U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
SCIENCE AND TECHNOLOGY
DENVER WILDLIFE RESEARCH CENTER
ANALYTICAL CHEMISTRY SECTION

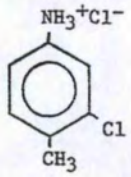
ANALYTICAL METHOD

Number: 28A	Effective Date: 8-3-90
Supersedes: ---------	Page 1 of 11

CPT HCl AQUATIC TOXICITY TEST SOLUTION ASSAY

I. CHEMICAL DATA

Common Name: CPT HCl
Alternate Names: 3-chloro-p-toluidine HCl, 3-chloro-4-methylaniline HCl, CL-47676, CPTH, DRC-1339.
Chemical Name: 3-chloro-4-methylbenzenamine hydrochloride
Structure:



Formula: $C_7H_8NCl_2$
MW: 178.06
Physical State: cream colored crystals.
MP: 220-230°C
Solubility: Soluble in alcohol and water; insoluble in acetone, benzene, ether, and hexane.

CPT HCl is light sensitive. It should be stored in the dark.

II. MATRICES

Soft blended water (for bluegill sunfish and rainbow trout aquatic toxicity tests) and hard blended water (for Daphnia magna aquatic toxicity tests) from Analytical Bio-Chemistry Labs (ABC), Aquatic Toxicity Division, 7200 ABC Lane, Columbia Missouri, 65205.

III. REAGENTS

	Name	CAS RN
1)	3-chloro-4-methylbenzenamine hydrochloride reference standard	7745-89-3
2)	Water, HPLC grade	7732-18-5
3)	Acetonitrile, HPLC grade	75-05-8

Developed By	Reviewed By	Approved By
[signature]	Margaret Goodall	Elizabeth A. Michelsen
Date 7-31-90	Date 7-31-90	Date 7-31-90

APHIS FORM 8050-R (Local Reproduction Authorized)

IV. STANDARD PREPARATION

Concentrated Standard Solution: Transfer about 25 mg of CPT HCl reference standard, accurately weighed, to a 25-mL volumetric flask. Dissolve in water, dilute with water to volume, and mix. The concentration of the resulting solution is about 1000 ppm.

Intermediate Standard: Transfer 1.00 mL of the concentrated standard solution to a 10-mL volumetric flask. Dilute with water to volume, and mix. The concentration of the resulting solution is about 100 ppm.

Working Standards: Prepare three working standards by making a serial dilution of the intermediate standard as follows:

I) Transfer 1.00 mL of the intermediate standard solution to a 10-mL volumetric flask. Dilute with water to volume, and mix. The concentration of the resulting solution is about 10 ppm.

II) Transfer 1.00 mL of Working Standard I to a 10-mL volumetric flask. Dilute with water to volume, and mix. The concentration of the resulting solution is about 1 ppm.

III) Transfer 1.00 mL of Working Standard II to a 10-mL volumetric flask. Dilute with water to volume, and mix. The concentration of the resulting solution is about 0.1 ppm.

V. ASSAY PREPARATION

Samples with nominal concentrations of 0.1 to 10 ppm CPT HCl do not require dilution.

Samples with nominal concentrations greater than 10 ppm CPT HCl must be diluted with HPLC grade water to obtain a concentration between 0.1 ppm and 10 ppm.

VI. PROCEDURE

Filter each working standard and assay preparation through separate 0.45 µm Teflon filters prior to injection into the HPLC. Repeatedly inject 100 µL of the 1 ppm working standard to determine the HPLC system suitability for analysis. Inject 100 µL of the assay preparation, and record the CPT chromatographic peak response in each chromatogram.

Developed By	Reviewed By	Approved By
J. O'Leno	Margaret Goodall	Elizabeth A. Michalerie
Date 7-31-90	Date 7-31-90	Date 7-31-90

APHIS FORM 8050-R (Local Reproduction Authorized)

VII. TYPICAL HPLC CONDITIONS

Mobile Phase: Prepare a mixture of water and acetonitrile (20:80), filter through a 0.45 μm membrane filter, and degas.

OR

Separately filter water and acetonitrile through a 0.45 μm membrane filter, and degas. Deliver the following from two reservoirs on the HPLC:

20% aqueous- water
80% organic- acetonitrile

Column: Alltech Econosil C-18, 5-μm, 4.6 x 250 mm column or equivalent. (Other types of C-18 packing materials may not be suitable and should be thoroughly tested before use.)

Flow Rate: 1.0 mL/min

Injection Volume: 100 μL

Column Temperature: ambient (20 - 25°C)

Detector: UV @ 241 nm

Operating and recording conditions should be adjusted to obtain optimum response and reproducibility.

VIII. SYSTEM SUITABILITY

System suitability is demonstrated when the relative standard deviation of the CPT chromatographic peak response is ≤ 2.0% for five consecutive injections of the 1 ppm working standard.

Developed By	Reviewed By	Approved By
[signature]	Margaret Goodall	Elizabeth A. Michalonie
Date 7-31-90	Date 7-31-90	Date 7-31-90

APHIS FORM 8050-R *(Local Reproduction Authorized)*

IX. DATA ANALYSIS AND CALCULATIONS

$$\text{ppm CPT HCl} = \frac{Au}{As} \times C_{std} \times \frac{Vf}{Vi}$$

where:

Au = the CPT chromatographic peak response from the assay preparation.

As = the CPT chromatographic peak response from the working standard.

C_{std} = the concentration of the CPT HCl working standard (ppm).

Vf = the final volume of the assay preparation after dilution, when dilution is required.

Vi = the sample aliquot volume used for dilution, when dilution is required.

Calculation Example

A CPT HCl/water sample with a nominal concentration of 98 ppm was assayed to confirm the CPT HCl concentration. The sample was diluted by taking a 1.00 mL aliquot and diluting to 10.0 mL with HPLC grade water. The CPT chromatographic peak height from the assay preparation was 199.5 mAU.

The working standard concentration was 10.0 ppm, and the CPT chromatographic peak height was 202.0 mAU.

The concentration of CPT HCl in the sample is calculated as follows:

$$\text{ppm CPT HCl} = \frac{199.5}{202.0} \times 10.0 \text{ ppm} \times \frac{10.0 \text{ mL}}{1.0 \text{ mL}} = 98.8$$

Developed By	Reviewed By	Approved By
[signature]	[signature]	[signature]
Date 7-31-90	Date 7-31-90	Date 7-31-90

U.S. DEPARTMENT OF AGRICULTURE ANIMAL AND PLANT HEALTH INSPECTION SERVICE SCIENCE AND TECHNOLOGY DENVER WILDLIFE RESEARCH CENTER ANALYTICAL CHEMISTRY SECTION **ANALYTICAL METHOD**	Number: 17A	Effective Date: 10-12-90
	Supersedes: None	Page 1 of 11

CPT HCl DETERMINATION IN BUFFERED AQUEOUS SOLUTIONS: ANALYTICAL METHOD FOR HYDROLYSIS STUDIES

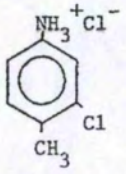
I. CHEMICAL DATA

Common Name: CPT HCl

Alternate Names: 3-chloro-4-methylaniline HCl, CL-47676, CPTH, DRC-1339.

Chemical Name: 3-chloro-*p*-toluidine hydrochloride

Structure:



Formula: $C_7H_9NCl_2$

MW: 178.06

Physical State: cream colored, flaky crystals.

MP: 220-230°C

Solubility: Soluble in alcohol and water; insoluble in acetone, benzene, ether, and hexane.

CPT HCl is light sensitive. It should be stored in the dark.

Developed By	Reviewed By	Approved By
Date 10-11-90	Date 10/11/90	Date 10-11-90

APHIS FORM 8050-R (Local Reproduction Authorized)

II. REAGENTS

	Name	CAS RN
1)	3-Chloro-*p*-toluidine hydrochloride reference standard, 97+% (w/w)	7745-89-3
2)	Acetonitrile, HPLC grade	75-05-8
3)	Water, HPLC grade	7732-18-5
4)	Sodium acetate, Enzyme Grade	127-09-3
5)	Acetic acid, glacial, 99.5+% (w/w)	64-19-7
6)	Buffer Solution- pH 7.00 Potassium phosphate monobasic/sodium hydroxide; 0.05 M. (Certified, Fisher Scientific); diluted with water to 0.01 M.	--------
7)	Buffer Solution- pH 9.00 Boric acid/potassium chloride/sodium hydroxide; 0.1 M. (Certified, Fisher Scientific); diluted with water to 0.01 M.	--------
8)	Hydrochloric acid (HCl), approx. 36% w/w Diluted with water to 0.01 M.	7647-01-0
9)	Sodium hydroxide (NaOH), Reagent Grade Prepare 0.01 M NaOH/water.	1310-73-2

III. BUFFER SOLUTION PREPARATION

pH 5 Buffer: Dissolve 0.27 g of sodium acetate in 500 mL of water. Add 94 µL of acetic acid. If necessary, adjust the pH to 5.00 ± 0.01 (25°C) with 0.01 M HCl or 0.01 M NaOH.

pH 7 Buffer: Dilute 100 mL of the 0.05 M pH 7 buffer with water to a final volume of 500 mL to obtain a 0.01 M buffer solution. If necessary, adjust the pH to 7.00 ± 0.01 (25°C) with 0.01 M HCl.

pH 9 Buffer: Dilute 50 mL of the 0.1 M pH 9 buffer with water to a final volume of 500 mL to obtain a 0.01 M buffer solution. If necessary, adjust the pH to 9.00 ± 0.01 (25°C) with 0.01 M HCl.

Developed By	Reviewed By	Approved By
Date 10-11-90	Date 10-11-90	Date 10-11-90

IV. STANDARD PREPARATION

Concentrated Standard Solution: Transfer about 100 mg of CPT HCl reference standard, accurately weighed, to a 100-mL volumetric flask. Dissolve in water, dilute with water to volume, and mix. The concentration of the resulting solution is about 1000 µg/mL.

Working Standards: Transfer 200 µL of the concentrated standard solution to a 10-mL volumetric flask. Dilute with 0.01 M pH 5 buffer to volume, and mix. The concentration of the pH 5 working standard is about 20 µg/mL.

Transfer 200 µL of the concentrated standard solution to a 10-mL volumetric flask. Dilute with 0.01 M pH 7 buffer to volume, and mix. The concentration of the pH 7 working standard is about 20 µg/mL.

Transfer 200 µL of the concentrated standard solution to a 10-mL volumetric flask. Dilute with 0.01 M pH 9 buffer to volume, and mix. The concentration of the pH 9 working standard is about 20 µg/mL.

V. SAMPLE PREPARATION

pH 5 Test Solution: Transfer 1.00 mL of the concentrated standard solution to a 50-mL volumetric flask. Dilute with the 0.01 M pH 5 buffer to volume, and mix. Prepare this solution in triplicate.

pH 7 Test Solution: Transfer 1.00 mL of the concentrated standard solution to a 50-mL volumetric flask. Dilute with the 0.01 M pH 7 buffer to volume, and mix. Prepare this solution in triplicate.

pH 9 Test Solution: Transfer 1.00 mL of the concentrated standard solution to a 50-mL volumetric flask. Dilute with the 0.01 M pH 9 buffer to volume, and mix. Prepare this solution in triplicate.

Transfer each test solution to a 50-mL screw cap glass culture tube, cap the tube, and seal it with Parafilm®.

VI. PROCEDURE

Prior to injection into the HPLC, filter the working standards and samples through separate 0.45 µm nylon filters.

Repeatedly inject 10 µL of one of the working standards to determine the HPLC system suitability for analysis. Inject 10 µL of each sample, and record the CPT peak response in each chromatogram.

VII. TYPICAL HPLC CONDITIONS

Mobile Phase: Prepare a mixture of water and acetonitrile (20:80), filter through a 0.45 µm nylon membrane filter, and degas.

OR

Separately filter water and acetonitrile through a 0.45 µm nylon membrane filter, and degas. Deliver the following from two reservoirs on the HPLC:

20% aqueous- water
80% organic- acetonitrile

Column: Alltech Econosil C-18, 5-µm, 4.6-mm x 25-cm column or equivalent.

Flow Rate: 1.0 mL/min

Injection Volume: 10 µL

Temperature: room temperature (about 22°C)

Detector: UV @ 241 nm

Operating conditions and integration parameters should be adjusted to obtain optimum response and reproducibility.

VIII. SYSTEM SUITABILITY

System suitability is demonstrated when the relative standard deviation of the CPT chromatographic peak response is ≤ 2.0% for five consecutive injections of the working standard.

Developed By	Reviewed By	Approved By
Date 10-11-90	Date 10-11-90	Date 10-11-90

APHIS FORM 8050-R (Local Reproduction Authorized)

IX. DATA ANALYSIS AND CALCULATIONS

The CPT HCl concentration in the test sample is calculated relative to the corresponding working standard (pH 5, 7, or 9) as follows:

$$\text{CPT HCl } (\mu g/mL) = \frac{Au}{As} \times C_{std}$$

where:

Au — is the CPT chromatographic peak response from the test solution.

As — is the CPT chromatographic peak response from the working standard.

C_{std} — the CPT HCl working standard concentration (pH 5, 7 or 9; about 20 µg/mL).

CALCULATION EXAMPLE

A 101.2 mg sample of CPT HCl was used to prepare a 1012 µg/mL concentrated standard solution. Test solutions were prepared with this concentrated standard solution by diluting 1.00 mL of the concentrated standard with 0.01 M pH 5, 7, and 9 buffers to 50.0 mL.

The % CPT HCl in the test solutions was determined at zero time and at various time intervals over a thirty day period. For example, one of the pH 5 samples was tested after fourteen days, and the CPT chromatographic peak area was 137,560.

A CPT HCl pH 5 working standard was prepared on the day of analysis. The concentration of the working standard was 20.4 µg/mL, and the CPT chromatographic peak area from this solution was 140,000.

Using the equation above, the concentration of CPT HCl in the test solution after fourteen days is calculated as follows:

$$\text{CPT HCl } (\mu g/mL) = \frac{137{,}560}{140{,}000} \times 20.4\ \mu g/mL$$

$$\text{CPT HCl } (\mu g/mL) = 20.0$$

Developed By	Reviewed By	Approved By
B. C. Nibbelink	[signature]	Elizabeth A. Nicholaisen
Date 10-11-90	Date 10-11-90	Date 10-11-90