STUDY TITLE

Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies

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STUDY INITIATION DATE

February 26, 2009

STUDY COMPLETION DATE

October 11, 2010

SPONSOR

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005

PERFORMING LABORATORY

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STUDY IDENTIFICATION

ABC Study No. 64403

STATEMENT OF GLP COMPLIANCE

Compound: Naphthenic Acids

Study Title: Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies

The study described in this report, with the following exceptions, was conducted in compliance with the following Good Laboratory Practice Standards:

Organization for Economic Co-operation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final].

U.S. Environmental Protection Agency. 1989. Toxic Substances Control Act; Good Laboratory Practice Standards; Final Rule (40 CFR, Part 792).

1) The test substance characterization was not conducted in accordance to the stated Good Laboratory Practices.

2) The latest water characterizations performed in August 2009 were not performed in accordance to the stated Good Laboratory Practices.

3) Analyses conducted by the University of Alberta were not conducted in accordance to the stated Good Laboratory Practices

These were the only exceptions to the stated GLP principles and they did not adversely affect the study integrity or the interpretation of the results generated from this study.

The original raw data and the study plan were provided to the American Petroleum Institute with the final report. Copies of all data in support of this report were retained at ABC Laboratories, Inc. along with original facility records and a copy of the final report and the study plan.

[redacted] Oct 11, 10
Date
ABC Laboratories, Inc.

[redacted] 11 Oct 2010
Date
ABC Laboratories, Inc.

Sponsor's Representative:

[redacted] 15 November 2010
Date
American Petroleum Institute

QUALITY ASSURANCE STATEMENT

ABC's Quality Assurance Unit reviewed Study No. 64403 entitled "Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies," for the American Petroleum Institute. The following inspections/audits were conducted on this study.

Date of Study-Based Inspection	Phase Inspected	Date Reported to Study Director	Date Reported to Management
27 Feb 09	Protocol	27 Feb 09	02 Mar 09
18 Jun 09	Procedure: Extraction	18 Jun 09	18 Jun 09
21-22 Jan 10	Raw Data & Draft Report	22 Jan 10	28 Jan 10
27 Sep 10	Final Report	27 Sep 10	27 Sep 10

These audits indicate that the report is an accurate reflection of the study as it was conducted by ABC Laboratories, Inc.

[redacted]

11 Oct. 2010
Date

ABC Laboratories, Inc.

STUDY PERSONNEL

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1.0 INTRODUCTION

The American Petroleum Institute contracted ABC Laboratories, Inc. to perform a method development and GLP equilibration trials for water accommodated fraction (WAF) preparations of naphthenic acids (CAS# 1338-24-5) to be used in toxicity tests. The purpose of the trials was to demonstrate the ability to prepare, maintain, and analyze a WAF of naphthenic acids and to determine the optimum mixing time to achieve the maximum dissolution of naphthenic acids in the different dilution media used in ecotoxicity tests.

2.0 MATERIALS AND METHODS

2.1 Equipment

Balances – Sartorius, Model R300S, Mettler AG245

Centrifuge – Beckman J2-21

pH Meter – WTW Model pH 330i

Glassware – Class A volumetric

Fourier Transform Infrared Spectroscope – Thermo Nicolet (see Section 2.6.4 for instrument details)

2.2 Test and Reference Substances

A sample of the test substance, naphthenic acids (CAS# 1338-24-5); EPL P/A #1203-000 (collected from Drum #2), was received from EPL Archives, Inc. on January 20, 2009 and was stored at room temperature. An expiration date of the sample was not provided. The sample was assigned ABC reference number TS-22856. The Material Sample Safety Data Sheet (MSDS) described the test substance as an amber-colored liquid and stable under normal storage conditions. The MSDS and a profile of physical-chemical specifications of the test substance provided by the original supplier are given in Appendix A. This material was used to prepare all test solutions, matrix spiking solutions, and analytical standards. All solution preparations were based on total product.

2.3 ABC Reagent Water

Reagent water for this study was purified using a Milli-Q Water System (Millipore Corporation). ABC reagent water is produced by passing reverse-osmosis water through a series of deionization tanks, a laboratory water purification system consisting of carbon, de-mineralization, and organic adsorption cartridges, and then through a 0.2-µm filter.

2.4 Reagents

Acetonitrile – Fisher HPLC/Residue/Pesticide grade

Anhydrous Sodium Sulfate – Fisher USP grade

Methylene Chloride – Fisher ACS grade

Sulfuric Acid – Fisher Technical grade

2.5 Test Medium

The test media were aged-blended freshwater and freshwater algal nutrient medium (FWAM) (1). The aged-blended freshwater was prepared by blending naturally hard well water with well water that was demineralized by reverse osmosis. These waters were blended to yield a total hardness of approximately 130 to 160 mg $CaCO_3$/L and biologically aged (held in a tank containing aquatic organisms). The water was then passed through a sediment filter, UV irradiated, and aerated prior to use. The FWAM was prepared by the addition of appropriate reagent grade salts to autoclaved ABC reagent water. After preparation, the medium was pH-adjusted to 7.5 ± 0.1 using 0.1 N NaOH and/or 0.1 N HCl and filtered through 0.45-µm Millipore® filters. Characterization of a representative sample of the base water, i.e., ABC well water, used to prepare the dilution water can be found in Appendix B.

2.6 Analytical Test Method

Test solutions were analyzed for the concentration of naphthenic acid, using Fourier transform infrared spectroscopy (FTIR). Analysis was accomplished based on a method developed at ABC Laboratories following Jivraj et al. (2). Details of the sample preparation and method of analysis are described below.

2.6.1 Preparation of Analytical Standard and Matrix Spiking Solutions

A 5,080 mg/L test substance solution was prepared on March 19, 2009 by weighing 507.7 mg of naphthenic acid into a 100-mL volumetric flask and bringing the flask to volume with methylene chloride. Dilutions of this solution were made in methylene chloride and used to prepare analytical standards.

A 100 mg/mL test substance solution was prepared on April 13, 2009 by weighing 10,001.0 mg of naphthenic acid into a 100-mL volumetric flask and bringing the flask to volume with acetonitrile. A dilution of this solution was prepared in acetonitrile and used as a spiking solution.

2.7 Method Validations

Test procedures followed the ABC test protocol entitled, "Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies," with amendments (Appendix C). Validations of the analytical method were performed from May 22 to June 5, 2009.

2.7.1 Method Validation in Freshwater

Nine 1,000-mL separatory funnels were filled with 500 mL volumes of aged blended freshwater. Three low validation spike samples (0.500 mg/L) were fortified with 0.250 mL of a 1.00 mg naphthenic acid/mL stock solution. Three high validation spike samples (100 mg/L) were fortified with 0.500 mL of a 100 mg naphthenic acid/mL stock solution. Three replicates were prepared for the control and the reagent blank. Samples were processed and analyzed following the methods described in Section 2.7.3.

2.7.2 Method Validation in Freshwater Algal Nutrient Medium

Nine 500-mL Nalgene bottles were each filled with 500 mL volumes of FWAM. Three low validation spike samples (0.500 mg/L) were fortified with 0.250 mL of a 1.00 mg naphthenic acid/mL stock solution. Three high validation spike samples (100 mg/L) were fortified with 0.500 mL of a 100 mg naphthenic acid/mL stock solution. Three replicates each were used as the control. Each of these 9 samples were then split evenly and placed into 250-mL Nalgene bottles and centrifuged for approximately 10 minutes at 3,000 rpm. Centrifugation was performed to check the solubility of naphthenic acid in FWAM. Three reagent blanks were also prepared.

Three non-centrifuged samples were prepared by filling 1,000-mL separatory funnels with 500 mL volumes of FWAM. Samples were fortified with 0.500 mL of a 100 mg naphthenic acid/mL stock solution.

Samples were processed and analyzed following the methods described in Section 2.7.3.

2.7.3 Test Solution Analysis

The supernatant resulting from the centrifugation described in Section 2.7.2 was then decanted into 1,000-mL separatory funnels. For the aged-blended water samples, a 500-mL volume was placed directly into the separatory funnels.

Each sample was acidified with concentrated sulfuric acid to a pH level of 2.5 ± 0.1. A 100-mL volume of methylene chloride was added to each sample and the samples were shaken to mix. After approximately one minute of shaking, the sample phases were allowed to separate. The methylene chloride (lower layer) was filtered through anhydrous sodium sulfate and collected in a 500-mL flat-bottomed flask. The above methylene chloride steps were repeated one more time. The methylene chloride extracts for each sample were combined in a single vessel. The samples were then evaporated to dryness using a rotary evaporator. In some cases, samples were quantitatively transferred to 15-mL culture tubes using two separate 5-mL aliquots of methylene chloride. In these cases, the samples were evaporated to dryness under a gentle stream of nitrogen and then reconstituted with an appropriate volume of methylene chloride. In other cases, samples were quantitatively transferred to an appropriate volume with methylene chloride without the culture tube evaporation step. Dilutions were made using methylene chloride, if necessary, to produce an analyte concentration that was within the range of the standard curve. The samples were vialed and analyzed by FT-IR. QC fortifications were prepared in a similar manner after control medium had been fortified with the test substance.

2.7.4 Instrumentation Conditions

Sample analysis was performed using a FTIR system equipped with the following analytical parameters:

Manufacturer: Thermo Nicolet
Model: Avatar 360
Software: Omnic 32
IR Cell: Thermo Scientific, KBr 1.0 mm sealed cell

Cell Holder: Thermo Scientific
Dry Nitrogen Gas Used to Protect the IR Cell Between Runs: Yes
Scan Times: 64
Scan Range: 4000-400 cm^{-1}
Scan Model: Absorbance
Resolution: 4 cm^{-1}
Wave Number of Interest: 1743 cm^{-1}
Solvent Used for Background Collection: Methylene chloride

2.7.5 Calculations

Naphthenic acid concentrations were determined directly from the standard curve by the following equation:

$$\frac{\left(\begin{array}{c}\mu g/L \text{ or } mg/L \text{ equivalents for} \\ \text{test substance from standard} \\ \text{curve equation}\end{array}\right)\left(\begin{array}{c}\text{sample volume} \\ \text{in mL for} \\ \text{chromatography}\end{array}\right)}{(\text{sample volume in mL before preparation})} = \begin{array}{c}\mu g/L \text{ or} \\ mg/L\end{array} = \begin{array}{c}\text{ppb or} \\ \text{ppm}\end{array}$$

The standard curve equation is of the form: y = mx + b

where:
y = peak response
m = slope of the standard curve
x = mg/L
b = y-intercept

Example calculation for the 2 mg/L aged-blended freshwater WAF sample at 18 hours:

Standard Curve: y = 0.000158351x +0.006116121
Sample Peak Response: 0.0800
Concentration from standard curve: 466.582 mg/L

Volume for Analysis: 2 mL
Sample Volume: 500 mL

The concentration of naphthenic acid in the sample was calculated by the following equation:

$$\frac{(466.582 \text{ mg naphthenic acid})(2 \text{ mL})}{500 \text{ mL}} = 1.90 \text{ mg/L}$$

Recovery was calculated as a percentage of the corresponding nominal concentration, as shown for the 2 mg/L WAF sample at 18 hours:

$$\frac{1.90 \text{ mg naphthenic acid/L}}{2 \text{ mg naphthenic acid/L}} \times 100 = 95\%$$

The minimum quantifiable limit (MQL) was determined from the following equation:

$$\frac{\left(\begin{array}{c}\text{low standard}\\ \text{concentration mg/L}\end{array}\right)\left(\begin{array}{c}\text{analysis}\\ \text{volume (mL)}\end{array}\right)}{\left(\begin{array}{c}\text{sample}\\ \text{volume (mL)}\end{array}\right)} = \text{MQL expressed as mg/L}$$

Lowest standard concentration: 75.0 µg/mL
Analysis volume: 2 mL
Sample volume: 500 mL
therefore:

$$\text{MQL} = \frac{(75.0\ \text{mg/L})(2\ \text{mL})}{(500\ \text{mL})} = 0.300\ \text{mg/L}$$

2.8 Equilibration Trials for Water Accommodated Fractions (WAFs)

Equilibration trials were performed in order to determine the optimal mixing times to achieve maximum dissolution of naphthenic acids in the aged-blended freshwater and FWAM. WAF solutions were prepared at loading rate concentrations of 2, 10, and 100 mg naphthenic acid/L by adding the appropriate amount of test substance to a volume of aged-blended freshwater or freshwater algal medium (FWAM) in a glass aspirator bottle containing a Teflon stir bar. Each container was covered with Parafilm while being stirred. Control WAF solutions were prepared using only aged-blended freshwater or FWAM. The WAF solutions were stirred at room temperature for 4, 18, 48, and 72 hours with the stirring adjusted to provide a vortex approximately 30 to 50% of the solution depth. After the prescribed time of stirring, stirring was stopped and the mixture allowed to sit undisturbed for approximately one hour before initiating draining/siphoning of the WAF solution. Solutions were drained from the outlet of the aspirator bottle into an appropriately sized glass container at each sample point. The first approximately 100 mL of solution from each preparation was allowed to drain into a waste container.

A total of 3 WAFs (in each test medium) were prepared at the 2 mg/L loading rate, 4 at the 10 mg/L loading rate, and 3 at the 100 mg/L loading rate. For the three WAFs prepared at 2 mg/L loading, one each was mixed for 4, 18, and 48 hours and sampled after a 1 hour settling period. For the four WAFs prepared at 10 mg/L loading, one each was mixed for 4, 18, 48, and 72 hours and again sampled after a 1 hour settling period. For the three WAFs prepared at 100 mg/L loading, one each was mixed for 18, 48, and 72 hours and sampled after a 1 hour settling period.

2.9 Characterization and Stability of Naphthenic Acids WAFs by Analysis of Z-number and Carbon Number Distribution

As part of the characterization of naphthenic acids in the WAF solutions, Dr. Phillip M. Fedorak (Department of Biological Sciences, University of Alberta, Edmonton, Alberta Canada) was retained by the Study Sponsor to perform Gas Chromatography-Mass Spectrometry (GC-MS) analysis of a representative WAF preparation. ABC Laboratories prepared a WAF at a loading

rate of 10 mg naphthenic acids/L similar to that described in Section 2.7, and samples were shipped to the University of Alberta where the analyses took place. The GC-MS technique allowed the discrimination of the naphthenic acids in the WAF into relative abundances of each ion corresponding to the general formula for naphthenic acids, $C_nH_{2n+z}O_2$, where n is the carbon number and Z is zero or a negative even number defining the hydrogen deficiency due to cyclization. For example, a Z-number of 0 represents a carboxylic acid having no rings, while a Z-number of 2 and 4 represents carboxylic acids having 1 and 2 naphthene rings, respectively. Although no analytical method exists whereby each individual naphthenic acid molecule is identified, the GC-MS method applied here results in a distribution of families of molecules having similar carbon numbers and Z-numbers.

Of interest to the ecotoxicity studies, the characterization of the WAF solutions by GC-MS reveals the naphthenic acids relative abundances as described above. As part of the validation study, the stability of the WAFs that would occur over a typical solution renewal cycle and shipping period anticipated to be used in the definitive study was investigated. Therefore, samples were analyzed over various time periods and shipped in either glass or plastic to assess whether the factor of time or type of sample container affected stability as evaluated by the carbon number and Z-number distributions. A report of these analyses is provided in Appendix D.

3.0 RESULTS AND DISCUSSION

3.1 Method Validation Results

3.1.1 Method Validation Results in Freshwater

The analytical method for recovering naphthenic acid from aged-blended freshwater was validated prior to initiating the WAF equilibration trials. Analysis of triplicate fortifications at a concentration of 0.500 mg naphthenic acid/L resulted in recoveries ranging from 120 to 128% of the nominal concentration (Table 1). Analysis of triplicate fortifications at a concentration of 100 mg naphthenic acid/L resulted in recoveries ranging from 102 to 105% of the nominal concentration (Table 1). Representative chromatography from the FTIR analysis set of the method validation in aged-blended freshwater is found in Appendix E.

3.1.2 Method Validation Results in Freshwater Algal Nutrient Medium

The analytical method for recovering naphthenic acid was validated in non-centrifuged and centrifuged freshwater algal nutrient medium. Analysis of triplicate centrifuged fortifications at a concentration of 0.500 mg naphthenic acid/L in FWAM resulted in recoveries ranging from 116 to 120% of the nominal concentration (Table 2). Analysis of triplicate centrifuged fortifications at a concentration of 100 mg naphthenic acid/L in FWAM resulted in recoveries ranging from 10 to 16% of the nominal concentration (Table 2). Analysis of triplicate non-centrifuged fortifications at a concentration of 100 mg naphthenic acid/L in FWAM resulted in recoveries ranging from 103 to 109% of the nominal concentrations (Table 3). The observed low recoveries when the 100-mg naphthenic acid/L samples were centrifuged was expected, as this demonstrated that the solubility of naphthenic acids had been exceeded at that level. Centrifugation segregated the insoluble test substance from the dissolved fraction, and without

centrifugation the total sample (including the insoluble fraction) was extracted. Quantitative recoveries with the total sample showed that the volume of extraction solvent used in the method was sufficient to produce a quantitative extraction without interferences. Thus the method was considered adequate to quantitatively extract dissolved naphthenic acids from WAF preparations. Inclusion of a centrifugation step in the analysis of WAF solutions was considered unnecessary.

3.2 WAF Equilibration Trial Results

3.2.1 WAF Freshwater Results

The measured concentrations of naphthenic acid in the 2 and 10 mg/L WAF preparations in aged-blended freshwater after 4 hours of stirring were 1.96 and 8.76 mg/L, representing 98 and 88% of the nominal concentrations, respectively. Measured concentrations in the 2, 10, and 100 mg/L WAF preparations after 18 hours of stirring were 1.90, 7.90, and 81.0 mg/L, representing 95, 79, and 81% of the nominal concentrations, respectively. The measured concentrations of naphthenic acid in the 2, 10, and 100 mg/L WAF preparations after 48 hours of stirring were 1.54, 7.48, and 66.5 mg/L, representing 77, 75, and 67% of the nominal concentrations, respectively. Measured concentrations in the 10 and 100 mg/L WAF preparations after 72 hours of stirring were 7.90 and 77.0 mg/L, representing 79 and 77% of the nominal concentrations, respectively. Recoveries from QC fortification samples ranged from 82 to 106%. Results from the equilibration trials in freshwater are presented in Table 4.

3.2.2 WAF Freshwater Algal Nutrient Medium Results

The measured concentrations of naphthenic acid in the 2 and 10 mg/L WAF preparations in freshwater algal nutrient medium (FWAM) after 4 hours of stirring were 1.84 and 7.89 mg/L, representing 92 and 79% of the nominal concentrations, respectively. Measured concentrations in the 2, 10, and 100 mg/L WAF preparations after 18 hours of stirring were 1.85, 8.61, and 59.9 mg/L, representing 92, 86, and 60% of the nominal concentrations, respectively. Measured concentrations in the 2, 10, and 100 mg/L WAF preparations after 48 hours of stirring were 1.77, 8.37, and 53.3 mg/L, representing 88, 84, and 53% of the nominal concentrations, respectively. Measured concentrations in the 10 and 100 mg/L WAF preparations after 72 hours of stirring were 8.97 and 59.5 mg/L, representing 90 and 60% of the nominal concentrations, respectively. Recoveries from the QC fortification samples ranged from 83 to 102%. Results from the equilibration trials in FWAM are presented in Table 5.

Comparison of the results between the 100 mg/L WAF preparations in aged-blended freshwater and FWAM suggest a slightly different solubility naphthenic acids in the two media. While the average naphthenic acid measured concentration was approximately 75 mg/L in age-blended freshwater, the average measured concentration in FWAM was 58 mg/L.

3.2.3 WAF Characterization by GC-MS

A summary of the report of the characterization by GC/MS for naphthenic acid C-number and Z-number profiles in a 10 mg/L WAF is given in Appendix D. Naphthenic acid profiles were substantially similar in the WAF stored and shipped in either glass or plastic. Additionally, the 10 mg/L WAF maintained for 48-h under simulated testing conditions for a Daphnia magna acute test showed similar C-number and Z-number profiles.

4.0 CONCLUSIONS

4.1 Method Development

Quantitative analysis of naphthenic acids in algal nutrient medium was demonstrated at concentrations of 0.5 and 100 mg/L, while analysis of naphthenic acids in ABC aged blended freshwater was slightly outside the acceptable range (80 – 120%) at the low level. This was attributed to an inherent matrix contribution to the absorbance originating in the aged blended freshwater. For this reason, the WAF equilibration trials were run at a low concentration of 2 mg/L.

4.2 WAF Equilibration Trial

Results of the WAF equilibration trial showed that naphthenic acids achieve a relative rapid dissolution equilibrium at the three loading rates of 2, 10, and 100 mg/L. Even at the shortest stirring time of 4 hours for the 2 and 10 mg/L loadings, concentrations in aged blended water appeared to have reached their equilibrium. This also appeared to be true for algal nutrient medium, but perhaps only at the 2 mg/L loading, as the dissolution equilibrium appeared to require a little more time at the 10 mg/L loading. Conversely, the slight rise in concentration between the 4 and 18 hour stirring may have been due to analytical variability. For both water types at the 100 mg/L loading, the shortest stirring time of 18 hours appeared to be sufficient to bring dissolved naphthenic acids to equilibrium with their loading rate. For aquatic toxicity testing, 18 hours appears to be a sufficient stirring time for naphthenic acids to achieve dissolution equilibrium in the two types of aqueous test media.

Analyses of the C-number and Z-number distribution profiles of naphthenic acids in a WAF solution were essentially similar whether stored in glass or plastic containers and maintained over a period of at least 48 hours. It was concluded from these analyses that sample containers made of either glass or plastic container would be appropriate for collection, storage, and shipping. From the analyses of the naphthenic acid profiles over time, it was concluded that WAFs of naphthenic acids are stable over a period of time expected to cover solution renewal cycles in aquatic toxicity testing, and shipping to the University of Alberta, Canada.

PROTOCOL DEVIATIONS

Protocol Section: 10.3 – Analytical Method Validation

The analytical method was not added to the protocol prior to the start of the equilibration trials.

Reason: Study director error.

Effect on Study Integrity: None. The method is described in the raw data.

Protocol Section: 10.3 – Analytical Method Validation

The MDL and PQL were not determined.

Reason: Study director error.

Effect on Study Integrity: None. Method limits are adequately defined by the MQL.

REFERENCES

(1) American Society for Testing and Materials (ASTM). 1997. Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae. ASTM E1218-97a. 14pp.

(2) Jivraj, M.N., M.D. MacKinnon, and B. Fung. 1991. Naphthenic acid extraction and quantitative analysis with FT-IR spectroscopy. Syncrude Canada Ltd. Research Department. Edmonton, AB. September 1991.

Table 1 Measured Concentrations of Naphthenic Acids During the Method Validation in Aged Blended Freshwater

Sample	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Recovery (%)
Blank1	0	<MQL	NA
Blank2	0	<MQL	NA
Blank3	0	<MQL	NA
Control s	0	<MQL	NA
Control t	0	<MQL	NA
Control u	0	<MQL	NA
Low spike s	0.500	0.638	128%
Low spike t	0.500	0.630	126%
Low spike u	0.500	0.600	120%
High spike y	100	102	102%
High spike z	100	102	102%
High spike aa	100	105	105%

MQL = 0.300 mg/L

Table 2 Measured Concentrations of Naphthenic Acids During the Method Validation in Freshwater Algal Nutrient Medium (Centrifuged)

Sample ID	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Recovery (%)
Blank1	0	<MQL	NA
Blank2	0	<MQL	NA
Blank3	0	<MQL	NA
Control p	0	<MQL	NA
Control q	0	<MQL	NA
Control r	0	<MQL	NA
Low spike p	0.500	0.580	116%
Low spike q	0.500	0.580	116%
Low spike r	0.500	0.600	120%
High spike p	100	13.6	14%
High spike q	100	10.1	10%
High spike r	100	16.1	16%

MQL = 0.300 mg/L

Table 3 Measured Concentrations of Naphthenic Acids During the Method Validation in Freshwater Algal Nutrient Medium (Non-Centrifuged)

Sample	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Recovery (%)
High spike v	100	103	103%
High spike w	100	103	103%
High spike x	100	109	109%

Table 4 Measured Concentrations of Naphthenic Acids During the WAF Equilibration Trials in Aged-Blended Freshwater

Sample ID	Sample Point	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Recovery (%)
64403-86 2ppm WAF, 4h	4-hours	2	1.96	98%
64403-87 10ppm WAF, 4h	4-hours	10	8.76	88%
64403-88, spike	4-hours	2	2.12	106%
64403-89 2ppm WAF, 18h	18-hours	2	1.90	95%
64403-90 10ppm WAF, 18h	18-hours	10	7.90	79%
64403-91, spike	18-hours	10	8.60	86%
64403-108 100ppm WAF, 18h	18-hours	100	81.0	81%
64403-109 Spike	18-hours	100	102	102%
64403-92 2ppm WAF, 48h	48-hours	2	1.54	77%
64403-93 10ppm WAF, 48h	48-hours	10	7.48	75%
64403-94, spike	48-hours	2	1.90	95%
64403-112 100ppm WAF, 48h	48-hours	100	66.5	67%
64403-113 Spike	48-hours	100	88.1	88%
64403-95 10ppm WAF, 72h	72-hours	10	7.90	79%
64403-96, spike	72-hours	10	8.20	82%
64403-116 100ppm WAF, 72h	72-hours	100	77.0	77%
64403-117 Spike	72-hours	100	98.2	98%

Table 5 Measured Concentrations of Naphthenic Acids During the WAF Equilibration Trials in Freshwater Algal Nutrient Medium

Sample ID	Sample Point	Nominal Concentration (mg/L)	Calculated Concentration (mg/L)	Recovery (%)
64403-97 2ppm WAF, 4h	4-hours	2	1.84	92%
64403-98 10ppm WAF, 4h	4-hours	10	7.89	79%
64403-99, spike	4-hours	2	1.93	97%
64403-100 2ppm WAF, 18h	18-hours	2	1.85	92%
64403-101 10ppm WAF, 18h	18-hours	10	8.61	86%
64403-102, spike	18-hours	10	8.94	89%
64403-110 100ppm WAF, 18h	18-hours	100	59.9	60%
64403-111 Spike	18-hours	100	102	102%
64403-103 2ppm WAF, 48h	48-hours	2	1.77	88%
64403-104 10ppm WAF, 48h	48-hours	10	8.37	84%
64403-105, spike	48-hours	2	1.96	98%
64403-114 100ppm WAF, 48h	48-hours	100	53.3	53%
64403-115 Spike	48-hours	100	87.6	88%
64403-106 10ppm WAF, 72h	72-hours	10	8.97	90%
64403-107, spike	72-hours	10	8.29	83%
64403-118 100ppm WAF, 72h	72-hours	100	59.5	60%
64403-119 Spike	72-hours	100	99.7	100%

APPENDIX A – TEST SUBSTANCE PHYSICAL-CHEMICAL SPECIFICATIONS FROM SUPPLIER

REVISION 4: March 28, 2008 — MATERIAL SAFETY DATA SHEET — Page 1/6

1 IDENTIFICATION OF THE PRODUCT AND OF THE COMPANY

1.1	Identification of the Product:	**Naphthenic Acids (Carboxylic Acids, Fatty Acids)**
1.2	Product Code:	NAP ACID
1.3	Company:	
1.4	Transportation Emergency:	USA 1-800-424-9300 (CHEMTREC)
1.5	Product Information:	1-205-556-1556 1-205-556-0568 (Fax)
1.6	Intended Use :	For industrial use only. No other use is intended.

2 HAZARDS IDENTIFICATION

2.1 Classification: Irritant (Xi), Harmful (Xn)

2.2 Warning Statements: Causes eye and skin irritation.
Harmful if swallowed – may enter lungs if swallowed or vomited.
High vapor concentrations may cause drowsiness and irritation of the eyes or respiratory tract.

2.3 Hazard Symbol(s):

2.4 Risk Phrase(s): R36/38 (Irritating to eyes and skin)
R65 (Harmful: May cause lung damage if swallowed)

2.3 Potential Health Effects:

Eye Contact:	Causes eye irritation. Exposure may cause irritation, redness and tearing.
Skin Contact:	Causes skin irritation. Exposure may cause redness, itching and inflammation.
Ingestion:	Expected to be a low ingestion hazard. Aspiration hazard. If swallowed, can enter lungs and cause damage.
Inhalation:	High vapor concentrations may cause drowsiness, respiratory tract irritation, coughing, asthmatic breathing and breathlessness.
Chronic Effects:	No known deleterious effects.

2.4 Other Hazards: Target organs: Eyes, skin and central nervous system.

3 COMPOSITION/INFORMATION ON INGREDIENTS

Substance	CAS No.	EC No.	% Present
Naphthenic Acid	1338-24-5	215-662-8	70 - 99
Petroleum Distillates	8008-20-6	232-366-4	1 - 30

4 FIRST-AID MEASURES

In case of contact with eyes:	Promptly flush eyes with running water for 15 minutes, including under eyelids. Seek medical assistance if irritation develops.
In case of contact with skin:	Wash affected area well with soap and water. Seek medical assistance if irritation develops.
In case of ingestion:	Do not induce vomiting. Give 2-3 glasses of milk or water to dilute. Contact physician or poison control center promptly for instructions. If vomiting occurs, keep head lower than hips to help prevent aspiration. Never give anything by mouth to an unconscious person.
In case of inhalation:	Remove to fresh air. Seek medical assistance if irritation develops.

5 FIRE-FIGHTING MEASURES

5.1	Suitable extinguishing media:	Water fog, fire fighting foam, dry chemical or carbon dioxide.
5.2	Unsuitable extinguishing media:	None
5.3	Specific hazards:	Combustion products are Carbon Oxides.
5.4	Personal protective equipment:	Wear Self Contained Breathing Apparatus and protective clothing appropriate for fire-fighting.
5.5	Other precautions:	Non-emergency personnel should be removed from the area immediately. Cool fire-exposed containers with water spray. Prevent water runoff from reaching drains, surface water and ground water.

6 ACCIDENTAL RELEASE MEASURES

6.1	Personal precautions:	Avoid unnecessary exposure by wearing personal protective equipment specified in Section 8. Remove material from eyes, skin and clothing.
6.2	Spill cleanup:	Suction up free liquids using non-sparking equipment. Liquid unable to be suctioned may be absorbed with a non-combustible material (vermiculite, sand, earth, etc.) and transferred to container(s) for later disposal. Remove contaminated sand, earth, etc. and transfer to container(s) for later disposal.
6.3	Environmental precautions:	Keep away from drains, surface water and ground water.

7 HANDLING AND STORAGE

7.1 Handling: Wear appropriate personal protective equipment (see Section 8).

Avoid breathing vapors, mists or spray. Use with adequate ventilation.

Avoid contact with eyes, skin and clothing.

Wash thoroughly after handling.

Do not taste or swallow.

7.2 Storage: Store in a sealed container in a clean, dry, well-ventilated area away from oxidizers, strong bases, heat and flame.

Avoid use of copper and brass alloys in storage and transfer equipment and process equipment.

8 EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Engineering controls: Use local exhaust ventilation to control emissions at source.

8.2 Eye/face protection: Wear safety glasses with side shields as minimum protection. Wear goggles or faceshield if a risk of splashing exists.

8.3 Skin protection: Wear chemical resistant gloves. Heavy PVC, butyl rubber or Viton are recommended.

8.4 Respiratory protection: An approved respirator must be worn if engineering controls do not maintain airborne concentrations below established exposure limits or, when limits have not been established, below irritant levels. Respirator selection must be based upon the airborne concentration. Consult a health and safety professional or manufacturer for specific recommendations.

8.5 Thermal hazards: None

8.6 Occupational Exposure Levels

Chemical Name	Source	Type	Exposure Limits	Notes
Cherosene (Non-Aerosol), Come Vapore totale Dell'Idrocarburo	Italy OEL's	TWA	200 mg/m^3	Skin Total Hydrocarbon Vapor
Kerosene	Poland MAC's	TWA	100 mg/m^3	
	Poland MAC's	STEL	300 mg/m^3	
Kerosine	Russian Federation MAC's	Ceiling	300 mg/m^3	As C
	Russian Federation MAC's	TWA	600 mg/m^3	As C
Kerosene (Non-Aerosol), As Total Hydrocarbon Vapor	ACGIH	TWA	200 mg/m^3	Irritation, CNS, Skin
Kerosene	NIOSH	REL	100 mg/m^3	

9 PHYSICAL AND CHEMICAL PROPERTIES

9.1 Appearance:	Amber color	9.2 Odour:	Hydrocarbon
9.3 pH:	5.2 (Saturated Solution)	9.4 Boiling Pt./range:	268°C (515°F)
9.5 Freezing Pt./Range:	Not established		
9.6 Flash point:	>149°C (300°F)		
9.7 Flammability:	See 9.6	9.8 Autoflammability:	See 9.6
9.9 Explosive properties:	Not applicable	9.10 Oxidizing properties:	Not an oxidizer
9.11 Vapor pressure:	0.005 mm Hg (37.8°C/100°F)	9.12 Relative density (H_2O = 1):	0.960 – 0.982 (15.6°C/60°F)
9.13 Apparent density:	Not applicable	9.14 Vapor density (Air = 1):	6.5

9.14 Solubility:

Water	-	<1% by weight (15.6°C/60°F)
Fat (type)	-	Not determined
Other solvents	-	Not determined

9.15 Partition coefficient: Log $P_{o/w}$ (Octanol/water) - Not determined

9.16 Other data: Not Applicable

10 STABILITY AND REACTIVITY

10.1 Reactivity: Not reactive under specified conditions of storage, shipment and use.

10.2 Stability: Stable under specified conditions of storage, shipment and use.

10.3 Conditions to avoid: Heat and ignition sources.

10.4 Incompatible materials: Strong oxidizers and strong bases.

10.5 Hazardous decomposition products: Carbon Oxides.

11 TOXICOLOGICAL INFORMATION

11.1 Acute: Eye and skin irritant. Not established as a respiratory tract irritant. May cause lung damage if aspirated.

Specified Substances

Chemical Name	Test Results
Kerosene	Dermal LD_{50} (Rabbit): >2000 mg/kg
	Oral LD_{50} (Rat): >5000 mg/kg
	Inhalation LC_{50}: >5000 mg/m^3, 4 H
	Skin (Rabbit): 500 mg (Severe Irritation)
	Skin (Rabbit): 100%/24 H (Moderate Irritation)
Naphthenic Acid	Oral LD_{50} (Rat): 3000 mg/kg
	Oral LD_{50} (Rat): 5880 mg/kg
	Dermal LD_{50} (Rabbit): >3160 mg/kg
	Eye (Rabbit): Moderate
	Skin Occluded (Rabbit): Moderate to Severe
	Skin (Rabbit): Slight

11.2 Chronic: Neither ingredient is listed by NTP, IARC or OSHA as a carcinogen. Kerosene (Non-Aerosol), as total hydrocarbon vapor, is listed by ACGIH as A3 (Confirmed Animal Carcinogen).

12 ECOLOGICAL INFORMATION

12.1 Ecotoxicity: No data available.

12.2 Persistence and degradability: No data available.

12.3 Bioaccumulation potential: No data available.

12.4 Mobility in soil: No data available.

12.5 Other adverse effects: No data available.

13 DISPOSAL CONSIDERATIONS

Generators of waste material are responsible for evaluating materials for compliance with all applicable procedures and regulations. Disposal of unused materials must be in accordance with all local, state and federal regulations. Containers should be cleaned of residual product and rinsed according to all local, state and federal regulations prior to disposal.

14 TRANSPORT INFORMATION

	UN Number	Proper Shipping Name	Hazard Class(es)	Packing Group
ADR/RID:	Not regulated			
IMO/IMDG:	Not regulated	Fatty Acids (Saturated C13+)		
IATA:	Not regulated			
DOT:	NA3082	Other Regulated Substances, Liquid, n.o.s., (Naphthenic Acid)	9	III

Note: Material is regulated by DOT only if shipped in a container containing an amount equal to or greater than the Reportable Quantity (RQ) of 100-pounds.

15 REGULATORY INFORMATION

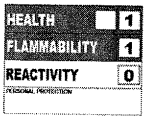
Warning symbol:

Warning words: Harmful. Irritant.

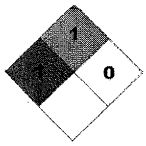
Risk phrases: R36/38: Irritating to eyes and skin
R65: May cause lung damage if swallowed

Safety phrases: S23: Do not breathe vapor
S24/25: Avoid contact with eyes and skin
S62: If swallowed, do not induce vomiting. Seek medical advice immediately and show this container or label

HMIS ratings (estimated):



NFPA ratings (estimated):



SARA : Section 302: None
Section 311/312: Immediate Health Hazard
Section 313: None

WHMIS: D2B

Inventories:

CAS Number	TSCA	DSL	EINECS
1338-24-5	Yes	Yes	Yes
8008-20-6	Yes	Yes	Yes

16 OTHER INFORMATION

Revision Date:	March 28, 2008
Supercedes Revision Date:	November, 2004
Revisions:	The latest informational changes are indicated by 20% shading.

The information on this form is furnished solely for the purpose of enabling those who transport, handle or use our products to ensure the safety and health of their employees and to comply with various laws and regulations (federal, state and local). This information is offered in good faith and is believed to be accurate. however, makes no guarantee or warranty, expressed or implied, regarding the accuracy of these data or the results to be obtained from the use hereof.

SAMPLE SUMMARY: SAMPLE FOR API/HPV TESTING

Specification	Results	Procedure
Acid number	**235mg KOH/gm**	ASTM D664-59
Unsaponifiables (Total)	**4.9%**	ASTM D322
Viscosity @ 40	**32cst**	ASTM D445-88
Specific Gravity @ 20C	**0.969**	ASTM D1298-85
Color (Gardner), GI	**4.5**	ASTMD1544-80
Water Content	**0.07%**	ASTM D95-83
Phenolic Content (acid)	**0.31%**	Standard Methods for the Examination of Water and Wastewater, 14th Edition (1975); Method 510, pp 574-592, APHA-AWWA-WPCF
Total Sulfur	**0.34**	ASTM D4294-83
CP - Flash Point °F (COC)	**343**	ASTM D92

APPENDIX B – DILUTION WATER CHARACTERIZATION

Chemical Characteristics of ABC Well Water Used by
ABC Laboratories' Chemical Services Group

August 2009 ABC Well Water Screen (non-GLP)

Organophosphate (µg/L)	2009	Historical Range 1998-2009 [1]
Azinphos ethyl	---	<1.0[5]
Azinphos-methyl	<0.200	<0.200[3]
Bolstar	<0.200	<0.200[4]
Chloropyrifos	<0.200	<0.200[4]
Coumaphos	<0.400	<0.400[4]
Demeton, Total	<0.200	<0.200-<1.0
Diazinon	<0.200	<0.200-<1.0
Dichlorvos	<0.200	<0.200[4]
Dimethoate	<1.00	<1.00[4]
Disulfoton	<0.200	<0.200-<1.0
EPN	<0.200	<0.200[4]
Ethion	---	<1.0[5]
Ethoprop	<0.200	<0.200[4]
Fensulfothion	<1.00	<1.00[4]
Fenthion	<0.200	<0.200[4]
Malathion	<0.200	<0.200-<1.0
Merphos	<0.200	<0.200[4]
Mevinphos	<1.00	<1.00[4]
Monocrotophos	<1.00	<1.00[4]
Naled	<2.00	<2.00[4]
Parathion:	---	<1.0[5]
Parathion, ethyl	<0.200	<0.200[3]
Parathion, methyl	<0.200	<0.200[3]
Phorate	<0.200	<0.200[4]
Ronnel	<0.200	<0.200[4]
Stirophos	<0.200	<0.200[4]
Sulfotepp	<0.200	<0.200[4]
TEPP	<0.200	<0.200[4]
Tokuthion	<0.200	<0.200[4]
Trichloronate	<0.200	<0.200[4]

Polychlorinated Biphenyls (µg/L)		
Aroclor 1016	<1.00	<1.00
Aroclor 1221	<1.00	<1.00
Aroclor 1232	<1.00	<1.00
Aroclor 1242	<1.00	<1.00
Aroclor 1248	<1.00	<1.00
Aroclor 1254	<1.00	<1.00
Aroclor 1260	<1.00	<1.00

Elements (mg/L)	2009	Historical Range 1998-2009 [1]
Aluminum	<0.0500	<0.0500[4]
Antimony	<0.0500	<0.0500[4]
Arsenic	<0.0250	<0.0250-<0.050
Barium	0.0189	0.0189[4]
Beryllium	<0.0010	<0.0010[4]
Boron	0.400	0.37-0.415
Cadmium	<0.0020	<0.0020-<0.0050
Calcium[2]	76.3	52-83.1
Chromium	<0.0100	<0.010
Cobalt	<0.0100	<0.0100[4]
Copper	<0.0100	<0.010
Iron	0.020	<0.0059-0.16
Lead	<0.0400	<0.0065-0.0400
Magnesium[2]	30.7	27-33.1
Manganese	<0.0050	<0.0050[4]
Molybdenum	<0.0100	<0.0100[4]
Mercury	---	<0.00060[5]
Nickel	<0.0100	<0.0100-<0.020
Potassium[2]	7.51	6.6-7.93
Selenium	<0.0500	<0.050
Silver	<0.0100	<0.010
Sodium[2]	29.0	27-32.2
Thallium	<0.0500	<0.0500[4]
Tin	<0.0200	<0.0200[4]
Vanadium	<0.0100	<0.0100[4]
Zinc	0.0197	0.0118-0.078

Chlorinated Hydrocarbons (µg/L)		
4,4'-DDD	<0.04	<0.040
4,4'-DDE	<0.04	<0.040
4,4'-DDT	<0.04	<0.040
Aldrin	<0.04	<0.040
α-BHC	<0.04	<0.040
β-BHC	<0.04	<0.040
Δ-BHC	<0.04	<0.040
Dieldrin	<0.04	<0.040
Endosulfan I	<0.04	<0.040
Endosulfan II	<0.04	<0.040
Endosulfan sulfate	<0.04	<0.040
Endrin	<0.04	<0.040
Endrin aldehyde	<0.04	<0.040

Chemical Characteristics of ABC Well Water Used by
ABC Laboratories' Chemical Services Group (continued)

August 2009 ABC Well Water Screen (non-GLP)

Miscellaneous (mg/L)	2009	Historical Range 1998-2009 [1]
Nitrite N	<0.01	<0.01-≤0.050
Nitrate N	0.328	<0.11-0.328
Total Phosphorus as P	0.12	<0.050-0.64
Chlorinated Herbicides (µg/L)		
2,4,5-TP (Silvex)	<0.200	<0.200-<50
2,4-D	<0.200	<0.200-<250

Chlorinated Hydrocarbons (µg/L) (continued)	2009	Historical Range 1998-2009 [1]
Endrin ketone	<0.04	<0.040
γ-BHC	<0.04	<0.040
Heptachlor	<0.04	<0.040
Heptachlor epoxide	<0.04	<0.040
Methoxychlor	<0.04	<0.04-<0.095
Toxaphene	<0.50	<0.50-<3.8
Chlordane	<0.05	<0.05-<0.48

[1] Data supporting these values are on file at ABC Laboratories. Less than (<) values indicate recovery was below the greatest limit of detection during these analyses.
[2] Historical Range is from 2003.
[3] Historical Range is from 2008.
[4] Historical Range is from 2009.
[5] Historical Range does not include 2009.

Note: ABC Well Water is the base water for ABC Reagent Water

APPENDIX C – PROTOCOL, AMENDMENTS, AND DEVIATION

Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies

ABC Study No. 64403

1.0 STUDY TITLE

Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies

2.0 OBJECTIVE

The Tasks of the study are in two parts. Task 1 includes method development and performance of GLP equilibration trials for water accommodated fraction (WAF) preparations to be used in the toxicity tests. Task 2 includes a GLP method validation to demonstrate the accuracy and precision for test solution concentration ranges to be used in the definitive toxicity tests.

3.0 STUDY SPONSOR

American Petroleum Institute
1220 L Street, NW
Washington, DC 20005
Phone: (202) 682-8480 Fax: (202) 682-8270
Sponsor Representative: [redacted]

Study Monitor: [redacted]
EcoTox Assessments LLC
506 Tennant Circle, Suite 100
St. Michaels, Maryland 21663
Tel: 410-745-6172 Fax: 410-745-9161
E-mail: [redacted]

4.0 TESTING FACILITY AND STUDY DIRECTOR ADDRESS

ABC Laboratories, Inc.
7200 E. ABC Lane
Columbia, Missouri 65202

Study Director: [redacted]
TEL: (573) 777-6050 FAX: (573) 777-6089
Email Address: [redacted]

5.0 PROPOSED SCHEDULE

PROPOSED EXPERIMENTAL START DATE: February 2009
PROPOSED EXPERIMENTAL COMPLETION DATE: March 2009

6.0 TEST PROTOCOL

This test protocol is intended to support environmental toxicity studies conducted in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing of chemicals and U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances (OPPTS).

7.0 TEST AND REFERENCE SUBSTANCES

7.1 Test Substance

The test substance will be Naphthenic Acids (CAS# 1338-24-5). The following sample information and chemical/physical properties should be provided with the test substance sample or before its shipment: batch/lot number, sample expiration date, physical description, purity (including certificate of analysis), stability, suggested storage conditions, water and organic solvent solubility, vapor pressure, available toxicity information, a Material Safety Data Sheet (MSDS) or its equivalent, and handling precautions.

7.2 Reference Substance

The reference substance will be Naphthenic Acids (CAS# 1338-24-5). The same information specified for the test substance sample in section 7.1 should be provided for the reference substance sample.

7.3 Sample Characterization and Retention

Characterization, stability, and solubility studies will be the responsibility of the Sponsor unless otherwise contracted to ABC Laboratories, Inc. The test and reference substances will be properly disposed of following completion of their use at ABC Laboratories, Inc., unless the Sponsor makes arrangements for retention or return. Archival of a retention sample will also be the Sponsor's responsibility.

7.4 Test Substance Preparation/Addition

Standard spiking solutions will be prepared in a suitable solvent. Chromatographic standards will be prepared in a solvent that is compatible with the chromatographic system employed. If possible, the choice of solvent for the spiking solution will be the solvent used in the aquatic tests. Test concentrations will be based on the whole product. Test solutions will be prepared as a water-accommodated fraction (WAF) as described in Section 10.2. The maximum loading of the test substance in a WAF preparation is not to exceed 1,000 mg/L.

8.0 TEST SYSTEMS

Various waters and defined aqueous mediums may be used to evaluate the analytical method and include, but are not limited to the following:

(a) Blended or aged blended laboratory freshwater [a blend of well water and reverse osmosis (RO) water] with a hardness of 130-160 mg $CaCO_3$/L.

(b) A freshwater synthetic plant nutrient medium

The various matrices to be used in the method validation study will be selected based on the type of environmental toxicity studies planned. If the studies require using more than one of the above matrices, each required matrix will be evaluated in the validation.

9.0 ORGANIC SOLVENTS

All organic solvents employed will be distilled in glass, suitable for HPLC, GC, residue analysis and spectrophotometry, or equivalent.

10.0 PROCEDURES

10.1 Equilibration Trials for Water Accommodated Fractions (WAFS)

Equilibration trials will be performed in order to determine the optimal mixing times to achieve maximum dissolution of naphthenic acids in the different dilution media. An equilibration trial will consist of a minimum of three WAF preparations made at loading rates of 10, 100, and 1,000 mg/L with each preparation being sampled at different mixing times (e.g., 6, 18, and 24 hours) for each of the dilution media. Dilution water will be placed into the preparation container(s) and the calculated amount of test substance will be added to achieve the nominal loading rates. Since no volatilization is expected with the test substance constituent components, efforts to minimize the head space in each WAF preparation container are not required, but may be used if requested by the Sponsor (WAF preparation containers partially filled with dilution water, dosed with the test substance, and dilution water added such that there is a minimal head space, then covered with a glass lid or plate). The size and type of the WAF preparation container and specifications for collection will be documented in the raw data and summarized in the report. Typically the containers will be similar to those used to prepare the WAF preparations for the individual studies (e.g., 2 to 4-L glass jar with dispensing outlet at the bottom for algae studies, etc.).

Each container will be equipped with a Teflon-coated stir bar. The stirring speed will be adjusted so that the vortex in each bottle is within the range of 30 to 50% of the container depth. Stirring will take place at ambient room temperature and lighting. After the prescribed time of stirring, stirring will be stopped and the mixture allowed to sit undisturbed for approximately 1 hour before initiating draining/siphoning of the WAF solution. The solution from each WAF will be drained/siphoned into a clean container for sample collection and subsequent analysis. If multiple WAF solutions are prepared, each WAF solution will be placed directly into the replicate test chambers for that treatment level. Portions of the WAF will also be drained/siphoned from the bottle for analytical and initial water quality measurements

10.2 Exposure System

If required, reduced pressure rotary evaporation or evaporation with dry nitrogen gas will be used to concentrate the extracts of test samples. The effect of this procedure (i.e., loss of test substance through volatility) will be assessed before its use.

A suitable chromatographic system equipped with the appropriate detector system and analytical column will be used to measure test substance concentrations.

If reasonably possible, the volume of solvent added should be consistent with the volumes of solvents used in the aquatic toxicity tests when fortifying the test dilution water with test substance.

All glassware employed will be labeled with the ABC Laboratories' study number and test level.

10.3 Analytical Method Validation

The analytical method for the test substance will be developed at ABC Laboratories using a FTIR spectroscopy analytical method provided by the Sponsor (1). If this methodology is determined to not be adequate for the analysis of the test substance, additional method development will be discussed with the Sponsor. Once determine to be adequate for the analytical confirmation of the test substance in the dilution media, the analytical method will be added to this protocol by amendment prior to the experimental start of the equilibration trials. If necessary to optimize analysis of the test substance in any of the media tested, modifications may be made to the analytical method.

The Method Detection Limit (MDL) will be based on 3.14 times the standard deviation determined from the analysis of 7 replicate samples of the lowest

standard in the calibration curve (2, 3). The Practical Quantitation Limit (PQL), the lowest detection limit that is routinely achievable among laboratories during routine laboratory operation, will be calculated as five times (MDL)(2). Additionally, the Minimum Quantifiable Limit (MQL) for a standard curve will be defined as the concentration of the lowest standard times the dilution factor of the control sample.

In general, the analytical method will be validated at concentration levels bracketing the anticipated test concentrations to be used in the definitive aquatic toxicity test(s). Unless there are analytical or physico-chemical reasons, the targeted high validation concentration will be approximately twice the highest test concentration and the targeted low validation concentration will be approximately one-half of the lowest test concentration, or the MQL (whichever is higher). It may be necessary to run a set of validation samples prior to the equilibration trials at a wide range of concentrations. Validation samples will include triplicate fortification samples at these two concentrations as well as triplicates of the appropriate matrix blank. If the concentrations of the fortified samples are above the analytical calibration curve concentrations, the samples may be diluted into the calibrated range and validated. Once the green algae and daphnid range-finding ecotoxicity tests are performed, an additional validation will be performed to bracket the range of the anticipated definitive test concentrations.

Chromatographic conditions will be evaluated with the selection of detector and column and operating parameters assigned to provide quality chromatography.

No bias is expected in this type of study.

10.3 Control and Assessment of Interferences

10.3.1 Method Interferences

Method interferences may be caused by contaminants in solvents, reagents, glassware, and other sample processing apparatus that lead to discrete artifacts or drifting baseline. In order to minimize potential method interferences, all reagents and solvents should be pesticide residue grade or better and all glassware and Teflonware will be cleaned in accordance with SOP. Appropriate blank analyses will be performed to demonstrate freedom from interferences with each analytical set.

10.3.2 Matrix Interferences

Matrix interferences may result from the co-extraction of contaminants from the sample matrix resulting in artificially low or high analytical

results. Sample preparation procedures can be employed to minimize this type of interference. Matrix interferences will be evaluated from the analysis of matrix blanks (controls) and matrix spikes.

10.4 Storage Stability

In general, the samples will be analyzed immediately upon collection thus eliminating the need for sample storage and storage stability. If analyses cannot be performed immediately, sample aliquots may be stored and analyzed periodically to determine storage stability. Samples must be stable (>80% recovery) for a period equal to or greater than the longest period stored to validate this procedure. If analyte recovery during stability studies falls below 80%, the samples will be considered unstable and storage will not exceed this time period.

10.5 Calculations

All calculations of the test substance concentrations will be accomplished using one or both of the following:

A normalized standard curve will be constructed using the detector responses observed for the external standards injected concurrently with each set of samples.

An external standard analysis will be performed using appropriate computer software.

Concentrations will be determined directly from the standard curve by the following equation:

$$\frac{\left(\begin{array}{c}\mu g/L \text{ or } mg/L \text{ equivalents for} \\ \text{test substance from standard} \\ \text{curve equation}\end{array}\right)\left(\begin{array}{c}\text{sample volume} \\ \text{in mL for} \\ \text{chromatography}\end{array}\right)}{(\text{sample volume in mL before preparation})} = \begin{array}{c}\mu g/L \text{ or} \\ mg/L\end{array} = \begin{array}{c}ppb \text{ or} \\ ppm\end{array}$$

10.6 Statistical Methods

Statistical methods (3) used during the study will include but not be limited to:

- Calculation of mean and standard deviation
- Calculation of a standard curve by linear regression using the least-squares method

10.7 Method Acceptability

The method will be considered valid if the recoveries of the fortified samples are in the range of 80% to 120%. Recovery values outside of this range may be used depending on the Sponsor or specific laboratory requirements.

11.0 REPORTING

Upon completion of the method development work, a summary report will be submitted to the Sponsor. The summary report will briefly describe the test methods and test results. A final report detailing all aspects of the equilibration trials and validation will be submitted to the Sponsor and will include, but not be limited to, the following:

- A certificate of analysis generated from the data collected
- Study dates, name, and address of test facility
- Objectives and test procedures as stated in approved protocol
- A description of the experimental design along with a description of and reference to any statistical methods used for data analysis
- Description of the test, control, and reference substance (date of receipt, storage conditions, purity, physical characteristics, and method of preparing stock and/or test solutions)
- Description of methods used during the study
- Description of all circumstance that may have affected the quality and integrity of the study
- Summary of all the data and a statement of the conclusions drawn from any data analyses, if appropriate
- Description of any protocol deviations
- A copy of the protocol as an appendix to the report
- Location of samples, raw data, and final report
- List of study personnel
- GLP compliance statement by the Study Director and a statement by ABC

Laboratories' Quality Assurance Unit

12.0 PROTOCOL AMENDMENTS AND DEVIATIONS

The Study Director, upon approval of the Sponsor Representative, may make amendments to this protocol. All amendments will describe the change(s), the reason(s) for the amendment, and the effect on the study, if any. All amendments will be signed and dated by at least the Study Director and maintained with the protocol.

In the event of a protocol deviation, a written description of the deviation including the reason for the deviation and any impact on the study as a result of the deviation will be submitted to the Sponsor Representative. All deviations will be signed and dated by at least the Study Director and maintained with the protocol.

13.0 QUALITY ASSURANCE

ABC's Quality Assurance Unit will inspect one or more critical phases to assure that equipment, personnel, procedures, and records conform to the guidelines listed in this protocol. The results of these inspections will be reported to the Study Director and ABC management. The draft and final reports will be reviewed for protocol and GLP compliance, as well as to assure that the methods and standard operating procedures used were followed. A signed statement will be included in the report specifying types of inspections made, the dates inspections were made, and the dates inspections were reported to the Study Director and management.

14.0 GLP COMPLIANCE

All test procedures, documentation, records, and reports will comply with the OECD Principles of Good Laboratory Practice (4) and the U.S. Environmental Protection Agency's Good Laboratory Practices as promulgated under the Toxic Substances Control Act (5). The report will contain a statement attesting to that fact.

15.0 RECORDS

Records to be maintained will include, but not be limited to, test substance receipt; solution preparations and dilutions; instrument logbooks detailing calibration and maintenance; facility records (kept at ABC); material control identification numbers for all instruments used; storage of test substance, solutions, and samples; and weights and volumes. All original raw data collected during this study will be maintained at ABC Laboratories until finalization of the study. Upon completion of the study, all original raw data will be submitted to the Sponsor along with the final report. A copy of the final report, copies of all raw data from the study, and all original facility records will be kept on file in ABC Laboratories' archives.

16.0 SPECIMEN DISPOSAL

Following finalization of the report, disposition of all specimens (i.e., any material derived from the test system for examination, analysis, or retention) generated during the conduct of the test will be completed in a timely manner. Retention specimens holding time will be based on stability information provided by the Sponsor or by stability data generated by ABC Laboratories. Retention specimens will be returned to the Sponsor unless archiving is contracted with ABC Laboratories. Documentation of specimen disposal will be retained with study records in ABC Laboratories' Archive.

17.0 REFERENCES

(1) Jivraj, M.N., M. MacKinnon, and B. Fung. 1996. Naphthenic acid extraction and quantitative analysis with FT-IR spectroscopy. Syncrude Canada Ltd. Report.

(2) American Public Health Association, American Water Works Association, Water Environmental Federation. 1998. *Standard Methods for the Examination of Water and Wastewater*. 1030 C. Method Detection Level, 1-17 to 1-18.

(3) U.S. Environmental Protection Agency. Appendix B to Part 136 - Definition and Procedure for the determination of the Method Detection Limit - Revision 1.11. 40 CFR. 1999; 136: 303-306.

(4) Snedecor, George W. and William G. Cochran. 1967. *Statistical Methods*. Iowa State University Press.

(5) Organization for Economic Cooperation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final].

(6) U.S. Environmental Protection Agency. 1989. Toxic Substances Control Act; Good Laboratory Practice Standards; Final Rule (40 CFR, Part 792). *Federal Register*, 54(158): 34043-34050.

PROTOCOL APPROVAL

ABC Laboratories' Study Director

Name (signed): [redacted] Date: Feb 26, 09

Name/Title: [redacted]

Sponsor Representative

Name (signed): [redacted] Date: 23 Feb 09

Name/Title: [redacted]

Test Facility Management

Name (signed): [redacted] Date: 3 Mar 09

Name/Title: [redacted]

QAU Protocol Review for GLP Compliance

Name (signed): [redacted] Date: 03 Mar 09

Name/Title: [redacted]

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE: Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies

TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64403
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	1	**EFFECTIVE DATE:**	June 11, 2009

1. Protocol Section: 10.1 Equilibration Trials for Water Accommodated Fractions (WAFS)

   This section is being reworded as follows:

   Equilibration trials will be performed in order to determine the optimal mixing times to achieve maximum dissolution of naphthenic acids in the different dilution media. The equilibration trials will be conducted as described in the following paragraphs.

   Dilution water will be placed into the preparation container(s) and the calculated amount of test substance will be added to achieve the nominal loading rates. Since no volatilization is expected with the test substance constituent components, efforts to minimize the head space in each WAF preparation container are not required. WAFS will be prepared in 2-L vessels that can be drained at the bottom. A total of 2 L of each of the two test media (aged blended water and freshwater algal medium) will be loaded at rates of 2, 10, and if necessary, 100 mg/L. The determination for the need of the 100 mg/L loading rate will be if there is a difference between the measured concentrations of the 2 mg/L and 10 mg/L loading rates, indicating that the maximum solubility may not have been achieved.

   A total of 3 WAFS (in each test medium) will be prepared at the 2 mg/L loading rate, 4 at the 10 mg/L loading rate, and 3 at the 100 mg/L loading rate. For the three WAFS prepared at 2 mg/L loading, one each will be mixed for 4, 18, and 48 hours and sampled after a 1 hour settling period. For the four WAFS prepared at 10 mg/L loading, one each will be mixed for 4, 18, 48, and 72 hours and again sampled after a 1 hour settling period. For the three WAFS prepared at 100 mg/L loading (if conducted), one each will be mixed for 18, 48, and 72 hours and sampled after a 1 hour settling period. Some loading rate/test medium combinations may be conducted simultaneously at the discretion of the study director.

   Each container will be equipped with a Teflon-coated stir bar. Each container will be covered with parafilm while being stirred. The stirring speed will be adjusted so that the vortex in each bottle is within the range of 30 to 50% of the container depth. Stirring will take place at ambient room temperature and lighting. After the prescribed time of

stirring, stirring will be stopped and the mixture allowed to sit undisturbed for approximately 1 hour before initiating draining/siphoning of the WAF solution. The solution from each WAF will be drained/siphoned into a clean container for sample collection and subsequent analysis. If multiple WAF solutions are prepared, each WAF solution will be placed directly into the replicate test chambers for that treatment level. Portions of the WAF will also be drained/siphoned from the bottle for analytical (and initial water quality measurements, if performed).

Reason: To identify the exact procedure for conducting the WAFS trials.

Effect on Study Integrity: None

STUDY DIRECTOR:	[redacted]	**DATE:** June 16, 09
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 16 Jun 09

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE:	Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64403
STUDY SPONSOR:	American Petroleum Institute		
AMENDMENT NO.:	2	**EFFECTIVE DATE:**	Jan 25, 2010

1. Protocol Section: 11.0 – Report

   A data summary of the method development and validation will be provided to the Sponsor and the results of the method development and validation test will be summarized in the final report. A final report detailing all aspects of the study will be submitted to the Sponsor and will include, but not be limited to, the following:

   - Study dates, name, and address of test facility.
   - Objectives and test procedures as stated in the approved protocol.
   - A description of the experimental design along with a description of and reference to any statistical methods used for data analysis.
   - Description of test substance (e.g., date of receipt, storage conditions, purity, physical characteristics, and method of preparing stock and/or test solutions) and identification of the reference substance, if applicable.
   - Description of test conditions during the study (e.g., dilution water, test temperature, lighting, and pH).
   - Description of methods used during the study.
   - Description of test system (e.g., source, culture conditions, etc.).
   - Summary of the data and a statement of the conclusions drawn from any data analyses, if appropriate.
   - Description of any protocol deviations.
   - Location of raw data.
   - List of all study personnel.

- GLP compliance statement by the Study Director and a statement by ABC Laboratories' Quality Assurance Unit.

Reason: To clarify how the results will be reported.

Effect on Study Integrity: None.

STUDY DIRECTOR:	[redacted]	**DATE:** Jan 27, 10
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 27Jan10

PROTOCOL AMENDMENT NOTIFICATION

PROTOCOL TITLE: Acute Toxicity of Water Accommodated Fractions of Naphthenic Acids to the Water Flea, *Daphnia magna*, Determined Under Static-Renewal Test Conditions

TEST FACILITY: ABC Laboratories, Inc. **STUDY SPONSOR:** American Petroleum Institute

AMENDMENT NO.: 3 **EFFECTIVE DATE:** Sept. 24, 2009

1. Protocol Section: 4.0 – Testing Facility and Study Director Address

   Additional analytical chemistry identification work will be performed at:
   Department of Biological Sciences
   Z-207 Biological Sciences Centre
   116th Street and 85th Avenue
   University of Alberta
   Edmonton, Alberta T6G 2R3 Canada

   Reason: To identify the location where additional analytical work will be performed.

   Effect on Study Integrity: None. This is additional work being contracted by the sponsor.

2. Protocol Section: 10.1 – Equilibration Trials for Water Accommodated Fractions (WAFS)

   Analytical samples will also be collected during the WAFS equilibration trials and sent to Dr. Fedorak at the University of Alberta for analysis.

   Reason: To describe the additional analytical samples to be collected for analysis by Dr. Fedorak.

   Effect on Study Integrity: None. This is additional work being contracted by the sponsor.

3. Protocol Section: 11.0 – Reporting

   The report from Dr. Fedorak's analysis will be presented in an appendix to the final report. Because ABC is not the sponsor of this additional work, ABC is not responsible for its GLP compliance or noncompliance and the corresponding data. A statement regarding these analyses will be added to the Statement of GLP Compliance page in the final report.

   Reason: To describe how Dr. Fedorak's analyses will be reported.

   Effect on Study Integrity: None. The analyses performed by Dr. Fedorak were additional work contracted by the sponsor.

STUDY DIRECTOR: [redacted] **DATE:** Aug 30, 10

TEST FACILITY MANAGEMENT: [redacted] **DATE:** 30 Aug 10

PROTOCOL DEVIATION NOTIFICATION

PROTOCOL TITLE:	Validation of Test Solution Preparations and Analytical Methods for Use in the Determination of Naphthenic Acids in Various Media Used in Environmental Toxicity Studies		
TEST FACILITY:	ABC Laboratories, Inc.	**ABC STUDY NO.:**	64403
STUDY SPONSOR:	American Petroleum Institute		
DEVIATION NO.:	1	**EFFECTIVE DATE:**	Jan 25, 2010

1. Protocol Section: 10.3 – Analytical Method Validation

   The analytical method was not added to the protocol prior to the start of the equilibration trials.

   Reason: Study director error.

   Effect on Study Integrity: None. The method is described in the raw data.

2. Protocol Section: 10.3 – Analytical Method Validation

   The MDL and PQL were not determined.

   Reason: Study director error.

   Effect on Study Integrity: None. Method limits are adequately defined by the MQL.

STUDY DIRECTOR:	[redacted]	**DATE:** Jan 27, 10
TEST FACILITY MANAGEMENT:	[redacted]	**DATE:** 27 Jan 10

APPENDIX D – CHARACTERIZATION OF NAPHTHENIC ACIDS IN WAF SOLUTIONS BY GC/MS

1.0 Stability Evaluation of Naphthenic Acids in Water Accommodated Fractions

1.1 WAF Equilibration and Sample Stability in Glass and Plastic Bottles

Prior to the aquatic toxicity testing, stability of the naphthenic acid profiles in a WAF was assessed by measuring the profile at different time periods using two different sample containers. This information was used to establish sample storage periods and type of sample containers used to ship future test samples. Therefore, the testing laboratory created a WAF at a loading rate of 10 mg/L. After the designated stirring period, the WAF was sub-sampled into one container of glass and one of plastic. Those samples were shipped to the U of A for GC-MS analysis. A third sample was held for 48 h under testing conditions that would exist during a *Daphnia magna* acute test. After the 48-h period, this sample was placed in a glass container and shipped to the U of A for analysis.

1.1.1 Samples and Methods

In May 2009, three naphthenic acids "water accommodated fraction" (WAF) samples were received in the Department of Biological Sciences at the U of A. Two of these samples were in glass bottles and one was in a plastic bottle. The samples were designated A, B, and C in our laboratory, and the verbatim text on each bottle label, is given below. The text in quotation marks was written by Analytical Bio-Chemistry Laboratories, Inc.

U of A Sample A:
"Naphthenic acids WAF preparation 10 mg/L Nominal Loading Rate
72 hour stir, 0 hour sample 500 mL
Prepared: 8May09 Collected: 11May09 Expire: 8June09
Store: Room temp. in a glass bottle"

U of A Sample B:
"Naphthenic acids WAF preparation 10 mg/L Nominal Loading Rate
72 hour stir, 0 hour sample 500 mL
Prepared: 8May09 Collected: 11May09 Expire: 8June09
Store: Room temp. in a plastic bottle"

U of A Sample C:
"Naphthenic acids WAF preparation 10 mg/L Nominal Loading Rate
72 hour stir, 48 hour samples
Prepared: 8May09 WAF Collected: 11May09 Collected: 13May09 Expire: 13June09
Store: Room temp. in a glass bottle"

A 10-mL subsample from each bottle was diluted to 1 L with distilled water and these were extracted individually as outlined by Merlin et al. (2007). Briefly, the diluted sample was acidified to pH 2 with concentrated HCl, and then 150 g NaCl was dissolved into the sample. The water sample was then extracted with three 60-mL portions of chloroform (HPLC grade). Free carboxylic acids were separated from lipids by extracting the chloroform phase with three 10-mL portions of an alkaline aqueous solution containing 4% sodium carbonate (pH 11.6). The free carboxylic (naphthenic) acids were recovered from the alkaline solution by acidifying with concentrated HCl to pH 2 and extracting three times with 10 mL of dichloromethane (DCM, HPLC grade). The combined DCM extracts were dried under nitrogen.

The residue was dissolved in 50 µL of DCM and the naphthenic acids were derivatized by adding 50 µL of Sigma MTBSTFA derivatizing agent (without 1% *t*-BDMCS, Young et al. in press) to each vial and heating at 60°C for 20 min.

The derivatized samples were analyzed by GC-MS (Young et al. 2008) and the total ion current mass spectra were collected. The data obtained were put into a Microsoft Excel spreadsheet (Holowenko et al. 2002) to prepare a table of the relative abundances of each ion corresponding to the general formula for naphthenic acids, $C_nH_{2n+Z}O_2$, where n is the carbon number and Z is zero or a negative even number defining the hydrogen deficiency due to cyclization. The distributions of ions summarized in each table was used to prepare a three-dimensional plots of the ion abundances for each n and Z value.

1.1.2 Results of Analyzing Water Accommodated Fractions

The tables of relative abundances of each ion (expressed as percentages) are summarized in Table 1.1.1 to 1.1.3. The values of the percentages reported in these tables are rounded to the nearest 0.1. The three-dimensional plots are shown in Figures 1.1.1 to 1.1.3.

Data from each analysis were compared to data from the other two sample analyses using the statistical method of Clemente et al. (2003). In this statistical method, the ion distributions from the GC-MS analysis are divided into three groups based on carbon numbers. These are: Group 1 (C_5 to C_{13}), Group 2 (C_{14} to C_{21}), and Group 3 (C_{22} to C_{33}). The ions in Group 3 constitute the "C22+ cluster" defined by Holowenko et al. (2002) and are commonly found in the analyses of Alberta oil sands process-affected waters (Holowenko et al. 2002; Clemente et al. 2003; Bataineh et al. 2006). However, ions in the "C22+ cluster" are much less abundant in commercial naphthenic acids preparations (St. John et al. 1998; Clemente et al. 2003; Biryukova et al. 2007). The carbon numbers in Group 1 and Group 2 were assigned somewhat arbitrarily.

For the group 1 naphthenic acids (C_5 to C_{13}), there were no statistically significant differences ($P > 0.05$) among the three samples. Similarly, for the group 2 naphthenic acids (C_{14} to C_{21}), there were no statistically significant differences ($P > 0.05$) among the three samples. The three samples were essentially devoid of group 3 naphthenic acids (C_{22} to C_{33}).

Table 1.1.1

API sample A: NA 10mg/L, 0 h (glass bottle)

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.2	0.0	0.0	0.0	0.0	0.0	0.0	1.2
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.0	1.1	0.0	0.0	0.0	0.0	0.0	2.1
8	0.6	0.5	0.0	0.0	0.0	0.0	0.0	1.1
9	2.5	0.5	0.0	0.0	0.0	0.0	0.0	3.0
10	1.0	0.9	0.8	0.0	0.0	0.0	0.0	2.7
11	0.8	3.4	3.8	0.0	0.0	0.0	0.0	8.0
12	4.1	6.3	8.6	1.0	0.0	0.0	0.0	19.9
13	1.2	6.4	9.1	1.6	0.0	0.0	0.0	18.3
14	2.0	4.2	6.6	1.6	0.4	0.0	0.0	14.9
15	0.8	2.0	3.1	1.2	0.3	0.0	0.0	7.4
16	6.0	1.0	1.5	0.7	0.2	0.1	0.0	9.4
17	0.5	0.3	0.6	0.4	0.1	0.1	0.0	2.0
18	3.1	0.7	0.4	0.3	0.1	0.2	0.1	4.8
19	0.3	0.0	0.1	0.1	0.0	0.1	0.3	0.8
20	0.1	0.1	0.1	0.0	0.0	0.2	0.1	0.6
21	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.3
22	0.1	0.5	0.0	0.0	0.0	0.2	0.1	0.9
23	0.1	0.0	0.0	0.0	0.0	0.1	0.2	0.5
24	0.1	0.0	0.0	0.0	0.0	0.2	0.3	0.6
25	0.1	0.0	0.1	0.0	0.0	0.1	0.1	0.4
26	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.2
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
28	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.2
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	26.1	28.0	34.7	6.9	1.2	1.7	1.3	100.0

Table 1.1.2

API sample B: NA 10mg/L 0 h (plastic bottle)

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.3	0.0	0.0	0.0	0.0	0.0	0.0	1.3
6	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.6
7	1.1	1.0	0.0	0.0	0.0	0.0	0.0	2.1
8	0.8	0.6	0.0	0.0	0.0	0.0	0.0	1.3
9	2.7	0.5	0.0	0.0	0.0	0.0	0.0	3.1
10	0.8	0.9	0.7	0.0	0.0	0.0	0.0	2.5
11	0.7	3.4	3.5	0.0	0.0	0.0	0.0	7.6
12	3.4	6.2	8.4	1.0	0.0	0.0	0.0	19.0
13	1.2	6.6	9.0	1.6	0.0	0.0	0.0	18.4
14	1.9	4.5	7.6	1.7	0.5	0.0	0.0	16.1
15	0.8	2.0	3.2	1.5	0.3	0.0	0.0	7.9
16	5.5	1.0	1.5	0.7	0.3	0.1	0.0	8.9
17	0.7	0.3	0.5	0.3	0.1	0.1	0.0	2.1
18	2.8	0.6	0.3	0.2	0.1	0.3	0.1	4.4
19	0.5	0.1	0.0	0.1	0.1	0.1	0.3	1.0
20	0.1	0.1	0.1	0.1	0.0	0.2	0.3	0.9
21	0.2	0.0	0.0	0.0	0.1	0.1	0.1	0.4
22	0.1	0.4	0.0	0.0	0.1	0.1	0.1	0.7
23	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.3
24	0.1	0.0	0.1	0.0	0.0	0.1	0.1	0.5
25	0.0	0.0	0.3	0.0	0.0	0.1	0.1	0.6
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	25.1	28.3	35.4	7.3	1.6	1.2	1.1	100.0

Table 1.1.3

API sample C: NA 10mg/L, 48 h (glass bottle)

C number	z number							
	0	2	4	6	8	10	12	% carbon no
5	1.3	0.0	0.0	0.0	0.0	0.0	0.0	1.3
6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5
7	1.0	0.9	0.0	0.0	0.0	0.0	0.0	2.0
8	0.8	0.4	0.0	0.0	0.0	0.0	0.0	1.2
9	1.5	0.3	0.0	0.0	0.0	0.0	0.0	1.8
10	0.4	0.5	0.4	0.0	0.0	0.0	0.0	1.3
11	0.4	2.5	2.8	0.0	0.0	0.0	0.0	5.7
12	2.6	6.0	8.3	1.0	0.0	0.0	0.0	17.8
13	1.2	7.7	10.3	1.8	0.0	0.0	0.0	20.9
14	1.6	5.9	9.5	2.2	0.6	0.0	0.0	19.8
15	0.8	3.0	4.6	1.7	0.4	0.0	0.0	10.5
16	3.4	1.3	2.2	1.0	0.3	0.1	0.0	8.4
17	0.7	0.4	0.8	0.5	0.1	0.1	0.0	2.6
18	1.6	0.6	0.3	0.2	0.1	0.2	0.1	3.1
19	0.3	0.0	0.1	0.1	0.0	0.0	0.1	0.7
20	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.6
21	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.5
22	0.0	0.3	0.0	0.0	0.2	0.0	0.0	0.5
23	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
24	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.2
25	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.5
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
33	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% by z No	18.2	30.0	40.2	8.5	2.2	0.6	0.4	100.0

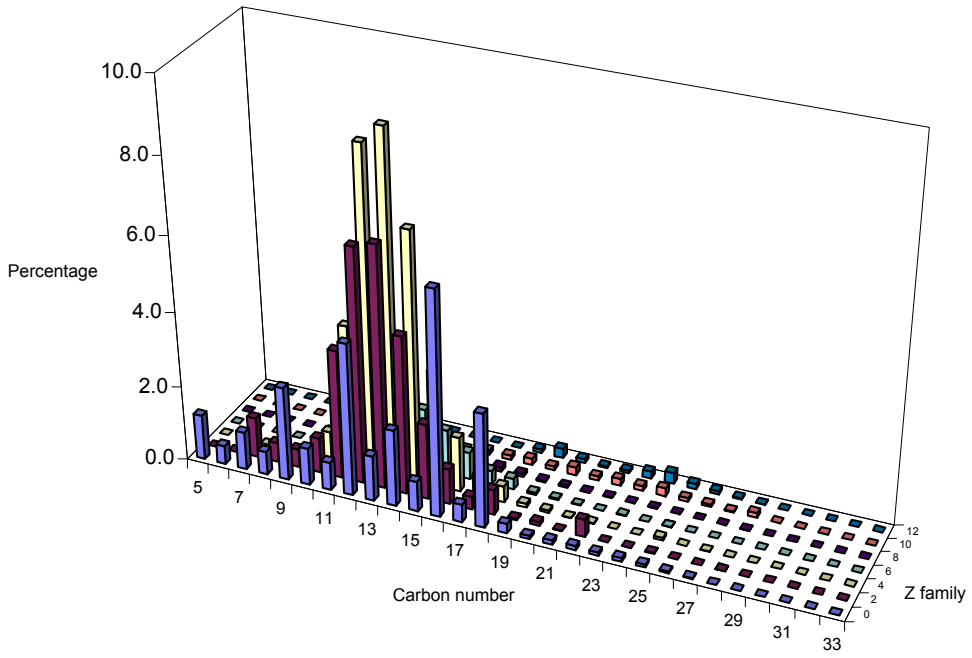


Figure 1.1.1 Three-dimensional plot of naphthenic acids sample from Sample A in the glass bottle (Table 2.1.1). The sum of all bars equals 100%.

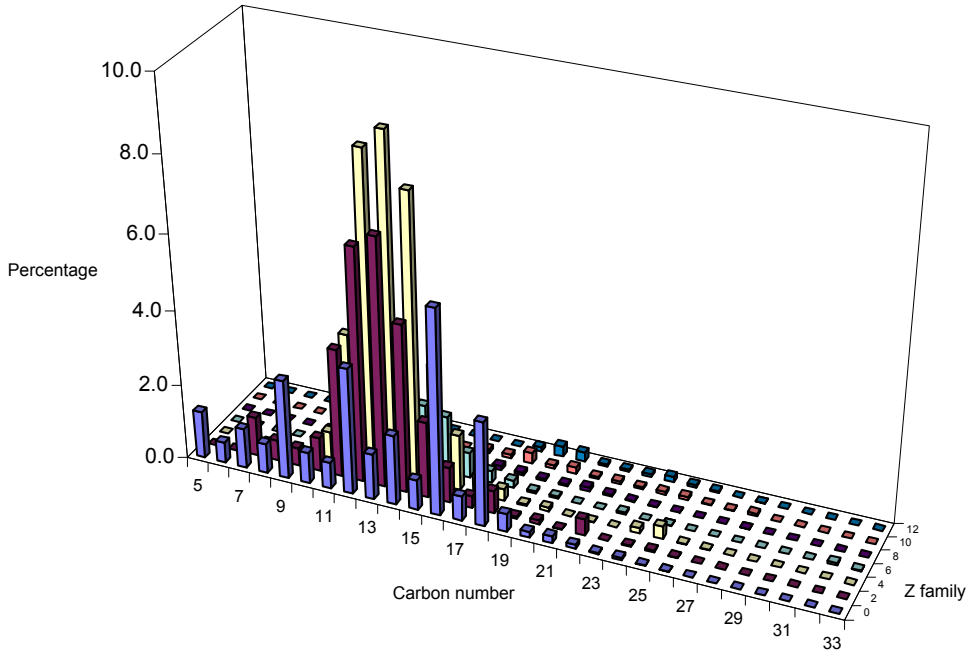


Figure 1.1.2 Three-dimensional plot of naphthenic acids sample from Sample B in plastic bottle (Table 2.1.2). The sum of all bars equals 100%.

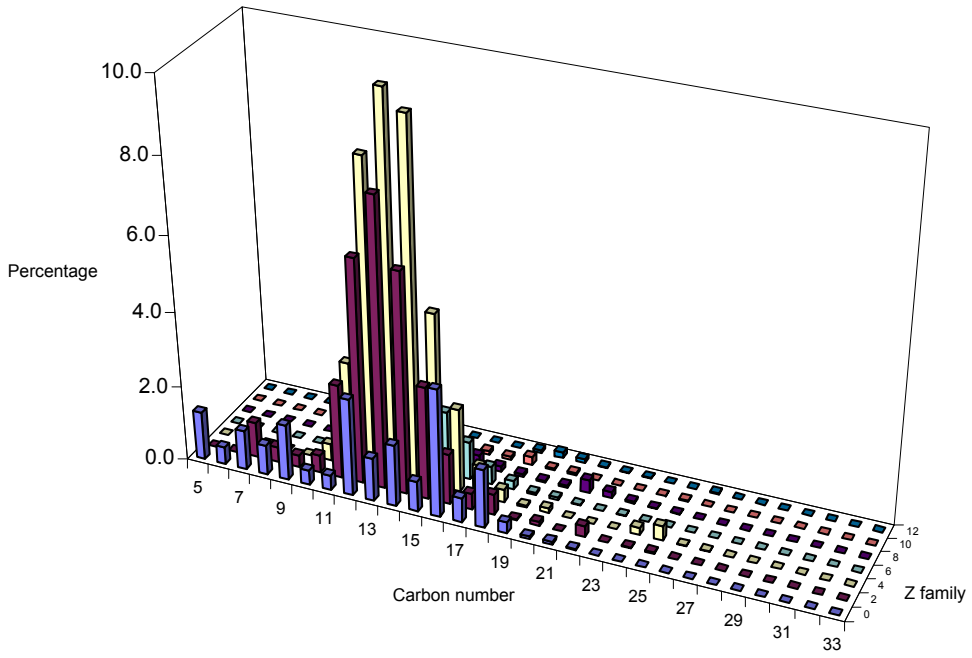


Figure 1.1.3 Three-dimensional plot of naphthenic acids sample from Sample C in glass bottle (Table 2.1.3). The sum of all bars equals 100%.

Overall, there were no statistically significant differences among the relative abundances of the ions in the three-dimensional plots from the three WAF samples that were analyzed. Thus, based on these qualitative analyses, there was no difference between shipping a sample in a plastic bottle or in a glass bottle. Similarly, there was no difference between the 0 h and the 48 h sample.

1.2 Conclusions on the Stability of Naphthenic Acids in Water Accommodated Fractions

Based on the results of the stability of the naphthenic acids profile when samples were stored and shipped in glass or plastic, either type of container was considered appropriate for use in collection and shipping test samples from the definitive aquatic toxicity tests. Additionally, once the WAF is prepared for use in a toxicity test, it is anticipated that no change would be expected over the duration of a WAF renewal period, as defined by the naphthenic acids C-number and Z-number profiles.

2.0 References

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APPENDIX E – REPRESENTATIVE CHROMATOGRAPHY

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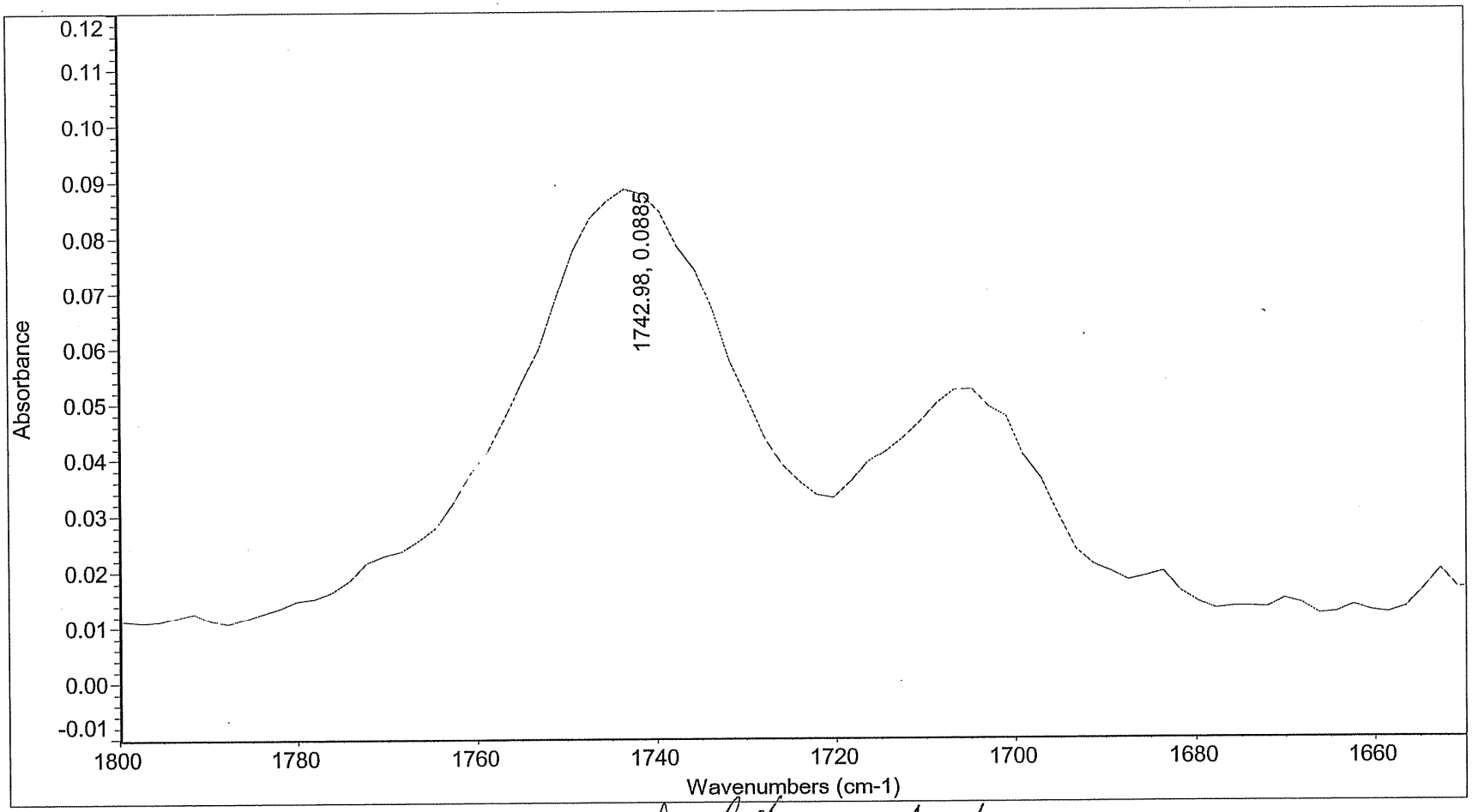


ABC Laboratories FT/IR Analysis of: Naphthenic Acids

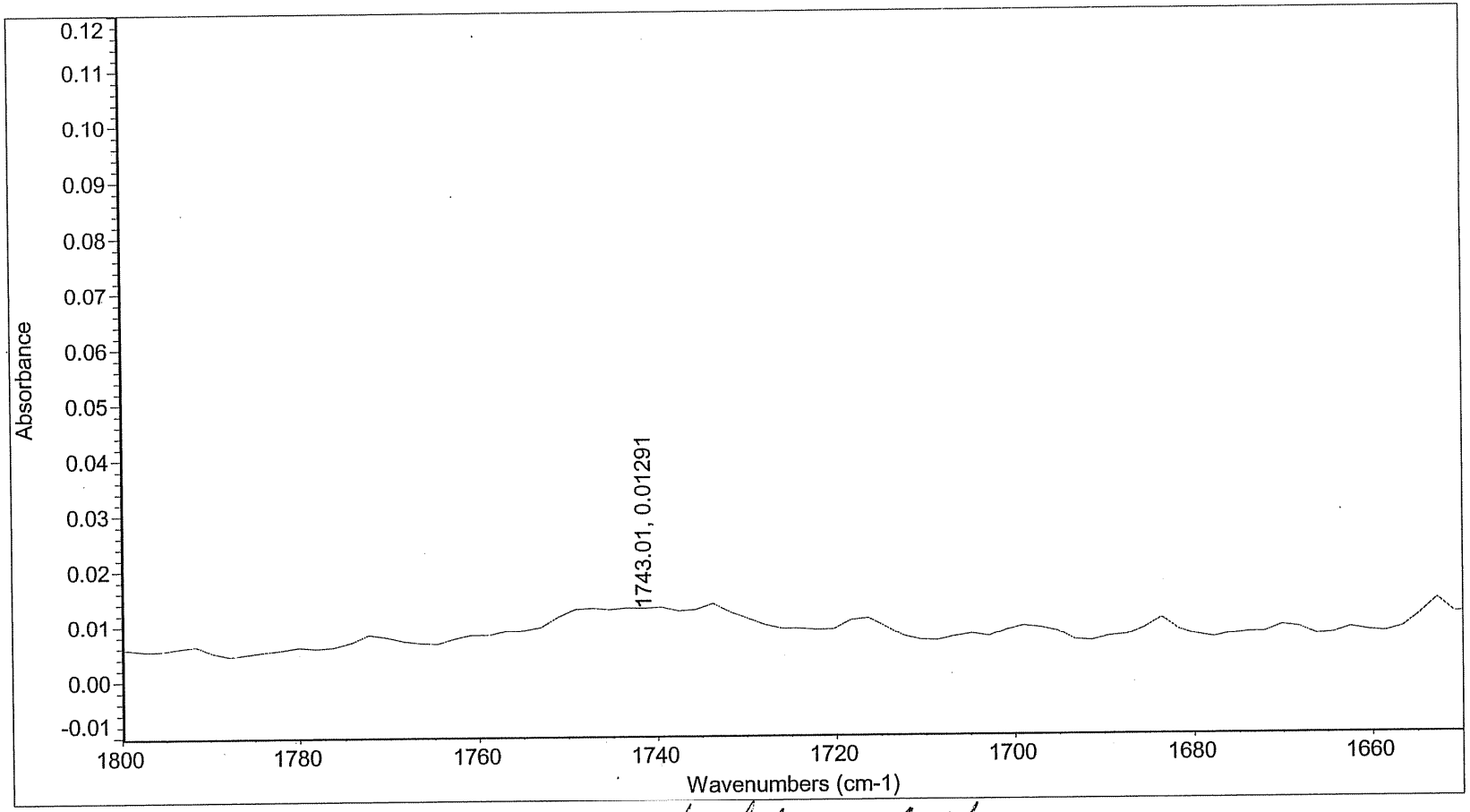
ABC Laboratories Study Number: 64403

Prepared By/Date: 05Jun09 **Checked By/Date:** 09JUN09

64403-77 Control S

ABC Laboratories Inc.

Fri Jun 05 16:36:12 2009 (GMT-05:00)

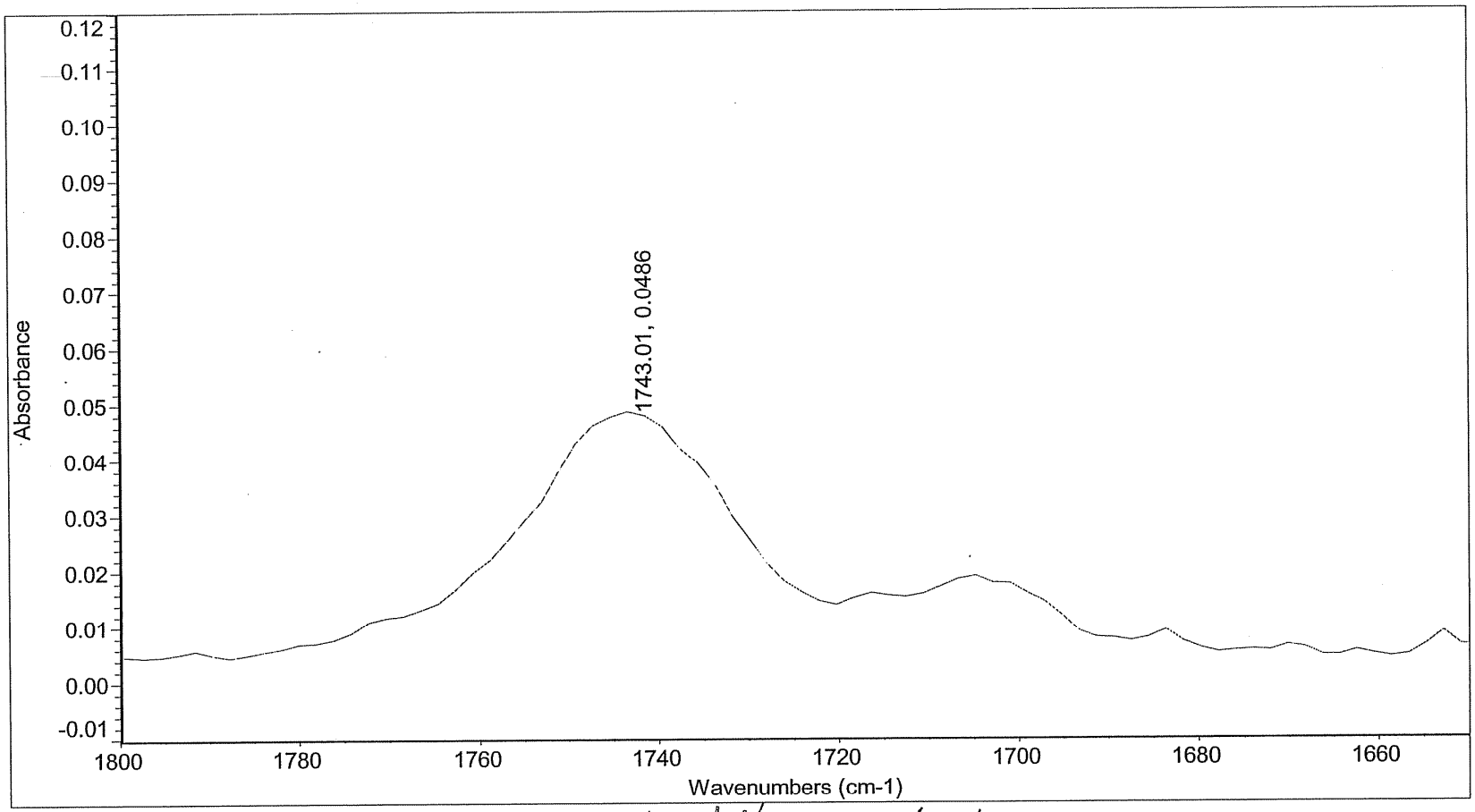


ABC Laboratories FT/IR Analysis of: Naphthenic Acids

ABC Laboratories Study Number: 64403

Prepared By/Date: 05Jun09 **Checked By/Date:** 09JUN09

64403-83 High Spike Y ABC Laboratories Inc. Fri Jun 05 16:39:48 2009 (GMT-05:00)



ABC Laboratories FT/IR Analysis of: Naphthenic Acids

ABC Laboratories Study Number: 64403

Prepared By/Date: [signature] 05Jun09 **Checked By/Date:** [signature] 09JUN09