

STUDY TITLE

**Method Development and Validation of Pentachloronitrobenzene and Two
Metabolites in One Sediment and Two Soil Matrices**

DATA REQUIREMENT

US EPA Good Laboratory Practice Standards (40 CFR Part 160), US EPA OPPTS 860.1340:
Residue Analytical Method, and US EPA OCSP 850.6100: Environmental Chemistry Methods
and Associated Independent Laboratory Validation

Satisfactory linearity, specificity, accuracy and precision data were achieved for all three ion masses monitored, demonstrating that the ion masses may be used for quantification and/or confirmation of residues.

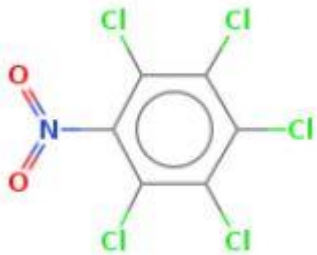
The method was successfully validated and is considered suitable for the determination of residues of pentachloronitrobenzene, pentachloroaniline, and pentachlorothioanisole in freshwater sediment, sandy loam and high organic soil over the concentration range tested.

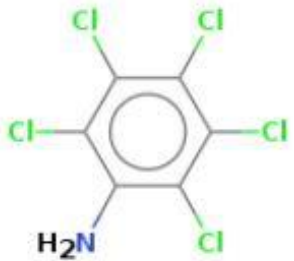
2. OBJECTIVE

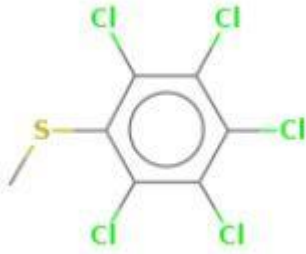
The objective of this study was to validate an analytical method for the determination of pentachloronitrobenzene and two metabolites, pentachloroaniline and pentachlorothioanisole, in freshwater sediment, sandy loam and high organic soil according to EPA Good Laboratory Practice Standards (40 CFR Part 160), US EPA OPPTS 860.1340: Residue Analytical Method, and US EPA OCSP 850.6100: Environmental Chemistry Methods and Associated Independent Laboratory Validation.

3. TEST ITEM

Common name:	Pentachloronitrobenzene (PCNB)
Chemical name (IUPAC):	Pentachloronitrobenzene
CAS number:	82-68-8
Molecular formula:	C ₆ Cl ₅ NO ₂

Molecular Weight:	295.335
Molecular structure:	
Batch number:	851201
Purity:	99.7%
Date of analysis:	January 31, 2017
Expiry date:	January 30, 2027
Storage conditions:	Store at room temperature under dry conditions

Common name:	Pentachloroaniline (PCA)
Chemical name (IUPAC):	2,3,4,5,6-Pentachloroaniline
CAS number:	527-20-8
Molecular formula:	C ₆ H ₂ Cl ₅ N
Molecular Weight:	265.352
Molecular structure:	
Batch number:	LB50O127
Purity:	98.1%
Issue date:	April 4, 2020
Expiry date:	Not Given [Assigned for one year.]
Purity:	96.2%
Storage conditions:	Store at room temperature under dry conditions

Common name:	Pentachlorothioanisole (PCTA)
Chemical name (IUPAC):	Pentachlorothioanisole
CAS number:	1825-19-0
Molecular formula:	C ₇ H ₃ Cl ₅ S
Molecular Weight:	296.429
Molecular structure:	
Batch number:	FCZ01
Purity:	99.8%
Certificate of analysis:	April 17, 2020
Expiry date:	Not Given [Assigned for one year.]
Storage conditions:	Store at room temperature

4. TEST SYSTEMS

The test system was sourced locally to the test facility as well as the state of Georgia, USA. The samples were frozen prior to homogenization with dry ice and allowed to sublime overnight in the freezer before use.

All samples were stored in a freezer at $\leq -20^{\circ}\text{C}$ when not in use.

5. METHOD VALIDATION

The method was developed by Battelle in the Norwell, Massachusetts laboratory, and was validated in terms of linearity, specificity, accuracy, and precision. The LOQ was established and the LOD was set at the lowest calibration standard of the method. Matrix effects and stability of the analytes in solvent and extract were also investigated.

The control samples were fortified as described in the following table:

Matrix	Untreated Control	Replicates at LOQ Fortification Level	Replicates at LOQ × 10 Fortification Level	Reagent Blank
Freshwater Sediment	2	7 at 0.01 mg/kg	7 at 0.1 mg/kg	1
Sandy Loam	2	7 at 0.01 mg/kg	7 at 0.1 mg/kg	1
High Organic Soil	2	7 at 0.01 mg/kg	7 at 0.1 mg/kg	1

5.1. Linearity

The linearity was investigated with matrix-matched solutions of PCNB, PCA, and PCTA prepared in a range of 3.0 ng/mL to 120 ng/mL (equivalent to 0.0030 to 0.120 mg/kg in samples).

5.2. Accuracy

The accuracy was determined from the analysis of seven replicates of fortified control samples at the LOQ and seven replicates of fortified control samples at 10 x LOQ.

5.3. Precision

The precision of the method was determined by measuring the relative standard deviation at each fortification level from replicates of soil and sediment samples spiked with PCNB, PCA, and PCTA at LOQ and at 10 x LOQ.

5.4. Specificity

Specificity was confirmed by use of the GC/MS which is a highly specific technique. One reagent blank and two untreated soil or sediment samples were analyzed for each matrix to demonstrate that no interferences greater than 30% of the LOQ were present at the retention times of the analytes.

5.5. Confirmation

The parent ion and two fragment ions were monitored for each target analyte, one for quantification purposes and two for confirmation purposes. Accuracy and precision were reported for all three ions.

5.6. Matrix Effects

Matrix effects were assessed at the LOQ and 10 x LOQ by comparing the peak area response between solvent solutions and matrix-matched calibration solutions prepared at the same concentrations.

5.7. Stability

One stock solution containing all analytes at 0.1 µg/mL was prepared and stored at 4°C for a period of 56 days and 70 days. After each storage period, the stored stock solutions were analyzed and a newly prepared stock solution containing all analytes was prepared at a concentration of 0.1 µg/mL and analyzed.

Matrix matched calibration solutions were prepared and analyzed over a concentration range of 0.003 µg/mL to 0.12 µg/mL and stored for a period of 16 day and 30 days. Following each storage period, new matrix matched calibration standards were prepared and analyzed with the stored calibration solutions.

Final sample extracts, at the 10xLOQ fortification level, were stored at a nominal temperature of 4°C for up to 36 days for freshwater sediment, sandy loam and high organic soil extracts and re-analyzed against a fresh set of matrix-matched calibration solutions to assess analyte stability in the presence of matrix.

5.8. Limit of quantification (LOQ) and Limit of detection (LOD)

The limit of quantification (LOQ) was set at 0.01 mg/kg for pentachloronitrobenzene, pentachloroaniline, and pentachlorothioanisole in freshwater sediment, sandy loam and high organic soil matrices. The limit of detection (LOD) was set at 0.003 mg/kg for pentachloronitrobenzene, pentachloroaniline, and pentachlorothioanisole in freshwater sediment, sandy loam and high organic content soil (equivalent to the lowest calibration point).

Satisfactory linearity, specificity, accuracy and precision data were achieved for all three ion masses monitored, demonstrating that the ion masses may be used for quantification and/or confirmation of residues.

6. EXPERIMENT AND REAGENTS

6.1. Equipment and Reagents

See **Appendix 4** for full details of all equipment, consumables, reagents, and prepared solutions.

6.2. Sample Preparation and Processing

Samples were sourced locally to the test facility as well as the state of Georgia, USA. The samples were frozen prior to homogenization with dry ice and allowed to sublime overnight in the freezer before use.

6.3. Standards, Fortifications, and Calibrations

Stock solutions

Individual stock solutions of PCNB, PCA, and PCTA, containing 500 µg/mL, were prepared by dissolving 5 mg of the reference item in 10 mL of acetonitrile. The stock solutions were prepared separately and then combined for fortification and calibration solutions.

Fortification Solutions

A combined fortification solution, containing PCNB, PCA, and PCTA at 20 µg/mL, was prepared by diluting appropriate amounts of the individual stock solutions with acetonitrile.

A fortification solution, containing PCNB, PCA, and PCTA at 2 µg/mL, was prepared by diluting appropriate amounts of the combined fortification solution with acetonitrile.

Fortifications

Fortification Standard Concentration (µg/mL)	Fortification Volume (µL)	Weight of Sample (g)	Fortification Level (mg/kg)
1	50	10	0.01 (LOQ)
10	50	10	0.1 (10 × LOQ)

Calibration

Intermediate solutions, containing PCNB, PCA, and PCTA at 1.2, 1.0, 0.6, 0.3, 0.1, 0.06 and 0.03 µg/mL, were prepared by diluting appropriate amounts of the combined stock solutions with acetonitrile. All standard solutions were stored at < 6°C but above freezing when not in use.

Calibration solutions in matrix were prepared at concentrations of 120, 100, 60, 30, 10, 6, and 3 ng/mL by diluting appropriate amounts of the 1.2, 1.0, 0.6, 0.3, 0.1, 0.05, and 0.03 µg/mL intermediate solutions with untreated sample extracts after d-SPE cleanup.

Stock, calibration and fortification solution preparation procedures are presented in **Table 1**.

7. ANALYTICAL PROCEDURE

A 10 g sample was weighed into a 50 mL Falcon tube for extraction. Recovery efficiency samples were fortified as necessary at the LOQ and 10xLOQ. 10 mL of acetonitrile was added, the samples were capped, and extracted on the GenoGrinder™ for 15 minutes at 1,500 RPM. Samples were removed from the GenoGrinder™ and the contents of one Q-Sep Q110 QuEChERS packet was added to each sample. Samples were capped, shaken, and extracted on the GenoGrinder™ for 15 minutes at 1,500 RPM. Extraction was followed by centrifugation at 4,000 RPM for 10 minutes. A 1 mL aliquot of the acetonitrile layer was transferred into the dispersive SPE tube (Q-Sep QuEChERS d-SPE), at this time, additional aliquots of the untreated controls were transferred to d-SPE for the matrix-matched calibration. The d-SPE tubes were shaken for 2 minutes followed by centrifugation at 3,500 RPM for 5 minutes. An aliquot of each extract was then transferred for analysis.

A flowchart of the procedure is presented in **Figure 1**.

7.1. Time Required for Analysis

For both matrices, the methodology is normally performed with a batch of 17 samples, one person can complete the processing of 17 samples in twelve hours, including the preparation of calibration standards and processing of data.

This does not include the time required for GC/MS analysis as this can be performed overnight.

7.2. GC/MS Analysis

All samples were analyzed by gas chromatography coupled with an electronic ionization mass spectrometer (GC/MS), monitoring the parent mass ion and two ion mass fragments.

Column:	Agilent DB-5 30 m x 0.25 mm (diameter) X 0.25 µm (film thickness) or equivalent
Injection Temperature:	250 °C
Injection Volume:	4.0 µL pulsed splitless mode (30 psi until 0.5 min)
Inlet liner	4mm focus liner or equivalent
Carrier gas:	Helium
Flow Rate:	1.0 mL/minute constant flow mode
Initial Oven Temperature:	120 °C hold for 1 minute
Ramp Rate:	15 °C/minute to 275 °C hold for 4 minutes
MS Transfer Line:	300 °C
MS Quad:	150 °C
MS Source:	230 °C
Acquisition Mode:	SIM
Group 1 ions:	237, 246, 263, 265, 267, 293, 294, 295, 296, 297, and 298
Group 1 dwell time:	20
Quant ions and Retention Times:	PCNB – 295, 297, 293, RT approximately 9.57 PCA – 265, 267, 263, RT approximately 10.2 PCTA – 295, 294, 246, RT approximately 10.8

7.3. CALIBRATION AND CALCULATIONS

Calibration curves were obtained from seven matrix calibration solutions containing PCNB, PCA, and PCTA, covering, at a minimum, a range of 3.0 ng/mL to 120 ng/mL (equivalent to 0.0030 to 0.120 mg/kg in samples). The curve was calculated by linear regression, with a weighting factor of 1/x. Correlation coefficients, r, greater than 0.99, demonstrating acceptable performance in terms of linearity.

The concentrations of PCNB, PCA, and PCTA in the final sample extract was made by comparison to the calibration curve using the EnviroQuant software.

The concentration in each sample was calculated as follows:

$$\text{Residue (mg/kg)} = \frac{M_p \times V_{\text{end}} \times (V_1 / V_2)}{G}$$

Where:

- M_p = analyte concentration in the final sample extract (µg/mL)
- V_{end} = volume of final extract for GC determination (1 mL)
- V_1 = volume of initial extraction (10 mL)
- V_2 = volume of extract removed for cleanup (1 mL)
- G = sample weight (10 g)

The limit of quantification (LOQ) was 0.01 mg/kg for pentachloronitrobenzene, pentachloroaniline, and pentachlorothioanisole in soil and sediment matrices. The limit of detection (LOD) was set at 0.003 mg/kg for pentachloronitrobenzene, pentachloroaniline, and pentachlorothioanisole in freshwater sediment, sandy loam and high organic content soil (equivalent to the lowest calibration point).

Satisfactory linearity, specificity, accuracy and precision data were achieved for all three ion masses monitored, demonstrating that the ion masses may be used for quantification and/or confirmation of residues.

9. CONCLUSIONS

The method was successfully validated and is considered suitable for the determination of residues of PCNB, PCA, and PCTA in freshwater sediment, sandy loam and high organic soil over the concentration range tested (0.003 – 1.2 mg/kg).

10. DEVIATIONS

A deviation from the study plan was noted for standard stability checks. The protocol listed intervals of 14 and 28 days for stock solutions. Stock solutions were verified as against freshly prepared stock solutions at 56 and 70 days. This deviation had no impact on the study.

11. SAFETY

Prior to working with test items, solvents, and reagents, analysts must familiarize themselves with the associated hazards by reviewing the SDS for all chemicals used in the methods. At all times, appropriate PPE must be used, including, but not limited to gloves, lab coats, and safety glasses. All transferring of solvents must be conducted in fume hood or other ventilated area. When working with instrumentation, care should be taken to avoid risk of electrical shock or burns related to heated areas on the instruments.

12. REFERENCES

1. U.S. EPA Residue Chemistry Test Guidelines, OPPTS 860.1340 Residue analytical Method, EPA 712-C-96-174, August 1996
2. U.S. EPA Ecological Effects Test Guidelines, OCSP 850.6100 Environmental Chemistry Methods and Associated Independent Laboratory Validation, EPA 712-C-001, January 2012

TABLES
Table 1: Typical Preparation of Calibration Standard Solutions in Matrix and Solvent
Stock solutions

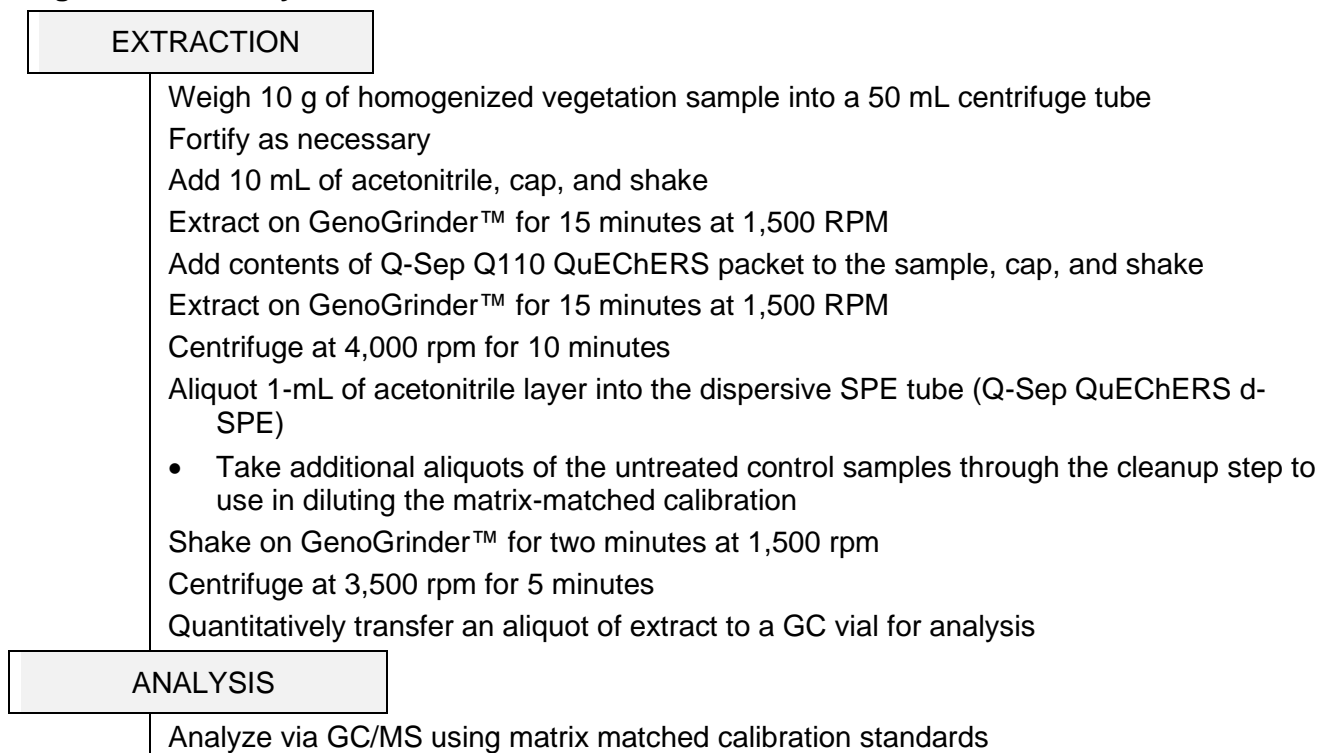
Standard ID	Purity (%)	Target Amount (mg)	Final Volume (mL)	Final Concentration(ug/mL)
Pentachloronitrobenzene (PCNB) Stock	99.7	5.02	10	500
Pentachloroaniline (PCA) Stock	97.0	5.15	10	500
Pentachlorothioanisole (PCTA) Stock	98.0	5.10	10	500

Fortification and Working Solutions

Standard ID	Stock ID	Stock Amount (mL)	Stock Concentration (ug/mL)	Final Volume (mL)	Final Concentration (ug/mL)
MLOQ Fortification	PCNB Stock	0.400	500	10	20
	PCA Stock	0.400	500		20
	PCTA Stock	0.400	500		20
LOQ Fortification	MLOQ Fortification	1.000	20	10	2.0
			20		2.0
			20		2.0
Working Solution 1	PCNB Stock	2.000	500	10	100.0
	PCA Stock	2.000	500		100.0
	PCTA Stock	2.000	500		100.0
Working Solution 2	Working Solution 1	1.000	100	10	10.00
			100		10.00
			100		10.00

Calibration Solutions

Calibration Standard Concentration (ng/mL)	Working Solution Used (µg/mL)	Aliquot of working solution (mL)	Aliquot of Matrix (mL)	Final Volume (mL)	Sample Equivalent (mg/kg)
3.00	0.030	0.030	0.270	0.300	0.003
6.00	0.060	0.030	0.270	0.300	0.006
10.0	0.100	0.030	0.270	0.300	0.010
30.0	0.300	0.030	0.270	0.300	0.030
60.0	0.600	0.030	0.270	0.300	0.060
100	1.00	0.030	0.270	0.300	1.00
120	1.20	0.030	0.270	0.300	1.20

Figure 1: Analytical Method Flow Chart

Appendix 4: Equipment, Consumables, Reagents and Prepared Solutions

Equipment	Manufacturer / Supplier
GenoGrinder™ 2010 or equivalent	Spex Sample Preparation
Vortex mixer	Benchmark
Pipettes	Rainin
Balances: Mettler XS-205 Mettler ML4002E	Mettler Toledo Mettler Toledo
Eppendorf Centrifuge 5810	Eppendorf

GC/MS:	
7890A GC 5975C Electronic Ionization MS Autosampler or equivalent	Agilent
GC Column: DB-5 30 m x 0.25 mm (diameter) X 0.25 µm (film thickness) or equivalent	Agilent
Helium	AirGas

Consumables	
Polypropylene centrifuge tubes	Fisher Scientific
Glass GC Vials	Fisher Scientific
Crimp seals	Microliter

Reagents	
Acetonitrile (Optima)	Fisher Scientific
Q-Sep Q110 dispersive SPE packets (4 g MgSO ₄ , 1 g NaCl, 1 g trisodium citrate dihydrate, and 0.5 g disodium hydrogencitrate sesquihydrate) or equivalent	Restek (Part No. 26236)
Q-Sep QuEChERS d-SPE (Restek part No. 26242, or equivalent) – vial contains 150 mg MgSO ₄ and 50 mg C18 or equivalent	Restek (Part No. 26242)
4 mm focus liner (GC inlet liner) or equivalent	Restek

Appendix 5: Abbreviations

Abbreviation	Definition
CAS	Chemical Abstracts Service
CFR	Code of Federal Regulations
CoA	certificate of analysis
conc.	concentration
DF	Dilution factor
d-SPE	Dispersive Solid Phase Extraction
EPA	Environmental Protection Agency
GC	Gas Chromatogram
GLP	Good Laboratory Practice(s)
i.d.	internal diameter
IUPAC	International Union of Pure and Applied Chemistry
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
MeCN	acetonitrile
MS	mass spectrometry
n	number of data
NA	not applicable
ND	not detected
OCSP	Office of Chemical Safety and Pollution Prevention
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PCA	Pentachloroaniline
PCNB	Pentachloronitrobenzene
PCTA	Pentachlorothioanisole
QAU	quality assurance unit
R (or r)	correlation coefficient
RPM	Rotations per minute
RSD	relative standard deviation
SD	standard deviation
STD	standard