INTRODUCTION

This report describes the validation of two analytical methods: 205G907A for the determination of nitrapyrin in drinking, surface, and ground water and 205G907B for the determination of 6-chloropicolinic acid (6-CPA) in drinking, surface, and ground water. Validation of the methods was conducted over the concentration range of 0.05-1.0 µg/L. Common names, chemical names and molecular formulae for the analytes used in this study are presented in Tables 1 and 2. The study protocol appears in Appendix A.

This study was conducted to fulfill data requirements outlined in the United States Environmental Protection Agency (US EPA) Guideline OCSPP 850.6100 (Reference 1) and EU requirements as specified in SANCO/825/00 rev. 8.1 (Reference 2) and SANCO 3029/99 rev. 4. (Reference 3).

ANALYTICAL

Storage and Characterization of Control Specimens

The control specimens were sourced directly by EPL BAS. On arrival, the specimens were placed in a refrigerator set to maintain a temperature of approximately 4°C where they were stored at all times unless removed for analysis. No sample preparation or homogenization was necessary prior to sample analysis. Full sample details are included in the raw data package.

The control specimens were characterized for pH, turbidity, conductivity, hardness, total suspended solids, alkalinity, calcium, phosphorous, total organic carbon and dissolved organic carbon. The control specimen certificates of analysis are found in Appendix B.

Reference Items

The reference item certificates of analysis can be found in Appendix C. The analytical grade materials were supplied by the Sponsor and maintained ambient.

Standard stock solutions, calibration standard solutions and fortification solutions were prepared as described in the analytical methods. Full details of these materials are included in the raw data package for the study along with details of the preparation of all analytical and fortification standards prepared from the primary reference item. The reference item will be retained until expiry and then disposed of following relevant disposal SOP's with the approval of the Sponsor's Representative.

Fortification of Recovery Samples

For each method, the following fortification scheme was used:

Matrix	Reference Item	Untreated Control Specimens	Replicates at Fortification Level (LOD)**	Replicates at Fortification Level (LLMV)***	Replicates at Higher Fortification Level (20x LLMV)
Drinking Water	Nitrapyrin	2	1	5	5
	6-CPA	2	1	5	5
Surface Water	Nitrapyrin	2	1	5	5
	6-CPA	2	1	5	5
Ground Water	Nitrapyrin	2	1	5	5
	6-CPA	2	1	5	5

**LOD – Limit of detection
***LLMV – Lower limit of method validation (Limit of Quantitation)

Nitrapyrin Method

Specimens were assayed according to EPL BAS method 205G907A. The detailed analytical procedure appears in Appendix D.

Method Principle:

Nitrapyrin was extracted from a 100 mL water sample with two 10 mL portions of 1:1 (v/v) hexane:toluene. The combined hexane:toluene extracts were evaporated to less than 0.5 mL and cleaned up on a deactivated silica solid-phase extraction (SPE) column. The SPE eluate was analyzed by gas chromatography with electron-impact mass spectrometry detection (GC/EI-MS). Selective ion monitoring (SIM) was used to detect nitrapyrin. The ions used were based on a full scan mass spectrum (Figure 1) obtained by analysis of a nitrapyrin standard solution. The ions chosen for SIM detection were at mass to charge ratios (*m/z*) of 194, 196 and 198. The ion at *m/z* 194 was assigned as the quantitation ion while the ions at *m/z* 196 and 198 were defined as the confirmatory ions.

Example Calculations:

GC/MS calibration curves were generated using linear regression analysis of the peak areas (y-axis) vs. concentration in ng/mL (x-axis) for nitrapyrin standard solutions analyzed with each batch of water samples. The calibration standard concentrations encompassed a range from 0.6 to 50 ng/mL. The amount found (ng/mL) of analyte was calculated from the linear regression equation of the calibration standards. Regression weighting was not used. The origin was excluded from the regression equation.

$y = mx + b$

Where:

y = Peak area
m = Slope of linear regression equation
x = Concentration of analyte in ng/mL
b = y-Intercept of linear regression equation

The linear regression equation was used to solve for x, which correlates to amount found in ng/mL.

Example: Set V001R, Drinking Water
Sample ID: 907-X001-S8R, 20x LOQ Fortification
Peak Area (*m/z* 194) Quantitation Ion: 3480
Calibration Regression Equation: y = 119.218 * x + 8.643
Amount Found (ng/mL) = (3480 – 8.643) / 119.218 = 29.118

The concentration found in each water sample was determined using the formula:
µg/L Found = Amount Found (ng/mL) * Elution Volume (mL) / Sample Volume (mL)

Example: Set V001R, Drinking Water
Sample ID: 907-X001-S8R, 20x LOQ Fortification
Amount Found: 29.118 ng/mL
Elution Volume: 3.0 mL
Sample Volume: 100.0 mL
µg/L Found = 29.118 * 3.0 / 100.0 = 0.8735

Recovery of fortified test portions was calculated as follows:

Fortification Level (µg/L) =

$$\frac{\text{Spiking Solution Concentration (µg/mL)} * \text{Volume Spiking Solution (mL)} * 1000}{\text{Sample Volume (mL)}}$$

Example: Set V001R, Drinking Water
Sample ID: 907-X001-S8R, 20x LOQ Fortification
Spiking Solution Concentration: 0.500 µg/mL
Volume Spiking Solution: 0.200 mL
Sample Volume: 100.0 mL
Fortification Level (µg/L) = 0.500 * 0.200 * 1000 / 100.0 = 1.00

Recovery (%) =
((µg/L Found Fortified Sample - µg/L Unfortified Control) / Fortification Level (µg/L)) * 100

Example: Set V001R, Drinking Water
Sample ID: 907-X001-S8R, 20x LOQ Fortification
µg/L Found: 0.8735
µg/L Found (Control): 0.000
Fortification Level: 1.00 µg/L
Recovery (%) = ((0.8735 – 0.00) / 1.00) *100 = 87.4

6-CPA Method

Specimens were assayed according to EPL BAS method 205G907B. The detailed analytical procedure appears in Appendix E.

Method Principle:

6-CPA was extracted from 20 mL water samples by C18 solid-phase extraction (SPE). 6-CPA was eluted from the SPE cartridge with acetonitrile. The acetonitrile eluent was evaporated to dryness. The dried residues were dissolved in 25/75 (v/v) methanol/DI water and submitted for liquid chromatography-tandem mass spectrometry analysis (LC/MS-MS).

Multiple reaction monitoring (MRM) was used to detect 6-CPA. The product ions used were based on the tandem mass spectrum (Figure 2) obtained by infusion of a 6-CPA standard solution. The transitions selected for MRM detection were *m/z* 156>112 and 158>114. The *m/z* 156>112 transition was assigned as the quantitation transition while *m/z* 158>114 was defined as the confirmatory transition. The use of LC/MS-MS constituted a change from the original protocol which specified GC-MS. The change was made to utilize the best available technology to achieve the target LOQ. A protocol deviation (Appendix F) to address this change was documented and authorized by the Study Director and Test Facility Management. The deviation had no negative impact on the study.

Example Calculations:

LC/MS-MS calibration curves were generated using linear regression analysis of the peak areas (y-axis) vs. concentration in ng/mL (x-axis) for 6-CPA standard solutions analyzed with each batch of water samples. The calibration standard concentrations encompassed a range from 0.5 to 100 ng/mL. The amount found (ng/mL) of analyte is calculated from the linear regression equation of the calibration standards. A weighting function of 1/x was used. The origin was excluded from the regression equation.

$y = mx + b$

Where:

y = Peak area
m = Slope of linear regression equation
x = Concentration of analyte in ng/mL
b = y-Intercept of linear regression equation

The linear regression equation was used to solve for x, which correlates to amount found in ng/mL.

Example: Set V006, Surface Water
Sample ID: 907-X003-S14, LOQ Fortification
Peak Area (*m/z* 156>112) Quantitation Ion: 176.611
Calibration Regression Equation: y = 45.9468 * x + 2.34565
Amount Found (ng/mL) = (176.611 – 2.34565) / 45.9468 = 3.793

The concentration found in each water sample was determined using the formula:

µg/L Found = Amount Found (ng/mL) * Final Volume (mL) / Sample Volume (mL)

Example: Set V006, Surface Water
Sample ID: 907-X003-S14, LOQ Fortification
Amount Found: 3.793 ng/mL
Final Volume: 0.5 mL
Sample Volume:20.0 mL
µg/L Found = 3.793 * 0.5 / 20.0 = 0.0948

Recovery of fortified test portions was calculated as follows:

Fortification Level (µg/L) =

$$\frac{\text{Spiking Solution Concentration (ng/mL)} * \text{Volume Spiking Solution (mL)}}{\text{Sample Volume (mL)}}$$

Example: Set V006, Surface Water
Sample ID: 907-X003-S14, LOQ Fortification
Spiking Solution Concentration: 100 ng/mL
Volume Spiking Solution: 0.010 mL
Sample Volume:20.0 mL
Fortification Level (µg/L) = 100 * 0.010 / 20.0 = 0.050

Recovery (%) =

((µg/L Found Fortified Sample - µg/L Unfortified Control) / Fortification Level (µg/L) * 100

Example: Set V006, Surface Water
Sample ID: 907-X003-S14, LOQ Fortification
µg/L Found: 0.0948
µg/L Found (Control): 0.0377 (Mean of duplicate control sample analyses)
Fortification Level: 0.050 µg/L
Recovery (%) = ((0.0948 – 0.0377) / 0.050) *100 = 114

Statistical Treatment of Data

The mean recoveries for the fortified samples were calculated using the "AVERAGE" function of the Microsoft Excel spreadsheet computer program, which divides the sum of the selected cells by the number of determinations. The standard deviation of the recoveries for a fortification level was calculated using the "STDEV" function of the same spreadsheet program, which sums the squares of the individual deviations from the mean, divides by the number of degrees of freedom, and extracts the square root of the quotient. Percent relative standard deviation, % relative standard deviation (RSD), was calculated by dividing the standard deviation by the mean, and then multiplying by 100.

Confirmation of Residue Identity

Confirmation was performed to demonstrate the selectivity of the primary method by monitoring two additional SIM ions for nitrapyrin and one additional MRM transition for 6-CPA simultaneous to the primary detection. Reagent blanks, unfortified matrix control samples and matrix control samples fortified at the lowest level of quantitation for each analyte/matrix combination were provided to demonstrate the selectivity of the method.

Problems Encountered, Changes or Modifications Made, and Critical Steps

Cautionary notes appear in method 205G907B (6-CPA) to adhere to a vacuum pressure of -10 inches Hg during the C18 SPE column drying step prior to elution of the analyte. Vacuum pressures below -10 inches of Hg or a drying time longer than 45 minutes can lead to loss of analyte. In addition, it is important that the heating block temperature for the solvent evaporation step following elution of 6-CPA from the SPE cartridge not exceed 35°C. It is also recommended to gently bring the eluates just to dryness. Leaving under nitrogen for extended periods of time will lead to loss of 6-CPA.

Sample Analysis Time Requirements

For method 205G907A (nitrapyrin), one set of thirteen samples and a reagent blank required approximately six person-hours to be completed in the laboratory, followed by the unattended overnight GC-MS analysis and approximately two hours of data evaluation and transcription. Thus, such a set can be completed in approximately one and a half calendar days.

For method 205G907B (6-CPA), one set of thirteen samples and a reagent blank required approximately four person-hours to be completed in the laboratory, followed by the unattended overnight LC-MS/MS analysis and approximately two hours of data evaluation and transcription. Thus, such a set can be completed in approximately one and a half calendar days.

Table 1: Nitrapyrin Structure

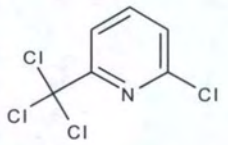
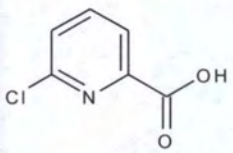
Common name:	**Nitrapyrin**
Chemical name (IUPAC):	2-Chloro-6-(trichloromethyl)pyridine
CAS-Registry-No.:	1929-82-4
Chemical structure:	
Molecular Formula:	$C_6H_3Cl_4N$
Purity:	>95.0%

Table 2: 6-Chloropicolinic Acid Structure

Common name:	**6-Chloropicolinic acid (6-CPA)**
Chemical name (IUPAC):	6-Chloropyridine-2-carboxylic acid
CAS-Registry-No.:	4684-94-0
Chemical structure:	
Molecular formula:	$C_6H_4ClNO_2$
Purity:	>95.0%

Determination of Nitrapyrin in Water by Gas Chromatography-Mass Spectrometry

EPL-BAS Method No. 205G907A

Method Summary:
Nitrapyrin is extracted from a 100 mL water sample with two 10 mL portions of 1:1 (v/v) hexane:toluene. The combined hexane:toluene extracts are evaporated to less than 1 mL and cleaned up on a deactivated silica solid-phase extraction (SPE) column. The SPE eluate (3 mL) containing nitrapyrin is analyzed by gas chromatography with electron-impact mass spectrometry detection (GC/EI-MS). The limit of quantitation (LOQ) for nitrapyrin in water is 0.05 µg//L.

I. Analytical Reference Standard

Common Name:	Nitrapyrin
Chemical Name:	2-chloro-6-(trichloromethyl)pyridine
CAS No.:	1929-82-4
Purity:	>95%
Storage Conditions:	Ambient

A. Stock and Working Standard Solutions (4:1 Hexane:Toluene Solvent)

Stock Standard, 1000 µg/mL:

Weigh 0.100 g of the neat nitrapyrin reference standard into a 100 mL volumetric flask and dilute to volume with 4:1 (v/v) hexane:toluene.

Working Standard, 50.0 µg/mL:

Pipette 10.0 mL of the 1000 µg/mL nitrapyrin stock solution into a 200 mL volumetric flask and dilute to volume with 4:1 (v/v) hexane:toluene.

Working Standard, 5.0 µg/mL:

Pipette 20.0 mL of the 50 µg/mL working solution into a 200 mL volumetric flask and dilute to volume with 4:1 (v/v) hexane:toluene.

Working Standard, 1.0 µg/mL:

Pipette 20.0 mL of the 5.00 µg/mL working solution into a 100 mL volumetric flask and dilute to volume with 4:1 (v/v) hexane:toluene.

B. Calibration Standards

Calibration standards are prepared by diluting the 1.0 µg/mL working solution prepared in Step I.A with toluene as follows:

Aliquot of 1.0 µg/mL Working Solution (mL)	**Final Volume (mL)**	**Calibration Soln. Final Conc. (ng/mL)**
0.060	100	0.60
0.150	100	1.50
0.250	100	2.50
0.750	100	7.50
1.500	100	15.00
2.500	100	25.00
3.500	100	35.00
5.000	100	50.00

C. Fortification Solutions (Acetonitrile Solvent)

Stock Standard, 100 µg/mL:

Weigh 0.0010 g of the neat nitrapyrin reference standard into a 10 mL volumetric flask and dilute to volume with acetonitrile.

Fortification Standard, 5.0 µg/mL:

Pipette 2.5 mL of the 100.0 µg/mL nitrapyrin stock solution into a 50 mL volumetric flask and dilute to volume with acetonitrile.

Fortification Standard, 0.5 µg/mL (500 ng/mL):

Pipette 0.5 mL of the 100 µg/mL nitrapyrin stock solution in acetonitrile into a 100 mL volumetric flask and dilute to volume with acetonitrile.

Fortification Standard, 0.05 µg/mL (50 ng/mL):

Pipette 10.0 mL of the 0.5 µg/mL nitrapyrin stock solution in acetonitrile into a 100 mL volumetric flask and dilute to volume with acetonitrile.

II. Reagents and Reagent Solutions

A. Reagents
Acetonitrile, HPLC Grade
Hexane, Capillary GC Grade
Deionized Water
Toluene, Capillary GC Grade
Sulfuric Acid, Concentrated, Reagent Grade
Silica Gel, Chromatographic, 100-200 mesh
Methanol, HPLC grade

B. Reagent Solutions

4:1 (v/v) Hexane:Toluene Solution: For each liter of solution prepared, combine 800 mL of hexane and 200 mL of toluene and mix well.

1:1 (v/v) Hexane:Toluene Solution: For each 4 liters of solution prepared, combine 2000 mL of hexane and 2000 mL of toluene and mix well.

6N Sulfuric Acid: For each 1 liter of solution prepared, slowly add 168 mL of concentrated sulfuric acid to a 1 L class A volumetric flask containing approximately 500 mL of deionized water. Slowly add deionized water to the base of the neck and mix. The solution will be very hot. Allow the solution to cool to room temperature and dilute to volume with additional deionized water. Mix well.

III. Equipment and Instrument

Balance, Analytical, capable of weighing to the nearest 0.1 mg
Balance, Top-loading, capable of weighing to the nearest 1 mg
Centrifuge
Culture Tubes, 25 mL capacity
Centrifuge Tubes, 50 mL plastic with screw-top caps
Desiccator with fresh desiccant
Forced-air oven capable of maintaining 105 °C
Cake pan, metal, 9 inches by 13 inches
Shaker, reciprocating
Glass wool
Gas Chromatograph, Model 7890, Agilent
Injector, Model 7693, Agilent
Mass Spectrometer, Model 5975, Agilent
Nitrogen Evaporation Unit, N-Evap, Organomation
Pipettes, Air-displacement, 10-100 µL capacity with disposable tips
Pipettes, Air-displacement, 100-1000 µL capacity with disposable tips
Transfer pipets, glass
Graduated Cylinders, various volumes up to 2000 mL
Class A Volumetric Pipettes and Flasks, various volumes
Autosampler Vials, 1.8 mL glass
Autosampler Caps, 11 mm crimp caps
Vial Crimper

IV. Methods

A. Silica Gel Deactivation

1. Weigh out approximately 350 g of silica gel into a 500 mL beaker. Spread the silica in a cake pan and dry in a forced-air oven at 100 °C (± 5 °C) for two hours.
2. Weigh out approximately 15.75 g of 6N sulfuric acid into a 500 mL Teflon bottle.
3. Add 300 g of silica to the bottle.
4. Immediately cap the bottle and place on mechanical shaker to shake vigorously for approximately 2 hours.
5. Transfer silica gel to a desiccator with fresh desiccant for storage. Silica should be stored tightly-capped in the 500 mL Teflon bottle.

B. Sample Extraction

1. Transfer a 100 mL aliquot of water sample into a 125 mL separatory funnel.
2. Fortify laboratory QC samples with the appropriate spiking solution.

LOD Fortification (0.015 µg/L): Pipet 30.0 µL of the 50 ng/mL fortification solution directly into the water sample aliquot.

LOQ Fortification (0.05 µg/L): Pipet 100.0 µL of the 50 ng/mL fortification solution directly into the water sample aliquot.

20x LOQ Fortification (1.0 µg/L): Pipet 200.0 µL of the 500 ng/mL fortification solution directly into the water sample aliquot.

3. Add 10 mL of 1:1 (v/v) hexane:toluene to the separatory funnel..
4. Shake for approximately 15 seconds and allow the phases to separate. Methanol may be added drop-wise to break up any emulsions.
5. Drain the lower aqueous layer into a beaker.
6. Using a glass transfer pipet, transfer the top organic layer to a 25 mL glass test tube. Take care not to transfer any of the aqueous phase that may be adhering to the sides of the separatory funnel.
7. Pour the aqueous phase in the beaker back into the separatory funnel and repeat steps 3-6 above, combining the organic layer with the previous extract in the 25 mL glass test tube.
8. Evaporate the extract to less than 0.5 mL under a stream of nitrogen (N-Evap). Use a heating block temperature setting of 35°C.

C. Silica Gel Cleanup

1. Place a small plug of glass wool in a disposable Pasteur pipette and add approximately 20 mm of deactivated silica gel. Place another small plug of glass wool on top of the deactivated silica gel.
2. Rinse the column with 2 mL of hexane. Do not collect the hexane as it comes through the silica column.
3. Place a 15 mL glass conical graduated tube beneath the silica column to collect the column eluate.
4. Add the concentrated extract from step IV.B.8 to the column and allow it to drain to the top of the bed.
5. Rinse the culture tube with 1 mL toluene and add it to the column. Allow the column to drain to the top of the bed.
6. Repeat the previous step using 1 mL of toluene.
7. Repeat the previous step using 0.5 mL of toluene.
8. Once the column stops dripping, force any remaining solvent from the silica column with a pipet bulb.
9. Adjust the eluate final volume in the graduated conical tube to 3 mL with toluene and vortex mix. Store sample solutions refrigerated when not needed in the laboratory.
10. Transfer a portion of the sample solution to an autosampler vial and cap. Analyze by GC/EI-MS.

D. GC/EI-MS Analysis

The following GC/EI-MS parameters are used to determine the concentration of nitrapyrin in sample extract solutions. The parameters may be modified to achieve adequate chromatographic resolution and/or detector sensitivity. The actual parameters used are documented with each GC/EI-MS sample sequence.

GC System: Agilent 7890N
Injector: Agilent Model 7693 with 150 sample capacity autosampler tray
Detector: Agilent Model 5975 mass spectrometer
Column: Agilent DB-5MS (30 m x 0.25 mm ID x 1.00 µm film)

A splitless column inlet liner and the specified column should be installed into the split/splitless injection port according to manufacturer's recommended procedures.

Oven Method:	
Column	130 °C for 1.0 minute 130 °C to 210 °C at 10 °C/minute 210 °C to 280 °C at 15 °C/minute 280 °C for 2.0 minute
Carrier Gas:	Helium
Constant Flow	1 mL/min
Injection:	Splitless
Injector Temperature	180 °C
Injection Volume	2 µL
Purge flow	40.0 mL/min
Detection Method:	Positive-ion Electron-impact ionization mode
Source Temperature	250 °C
Quad Temperature	180 °C

Ions Monitored:

	Ion	Dwell
Quantitation	*m/z* 194	50 msec
Confirmation (primary)	*m/z* 196	50 msec
Confirmation (secondary)	*m/z* 198	50 msec

V. Calculations

Calibration curves are generated using linear regression analysis of the area of the nitrapyrin peak (y-axis) and nitrapyrin concentration in ng/mL (x-axis) for calibration standards injected in each sample sequence. The calibration standard concentrations encompass a range from 0.6-50 ng/mL.

The amount found (ng/mL) of analyte is calculated from the linear regression equation of the calibration standards.

$y = mx + b$

Where:

y = (Area of analyte peak)
m = Slope of linear regression equation
x = (Conc. of analyte in ng/mL)
b = Intercept of linear regression equation

The linear regression equation was used to solve for *x*, which correlates to amount found in ng/mL.

µg/L Found =

$$\frac{\textit{Amt Found (ng/mL)} * \textit{Elution Volume (mL)} * \textit{Dilution Factor}}{\textit{Water Sample Volume (mL)}}$$

Fortification Level (µg/L) =

$$\frac{\textit{Volume Spiking Soln. (mL)} * \textit{Spiking Soln. Concn. (ng/mL)}}{\textit{Water Sample Volume (mL)}}$$

Recovery (%) =

*(µg/L Found – µg/L Found in Control) / Fortification Level (µg/L) * 100*

Determination of 6-Chloropicolinic Acid (6-CPA) in Water by Liquid Chromatography with Tandem Mass Spectrometry Detection

EPL-BAS Method No. 205G907B

Method Summary:

6-CPA is extracted from water samples (20 mL) by C18 solid-phase extraction (SPE). 6-CPA is eluted from the SPE cartridge with acetonitrile. The acetonitrile eluate is evaporated to dryness. The dried residues are dissolved in 25/75 (v/v) methanol/DI water and submitted for liquid chromatography-tandem mass spectrometry analysis. The limit of quantitation for 6-CPA in water is 0.05 µg/L. The limit of detection is 0.015 µg/L.

I. Reference Substance and Reference Substance Solutions

Common Name	6-Chloropicolinic acid (6-CPA)
CAS Number	4684-94-0
Purity (%)	>95
Storage Conditions	Ambient

Volumes given may be adjusted as necessary as long as proportionality is maintained. Additional working and calibration standard concentrations may be used as needed.

Stock Standard Solution, 1,000 µg/mL

A stock solution of 6-CPA is prepared at a target concentration of 1,000 µg/mL. Weigh 0.100 g of the neat reference substance into a 100 mL volumetric flask and dilute to volume with methanol. Maintain refrigerated up to 365 days when not needed in the laboratory.

Working Standard Solution, 10 µg/mL

Pipet 1.0 mL of the 1,000 µg/mL stock solution into a 100 mL volumetric flask and bring to volume with methanol. Maintain refrigerated up to 365 days when not needed in the laboratory.

Working/Fortification Standard Solution, 1 µg/mL (1,000 ng/mL)

Pipet 0.1 mL of the 1,000 µg/mL stock solution into a 100 mL volumetric flask and bring to volume with methanol. Maintain refrigerated up to 365 days when not needed in the laboratory.

Fortification Standard Solution, 0.1 µg/mL (100 ng/mL)

Pipet 1.0 mL of the 10 µg/mL working solution into a 100 mL volumetric flask and bring to volume with methanol. Maintain refrigerated up to 365 days when not needed in the laboratory.

Fortification Standard Solution, 0.03 µg/mL (30 ng/mL)

Pipet 0.3 mL of the 10 µg/mL working solution into a 100 mL volumetric flask and bring to volume with methanol. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 100 ng/mL

Pipet 1.0 mL of the 10 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 50 ng/mL

Pipet 0.5 mL of the 10 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 10 ng/mL

Pipet 0.1 mL of the 10 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 5 ng/mL

Pipet 0.05 mL of the 10 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 2 ng/mL

Pipet 0.2 mL of the 1 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 1 ng/mL

Pipet 0.1 mL of the 1 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory.

Calibration Standard Solution, 0.5 ng/mL

Pipet 0.05 mL of the 1 µg/mL working solution into a 100 mL volumetric flask and bring to volume with 25/75 (v/v) methanol/DI water. Maintain refrigerated up to 365 days when not needed in the laboratory

II. Reagents and Reagent Solutions

A. Reagents

Acetic acid, glacial, reagent grade
Acetonitrile, HPLC grade
Methanol, HPLC grade
Deionized (DI) water
6N Hydrochloric acid, reagent grade (used to prepare 1N HCl below)
0.1N Hydrochloric acid, reagent grade
1.0N Hydrochloric acid, prepared internally or purchased from a commercial source.

B. Reagent Solutions

1N Hydrochloric Acid: Add 400 mL of 6N HCl to 2000 mL of DI water and mix well. The solution is stored ambient for up to 365 days.

25/75 (v/v) Methanol/DI Water

Combine 250 mL of methanol with 750 mL of DI water. The solution is stored ambient for up to 365 days.

0.01% (v/v) Acetic Acid

Pipet 0.1 mL of glacial acetic acid into 1,000 mL of DI water and mix. The solution is stored ambient for up to 180 days.

III. Laboratory Equipment

Balance, analytical, capable of weighing to the nearest 0.1 mg
Centrifuge tubes, 50 mL, polypropylene
Culture tubes, 15 mL, glass with PTFE-lined screw caps
SPE extraction manifold with vacuum pressure gauge pump
SPE Columns, Bakerbond Octadecyl (C18), 6 mL/1 g, cat. No. 7020-07
Pipettes, air-displacement, with disposable tips
Nitrogen evaporation apparatus, N-Evap, Organomation
Vortex mixer
Volumetric flasks and pipets
Autosampler vials with caps, low volume recovery

IV. Methods

A. Sample Extraction

1. Dispense 20 mL of water sample into a 50 mL plastic centrifuge tube.
2. Fortify laboratory QC samples (20 mL) with the appropriate spiking solution.

   LOD Fortification (0.015 µg/L): Pipet 0.01 mL (10.0 µL) of the 30 ng/mL fortification standard solution into the water sample.

   LOQ Fortification (0.05 µg/L): Pipet 0.01 mL (10.0 µL) of the 100 ng/mL fortification standard solution into the water sample.

   10x LOQ Fortification (0.5 µg/L): Pipet 0.01 mL (10.0 µL) of the 1,000 ng/mL fortification standard solution into the water sample.

   20x LOQ Fortification (1 µg/L): Pipet 0.02 mL (20.0 µL) of the 1,000 ng/mL fortification standard solution into the water sample.

3. Add 2.0 mL of 1N hydrochloric acid. Cap and mix.

B. Solid-Phase Extraction

1. Place a C18 SPE column on an SPE vacuum manifold.
2. Rinse the column with 5.0 mL of acetonitrile and discard the rinse solvent.
3. Condition the SPE column with 5.0 mL of 0.1 N HCl. Do not allow the column bed to dry. Discard the conditioning solution.
4. Transfer the entire sample from Step IV.A.3 to the SPE column and draw through at approximately 1-2 mL/minute using a vacuum if gravity flow is inadequate to achieve the approximate flow rate of 1-2 mL/minute. Discard the column effluent.
5. Add 2.0 mL of 0.1 N HCl to the SPE column and draw through at approximately 1-2 mL/minute. Discard the column effluent.
6. Use vacuum (-10 inches Hg) to dry the SPE column for 40-45 minutes. **Do not use a vacuum pressure less than -10 inches Hg and do not exceed the 45 minute drying time or analyte loss may occur.**
7. Elute 6-CPA into a clean 15 mL glass culture tube with 5.0 mL of acetonitrile using a flow rate of approximately 1 mL/minute.
8. Once the column stops dripping, apply vacuum to collect the remaining eluent from the SPE column.
9. Evaporate the acetonitrile eluate just to dryness under a stream of nitrogen (N Evap). A heating block temperature setting of 35°C may be used. **Do not exceed the 35°C heating block temperature and do**

not over dry or analyte loss will occur. Evaporating the solvent to less than 100 µL then manually taking to dryness is recommended.

10. Redissolve the dried sample residue by pipetting 0.5 mL of 25/75 (v/v) methanol/DI water into the culture tube.
11. Vortex mix for about 10 seconds at a speed adequate to thoroughly wash the sides of the culture tube.
12. Transfer the sample solution to a low volume recovery autosampler vial and cap. Store refrigerated if LC-MS/MS analysis cannot be initiated the same day.

C. LC-MS/MS Analysis

The following LC-MS/MS parameters are used to determine 6-CPA concentrations. The parameters may be modified to achieve adequate chromatographic resolution or detector sensitivity and selectivity. The actual parameters used are documented with each analysis sequence.

LC System: Acquity UPLC, Waters Corporation
MS Detector: Acquity TQ, Waters Corporation
Column: HSS T3, 1.8 µm, 2.1 x 100 mm, Waters Corporation
Column Temperature: 40°C
Injection Volume: 5.0 µL

Mobile Phase: A: 0.01% (v/v) Acetic Acid in DI Water
B: Methanol

Gradient Program:

Time (minutes)	%A	%B	Curve	Flow (mL/minute)
0.00	95	5	1	0.20
0.10	95	5	1	0.20
4.00	30	70	6	0.20
4.50	30	70	1	0.20
4.51	5	95	1	0.40
5.50	5	95	1	0.40
5.51	95	5	1	0.40
6.50	95	5	1	0.40
6.51	95	5	1	0.20

Divert flow to waste from 0.00 to 3.80 minutes. Direct flow to the MS detector from 3.80 to 5.00 minutes. Divert flow to waste again after 5.00 minutes.

MS Source: Electrospray Ionization
Source Temperature: 120-130°C
Desolvation Temperature: 400°C
Nebulizer Gas (N_2) Flow: 550 L/hour
Cone Gas (N_2) Flow: 30 L/hour
Spray Voltage: 2.8 kV
Scan Type: Multiple Reaction Monitoring (MRM)
MRM Program: The following MRM transitions are collected from 4.00 to 5.00 minutes post-injection.

Precursor Ion (m/z)	Product Ion (m/z)	Dwell (s)	Cone (V)	Collision Energy
156 (Q)	112	0.400	20	10
158 (C)	114	0.400	20	10

(Q) = Quantitation
(C) = Confirmatory

Expected retention time: Approximately 4.65 minutes

Calibration standard solutions ranging in concentration from 0.5 – 100 ng/mL are injected at least once over the course of the analysis sequence, interspersed with sample solutions. The instrument data system is used to construct a linear regression external standard calibration curve of the calibrant peak areas (y-axis) vs. the standard concentrations in ng/mL (x-axis). A weighting factor of 1/x is used. The curve origin is not included in the linear regression equation.

D. Calculations

The amount found (ng/mL) of analyte is calculated from the linear regression equation:

$$y = mx + b$$

Where:

y = (Peak area)
m = Slope of linear regression equation
x = (Concentration in ng/mL)
b = y-intercept of linear regression equation

The linear regression equation is used to solve for x, which is correlated to amount found in ng/mL.
µg/L (ng/mL) Found =

$$\frac{\textit{Amount Found (ng/mL)} * \textit{Final Volume (mL)}}{\textit{Sample Volume (mL)}}$$

Where:

Final Volume = 0.5 mL
Sample Volume = 20 mL

Fortification Level (µg/L or ng/mL) =

$$\frac{\textit{Volume Spiking Soln. (mL)} * \textit{Spiking Soln. Concentration. (ng/mL)}}{\textit{Sample Volume (mL)}}$$

Recovery (%) =

$$\textit{(µg/L Found – µg/L found in unfortified control) / Fortification Level (µg/L)} * \textit{100}$$

Limit of Quantitation (LOQ): The method LOQ is 0.05 µg/L.
Limit of Detection (LOD): The method LOD is 0.015 µg/L