**Designation: ~~D7304 – 06~~ D7304 – 14**

# 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 ~~ion~~ 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..~~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

## 3. Summary of Test Method



3.1 ~~A sample volume of working sample is prepared by dilution of the sample with water. A high-pressure pump forces the mobile phase, eluant, through the HPLC columns (a guard and analytical column) at typical flow rates of 0.1- 2 mL/min. A sample to be separated is introduced in the mobile phase by an injection device prior to the column. The analytes are separated as they pass through the column. An optical sensor detects the changes in characteristics of the eluant stream and converts the signal into an absorbance spectrum. The data system compares this response with an external calibration curve and the results of the concentration of analyte reported as ppm or milligrams per litre (mg/L). Refer to~~ 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.~~Appendix X1 for a HPLC flow diagram..~~

## 4. Significance and Use

4.1 This test method provides for the qualitative and quantitative determination of denatonium ~~ion~~benzoate in engine coolant in milligrams per ~~liter~~litre to low percent range and requires approximately ~~100~~1 mL per test, with results available in less than

[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.

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[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.

30 min. Acceptable levels of denatonium vary with manufacturer's blending specifications and applicable minimum or maximum industry and state specifications. Typically specification ranges from 30 to 50 ppm denatonium ion.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. Sample dilution and optimized gradient elution 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 minimizereduce or solve most interference problems.

5.2 A water dip (solvent system peak) can cause interference with some integrators. This is eliminated by dilution with the eluant if the sample dilution factor is 4+1 (v/v) or greater.

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 levellevel.

## 6. Apparatus

6.1 *HPLC System*—Analytical High Performance Liquid Chromatograph system equipped with all required accessories including syringes, gasses, columns, pumps, detectors 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,* ZORBAX RX-C8 or equivalent column, capable of producing analyte separation equivalent to or better than that shown in Kinetex C-18, 2.6 µm packing, 75 mm x 4.6 mm or equivalent column.Fig. 1.

6.1.5 *Guard Column,* for ZORBAX RX-C8 or equivalent, for protection of the analytical column from strongly retained constituents.Ultra Cartridge UPLC C-18 for 4.6 mm or equivalent.

6.1.6 *Integrator or Chromatography Data System Software* capable of obtaining approximately the same detection limits as listed in Table 1.

6.2 *Analytical Balance,* 0.0001 g precision.

6.3 *Volumetric Flasks,* 50 mL, 100 mL, 200 mL. 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.

**TABLE 1 Minimum Detection Limits**

| colwidth="1.91in">colwidth="1.09in"> | |
|---|---|
| Analyte | Detection Limits, ppm[A] |
| Denatonium ion | 1.0 |

**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 |

[A] Determination using 100-µL sample volume. Sample diluted 1+3 (wt/v) with type II water, full scale, UV/Vis detector set at 210 nm wavelength, Zorbax RX-C8 analytical column with Zorbax RX-C8 guard columns. Other systems will require MDL determination using chosen dilution factors, eluants, columns and detectors.

## 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, ~~Reagent Grade.~~98 % purity.

7.5 Ethylene Glycol, Reagent Grade.

7.6 ~~Deionized~~De-ionized water, ~~Type~~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 ~~Buffer Stock~~Solution $KH_2PO_4$ ~~Solution—~~(0.5M):* ~~Weigh 650 mg of Monobasic Potassium Phosphate and transfer it into a 1 L volumetric flask, add 0.5 mL Phosphoric acid and fill to the mark with Type II water. Mix this solution thoroughly. Filter the solution with 0.45 µm filter before use.~~

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 ~~*Denatonium Ion Standard Stock Solution—pH 4.3 Mobile Phase, 30 % Acetonitrile 70 % of Potassium Phosphate Solution $KH_2PO_4$ (0.1M):* Weigh 0.1372 g of Denatonium Benzoate into 100-mL volumetric flask and dilute with ethylene glycol to 100 g. This stock solution is 1000 ppm of denatonium and is stable for one month. Make-up working standard solutions in 25% v/v ethylene glycol/Type II water to desired concentrations. Example: 5 ppm denatonium standard solution - weigh 0.5000 g of stock denatonium standard solution into a 100 mL volumetric flask and fill to 100 mL mark with 25 % v/v ethylene glycol water solution.~~

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 minutes 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)~~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 MSDS 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. Preparation of Apparatus**~~

~~10.1 Over the course of the HPLC run, the concentration of the eluant will change to improve the separation of the analytes. The HPLC system is programmed to handle the changing mix of eluant. Two different eluant gradient programs are given below, for a borate-silicate based engine coolant and a silicate free, organic acid based engine coolant. The programming of the eluant gradient will vary with the HPLC system used and the inhibitor package utilized in the engine coolant.~~

~~10.2 Program Gradient 1: (for a borate, silicate based engine coolant)~~

| ~~Time~~ | ~~Flow, mL~~ | ~~Acetonitrile, Vol %~~ | ~~Phosphate Buffer, Vol %~~ | ~~Curve[A]~~ |
|---|---|---|---|---|
| ~~Initial~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~–~~ |
| ~~0.1~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~1~~ |
| ~~10.0~~ | ~~1.9~~ | ~~45~~ | ~~55~~ | ~~6~~ |
| ~~20.0~~ | ~~1.9~~ | ~~90~~ | ~~10~~ | ~~6~~ |
| ~~23.0~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~6~~ |
| ~~27.0~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~1~~ |

~~[A] The Curve Number entered here specifies the manner in which successive gradient steps are connected.~~
~~Curve 1 Go to end condition immediately~~
~~2-5 Convex~~
~~6 Linear~~
~~7-10 Noncave~~
~~11 Maintain start conditions until end of segment~~

~~10.3 Program Gradient 2: (for a silicate free, organic acid based engine coolant)~~

| ~~Time~~ | ~~Flow, mL~~ | ~~Acetonitrile, Vol %~~ | ~~Phosphate Buffer, Vol %~~ | ~~Curve[A]~~ |
|---|---|---|---|---|
| ~~Initial~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~–~~ |
| ~~0.1~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~1~~ |
| ~~10.0~~ | ~~1.9~~ | ~~40~~ | ~~60~~ | ~~6~~ |
| ~~15.0~~ | ~~1.9~~ | ~~80~~ | ~~20~~ | ~~6~~ |
| ~~23.0~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~6~~ |
| ~~27.0~~ | ~~1.9~~ | ~~20~~ | ~~80~~ | ~~1~~ |

~~[A] The Curve Number entered here specifies the manner in which successive gradient steps are connected.~~
~~Curve 1 Go to end condition immediately~~
~~2-5 Convex~~
~~6 Linear~~
~~7-10 Noncave~~
~~11 Maintain start conditions until end of segment~~



**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. Calibration and Standardization**~~

~~11.1 Analyze each standard solution separately to determine the analyte's retention time.~~

~~11.2 Set the chromatograph up in accordance with the conditions specified in 11.9. The use of other equipment, eluants, or flows requires calculation of suitable dilution factors and instrument settings that permit the analyst to obtain the resolution and detection limits given in Fig. 1 and Table 1, respectively.~~

~~11.3 Prepare concentrations of denatonium ion at 0.005, 0.025 and 0.055 g/L (5, 25 and 55 ppm) from the standard stock solution. Additional concentrations can be prepared for a more comprehensive calibration curve. All final solutions should be made with Denatonium Ion Standard Stock solution as described in 7.10. Calibrate chromatograph with a minimum of at least three~~

levels of the analyte, starting near but above the minimum detection limit (MDL) and further defining the work range in samples subsequent to dilution. These denatonium analyte examples reflect a dilution of 3+1 (wt/v%) with Type II water.

NOTE 1—HPLC equipment other than that described in this test method may require that standards be prepared at higher or lower levels.

11.4 Analyze a blank containing only the eluant as described in Section 12.

11.5 A sample of known concentration should be run every 10 injections to make sure the instrument is working correctly.

11.6 The analytical calibration curve must be established at only one detector scale setting in order to prevent a change of slope affecting the analytical curve.

11.7 The analytical calibration curve and an eluant blank shall be verified daily prior to the analysis of samples to verify the system resolution, calibration and sensitivity.

11.8 The analytical calibration curve, analyte retention times and resolution, and an eluant blank shall be verified subsequent to a change of the system eluant.

11.9 Conditions (or equivalent conditions to meet requirements of Table 1 and Fig. 1):

*(a)* Detector Setting: 210 nm wave length
*(b)* Analytical Column: Zorbax RX-C8 with Guard Column: Zorbax RX-C8
*(c)* Pump: High Pressure limits 3000 psi; Low Pressure limits 100 psi
*(d)* Flow Rate: 1.9 mL/min
*(e)* Gradient Program: See Section 10
*(f)* Retention Time: 10 to 12 min depending on test sample and gradient program

NOTE 2—These conditions may vary depending on the dimensions of the column used.

## 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. Procedure

12.1 Set up the HPLC (Waters Chromatographic System) in accordance with the manufacturer's instructions.

12.2 Check the instruments setting: see 11.9.

12.3 Fill eluant containers (acetonitrile and phosphate buffer), sparge and pressurize with helium to 5 psi.

12.4 Prime the pump, initiate flow and equilibrate the column for 20-25 min with initial settings in program gradient, Section 10.

12.5 Turn on HPLC and detector. Allow the instrument to equilibrate fro 20-25 min before starting a run.

12.6 At least one duplicate and one spiked coolant sample must be analyzed with each batch of ten or fewer samples. It is recommended that all test samples be filtered through a 0.45 µm filter prior to injection. Results must be recorded in the laboratory manual or be electronically maintained. The spiked sample is to be prepared by addition of a mixed analyte mid-range standard.

NOTE 3—Once the phosphate buffer has run through the system, do not purge with 100 % acetonitrile. This will precipitate buffer in the pump, column or detector and result in total blockage and pressure overload.

12.7 The column and entire system should be flushed with 50-50 %(v/v) water/acetonitrile solution after an analysis to free from phosphate salts.

## 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.

**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. Calculation or Interpretation of Results**~~

~~13.1 Integrate the peak area for determination of the concentrations. Plot the peak area against concentration. A linear calibration curve is generated for the denatonium ion. Calibration is in the concentration range of interest (5 to 60 ppm) and must have a linear least squares correlation coefficient of 0.9990 or greater.~~

~~13.2 The concentration of analyte is given by the concentration read from the calibration curve multiplied by the dilution factor used.~~

~~DB concentration = DB concentration from curve·dilution factor (1)~~

~~DB = denatonium ion~~

~~13.3 Relative percent difference (RPD) of duplicate runs:~~

~~$RPD = \text{Absolute Value}\,[((A - B)/A)\cdot 100]$ (2)~~

~~*where:*~~

~~*A* = concentration found in Analysis Run 1, and~~
~~*B* = concentration found in Analysis Run 2.~~

~~Results must be within 20 %.~~
~~Spike recovery (*SR*) as percent:~~

~~$SR = ((C + B)/A)\cdot 100$ (3)~~

~~*where:*~~

~~*A* = concentration found in analysis of spiked sample,~~
~~*B* = concentration found in analysis of sample, and~~
~~*C* = concentration of analyte (denatonium ion) added to sample.~~
~~Spike recovery must be within a 80 to 120 % range.~~



**13. Sample Preparation**

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

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 to 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:

DNB Concentration (ppm wt./wt.)= Instr. concentration in mg/L × Dilution Factor) (1)

where:

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