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Immunoassay Method for the Determination of Pentachlorophenol in Soil and Sediment

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Pentachlorophenol (PCP) does not occur naturally but is manufactured for several uses. Pure PCP is synthesized as colorless crystals. Impure PCP (the form usually found at hazardous waste sites) is dark gray to brown and exists as dust, beads, or flakes. PCP was widely used as a pesticide and wood preservative. Since 1984, the purchase and use of PCP have been restricted to certified applicators and is no longer available to the general public. Wilson et al. found PCP in samples of air, water, dust, soil, and food (2001 and 2002). The compound can enter the microenvironment through evaporation from treated wood surfaces, industrial spills, and inappropriate disposal at hazardous waste sites. The acceptable daily intake (ADI) for PCP is 0.03 mg/kg/day for humans (Choudhury et al. 1986). At this PCP level, no deleterious effects are seen based on extended daily exposure.

Immunoassay detection methods are generally sensitive, specific, and cost effective (Van Emon et al. 1992). They facilitate a high sample throughput and can be used as either qualitative or quantitative tools. Several immunoassay methods have been developed for the detection of environmental pollutants in various sample media (Van Emon et al. 2000, 2001a and 2001b, Lopez-Avila et al. 1996). Immunoassy method performance data have been reported for real-world samples for several ELISA testing kits (Chuang et al. 1998, 2003 and 2004). An immunoassay method evaluated for monitoring PCP in drinking water, surface water and ground water provided favorable results when compared to GC analysis (Van Emon et al. 1992). This previous study was conducted under the U.S. EPA Superfund Innovative Technology Evaluation (SITE) program. Another SITE project evaluated three commercially available PCP ELISA testing kits as field screening tools for monitoring PCP in soil and water (Van Emon et al. 1995). The antibodies utilized in these methods were not highly selective to PCP and had cross reactivities for tetrachlorophenols, trichlorphenols, and other structurally related chemicals. For example, the antibody in one testing kit exhibited 50% cross activity for 2,3,5,6-tetrachlorophenol.

We report here, an evaluation of a prototype ELISA (magnetic particle format) testing kit (Abraxis, Warminster, PA) for monitoring PCP in soil and sediment samples. The polyclonal antibody employed in this PCP ELISA testing kit (Noguera et al. 2002), showed only a 9.4% cross reactivity for 2,3,5,6-tetrachlorophenol which is lower than previously reported ELISA methods for PCP (Van Emon et al. 1992 and 1995). Two extraction techniques, accelerated solvent extraction (ASE) and a simple shaking procedure were also evaluated for coupling with the ELISA. Sample extracts were analyzed by both gas chromatography/ mass spectrometry (GC/MS) and ELISA. Solid phase extraction (SPE) cleanup methods for ASE sample extracts were evaluated for ELISA to minimize the interferences of other compounds present in soil and sediment. The

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GC/MS method always requires a derivatization step, with an additional cleanup procedure necessary for complex sample matrices. Analytical results were compared for the measurement of PCP in real-world soil and sediment samples to determine the suitability of the magnetic particle immunoassay as a qualitative or quantitative method for determining PCP in soil and sediment samples.

MATERIALS AND METHODS

Environmental soil and sediment samples were obtained from an exposure field study and an EPA Superfund Innovative Technology Evaluation (SITE) dioxin demonstration study (Chuang et al. 2003 and EPA 2005). A total of 48 residential back yard soil samples and 15 SITE sediment and soil samples were analyzed in this study. Accelerated solvent extraction (ASE) of PCP from soil or sediment was performed using a Dionex ASE 200 system. A known amount (2 to 10 g) of the sample was mixed thoroughly with an equal amount of clean Ottawa sand prior to placement into the extraction cell. If the soil or sediment sample contained an excess amount of moisture, the sample was mixed with Extrelut (1 to 2 g, EM Scientific) prior to ASE. The bottom of the extraction vessel was covered with a cellulose filter, followed by a layer of the Ottawa sand. The sample mixture was next placed in the extraction vessel and additional sand was added to completely fill the vessel. For spiked samples, a known amount of a PCP standard solution was spiked onto the sample mixture. The initial extractions were performed with methanol at 2000 psi and 125°C for 3 cycles of 5 minutes with 100% flush. Two additional extraction solvents, dichloromethane (DCM) and acetone were evaluated for extraction efficiency of randomly selected samples and analyzed by GC/MS. The methanol extracts contained more co-extracted interferences for GC/MS. The DCM and acetone extracts were comparable based on GC/MS analysis. Thus, DCM was used for the remainder of the samples since it could more easily be removed by evaporation for the methanol exchange step necessary for the ELISA. A simple shaking method easily adapted to field operations was used for an inter-laboratory comparison for the ELISA. An aliquot (10 g) of each SITE soil and sediment sample was extracted with 20 mL of methanol by a mechanical shaker for 1 hour for ELISA analysis.

The ASE DCM extract was treated with anhydrous Na_2SO_4; filtered through a muffled quartz fiber filter; and concentrated to a final volume of 10 mL. The extract was divided into two portions: one for GC/MS and one for ELISA analysis. For GC/MS, the extract was further concentrated to 1 mL; spiked with 10 µL of the internal standard dicamba-d_3 (10 ng/µL) and 50 µL of methanol; and methylated with diazomethane. For the ELISA analysis, the DCM extract was simply solvent exchanged into methanol. A known amount (100 µL) of the methanol extract was diluted with 900 µL of assay buffer (1 to 10 dilution) prior to ELISA. If the ELISA results were outside the calibration curve, further dilutions were performed and samples were reassayed. To remove potentially cross-reacting PCBs and chlorobenzenes prior to ELISA, selected sample extracts were processed through a silica SPE column. Each SPE column (1g of silica) was conditioned with 6 mL of DCM followed by 6 mL of hexane. The DCM sample extract was solvent exchanged into 1 mL of hexane and applied to the SPE column. The first fraction was eluted with 18 mL of hexane (F1) and the second fraction was eluted with 24 mL of DCM (F2). The F2 fraction was solvent exchanged into methanol for ELISA analysis.

The ELISA analysis was performed using the magnetic particle PCP ELISA prepared by Abraxis (Warminster, Pennsylvania). An aliquot (200 µL) of each calibration solution (0, 1.0, 2.5, 10, 25, 50, and 100 ng/mL), control solution (10 ng/mL), and diluted sample extract was carefully placed in the bottom of individually labeled test tubes. The test tubes were secured to the test tube rack of a magnetic separation system. An aliquot (250 µL) of a PCP enzyme (horseradish

peroxidase) conjugate and an aliquot (500 µL) of the PCP antibody-coupled paramagnetic particles were added to the inside wall of each tube and allowed to flow to the bottom. This solution was mixed by vortexing (Vortex®, Scientific Industries, Bohemia, NY) and allowed to incubate at room temperature for 60 min. The test tube rack was then affixed to the magnetic base. The samples were allowed to stand for 2 min for the magnetic particles to separate and adhere to the wall of the tube. The rack assembly was inverted over a waste container to decant unbound reagents. The rims of the test tubes were gently blotted on several layers of clean paper towels. An aliquot (1 mL) of a buffered washing solution was added down the inside wall of each test tube. The solution was allowed to stand for 2 min at room temperature and was decanted as before. This washing step was repeated. The magnetic separation rack was then removed from the magnetic base. An aliquot (500 µL) of the color reagent was added down the inside wall of each tube and mixed by vortexing. The solution was allowed to incubate for 20 min at room temperature. At the end of the incubation period, an aliquot (500 µL) of an acidic stopping solution was added down the wall of each tube without mixing. Each test tube was analyzed on a RPA-I RaPID photometric analyzer (SDI, Newark, DE) within 15 minutes of the addition of stopping solution.
Sample extracts and standard solutions were analyzed by 70 eV electron impact GC/MS. The GC/MS method was performed similar to that described in other studies with monitoring ions specific for the target analytes (Chuang et al. 2004). Peaks monitored were the molecular ion peaks and their associated characteristic fragment ion peaks (278, 280, 282 for PCP and 237 and 239 for dicamba-d_3).

RESULTS AND DISCUSSION

Sediment and soil samples from the dioxin SITE demonstration study were extracted using ASE with either DCM or acetone then analyzed by GC/MS. Levels of PCP ranged from 0.005 to 2600 µg/g (ppm) when DCM was used as the extraction solvent and from 0.004 to 2600 ppm when acetone was the extraction solvent. Thus, similar PCP concentrations were obtained for a given sample for both DCM and acetone. Since the boiling point of DCM is lower than acetone and it is easily solvent exchanged into methanol for ELISA, DCM was used as the extraction solvent for the subsequent experiments. Selected SITE sediment and residential yard soil samples were spiked with PCP (400 ppb for sediment and 5-15 ppb for yard soil); extracted with DCM using ASE and analyzed by GC/MS. Quantitative recoveries (86-103%) were obtained for these spiked sediment and soil samples. Recoveries of spiked PCP in two of the sediment samples were less than 50% when shaking was used as the extraction method followed by ELISA. The shaking method is simple but can not quantitatively remove PCP from sediment samples. Extraction efficiency of the shaking method should be documented when used for field operations.

In this study, GC/MS was treated as the standard method and the data derived from the GC/MS method was use to evaluate the data derived from the ELISA. The GC/MS analysis acceptance criteria were: (1) <20% in relative standard deviation (RSD) value of the average response factor of PCP to the internal standard, dicamba-d_3; (2) <20% in percent difference (%D) value of the measured and expected value of the standard solution; and (4) 80-120% recovery of a matrix spiked sample. The GC/MS data met all of these QA requirements. Forty yard soil samples and 15 SITE sediment and soil samples were extracted with ASE using DCM and analyzed by GC/MS. Levels of PCP ranged from <0.001 to 2600 (ppm) in the soil and sediment samples. Quantitative recoveries were obtained in the spiked soil samples (96±8.9%). The overall precision of the GC/MS method was within ±20%, with an accuracy greater than 80%.

The organic solvent tolerance for the PCP ELISA was investigated. Aqueous standard solutions for PCP were prepared in 5%, 10%, and 20% methanol and

5%, 10% and 20% acetonitrile (ACN). These standard solutions were assayed simultaneously with standard solutions prepared in 100% water. The results demonstrated that the PCP ELISA could withstand up to 20% methanol without adversely affecting assay performance. The ELISA tolerance to ACN was much lower. Therefore, the calibration standard solutions and control standard solutions were prepared in 10% methanol in water for the ELISA. The soil and sediment samples were solvent exchanged from DCM into methanol and diluted to 10% methanol in water prior to the ELISA.

A comparison of the PCP ELISA and the two extraction procedures was performed by analyzing different aliquots of the selected soil and sediment samples in two different laboratories. Table 1 summarizes the ELISA results obtained from the two laboratories. Overall, there was a good correlation between the two laboratories for this set of samples. The linear regression equation was (ELISA Lab #1) = 90.625 + 0.5362 x (ELISA Lab #2) with a correlation coefficient of 0.99 for the linear regression line. The slope of 0.5362 demonstrates that the ELISA-derived concentrations from Lab #1 are approximately twice of those from Lab #2. This difference was partly attributed to differences in sample extraction procedures (ASE for Lab #1 and shaking for Lab #2). The ASE extraction method is more vigorous than the shaking method and offers better extraction efficiency for PCP.

All the data discussed below were generated from Lab#1 using ASE as the extraction method. Each soil and sediment sample was quantitated using a calibration curve generated from duplicate analyses of the standard solutions at six concentration values (0, 1.0, 2.5, 10, 25, 50, and 100 ng/mL). The acceptance criteria established for the PCP ELISA were: (1) the %CV (coefficient of variance) of the absorbance values of the standard solution be less than 10%, where %CV is based on the ratio of the relative

Table 1. Summary of PCP ELISA data from two laboratories.

Summary Statistics	Lab #1, µg/g	Lab #2, µg/g
Sample Size	15	15
Minimum	0.70	0.41
25th Percentile	1.8	0.78
50th Percentile	2.7	1.9
75th Percentile	237	286
95th Percentile	9404	5802
Maximum	21000	11200

a correlation coefficient greater than 0.99 for the calibration curve is obtained. All the results from the RaPID assays reported here met the acceptance criteria. The percent difference (%D) of the derived concentrations of the standard solution from duplicate analyses ranged from 0 to 39.9%. The %D values for all but one standard solution (1.0 ng/mL from duplicate assays) were within 30%. The measurement of a control solution (10 ng/mL) after analysis of the calibration standards was performed for quality assurance (QA). The average measured value for the control solution for each ELISA analysis of the ten assay sets was 11.7 ±1.7 ng/mL (15%). These measured values were in agreement with the expected value (10.0 ng/mL). The percent relative standard deviation (%RSD) was 15% for the control solution among the ten assays from different days. Method blanks were analyzed with each sample set yielding all non-detectable values. The estimated assay detection limit determined in this study was 1.0 ng/mL.

Duplicate ELISA analyses were conducted for all soil and sediment samples. The means of the duplicate assay values were used to calculate each concentration. The %D values of the duplicate assays ranged from 0 to 39.8% for these samples. Concentrations derived from the raw extracts (without cleanup) by ELISA ranged from 0.015 to 21,000 ppm in the soil and sediment samples. Selected soil methanol extracts were diluted with diluent and spiked with 10 ng/mL of PCP and analyzed by the ELISA yielding satisfactory recoveries. Average recoveries were 137±13 % for soil sample extracts. Selected soil and sediment samples were spiked with known amounts of PCP prior to ASE to assess the overall method accuracy including extraction. Without the cleanup step, recoveries of PCP were not acceptable (well above 100%) in matrix spike samples. The over recovery of the matrix spike samples by ELISA was mainly due to the cross-reactivities of contaminants other than PCP such as chlorobenzenes, other chlorophenols, and PCBs.

The same extraction method (ASE with DCM) was used for the soil and sediment samples for both the ELISA and GC/MS methods. Thus, the discrepancies between the ELISA and the GC/MS data are essentially due to the detection techniques. Summary statistics (sample size, mean, standard deviation, minimum, and maximum) for the ELISA (without cleanup procedure) and GC/MS results are shown in Table 2. In general, the ELISA data derived from the raw extracts were two to three order of magnitude higher than the GC/MS data for all the soil and sediment samples. These results showed the importance of incorporating good analytical practices prior to ELISA detection to obtain quantitative data when comparing with a standardized quantitative method. The PCP antibody is not entirely selective to PCP and has cross-reactivity to other chemicals and contributed to higher ELISA-derived PCP concentrations. If the data quality objective of the study is to monitor PCP and related compounds, then a cleanup step is not necessary. Thus, the ELISA would be a good screening method prior to a more in-depth analysis (Van Emon et al. 1992).

Florisil and silica SPE columns were evaluated as cleanup methods for the soil and sediment extracts to remove most non-polar pollutants including chlorobenzenes and PCBs from the extracts. The GC/MS results showed that a silica SPE column cleanup method offered quantitative recoveries (85-103%) of PCP in spiked soil and sediment sample extracts while recoveries of PCP were less than 50% when Florisil SPE columns were used. Quantitative recoveries (86 to 157%) were obtained when the silica SPE cleanup procedure was employed for spiked and nonspiked sample extracts prior to ELISA. The cleanup procedure was applied to a subset of samples (8 yard soil samples and 15 SITE soil and sediment samples) and the target fraction was analyzed by ELISA. Summary statistics of the ELISA and GC/MS data for this sub set of samples are given in Table 3.

ELISA derived PCP concentrations (with the cleanup procedure) were lower than those derived from raw extracts (without cleanup procedure). This was mainly because the silica SPE column cleanup procedure removed the nonpolar pollutants such as PCBs and chlorobenzenes. However, the target fractions (F2) analyzed by ELISA could still contain other polar pollutants including mono-, di-, tri-, and tetra-chlorophenols that have cross-reactivities in the assay. Thus, the ELISA results derived from the SPE fractions were higher than the GC/MS results.

Figure 1 displays the relationship of the ELISA (with cleanup procedure) and GC/MS data. The data points (N=23) for Figure 1 were summarized in Table 3. The linear regression equation and the square of the correlation coefficient, R^2 (R^2 = 0.9999), are also given. The fitted equation is: (GC/MS PCP) = 0.7115 x (ELISA PCP) - 3.7602. Note that the slopes are not equal to 1 indicating that the results from the ELISA and the GC/MS methods are highly correlated but not identical. Additionally, the correlation was highly influenced by six out of the 23

Table 2. Summary statistics for PCP concentrations (ppm) measured by ELISA (without cleanup procedure) and GC/MS.

Summary Statistics	Yard Soil		Sediment		All Samples	
	ELISA	GC/MS	ELISA	GC/MS	ELISA	GC/MS
Sample Size	48	48	15	15	63	63
Mean	1.34	0.002	1,880	183	458	45.1
Std. Dev.	1.14	0.003	5,490	669	2770	333
Maximum	4.57	0.014	21,000	2,600	21,000	2,600
Minimum	0.015	<0.001	0.70	0.006	0.015	<0.001

Table 3. Summary statistics for PCP concentrations (ppm) measured by ELISA (without and with cleanup procedure) and GC/MS.

Summary Statistics	ELISA (no cleanup)	ELISA (cleanup)	GC/MS
Sample Size	23	23	23
Mean	1200	290	210
Standard Deviation	4500	1300	950
Maximum	21000	6400	4500
Minimum	0.14	0.039	0.00004

samples that had ELISA results greater than 3 ppm. The relationship of the ELISA and GC/MS data in these 17 samples (PCP concentrations < 3 ppm) is much weaker ($R^2 = 0.1983$) than the one including all data.

The Preliminary Remediation Goal (PRG) Table established by EPA Region 9 is based on human health toxicity values known as cancer slope factors or non-cancer reference doses (Region 9, 2004). The PRG values for PCP is 3 ppm in residential soil and 9 ppm in industrial soil. We used the 3 ppm level as the threshold level for the evaluation of the performance of the PCP ELISA. Four performance measures evaluated were sensitivity (or true positive rate), specificity (or true negative rate), positive predictive value (PPV), and negative predicative value (NPV). Table 4 shows the frequency distributions for ELISA and GC/MS measurements on the 2 x 2 tables along with the Fisher's Exact test results, and the four performance characteristics measurements for ELISA (without cleanup) versus GC/MS and ELISA (with cleanup) versus GC/MS. The performance measurements except for true positive rate are greater than 80%. The true positive rate is 33% for the raw extracts and improved to 67% when a cleanup procedure was used. Note that two of the SITE sediment samples contained over 5000 ppm of Aroclors but less than 1 ppm of PCP, however, the cleanup procedure may not have removed all the PCBs and still contributed to the ELISA response. If we excluded these two samples, the true positive rate would improve to 40% (without cleanup) and 100% (with cleanup). The true negative rate is 100% for both the raw extracts and the clean fractions. Fisher's Exact test results indicated a high degree of statistical dependence between ELISA and GC/MS data ($p<0.0001$). Thus, ELISA can be used as a screening tool for PCP in soil and sediment.

In summary, there is a positive relationship between the ELISA and GC/MS data. The ELISA derived PCP concentrations were different from and higher than the GC/MS data. The concentrations derived from the ELISA method should not be treated as absolute accurate measurements but rather as relative comparisons to

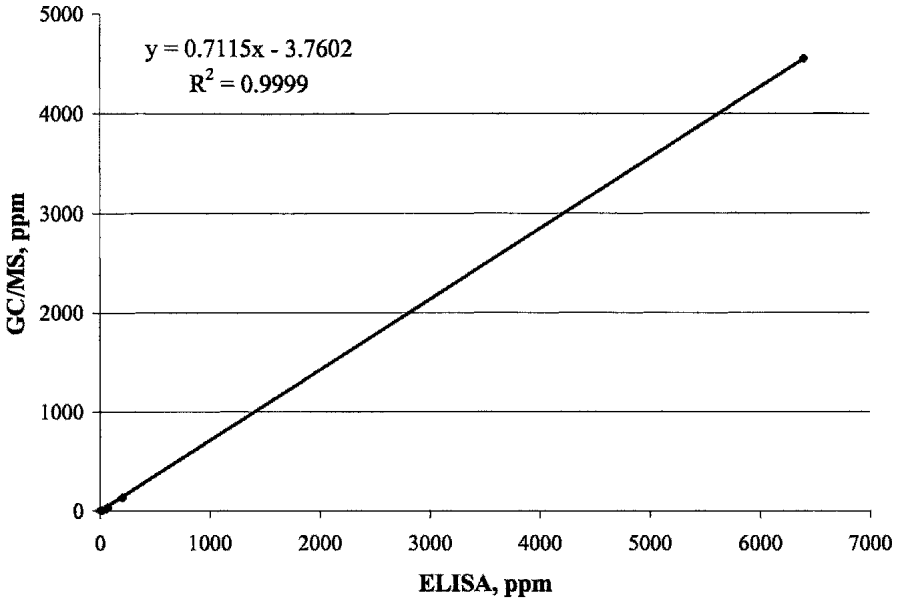

Figure 1. Correlation of the ELISA (with cleanup procedure) and GC/MS data for soil and sediment samples.

Table 4. Frequency distribution of ELISA and GC/MS results for soil and sediment.

ELISA (without cleanup)[1]	GC/MS		Total
	< 3 ppm	≥ 3 ppm	
< 3 ppm	49	0	49
≥ 3 ppm	8	4	12
Total	57	4	61
ELISA (with cleanup)[2]	GC/MS		Total
	< 3 ppm	≥ 3 ppm	
< 3 ppm	17	0	17
≥ 3 ppm	2	4	6
Total	19	4	23

[1] Fisher's Exact test: $P < 0.001$, true positive rate: 33% (4/12), true negative rate: 100% (49/49), PPV: 100% (4/4), NPV: 86% (49/57).
[2] Fisher's Exact test: $P = 0.0017$, true positive rate: 67% (4/6), true negative rate: 100% (17/17), PPV: 100% (4/4), NPV: 89% (17/19).

assist in sample ranking. With the 100% true negative rate obtained in this study, the PCP ELISA method could serve as a useful field monitoring tool in conjugation with GC/MS for the identification of soil or sediment samples above the PRG level in large scale site monitoring studies.

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