SUMMARY

An analytical method for the quantitation of 3-Chloro-p-Toluidine Hydrochloride (CPTH) in surface water test systems was successfully independently validated at 1 ng/mL (LOQ), 20 ng/mL (20X LOQ) and 300 ng/mL (300X LOQ) from two different sources of surface water, using the method as written.

The method is described in "Determination of 3-Chloro-p-Toluidine Hydrochloride (CPTH) in Environmental Surface Water by GC/MS/MS", USDA-APHIS National Wildlife Research Center, Analytical Method Number 175A (Effective Date: February 22, 2016).

Quantitation analysis of the test substance, CPTH was performed by using Gas Chromatography with triple quadrapole mass spectrometer detection (GC-QQQ). The surface water test systems were collected from North Dakota; Golden Lake was collected from Steele County and Goose River was collected from Grand Forks County. Aliquots from each test system were fortified with CPTH at 1 ng/mL (LOQ), 20 ng/mL (20X LOQ) and 300 ng/mL (300X LOQ).

The experiment for each water test system was conducted with one solvent blank, one reagent blank, two untreated controls, and five control samples spiked for each fortification level: one at the LOQ level, one at 20X LOQ level and one at 300X LOQ level, and analyzed by GC/MS/MS.

The CPTH analysis methods as described and explained in the original method validation report were used for analyte separation. CPTH content was quantitated against a second order quadratic curve of the reference substance CPTH for both the quantitation and confirmation ions, with concentrations ranging from 5 ng/mL to 5000 ng/mL. The calibration for the CPTH quantitation and confirmation analysis methods yielded acceptable linearity (correlation coefficients $r > 0.99$) over the range examined. The quantitation of CPTH was based on the ratio of peak area responses of CPTH and d_6-CPTH and the ratio of CPTH and d_6-CPTH concentrations of the calibration standards. The amount of CPTH was determined by GC/MS/MS for both quantitation and

confirmation analyses. Method recovery from fortified samples was determined by calculating the found concentration of CPTH and dividing the concentration by the relevant fortification level.

The method limit of quantitation (MLOQ) was estimated to be 1.1 ng/ml for CPTH in both the Golden Lake and Goose River surface water systems.

The method limit of determination (MLOD) was estimated to be 0.3 ng/mL for CPTH in both the Golden Lake and Goose River surface waters.

No direct interferences or residues were detected in the surface water systems for the quantitation and confirmatory analysis methods.

INTRODUCTION

The purpose of this study was to conduct an independent laboratory validation (ILV) for the determination of CPTH in two surface water test systems. The analysis of CPTH was performed by Gas Chromatography with tandem Mass Spectrometric Detection (GC-MS/MS, based on the method described in "Determination of 3-Chloro-p-Toluidine Hydrochloride (CPTH) in Environmental Surface Water by GC/MS/MS", USDA-APHIS National Wildlife Research Center, Analytical Method Number 175A (Effective Date: February 22, 2016) [1].

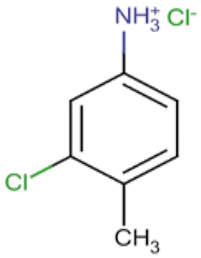
This study was designed to satisfy US EPA Guideline requirements described in OCSPP 850.6100 [2]. The study was initiated on November 9, 2016. The experimental work was conducted from November 28, 2016 through February 7, 2017 at EAG Laboratories-Hercules, 625-B Alfred Nobel Drive, Hercules, CA 94547 under the approved protocol (Appendix A) according to the US EPA FIFRA Good Laboratory Practice Standards, 40 CFR §160.

MATERIAL AND METHODS

Reference and Test Substances

Common Name:	**CPTH; DRC-1339**
Chemical Name:	3-chloro-*p*-toluidine hydrochloride
CAS Registry No.:	7745-89-3

Chemical Structure:



Molecular Formula:	$C_7H_9NCl_2$
Molecular Mass:	178.06 grams/mole

Lot No.:	040159
Purity:	98.0%
Date of Expiry:	04-08-17
Storage Conditions:	Room Temperature

A copy of the Certificate of Analysis for the test/reference substance is provided in Appendix B.

Other Chemicals

Chemicals		
Reagent	**Grade**	**Manufacturer**
d6-CPTH	na	National Wildlife Research Center
Water	HPLC	Burdick & Jackson
n-butyl acetate	Reagent	Acros Organics
Hexanes	HPLC	Burdick & Jackson
Sodium Chloride	Reagent	EMD
Sodium Hydroxide	Reagent	EMD

Equipment List

Equipment	
Instrument	**Description**
Volumetric Flasks	Appropriate Size, glass flasks
Centrifuge	Sorvall RT-7 plus and Mistral
Vortex Mixer	Fisher Scientific
SPE Vacuum Manifold	Glass Chamber
Weigh Boats	Glass
Automatic Calibrated Pipette	Various size, disposable tips
Centrifuge Tubes	50 mL plastic
Graduated Cylinders	Glass, various sizes
Screw Top Test Tube	25 mL glass tube
SPE Cartridge	IST-Si (1g / 6 mL)
Gas Chromatograph	Agilent 7890 Series
Detector	Agilent 7000B GC-MS/MS triple quad
Analytical Balance	Sartorius or Equivalent

Test System

Source of Test System

The test systems that were used in this study were purchased by the Sponsor, characterized by Agvise Laboratories and shipped directly to EAG Laboratories-Hercules. The Golden Lake surface water (2870W-003) was collected from Steele County, North Dakota and the Goose River surface water (2870W-004) was collected from Grand Forks County, North Dakota. The test systems were maintained under refrigerated conditions while not in use. The water characterization details are presented below and the characterization reports are provided in Appendix C.

Test Systems		
Description	**Golden Lake**	**Goose River**
PTRL Identification	2870W-003	2870W-004
pH	8.7	8.3
Potassium (ppm)	18	10
Calcium (ppm)	93	105
Magnesium (ppm)	106	62
Sodium (ppm)	133	139
Hardness (mg equivalent $CaCO_3$/L)	674	521
Conductivity (mmhos/cm)	1.48	1.26
Sodium Adsorption (SAR)	2.24	2.66
Total Dissolved Solids (ppm)	1282	1016
Turbidity (NTU)	11.7	18.1
Alkalinity (mg $CaCO_3$/L)	199	323
Carbonates (meq/L)	1.47	0.88
Bicarbonates (meq/L)	2.79	5.88
Sulfate-Sulfur (ppm)	224	145
Nitrate-Nitrogen (ppm)	*	0.2
Chloride (ppm)	27.3	12.6

*below dectection limit of 0.1 ppm

Test Method

The analytical method for the analysis of CPTH in surface water was independently validated at EAG Laboratories-Hercules by Gas Chromatography with tandem mass

spectrometric detection (GC-MS/MS) for CPTH and is described in "Determination of 3-Chloro-p-Toluidine Hydrochloride (CPTH) in Environmental Surface Water by GC/MS/MS", USDA-APHIS National Wildlife Research Center, Analytical Method Number 175A (Effective Date: February 22, 2016) [1] and is provided in Appendix D.

The water samples were spiked with known concentrations of CPTH and extracted with hexane following the procedure as described below. An aliquot of the final sample solution was injected onto the gas chromatograph with tandem mass spectrometric detection (GC-MS/MS), for both quantitation and confirmation analysis of CPTH. The quantitation of CPTH was based on the ratio of peak area responses of CPTH and d_6-CPTH and the ratio of CPTH and d_6-CPTH concentrations of the calibration standards. The amount of CPTH was determined by GC/MS/MS. Method recovery from fortified samples was determined by calculating the found concentration of CPTH and dividing the concentration by the relevant fortification level.

Preparation of Reagent Solutions

2.0 M NaOH:

In a glass container, dissolve 20 g of sodium hydroxide with 250 mL of HPLC grade water.

Water Saturated with NaCl:

In a glass container, dissolve 20 g of sodium chloride with 50 mL of HPLC grade water.

2 M NaOH saturated with NaCl:

In a glass container combine 40 g of sodium chloride with 100 mL of 2 M sodium hydroxide.

Preparation of Standard Solutions

Concentrated Standard Stock Solution:

A single stock solution was prepared for CPTH, by weighing an aliquot approximately 10 mg of the reference substance into a 10-mL volumetric flask and brought to volume with HPLC water and vortexed. The concentration of the stock solution was adjusted for purity to a final concentration of 1 mg/mL by adding 74.4 µL of water using a 100 µL calibrated automatic pipette. The stock solution was transferred to a 12-mL amber vial and stored in the refrigerator when not in use.

PTRL West No.	Stock ID	Standard Name	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical Conc. (mg/mL)
2870W-001	1 mg/mL	CPTH	10.28	10.0744	98.0	1.00

Data transcribed from NB.3424 p.13

Intermediate Standard Solution A:

A single 100 µg/mL solution was prepared from the CPTH standard stock solution, by removing a 1.0 mL aliquot using a 1-mL volumetric pipette and transferring to a 10-mL volumetric flask. The solution was brought to volume with HPLC water, vortexed and then transferred to a 12-mL amber vial and stored in the refrigerator when not in use.

Intermediate Standard Solution B:

A single 10 µg/mL solution was prepared from the CPTH standard stock solution, by removing a 0.1 mL aliquot using an automatic calibrated pipette and transferring to a 10-mL volumetric flask. The solution was brought to volume with HPLC water, vortexed and then transferred to a 12-mL amber vial and stored in the refrigerator when not in use.

Intermediate Standard Solution C:

A single 1 µg/mL solution was prepared from Intermediate Solution A, by removing a 0.1 mL aliquot using an automatic calibrated pipette and transferring to a 10-mL

volumetric flask. The solution was brought to volume with HPLC water, vortexed and then transferred to a 12-mL amber vial and stored in the refrigerator when not in use.

d_6-CPTH Surrogate Stock Solution:

A single stock solution was prepared for d_6-CPTH, by weighing an aliquot approximately 5 mg of the reference substance into a 25-mL volumetric flask, brought to volume with HPLC water and vortexed. The concentration of the stock solution was 209 µg/mL. The stock solution was transferred to a 30-mL amber vial and stored in the refrigerator when not in use.

PTRL West No.	Stock ID	Standard Name	Weight (mg)	Final volume (mL)	Purity (%)	Theoretical Conc. (µg/mL)
2870W-002	D_6 Stock	*d6*-CPTH	5.22	25	na	209

Data transcribed from NB.3424 p.1

d_6-CPTH Fortification Solution:

A single 4 µg/mL solution was prepared from the d_6-CPTH stock solution, by removing a 0.1914 mL aliquot using an automatic calibrated pipette and transferring to a 10-mL volumetric flask. The solution was brought to volume with HPLC water, vortexed and then transferred to a 12-mL amber vial and stored in the refrigerator when not in use.

Standard Extraction

1. Prepare in 12-mL amber glass vials.
2. Add appropriate volumes of standard solutions as described below.
3. Add 0.3 mL of water saturated with NaCl, 0.25 mL of d_6-CPTH fortification solution, and 2 mL of 2M NaOH saturated with NaCl.
4. Add 3 mL of *n*-butyl acetate and cap tightly. Vortex mix for 5-7 seconds.
5. Centrifuge for 1 minute at approximately 4000 RPM.
6. Transfer upper layer (*n*-butyl acetate) to 10-mL volumetric flask.
7. Repeat steps 4 to 6, 2 additional times, combining extracts.

8. Dilute to 10 mL with *n*-butyl acetate solution. Vortex and transfer to 12 mL amber glass vials.

Intermediate Solution Identification	Aliquot (µL)	Water Volume (µL)	Final CPTH Concentration (ng/mL)	Level Name
C	50	450	5	1
C	100	400	10	2
B	50	450	50	3
B	100	400	100	4
B	500	0	500	5
A	100	400	1000	6
A	500	0	5000	7

Data transcribed from NB.3424 p.14

Fortification Procedure

Fortification of untreated water samples was conducted at the following three fortification levels as shown below using a calibrated automatic pipette:

Fortification Level (ng/mL)	Fortification Solution
1	25 µL of 1 µg /mL in 25 mL of water
20	50 µL of 10 µg /mL in 25 mL of water
300	75 µL of 100 µg /mL in 25 mL of water

Fortification was conducted to determine the percent recovery, and accuracy within the method validation. This procedure was performed in quintuplicate during method validation at each fortification level.

Sample Extraction

1. For method validation, the 25 ml fortification levels of each water sample were prepared for extraction.
2. Add 50 µL of d_6-CPTH fortification solution.

3. Add a drop of water soluble food coloring.
4. Add 2.5 mL 2.0 M NaOH and approximately 5 g NaCl to each sample.
5. Add 10 mL hexane to sample and vortex for 10 seconds.
6. Centrifuge at approximately 4000 RPM for 1 minute.
7. Transfer the upper hexane layer to a clean 25-mL screw-cap test tube.
8. Repeat steps 6 through 8 two additional times with 5 mL of hexane.
9. Prepare an IST-Si (1 g/6 mL) SPE cartridge for each sample:
    a. Add 2 mL *n*-butyl acetate.
    b. Add 5 mL hexane.
    c. Vacuum dry the SPE cartridge
    d. Load the sample extract (do not allow to dry).
    e. Gravity elute hexane through the column into a 10-mL tube; discard to waste.
    f. Vacuum dry the SPE cartridge.
    g. Elute CPTH by vacuum with 2 mL of n-butyl acetate into clean 10-mL glass screw-top tubes.
10. Transfer to an auto sampler vial for analysis.

A schematic diagram of the water extraction method is presented in Figure 1.

Gas Chromatography with Tandem Mass Spectrometric Detection Analytical Method (GC-MS/MS)

Parameters

Column 1	Agilent HP-5MS, 15m x 0.25mm x 0.25 µm
Column 2	Restek guard column 1m x 0.15mm
Injector Temperature	250ºC; Splitless, ~12.77 psi
Inj. Pulse Press.	70 psi Until 1.0 min.
Purge Flow to Split Vent	60 mL/min at 2.5 min
He Carrier Gas Flow Rate	1.2 mL/min
He Quench Gas	2.25 mL/minute
N_2 Collision Gas	1.5 mL/minute
Temperature program	Initial Temperature: 70°C hold for 2 min Ramp 20ºC/minute to 175°C, no hold, Ramp 100°C/minute to 300°C Backflush 2.00 min at 300°C
Injection volume	1 µL
GC liner	Single gooseneck (inert)
Ionization Source	Electron impact
Source Temperature	230°C
Retention Time	Approximately 5.9 minutes
Run time	8.5 minutes

Multiple Reaction Monitoring (MRM)						
Compound Name	Precursor ion	MS1 resolution	Product ion	MS2 resolution	Dwell (ms)	CE (V)
d_6-CPTH	148.9	Wide	112.2	Unit	20	19
d_6-CPTH	146.9	Wide	112.2	Unit	40	15

d_6-CPTH	112.2	Wide	81.2	Unit	20	19
CPTH	140.9	Wide	106.2	Unit	50	15.5
CPTH	139.9	Wide	105.2	Unit	20	16
CPTH	139.9	Wide	77.2	Unit	20	19

GC Analysis

Samples were analyzed in a set consisting of calibration standards, a solvent blank, reagent blank, two control extracts, five 1 ng/mL fortified controls, five 20 ng/mL fortified controls, and five 300 ng/mL fortified controls. In addition, to ensure accuracy was maintained over the course of the analytical set, quality control (QC) calibrants were added to the sequence. The final QC calibrant response was within 20% when compared to its initial response. Calibrants and samples were analyzed in a single sequence of injections.

Methods of Calculation

Quantitation

The quantitation of CPTH was conducted using the peak area ratio of CPTH and d_6-CPTH and the ratio between theoretical CPTH and d_6-CPTH concentrations of the calibrants. The content of CPTH in samples was quantitated against a 1/x weighted quadratic curve. An example for the quantitation of CPTH is presented below where:

Weighting of the calibration curve was applied to provide better curve fit at the lower concentration levels of CPTH.

The calculation of the curve equations (quadratic) and concentrations (ng/mL) present in samples and calibrants was conducted using MassHunter® software.

The residue of the analyte in the sample is determined as follows:

$$\text{Response Ratio} = a\left(\frac{\text{Concentration}_{\text{CPTH}}}{\text{Concentration}_{\text{CPTH-d6}}}\right)^2 + b\left(\frac{\text{Concentration}_{\text{CPTH}}}{\text{Concentration}_{\text{CPTH-d6}}}\right) + c$$

The response ratio (y) for sample F1-A is calculated as follows:

$$\text{Response Ratio} = \left(\frac{\text{Response}_{\text{CPTH}}}{\text{Response}_{\text{CPTH-d6}}}\right) =$$

Response Ratio = 2944 ÷ 10607 = 0.2776

The calibration curve determined by the software for the golden lake surface water sample set is:

$y = 0.002982x^2 + 2.367504x - 0.025416$

The concentration ratio (x) is determined from the quadratic formula:

$$x = \frac{-b \pm \sqrt{b^2 - 4a(c - \text{ResponseRatio})}}{2a}$$

a = 0.002982

b = 2.367504

c = -0.025416 + - 0.2776

Solving for x =

x = 0.12797 = (Concentration $_{\text{CPTH}}$ ÷ Concentration $_{d6\text{-CPTH}}$)

x = 0.12797 (100)

= 12.797 ng/mL Concentration $_{\text{CPTH}}$

The CPTH residue (ng) for F1-A =

12.797 ng/mL x 2 mL (final volume)

= 25.59 ng

The Percent Recovery of a fortified sample is determined as follows:

$$\frac{\text{Residue (ng) - Average Residue of Controls (ng)}}{\text{Fortification Level (ng)}} \text{ x } 100$$

The percent recovery of fortified sample F1-A (CPTH):

{[25.59 ng – 0.000 ng (avg. control residue)] ÷ 25 ng (fort. level)} x 100%

= 102%

Note: values rounded for presentation and may differ slightly from reported values.

Calibration Range

The calibration curve, ranging from 5 ng/mL to 5000 ng/mL for CPTH, was generated by MassHunter® software for the water method validations.

Method Limit of Quantitation

The method limit of quantitation (MLOQ) was estimated to be 1.1 ng/mL for CPTH in both Golden Lake and Goose River surface waters. Data for the MLOQ determination is presented in Table 2.

Method Limit of Detection

The method limit of detection (MLOD) was estimated to be 0.3 ng/mL for CPTH in both Golden Lake and Goose River surface waters. Data for the MLOD determination is presented in Table 2 and Table 3.

Time Required for Completion of a Sample Set

A sample set consisted of one solvent blank, one reagent blank, two controls (untreated water samples), and five fortified water samples (at each level i.e. LOQ, 20X LOQ and 300X LOQ). The time required for one sample set from preparation of standard solutions,

initiation of extraction, until the completion of instrumental analysis and data evaluation is as follows:

- Preparation of standard solutions takes approximately 4 hours
- Sample preparation takes approximately 4 hours
- GC-MS/MS analysis and data processing takes approximately 2 hours, not including automated sample analysis

TOTAL = approximately 10 hours for one analyst to complete a set to satisfy the validation requirements.

Statistical Methods

Mean, standard deviation, relative standard deviation, and quadratic fit were the only statistical methods employed in this study.

The MLOQ was estimated to be 1.1 ng/mL for both the golden lake and goose river test systems. The MLOD was estimated to be 0.3 ng/mL for both the golden lake and goose river test systems.

Method Modification

The following minor method modifications occurred during conduct of the study:

Modification	Impact
SPE cartridge was vacuum dried prior to addition of extract.	No negative impact, results show good comparison with method validaton.
GC Column used was an Agilent HP-5MS instead of an Agilent HP-5MS UI (ultra-inert)	No negative impact, results show good comparison with method validaton.
GC injection port liner was a double gooseneck liner instead of the splitless, single taper liner with wool	No negative impact, results show good comparison with method validaton.

Correspondence

There was no contact with the laboratory that developed the original method during the conduct of this study.

CONCLUSIONS

An independent laboratory validation for the analysis of CPTH in two surface waters was successfully validated at the defined LOQ, 20X LOQ and 300X LOQ levels by GC-MS/MS. However, the following proposed recommendation should be considered by the method developers in order to clarify methodology and reduce potential mis-interpretations of the validated method.

2870W Method Recommendations		
Method Validation Section	**Recommendation**	**Reason**
Typical GC Conditions	Change approximate retention time to 4.8 minutes	Typographical error based on provided chromatograms.

The MLOQ was estimated to be 1.1 ng/mL for both the Golden Lake and Goose River test systems. The MLOD was estimated to be 0.3 ng/mL for both the Golden Lake and Goose River test systems.

The recovery data for the independent laboratory validations in the two surface water test systems at the LOQ, 20X LOQ and 300X LOQ levels demonstrated acceptable precision and accuracy of the analytical method. Therefore, the analytical method independently validated in this study was demonstrated to be suitable for the determination of CPTH in surface water.

No interference residues were detected above the lowest calibration standard (5 ng/mL) in the control surface water test systems for CPTH.

This study has met the requirements and acceptance criteria outlined in US EPA guideline OCSPP 850.6100 [2]. The study was also in compliance with Good Laboratory Practices (GLP) as stated in 40 CFR Part 160.

REFERENCES

1. USDA-APHIS National Wildlife Research Center, 2-22-16. Determination of 3-Chloro-p-Toluidine Hydrochloride (CPTH) in Environmental Surface Water By GC/MS/MS. Analytical Method Number 175A

2. EPA Ecological Effects Test Guidelines OCSPP 850.6100, Environmental Chemistry Methods and Associated Independent Laboratory Validation, EPA 712-C-001, January 2012.

Figure 1. Schematic Diagram for the Extraction of CPTH from Water.