOP No. NCTR DBT CHEM 549.00	Date Approved: 08/23/2012
Page 1 of 4	Effective Date: 08/23/2012

Determination of Oxybenzone in Animal Diet for Dose Certification, Homogeneity, Stability, or Dose Verification

1. PURPOSE:

To describe the procedure used for the analysis of 2-Hydroxy-4-Methoxybenzophenone (Oxybenzone) by HPLC-UV in animal meal diets for dose certification, homogeneity, stability or dose verification at concentrations from 0 to 50,000 mg/kg.

2. SCOPE:

This procedure applies to evaluation of Oxybenzone in animal diets and personnel responsible for conducting and supporting GLP-designated protocols as required for regulatory compliance with 21 CFR Part 58.

3. 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.

3.1 Chemicals

- 3.1.1 Oxybenzone, Aldrich 98 %, Cat. H3,620-6, Lot S42088
- 3.1.2 Acetonitrile, (J.T. Baker, HPLC grade)
- 3.1.3 Deionized water (18 MΩ cm^{-1}, 0.2μ filtered. Barnstead NANOPure or equivalent)
- 3.1.4 Formic acid, 0.05% pH=2.3, (Fluka)
- 3.1.5 Methanol, (J.T.Baker, HPLC grade)

3.2 Equipment

- 3.2.1 Analytical balance, Ohaus, Model GA200D or equivalent
- 3.2.2 Pipettes, volumetric (Class A)
- 3.2.3 Graduate cylinder
- 3.2.4 LC autosampler vials (1.5ml, amber) with Teflon-lined septum screw-cap
- 3.2.5 Culture tubes, 50ml screw-cap
- 3.2.6 Pasteur 9cm glass transfer pipettes (2ml with latex rubber bulbs)
- 3.2.7 Syringeless filter system, 0.45 μm, (Whatman Mini-UniPrep™)
- 3.2.8 Eppendorf Centrifuge Mod 5424

3.3 Oxybenzone Standard solutions

3.3.1 Stock Solution: Weigh 0.1000 ± 0.0004g of Oxybenzone into a 20ml borosilicate glass scintillation vial on an analytical balance and dissolve in 10 ml of Methanol (i.e. 10.0 mg/ml stock solution).

3.3.2 Analytical Standards: For HPLC analysis. The Oxybenzone Stock solution is diluted with MeOH to yield 10.0 to 100 µg/ml Oxybenzone analytical standards.

3.4 Oxybenzone Stability in Dosed Diet

Oxybenzone in 5K96 diet is formulated by Diet Preparation, at a concentration of 1000 mg/kg. Diet samples are stored in the dark and refrigerated (2-8°C) for the duration of stability testing. For the stability study, samples are analyzed at weekly intervals for up to 7 weeks (49 Days) according to the procedure described in 3.5.

3.5 Dose Certification or Homogeneity of Oxybenzone in Diet

For certification of dose (or stability), dosed diets are delivered as 3 ~10g aliquots of diet in specimen cups designated, Top, Middle & Bottom, for HPLC-UV analysis. Each diet sample is prepared for HPLC analysis by extraction of 0.500 ± 0.005g diet with 50ml MeOH for 1 hour on a flat bed reciprocating shaker. Following extraction, the samples are left for about 2 hours to settle the solid materials. An aliquot of approximately 2 mL of the upper phase is centrifuged for 10 minutes at 20,000 rpm. If necessary, the sample may be filtered through a 0.45 µM filter. After centrifugation an aliquot of the upper phase is directly injected into HPLC system for analysis. For evaluation of homogeneity, each diet Top, Middle & Bottom aliquots are submitted in triplicate (n=9).

Dose Level	Extract Oxybenzone Conc.	Additional Dilution	Standard Concentration Required
Vehicle 0 mg/kg	0 µg/ml	-NA	--
1000 mg/kg	10 µg/ml	-NA	10.0 µg/ml
3000 mg/kg	30 µg/ml	-NA	30.0 µg/ml
10,000 mg/kg	100 µg/ml	-NA	100 µg/ml
25,000 mg/kg	250 µg/ml	1:5 (300µl Ext + 1.2ml MeOH)	50.0 µg/ml
50,000 mg/kg	500 µg/ml	1:5 (300µl Ext + 1.2ml MeOH)	100 µg/ml

3.6 Analysis of Oxybenzone by HPLC-UV

HPLC analyses for quantitation of Oxybenzone are performed utilizing a Waters 2695 Alliance HPLC system equipped with a Waters 2996 PDA detector with the 288nm wavelength extracted for sample quantification. A similar Waters HPLC-PDA system instrumentation could also be utilized and adapted for performing the HPLC chormatography.
Sample and standard injections were 10µl from 1.5ml amber screw-capped vials. A Phenomenex Synergi Fusion RP 80A, 4µm particle size, 4.6 mm x 250 mm length column was used for analysis. The mobile phase was programmed from 100% A (5% acetonitrile: 95% formic acid (0.05%, pH 2.3) to B (95% acetonitrile: 5% formic acid (0.05%, pH 2.3) and delivered at a flow of 1 ml/min. in the following manner.

Time	Flow	%A	%B	Gradient
0	1.00	100	0	Initial
1.00	1.00	90	10	Step
10.00	1.00	10	90	Linear
15.00	1.00	10	90	Linear
20.01	1.00	100	0	Step

The HPLC sample sequence is arranged such that each 3 sample injections are bracketed by injections of the specified Oxybenzone quantitation standards.

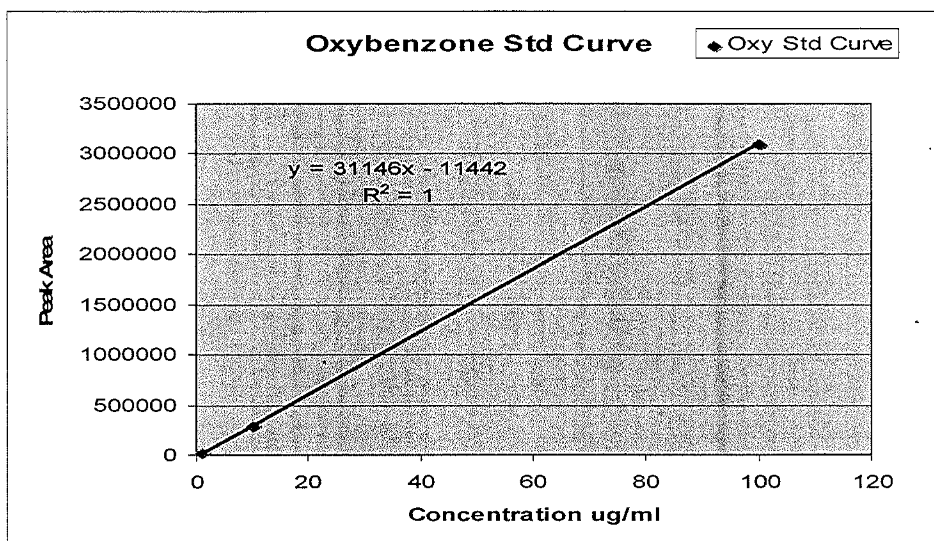
3.7 Calculation of Diet Oxybenzone Content

Bracketed matched standard peak areas are averaged to quantify Oxybenzone content of the bracketed sample(s). Calculate the concentration of Oxybenzone in samples by dividing the peak area of the sample by the average area of the standard then multiplying by the concentration of the standard in µg/ml Oxybenzone and the total dilution factor.

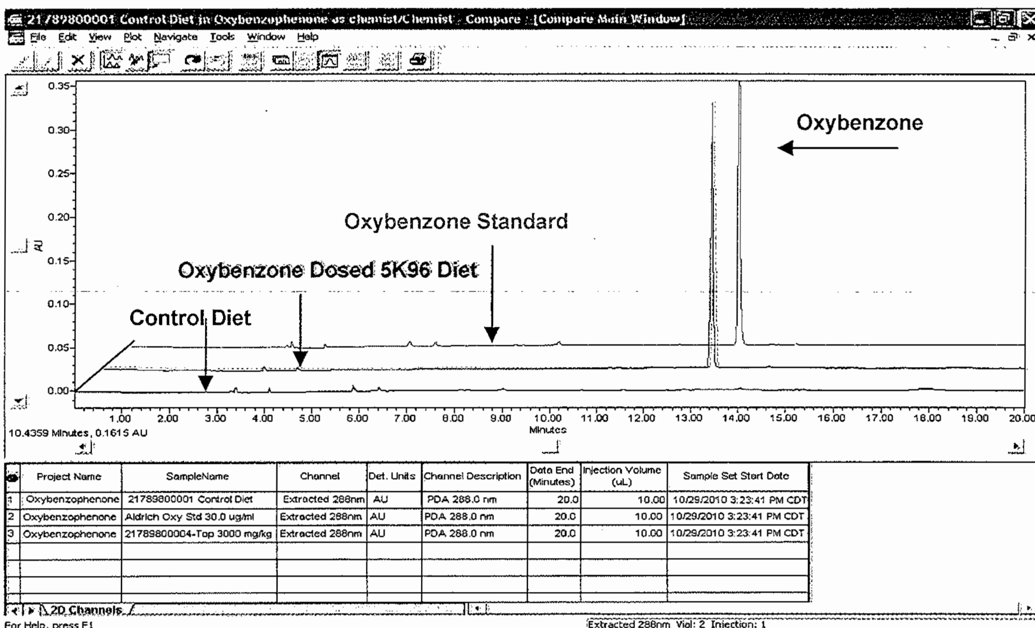
Example Calculation:
(Sample area/Std. area)(Std. µg/ml)(Dilution Factor) = Oxybenzone µg/g

4. METHOD VALIDATION

4.1 Injections of 10µl were made of a range of concentrations of oxybenzone standards in methanol from 1 to 1000 µg/ml and the PDA response from 190 to 400nm was collected. The spectrum from the oxybenzone peak indicated that an optimal absorbance maximum occurred at 288nm. The 288nm wavelength was extracted for multiple standards over this range and a linear relationship was demonstrated from 1 to 100 µg/ml as shown below:



4.2 Example of Overlaid Chromatograms



4.2 Recovery of Oxybenzophenone from Fortified Diet: Basal 5K96 irradiated diet was fortified in triplicate with 1000 mg/kg and 50,000 mg/kg Oxybenzophenone. Samples were prepared for analyses as described in 3.5 above.

Results of Analyses for Recovery of Oxybenzone

Sample	Fortification Level µg/g Oxybenzone	Oxybenzone Recovered	Percent Recovery
1000 (1)	1000	1010	101
1000 (2)	1000	1001	100
1000 (3)	1000	1020	102
50,000 (1)	49,800	49,550	99.5
50,000 (2)	50,400	50,070	99.3
50,000 (3)	50,000	49,980	100

5. References

5.1 Battelle Technical Report 13-292-DFD-164, Dose Formulation Study Report for 2-HYDROXY-4-METHOXYBENZOPHENONE, July 31, 2009.

5.2 The MERCK INDEX, Twelfth Edition, 1996, by Merck & Co., Inc.

Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

Appendix 2

NCTR DBT CHEM SOP No. 547.02

National Center for Toxicological Research US Food and Drug Administration, Jefferson, AR Division of Biochemical Toxicology Laboratory Operating Procedure	
SOP No: NCTR DBT CHEM 547.02 Prepared by: Mani Chidambaram	Approved by: DBT/Chemistry Team Leader
Supersedes: NCTR DBT CHEM 547.01	Date
Page 1 of 4	Effective Date: 5/12/10

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.

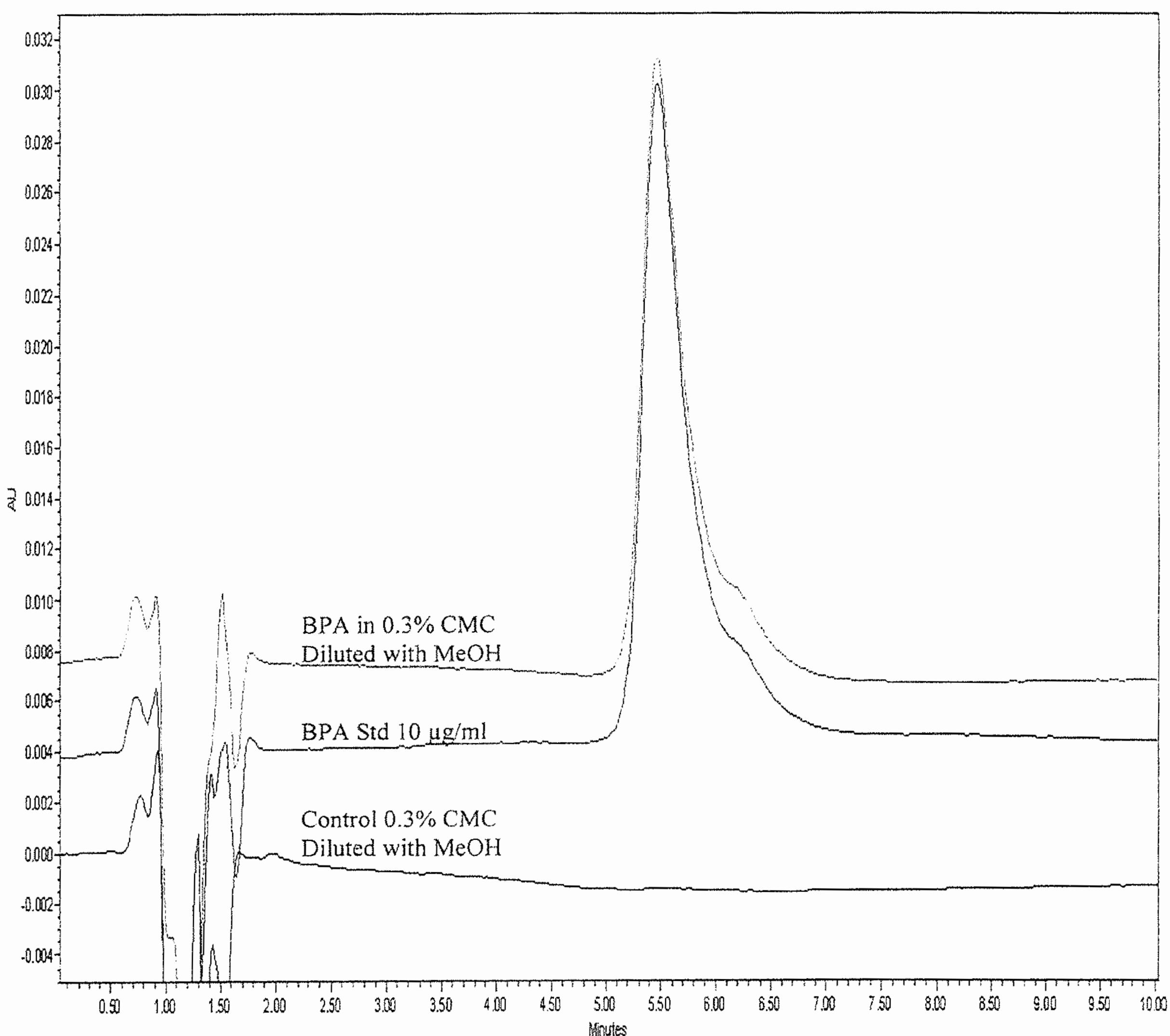
5.3 Transfer samples (5.2.3.9 – 5.2.6.9) to DBT Mass Spectrometry Support personnel for analyses.

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



Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

Appendix 3

Mass Spectrometry Report for Oxybenzone Test Article

Mass Spectrometry Laboratory
NCTR 870-543-4114

Mass Spectrometry Report

SAMPLE:	Oxybenzone test article; oxybenzone reference standard Aldrich H36206, Lot#S42088	REQUESTER:	Matt Bryant
FILES:	2188m208-234	DATE:	25 June 2012
INSTRUMENT:	Quantum Ultra	INLET/MODE:	**HPLC/- ESI-MS/MS**
SCAN:	Q1 90-700/1 sec	OPERATOR:	Kellie A. Woodling
Energy	20 eV	METHODS:	Test article certification Test article certification MSMS 20 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

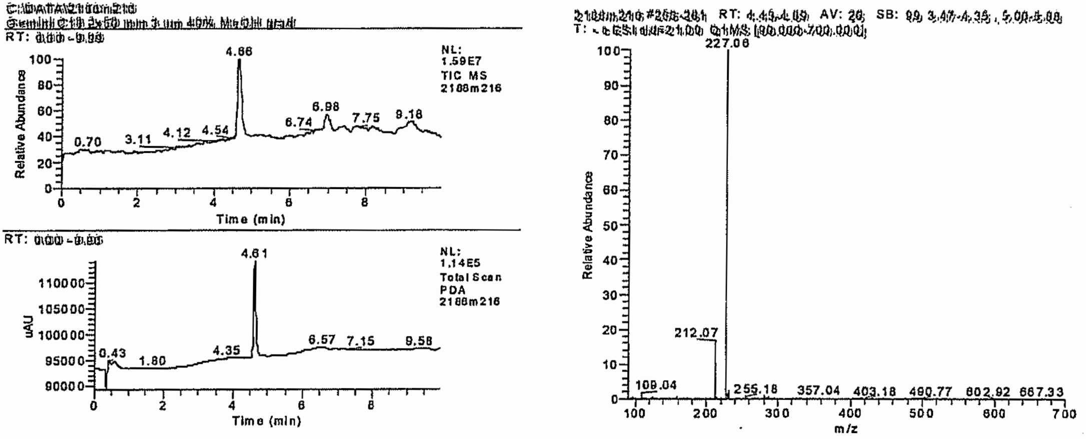
Sample preparation

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.* 4.6 min in all samples, with similar UV absorption spectra. A base peak with m/z = 227, consistent with the deprotonated oxybenzone molecule was observed at that same retention time (Figure 1) in the TIC (Total Ion Chromatogram).

Oxybenzone Test Article



Oxybenzone Aldrich Lot # S42088

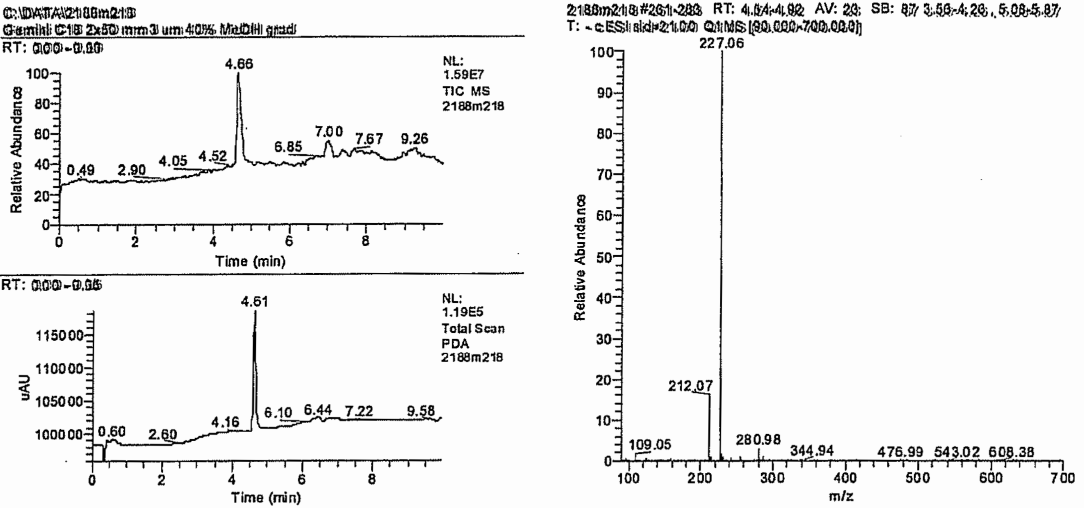
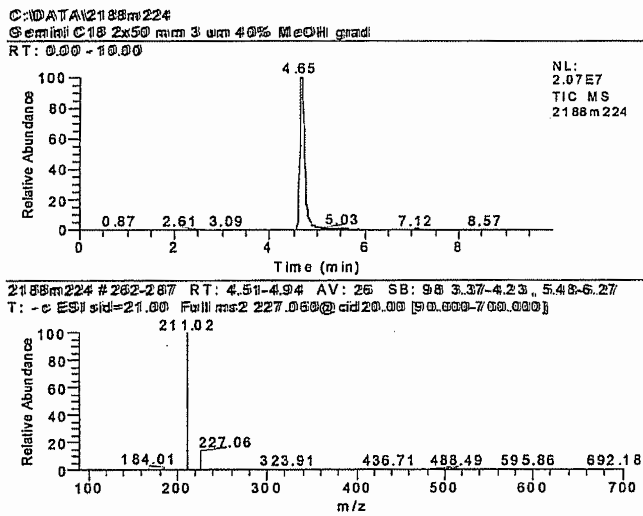


Figure 1

HPLC-PDA / -ESI-MS/MS results

The analyses of the Aldrich oxybenzone reference standards (Lot # S42088) and the test article revealed a deprotonated molecule at m/z = 227, consistent with the deprotonated oxybenzone molecule and a fragment at m/z = 211, consistent with the loss of 16 Da. This can be seen in Figure 2.

Oxybenzone
Test article



Oxybenzone
Aldrich Lot#S42088

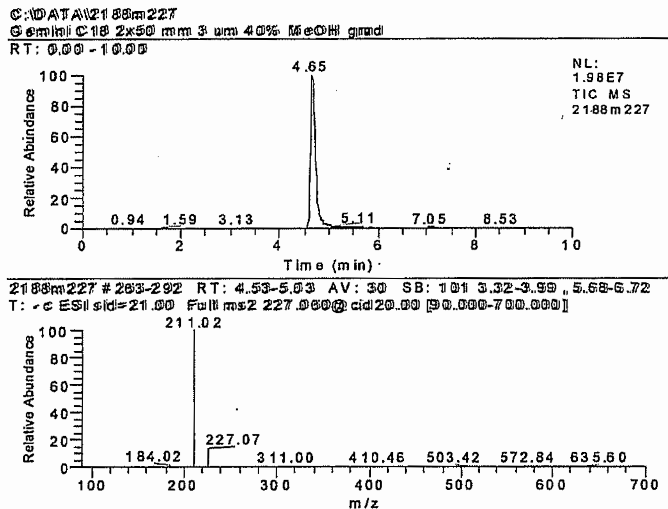


Figure 2.

Conclusion

Considering the coincidence of the chromatographic and spectrometric analytical data of the test compound with the Aldrich standards [Oxybenzone Lots # S42088], it is concluded that within the limitations inherent to the technique utilized, the main component of the test compound submitted for analysis is oxybenzone.

Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

Appendix 4

Mass Spectrometry Report for EE2 Test Article

Mass Spectrometry Laboratory
NCTR 870-543-4114

Mass Spectrometry Report

SAMPLE:	EE2 test article; EE2 reference standard, Steraloids Batch G745	REQUESTER:	Matt Bryant
FILES:	2188m208-234	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

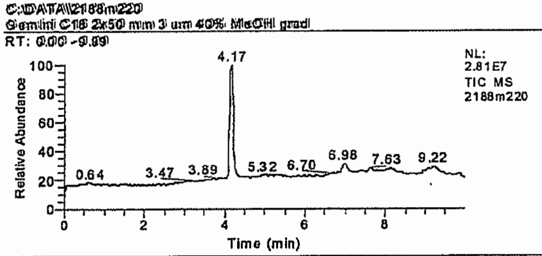
Sample preparation

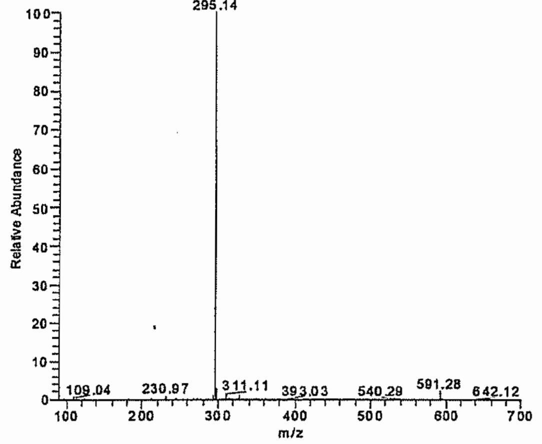
Dissolved the test articles in 1 mL of acetonitrile. Diluted appropriately in 50/50 methanol/water to afford a concentration of *ca.* 0.1 mg/mL. Injection volumes were 1 µl.

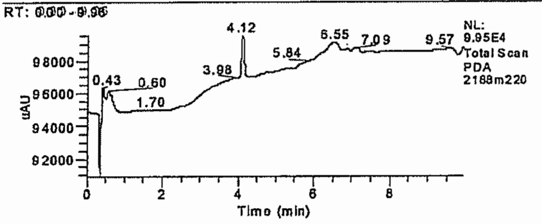
HPLC-PDA / -ESI results

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).

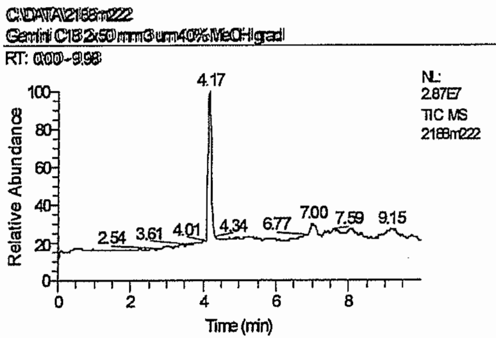
Ethinylestradiol Test Article Lot # 071M1492V

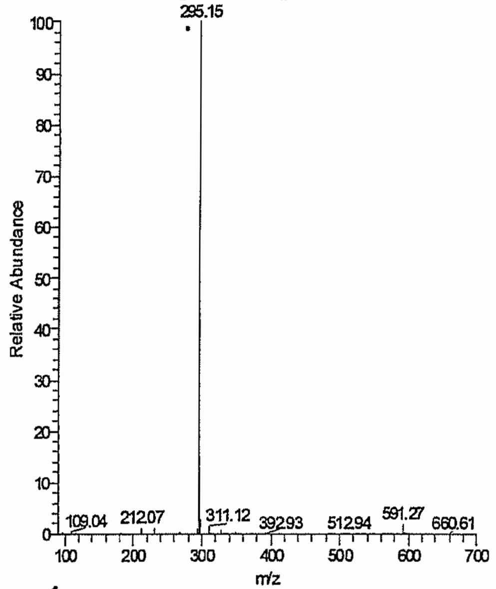






Ethinylestradiol Steraloids Batch G745





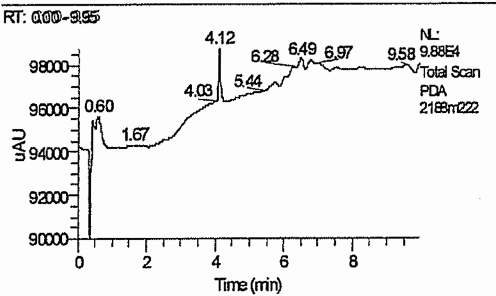


Figure 1

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.

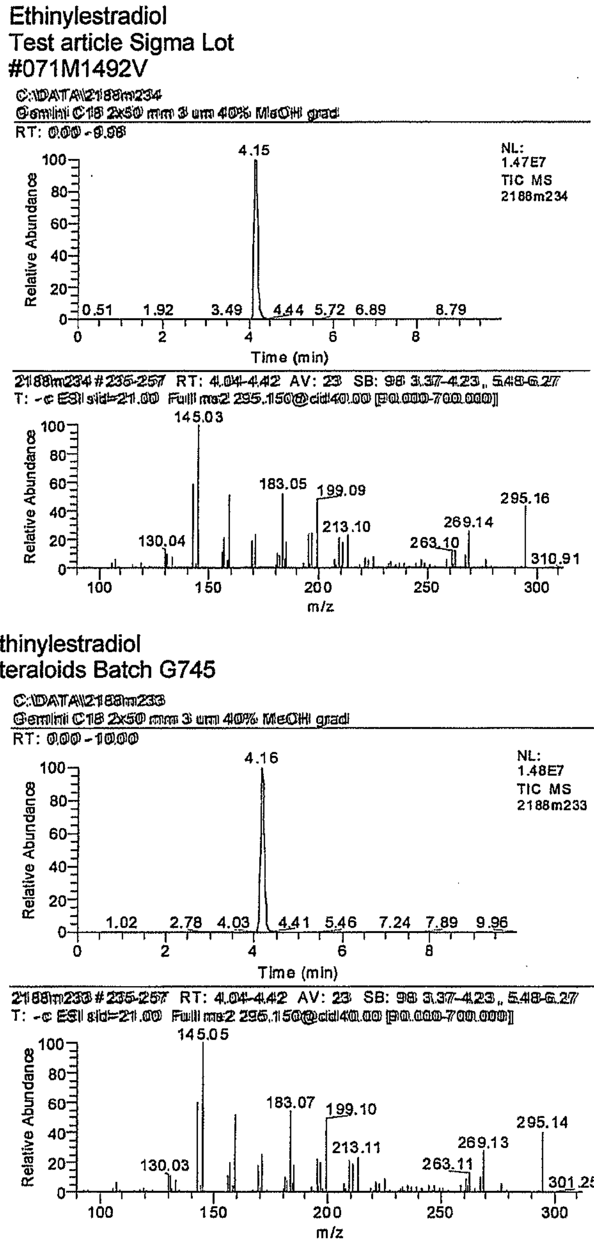


Figure 2.

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.

Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

Appendix 5

NMR Report for Oxybenzone Test Article

Laboratory of Nuclear Magnetic Resonance
Division of Biochemical Toxicology
NCTR
NMR Report

SAMPLES: Oxybenzone test article (2.5 mg); Oxybenzone reference standard (Aldrich H36206, Lot S42088; 9.5 mg)	**REQUESTER:** Matt Bryant
	DATE: 21 August 2012
SOLVENT: DMSO-d_6 (100%, Cambridge Isotope Laboratories, Andover, MA)	**OPERATOR:** Gonçalo Gamboa da Costa

Sample preparation

Dissolved each sample in *ca.* 0.7 mL of DMSO-d_6 and transferred each sample into a standard 5 mm NMR tube.

Analytical conditions

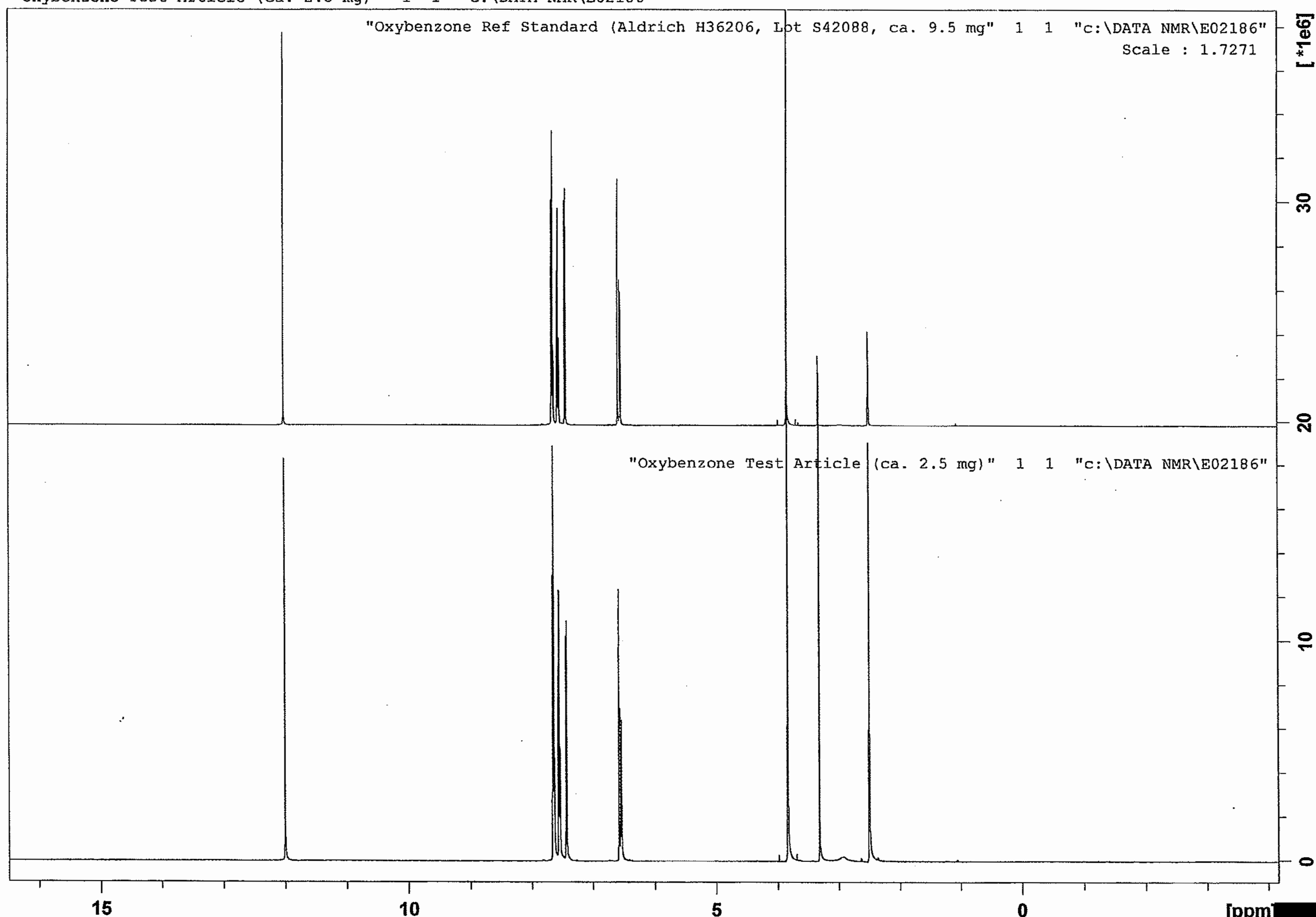
Analyzed the samples according to the procedures described in NCTR NMR-04-00 "Test article analysis". The spectra were referenced to the residual non-deuterated solvent (2.50 ppm).

Results

The ^{1}H NMR spectrum of the test article was identical to that of the reference standard. The spectra were consistent with the structure of oxybenzone. Other than the resonances of the residual non-deuterated solvent (2.50 ppm) and water (*ca.* 3.3 ppm) no other significant traces of contaminants were detected.

Conclusions

Based on the results of these analyses, and within the limitations of the technique, it is concluded that the test article is consistent with the specifications indicated by the manufacturer.


"Oxybenzone Test Article (ca. 2.5 mg)" 1 1 "c:\DATA NMR\E02186"
"Oxybenzone Ref Standard (Aldrich H36206, Lot S42088, ca. 9.5 mg" 1 1 "c:\DATA NMR\E02186"
Scale : 1.7271
"Oxybenzone Test Article (ca. 2.5 mg)" 1 1 "c:\DATA NMR\E02186"
[*1e6]
30
20
10
0
15
10
5
0
[ppm]

"Oxybenzone Test Article (ca. 2.5 mg)" 1 1 "c:\DATA NMR\E02186"

"Oxybenzone Test Article (ca. 2.5 mg)" 1 1 "c:\DATA NMR\E02186"

Oxybenzone Test Article (ca. 2.5 mg)

12.01

7.65 7.65 7.64 7.64 7.56 7.55 7.44 7.42 6.58 6.57 6.55 6.54

3.84

3.32

2.50 2.50 2.50

0.97 6.00 1.99 3.18

[*1e6]

80 60 40 20 0

15 10 5 0 [ppm]

"Oxybenzone Ref Standard (Aldrich H36206, Lot S42088, ca. 9.5 mg" 1 1 "c:\DATA NMR\E02186"

12.01
7.65 7.65 7.64 7.64 7.62 7.62 7.56 7.56 7.55 7.54 7.53 7.53 7.53 7.44 7.42
6.58 6.57 6.55 6.55 6.53 6.53
3.84
3.33
2.50 2.50 2.50

0.98
6.00
1.99
3.14

[*1e6]
40
30
20
10
0

15
10
5
0
[ppm]

Summary Report of the Analytical Chemistry Support Provided by the NCTR Division of Biochemical Toxicology for the Oxybenzone Experiments E02178.01, E02186.01, E02187.01 and E02188.01

Appendix 6

NMR Report for EE2 Test Article

Laboratory of Nuclear Magnetic Resonance
Division of Biochemical Toxicology
NCTR

NMR Report

SAMPLES: 17α-Ethinylestradiol (EE2) test article (Sigma E4876, Lot no. 071M1492V; 1.7 mg); EE2 reference standard (Steraloids, Inc., Newport, RI, Batch G745; 4.1 mg)	**REQUESTER:** Matt Bryant **DATE:** 21 August 2012
SOLVENT: DMSO-d_6 (100%, Cambridge Isotope Laboratories, Andover, MA)	**OPERATOR:** Gonçalo Gamboa da Costa

Sample preparation

Dissolved each sample in *ca.* 0.7 mL of DMSO-d_6 and transferred each sample into a standard 5 mm NMR tube.

Analytical conditions

Analyzed the samples according to the procedures described in NCTR NMR-04-00 "Test article analysis". The spectra were referenced to the residual non-deuterated solvent (2.50 ppm).

Results

The ^{1}H NMR spectrum of the test article was identical to that of the reference standard. The spectra were consistent with the structure of 17α-Ethinylestradiol. Other than the resonances of the residual non-deuterated solvent (2.50 ppm) and water (*ca.* 3.3 ppm) no other significant traces of contaminants were detected.

Conclusions

Based on the results of these analyses, and within the limitations of the technique, it is concluded that the test article is consistent with the specifications indicated by the manufacturer.

"EE2 Test Article (Sigma E4876, Lot #071M1492V; ca. 1.7 mg)" 1 1 "c:\DATA NMR\E02186"

"EE2 Reference Standard (Steraloids, Inc., Newport, RI, Batch G7" 1 1 "c:\DATA NMR\E02186"

"EE2 Test Article (Sigma E4876, Lot #071M1492V; ca. 1.7 mg)" 1 1 "c:\DATA NMR\E02186"

Scale : 1.1353

[*1e6]

80

60

40

20

0

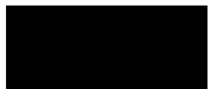
8

6

4

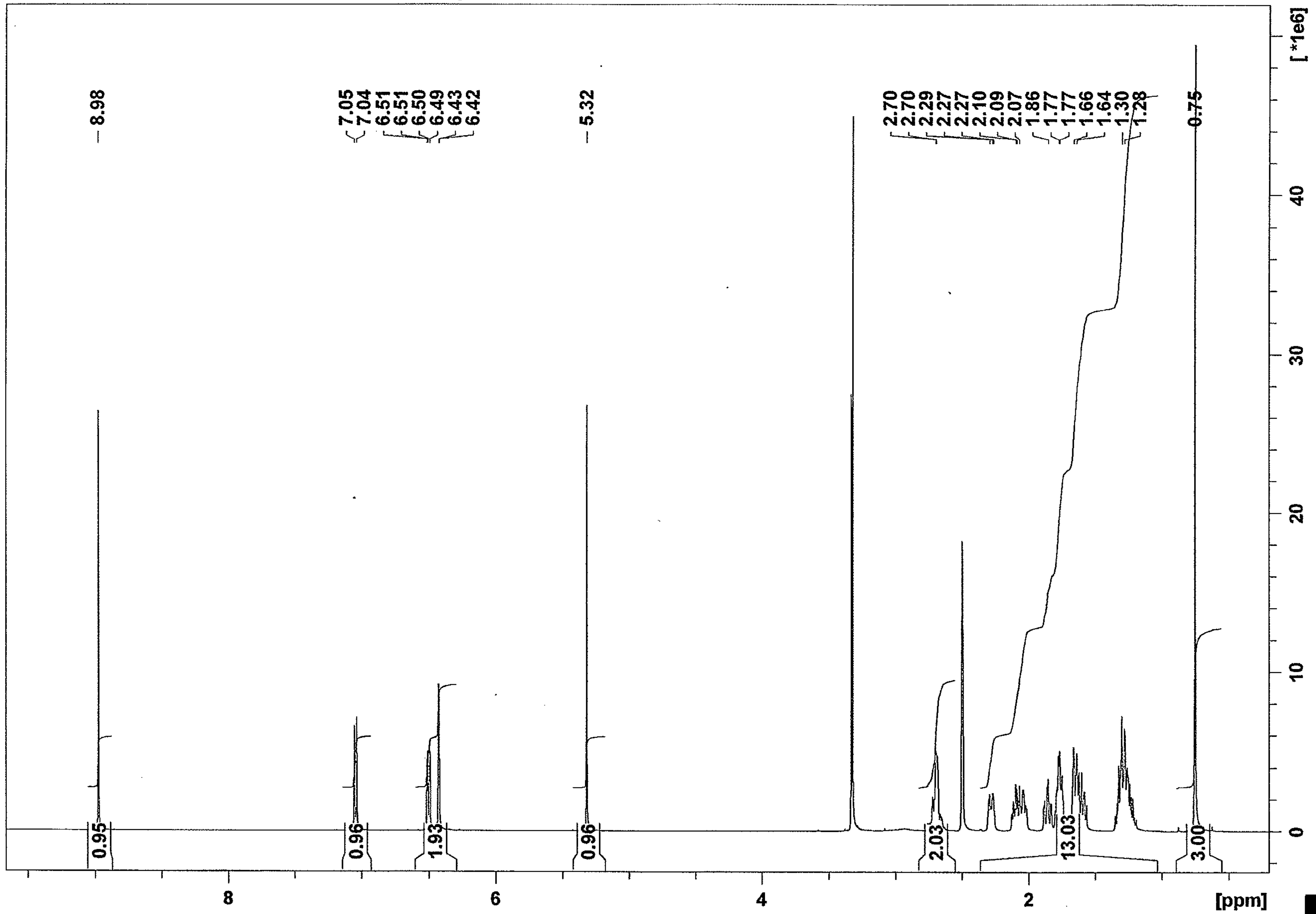
2

[ppm]



"EE2 Test Article (Sigma E4876, Lot #071M1492V; ca. 1.7 mg)" 1 1 "c:\DATA NMR\E02186"

EE2 Test Article (Sigma E4876, Lot #071M1492V; ca. 1.7 mg)


"EE2 Reference Standard (Steraloids, Inc., Newport, RI, Batch G7" 1 1 "c:\DATA NMR\E02186"
8.98
7.05
7.04
6.51
6.51
6.50
6.49
6.43
6.42
5.32
2.70
2.70
2.29
2.27
2.27
2.10
2.09
2.07
1.86
1.77
1.77
1.66
1.64
1.30
1.28
0.75
[*1e6]
40
30
20
10
0
0.95
0.96
1.93
0.96
2.03
13.03
3.00
8
6
4
2
[ppm]