Standard Test Method for Determination of Denatonium Ion in Engine Coolant by HPLC[1]

This standard is issued under the fixed designation D7304; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the chemical analysis of engine coolant for denatonium benzoate (DNB) by high-performance liquid chromatography (HPLC). DNB is added to potentially render engine coolant unpalatable to animals and humans. This analytical method was designed for the analysis of DNB and is not valid for any other bittering agents such as denatonium saccharide.

1.2 This test method is applicable to both new and used coolants.

1.3 Coelution of other ions may cause interferences in the detection of the denatonium cation. In the case of unfamiliar formulations, identification verification should be performed by either or both fortification and dilution of the sample matrix with denatonium ion.

1.4 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

D1176 Practice for Sampling and Preparing Aqueous Solutions of Engine Coolants or Antirusts for Testing Purposes

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

3. Summary of Test Method

3.1 The denatonium benzoate analysis is achieved by an HPLC method, where a weight of engine coolant is placed in an auto-sampler vial and mixed with a known volume of de-ionized water. The auto-sampler vial is placed in a HPLC autosampler and the measurement of denatonium benzoate is performed using a C-18 reverse phase column attached to an ultraviolet detector. The ultraviolet detector is used to measure the response of the DNB active ingredients (denatonium and benzoate) in the engine coolant after they have been separated in the reverse phase column. The denatonium and benzoate responses are compared to responses of known concentrations and the HPLC's computer calculates the amount of DNB present in the coolant.

4. Significance and Use

4.1 This test method provides for the qualitative and quantitative determination of denatonium benzoate in engine coolant in milligrams per litre to low percent range and requires approximately 1 mL per test, with results available in less than 10 min. Denatonium benzoate is a compound composed of a quaternary ammonium cation, denatonium and an inert anion, benzoate. In solution the denatonium benzoate exists in equilibrium between the denatonium benzoate compound, the denatonium cation and benzoate anion. By slightly adjusting the pH of the solution to be more acidic (≈ pH 4.6) the equilibrium will be shifted to the direction of forming more denatonium and benzoate ions in the solution.

5. Interferences

5.1 Interferences can be caused by substances with similar retention times, especially if they are in high concentration compared to the analyte of interest, denatonium ion. Known chromatographic interferences have been determined and the analysis modified to minimize any co-elution of interfering peaks. The eluent strength and flow rate can be used to reduce or solve most interference problems.

---

[1] This test method is under the jurisdiction of ASTM Committee D15 on Engine Coolants and Related Fluids and is the direct responsibility of Subcommittee D15.04 on Chemical Properties.

Current edition approved May 15, 2016. Published July 2016. Originally approved in 2006. Last previous edition approved in 2014 as D7304–14. DOI: 10.1520/D7304-16.

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

5.2 Method interferences can also be caused by the contamination of glassware, eluant, reagents, etc. Great care must be taken to ensure that contamination is kept at the lowest possible level.

6. Apparatus

6.1 *HPLC System*—High Performance Liquid Chromatograph system equipped with appropriate computer and software.

6.1.1 *Gradient Pump.*

6.1.2 *UV/VIS-Mulitple Wavelength Detector.*

6.1.3 *Eluant Degas System.*

6.1.4 *Analytical Column,* Kinetex C-18, 2.6 µm packing, 75 mm × 4.6 mm or equivalent column.

6.1.5 *Guard Column,* Ultra Cartridge UPLC C-18 for 4.6 mm or equivalent.

6.2 *Analytical Balance,* 0.0001 g precision.

6.3 *Volumetric Flasks,* 50 mL, 100 mL, 200 mL, 500 mL, 1 L.

6.4 *pH Meter,* capable of pH/mV/temperature measurements.

6.5 *Glassware,* class "A" laboratory glassware and plastic ware.

6.6 *Eppendorf Series 2100 Pipetter,* capable of delivering 1000 µL.

6.7 *Electronic Pipetter,* capable of delivering between 30 → 300 µL.

7. Reagents and Materials

7.1 Acetonitrile, (ACN), Reagent Grade.

7.2 Ortho Phosphoric acid, H_3PO_4, 85 %, Reagent Grade.

7.3 Potassium Phosphate, Monobasic, KH_2PO_4, 99.995 % purity.

7.4 Denatonium Benzoate, 98 % purity.

7.5 Ethylene Glycol, Reagent Grade.

7.6 De-ionized water, Type II water.

7.7 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

7.8 Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type II. It is recommended that all water be filtered through a 0.45 µm filter. For eluant preparation, degas the water by sparging with helium or vacuum degassing and sonication.

7.9 *Potassium Phosphate Solution* KH_2PO_4 *(0.5M):*

7.9.1 Weigh 34.022 g of the KH_2PO_4 into a 500 mL volumetric flask.

7.9.2 Add 250 mL of de-ionized water and mix until the KH_2PO_4 has dissolved.

7.9.3 Dilute to the mark with de-ionized water and thoroughly mix.

7.10 *Sample Dilution Solution (pH 4.6): Potassium Phosphate Solution* KH_2PO_4 *(0.1M):*

7.10.1 Transfer 200 mL of Potassium Phosphate Solution KH_2PO_4 (0.5M) to 1000 mL volumetric flask.

7.10.2 Dilute to mark with de-ionized water.

7.10.3 Measure the pH of the de-ionized water with a pH meter.

NOTE 1—The pH of the sample dilution water should be checked bi-weekly and if the pH has changed the dilution water should be re-prepared.

7.11 *pH 4.3 Mobile Phase, 30 % Acetonitrile 70 % of Potassium Phosphate Solution* KH_2PO_4 *(0.1M):*

7.11.1 Add 300 mL of acetonitrile to a 1000 mL volumetric flask.

7.11.2 Dilute to the mark with Potassium Phosphate Solution KH_2PO_4 (0.1M).

7.11.3 Stopper and mix.

7.11.4 Degas the solution for 10 min in an ultra-sonic bath as a large amount of dissolved gas will be present in the solution.

7.11.5 Place solution in a 1000 mL beaker and measure the solution's pH.

7.11.6 Adjust the pH of the mobile phase solution with 0.5M KH_2PO_4 or 85 % phosphoric acid until it reaches pH 4.3.

7.11.7 Transfer the solution to the HPLC reagent bottle.

NOTE 2—The final pH of the Mobile Phase should be approximately 4.3 pH units.

8. Hazards

8.1 Personnel protective equipment (such as eye protections, gloves, laboratory coat, etc.) should be used in the handling of all chemicals. Special care should be taken when handling solutions around electrical equipment. All solution should be prepared in a hood.

8.2 Read all equipment manuals before attempting to operate HPLC instrumentation. Special attention should be given to all warnings.

8.3 Be familiar with the SDS for all chemicals used in this procedure. A dust mask is recommended for the handling of Denatonium Benzoate. Review your company's policy concerning the use of a dust mask. Prepare standards in a hood.

9. Sampling, Test Specimens and Test Units

9.1 Collect the sample in a scrupulously clean glass or polyethylene bottle in accordance with Test Method D1176. Collect at least 100 mL of sample.

10. Calibration Solutions:

10.1 *Stock Denatonium Benzoate Calibration Solution (500 mg/L DNB):*

10.1.1 Weigh 0.0500 ± 0.0001 g of denatonium benzoate into a 100 mL volumetric flask and record the weight.

10.1.2 Add de-ionized water, stopper and mix until solid dissolves.

10.1.3 Dilute to mark with de-ionized water and mix.

10.2 *Working Standard (20 mg/L DNB):*

10.2.1 Pipette 4 mL of the Stock Calibration Solution in a 100 mL volumetric flask.

10.2.2 Dilute to mark with de-ionized water.

10.2.3 Stopper and mix.

10.3 *Calibration Standards:*

10.3.1 Prepare a series of calibration standards by placing the appropriate amount of Stock Solution (see Table 1) into a 2000 µL auto-sampler vial.

10.3.2 Dilute to volume with Sample Dilution Solution (pH 4.6).

10.3.3 Place vials in the instrument's auto-sampler.

10.3.4 Repeat for each calibration standard.

10.3.5 Analyze standards and calibrate instrument using the method's external calibration function.

11. Analytical Conditions

11.1 *Instrument:*

Column:	75 mm × 4.6 mm
Column Temperature:	40°C
Eluent "A":	30 % Acetonitrile 70 % of KH_2PO_4 (0.1M)
Analysis Program:	Isocratic
Flow Rate:	1.95 mL/min
System Pressure:	3100 psi or 214 bar
Injection Size:	5 µL

11.2 *Ultra-Violet Detector:*

Signal:	Absorbance
Wavelength:	210 mm
Response:	0.02 ABU
Cell Size:	12 µL
Signal Rate:	10.0 pts/sec

12. Gradient Program

12.1 See Table 2.

NOTE 3—If the coolant contains 4-Tert-Butylbenzoic acid (TBBA) the analysis time will be 9 min.

13. Sample Preparation

13.1 Place a 2000 µL auto-sampler vial on an analytical balance and zero the balance.

TABLE 1 Denatonium Benzoate Calibration Standards

Standard	Concentration of Standard	Volume of Working Standard (20 mg/L)	Volume of Monoethylene Glycol	Volume of pH 4.6 Water Added to Auto-sampler Vial
Unit	(mg/L)	µL	µL	µL
DNB Std 1	0.25	25	50	1925
DNB Std 2	0.50	50	50	1900
DNB Std 3	0.75	75	50	1875
DNB Std 4	1.00	100	50	1850
DNB Std 5	1.25	125	50	1825
DNB Std 6	1.50	150	50	1800

TABLE 2 Gradient Program

Program Step	Time (min)	Flow (mL/min)	30 % Acetonitrile 70 % of KH_2PO_4 (0.1M) (adjusted pH 4.3)
Equilibration	0.5	1.95	100.0
1	5.0	1.95	100.0

13.2 Pipette 50 µL of sample into the auto-sampler vial and record the sample weight in milligrams (mg).

13.3 Pipette 1950 µL of pH 4.6 Sample Dilution Solution into the auto-sampler vial.

13.4 Cap the vial, shake and place vial in the auto-sampler.

13.5 Repeat Steps 13.1 – 13.4 for each sample.

13.6 Calculate the sample dilution factor using the formula in Eq 1.

13.7 Prepare an auto-sampler sequence and analyze samples.

14. Calculation

14.1 *Instrument Results:*

14.1.1 The instrument denatonium benzoate results are calculated by comparing the sample denatonium peak area response to the calibration curve for the denatonium benzoate. This calibration curve is part of the instrument method.

14.2 *Sample Denatonium Benzoate (DNB) Results:*

14.2.1 The concentration of the denatonium benzoate is determined by multiplying the denatonium benzoate's instrument concentration by the sample's dilution factor using the following formula:

$$\text{DNB Concentration (ppm wt./wt.)} = (\text{Instr. concentration in mg/L} \times \text{Dilution Factor}) \tag{1}$$

where:

Dilution Factor = 2000 µL / weight of 50 µL of sample (mg)

14.3 *Limit of Detection and Limit of Quantitation:*

14.3.1 The Limit of Detection (LOD) is the point at which the analyte cannot be determined.

14.3.2 The Limit of Quantitation (LOQ) is the lowest concentration of analyte that can be reported with confidence and is defined as 3.3 times the LOD.

14.3.3 The instrument and method LOD and LOQ values are reported in Table 3.

NOTE 4—The Method LOD and LOQ are determined by multiplying the Instrument LOD and LOQ by the Sample Dilution Ratio.

TABLE 3 Limit of Detection and Limit of Quantitation Data

	Instrument LOD ppm (wt/vol)	Instrument LOQ ppm (wt/vol)	Method LOD ppm (wt/wt)	Method LOQ ppm (wt/wt)
Bittering Agent (Denatonium Peak)	0.050	0.165	2.0	6.6

15. Report

15.1 Convert and report the concentration of denatonium ion, ppm found in the sample using the following formula:

$$[\text{Denatonium Ion}] = [\text{DNB}] * 0.7287 \qquad (2)$$

15.2 Include in the report: the sample analysis date, analyst, and the lot number or other identification number for the sample.

16. Precision and Bias[3]

16.1 *Precision*—The precision of this test method is based on an interlaboratory study of ASTM D7304, Standard Test Method for Determination of Denatonium Ion in Engine Coolant by HPLC, conducted in 2014. Each of seven laboratories tested fifteen different materials. Every "test result" represents an individual determination, and all participants reported duplicate test results. Practice E691 was followed for the design and analysis of the data; the details are given in ASTM Research Report No. RR:D15-1035.

16.1.1 *Repeatability (r)*—The difference between repetitive results obtained by the same operator in a given laboratory applying the same test method with the same apparatus under constant operating conditions on identical test material within short intervals of time would, in the long run, in the normal and correct operation of the test method, exceed the following values only in one case in 20.

16.1.1.1 Repeatability can be interpreted as maximum difference between two results, obtained under repeatability conditions, that is accepted as plausible due to random causes under normal and correct operation of the test method.

16.1.1.2 Repeatability limits are listed in Table 4.

16.1.2 *Reproducibility (R)*—The difference between two single and independent results obtained by different operators applying the same test method in different laboratories using different apparatus on identical test material would, in the long run, in the normal and correct operation of the test method, exceed the following values only in one case in 20.

16.1.2.1 Reproducibility can be interpreted as maximum difference between two results, obtained under reproducibility conditions, that is accepted as plausible due to random causes under normal and correct operation of the test method.

16.1.2.2 Reproducibility limits are listed in Table 4.

16.1.3 The above terms (repeatability limit and reproducibility limit) are used as specified in Practice E177.

TABLE 4 Repeatability and Reproducibility Limits

Analysis	Average		
	r	R	% Recovery of Denatonium Ion Spike
Day 1 - 3: Denatonium ion (ppm)[A]	1.89	4.06	99.9
Day 1 - 3: Denatonium Benzoate (ppm)[A]	2.36	5.55	...
Day 1 - 3: Determined Water content (wt%)[A]	0.15	0.46	...

[A] Each calculation is the average of 15 samples tested over the course of three days.

16.1.4 Any judgment in accordance with statements 16.1.1 and 16.1.2 would have an approximate 95 % probability of being correct.

16.2 *Bias*—At the time of the study, there was no accepted reference material suitable for determining the bias for this test method, therefore no statement on bias is being made.

16.3 The precision statement was determined through statistical examination of 945 results, from seven laboratories, on fifteen materials described below:

Sample Code	Denatonium Benzoate Concentrate	Sample Description
1	60 ppm	2-EHA based coolant concentrate
2	30 ppm	Ethylene glycol
3	40 ppm	Sodium benzoate based coolant concentrate
4	80 ppm	Traditional coolant concentrate without phosphate
5	40 ppm	Ethylene glycol
6	30 ppm	Tert butylbenzoic acid based coolant premix
7	40 ppm	Benzoate and Tert butylbenzoic based coolant premix
8	50 ppm	Ethylene glycol
9	50 ppm	2-EHA based hybrid coolant premix
10	40 ppm	Benzoate and toluic acid based coolant premix
11	60 ppm	Ethylene glycol
12	25 ppm	Phosphate based traditional coolant premix
13	10 ppm	Phosphate based OAT coolant premix
14	10 ppm	Ethylene glycol
15	35 ppm	Phosphate based hybrid coolant premix

16.4 To judge the equivalency of two test results, it is recommended to choose the material closest in characteristics to the test material.

17. Keywords

17.1 analytical method; antifreeze; Bitterant; chromatography; denatonium benzoate; denatonium ion; engine coolant; HPLC

---

[3] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D15-1035. Contact ASTM Customer Service at service@astm.org.

APPENDIX

X1. SAMPLE CHROMATOGRAMS AND CALIBRATION CURVE

Nonmandatory Information

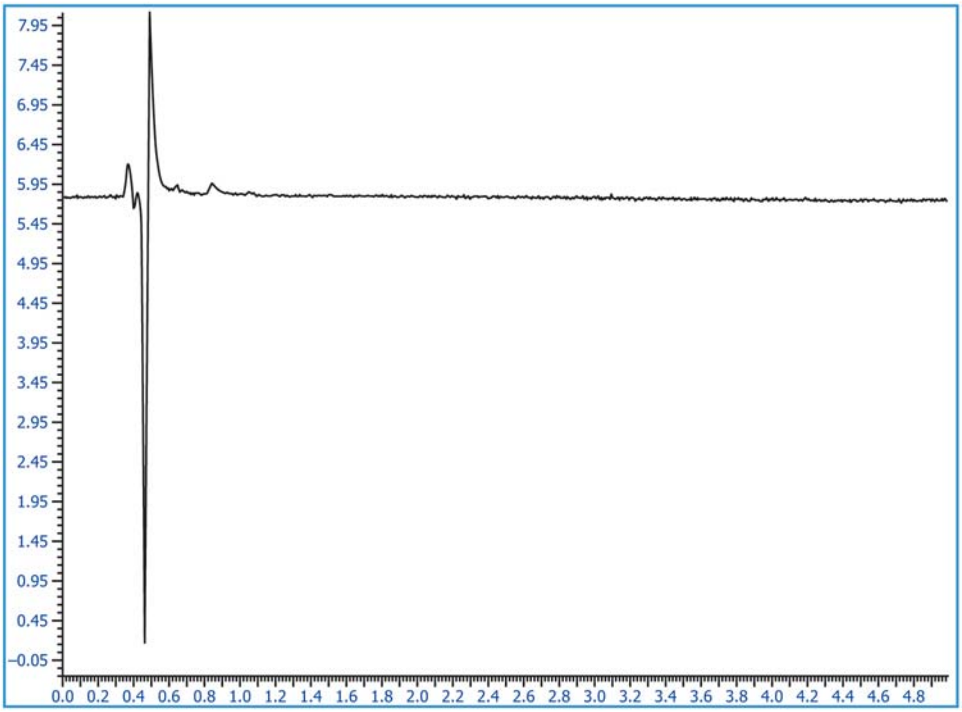

FIG. X1.1 Chromatogram of pH 4.6 Water Blank

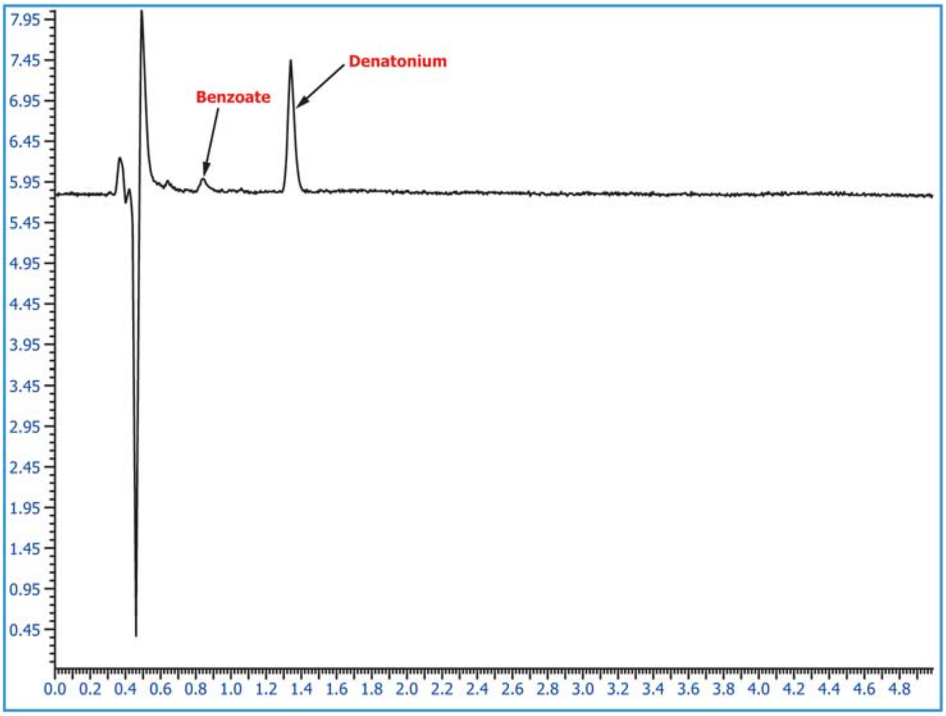

FIG. X1.2 Chromatogram of 0.600 mg/L Bittering Agent Standard + Glycol

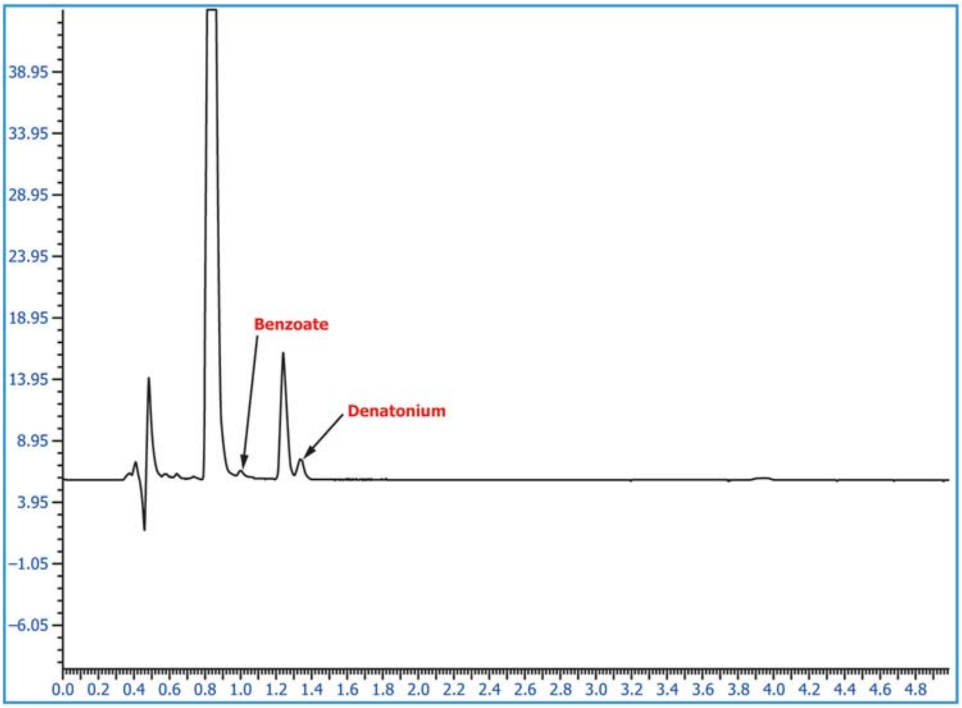

FIG. X1.3 Chromatogram of Coolant "A"

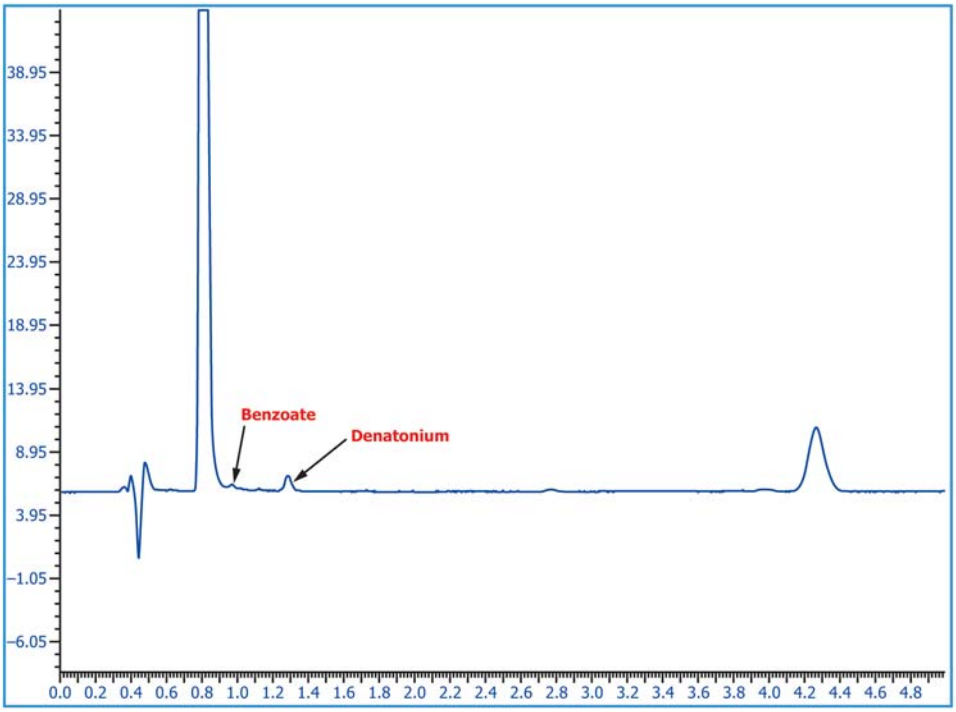

FIG. X1.4 Chromatogram of Coolant Sample "B"

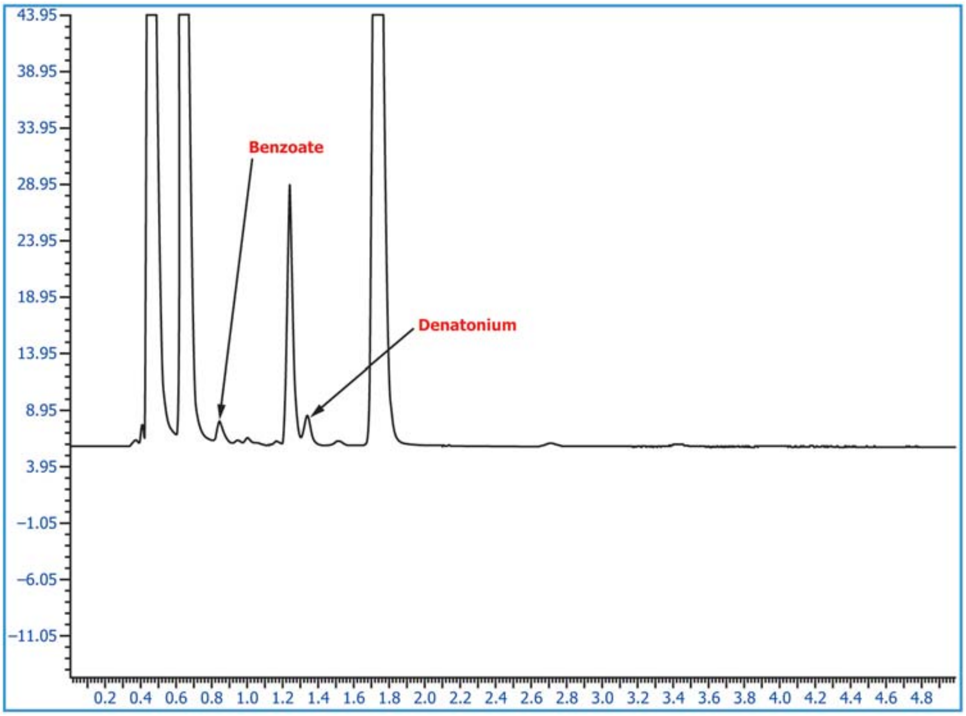

FIG. X1.5 Chromatogram of Coolant Sample "C"

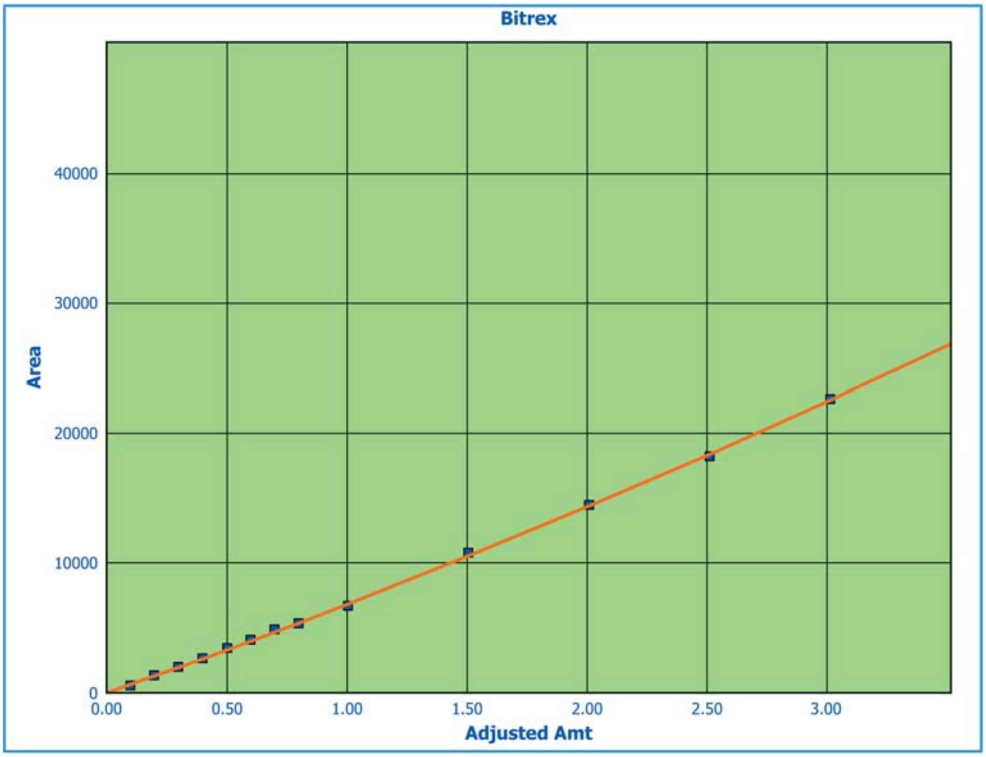

FIG. X1.6 Bittering Agent (DNB) Calibration Curve

ASTM International takes no position respecting the validity of any patent rights asserted in connection with any item mentioned in this standard. Users of this standard are expressly advised that determination of the validity of any such patent rights, and the risk of infringement of such rights, are entirely their own responsibility.

This standard is subject to revision at any time by the responsible technical committee and must be reviewed every five years and if not revised, either reapproved or withdrawn. Your comments are invited either for revision of this standard or for additional standards and should be addressed to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend. If you feel that your comments have not received a fair hearing you should make your views known to the ASTM Committee on Standards, at the address shown below.

This standard is copyrighted by ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959, United States. Individual reprints (single or multiple copies) of this standard may be obtained by contacting ASTM at the above address or at 610-832-9585 (phone), 610-832-9555 (fax), or service@astm.org (e-mail); or through the ASTM website (www.astm.org). Permission rights to photocopy the standard may also be secured from the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, Tel: (978) 646-2600; http://www.copyright.com/