CAPE Technologies

High Performance Dioxin/Furan Immunoassay Kit

Application Note AN-007

Analysis of PCDD/Fs in soil at 500 ppt using rapid extraction and rapid one-step cleanup

Contents of this Application Note

A. Introduction

This Application Note describes a rapid immunoassay specific extraction and rapid one step cleanup to prepare soil samples for screening analysis at 500 ppt (pg/g) using the CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit. The method described here includes a much faster and simpler extraction and cleanup than typically required for GC-MS analysis. This procedure has been submitted to the US EPA for review as Method 4025. Using this method, one analyst can screen up to 20 samples per day in a facility as simple as a small mobile laboratory. This Application Note is intended to be used in conjunction with the DF1 Dioxin/Furan Immunoassay Kit and its insert (IN-DF1) and the SP1 and/or SP2 Sample Preparation Kits (12 or 60 sample). Please read this Application Note carefully as part of planning your sample preparation and analysis. Important recommendations for quality assurance samples are noted at certain points in section F. The documents cited in this Application Note are available at the CAPE Technologies web site (www.cape-tech.com).

B. Summary of Procedure

1. Add sodium sulfate to soil sample and mix. Add dimethylformamide (DMF) and extract soil sample by shaking 2 hours. Remove the supernatant DMF extract.
2. Add hexane to an aliquot of the DMF extract, then shake 15 minutes with 15% SO_3 in conc. H_2SO_4.
3. Remove the supernatant hexane and evaporate to exchange sample into water-miscible keeper solution.
4. Perform the immunoassay procedure as described in the kit insert IN-DF1.
5. Interpret the immunoassay results as described in section G of this Application Note.

C. Reagents Required

1. Anhydrous sodium sulfate, approx. 20 g per sample; reagent grade
2. Dimethylformamide (DMF), approx. 15 mL per sample; HPLC grade or better
3. Solution of 15% SO_3 in conc. H_2SO_4, approx. 2 mL per sample (fuming sulfuric acid, CASRN 8014-95-7; can be purchased from Aldrich Chemical Company as 15% [catalog number 37,561-6]; NOTE- fuming sulfuric acid can not be shipped by air; inquire about time required for shipment and plan accordingly)

4. Hexane, approx. 6 mL per sample; HPLC grade or better of mixed isomer type (CASRN 73513-42-5; this is typically 85% n-hexane with remainder methylcyclopentane and other hexane isomers; do not use n-hexane; all development and validation work for this method is based on mixed isomer hexane)

Additional reagents needed for carbon column cleanup (see section H to determine if this is required):

5. Toluene, approx. 25 mL per sample; ultrapure or residue grade (such as Burdick & Jackson) or better
6. Increase hexane (C4) by approx. 25 mL per sample
7. Increase sodium sulfate (C1) by approx. 2 g per sample

D. Equipment Required

1. Equipment for performing the DF1 Immunoassay; summarized in section G of the kit insert (IN-DF1) and described in detail in Equipment List (EL-001)
2. Balance for weighing sample (0.1 g or better readability; see Equipment List EL-002)
3. Orbital platform shaker for mixing during extraction and oxidation (see Equipment List EL-002)
4. Centrifuge with capacity for holding 40 mL vials (28 mm outside diameter x 98 mm high, flat bottoms; see Equipment List EL-002)
5. Fume hood and solvent exchange system (refer to IN-DF1, section I3; also see Equipment List EL-002)
6. Computer with Microsoft Excel (for Win97 or later, or for Mac OS9 or later) for data analysis

E. Supplies Required

1. DF1 Immunoassay Kit and supplies specified in section G of the kit insert (IN-DF1)
2. Pipet bulbs and glass Pasteur pipets for transfer of oxidized extracts
3. Pipettors and glass pipets for items C2 to C7 above
4. Glass vials (2 to 12 mL) with Teflon lined caps for storage of sample extracts
5. Glass tubes (10-15 mL) for evaporation of oxidized extracts
6. SP1 or SP2 Sample Preparation Kit (12 or 60 samples). The SP1 Kit is sufficient for most situations. The SP2 Kit may be required for cleanup of samples which are highly contaminated with oil. See section H below for more detailed discussion.

Additional supplies needed for carbon column cleanup (see section H to determine if this is required):

7. SP2 Sample Preparation Kit containing carbon mini-columns
8. SP2-ST Starter Sample Preparation Kit containing reusable hardware for manual execution of the column cleanup procedure described in steps H1-6 below
9. SP2-RK, rack for holding SP2-ST reservoirs (glass columns like 25 mL pipets; 16 mm dia. by 30 cm long)
10. Basin or other receptacle to catch waste from carbon mini-column procedure

F. Detailed Sample Preparation Procedure

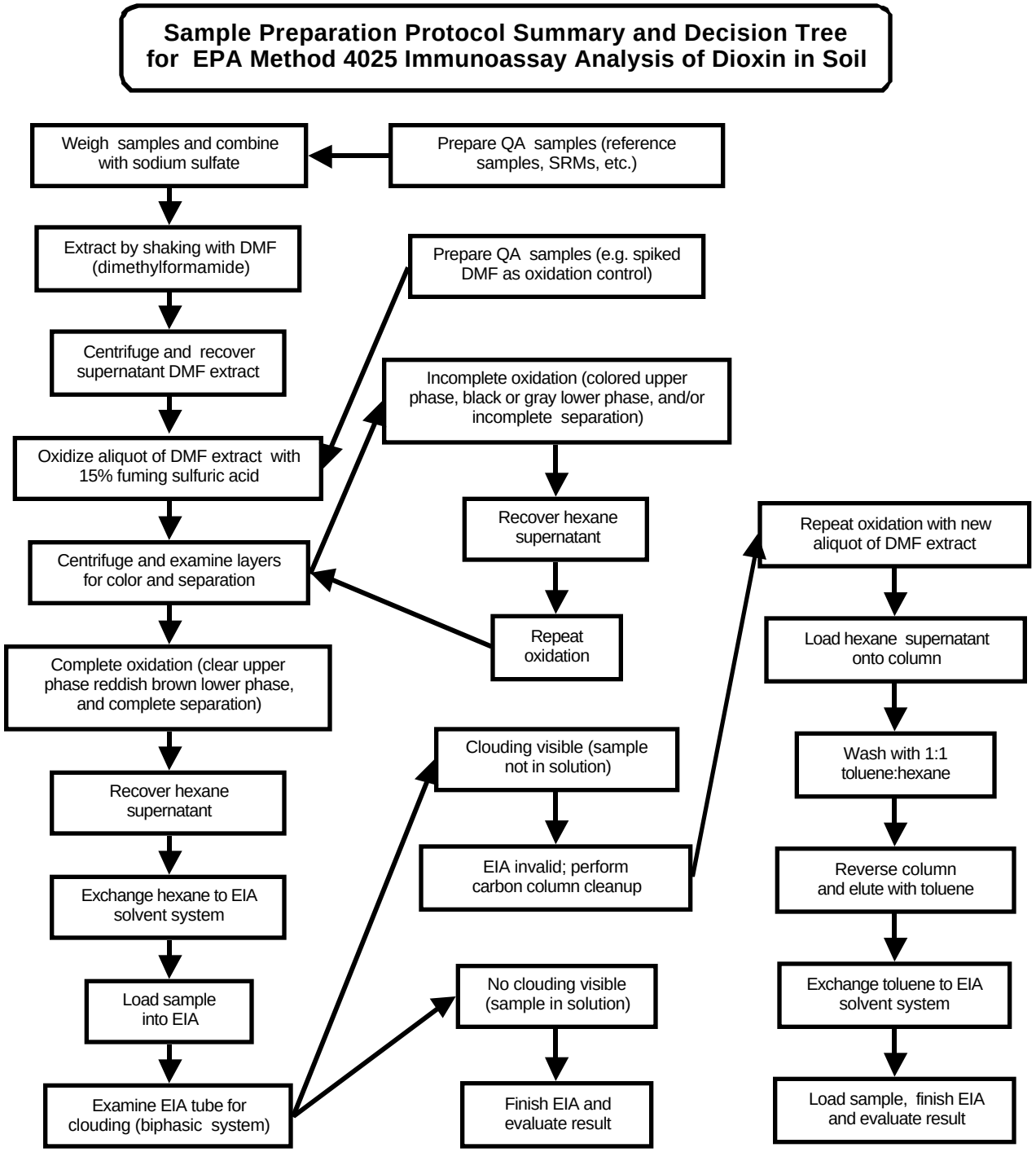
Before beginning this procedure, please read section F carefully, especially step F9. This section has several references to the DF1 Kit Insert (IN-DF1), which has a detailed description of the EIA procedure in section J. Steps I1 and J1-2 of the EIA procedure should be performed before step F8 below. Steps J3-6 of the EIA procedure should be done at the end of step F8 below. Steps F1-3 below are the same with or without the additional carbon column cleanup. Before beginning step F4, please first read section H about the possible need for carbon column cleanup. The sample preparation process, including the key points at which the analyst must assess the need for additional cleanup, is also summarized in the flow chart at the end of this section.

1. **Weigh sample**: Use CAPE Technologies Sample Preparation Kit (SP1 or SP2, for 12 or 60 samples; see Section H below for discussion of choice). Using wooden spatula from Sample Preparation Kit, mix sample thoroughly and weigh 5 g into 40 mL extraction vial from Sample Preparation Kit. Quality assurance samples to verify method performance (unspiked and spiked method blanks and reference soils) should be included at this point.

2. **Extract sample**: Add 15-20 g anhydrous sodium sulfate to extraction vial and mix with wooden spatula until sample is free flowing. Add 3 steel mixing balls from the Sample Preparation Kit, then 15 mL DMF. Read and follow precautions and other instructions in Sample Preparation Kit insert (IN-SP1/2). Cap vials tightly and extract by shaking 2 hours at 350 rpm on orbital platform shaker. Extraction vials should lie flat on their sides for maximum agitation.
3. **Spin extract and store**: Centrifuge extraction vial for 10-15 minutes at 1000 x g or less and remove a portion of the supernatant DMF extract to a clean vial with Teflon lined cap for storage. **Caution**: Exceeding this force during centrifugation can cause breakage of glass vials. The concentration of soil matrix in the extract will be 0.33 mg soil equivalent per µL. Extracts are stable for months at room temp. in tightly sealed vials.
4. **Remove aliquot of extract**: Place 180 µL of DMF extract (equivalent to 60 mg of sample) into an 8 mL oxidation vial from Sample Preparation Kit SP1 or SP2. This procedure allows analysis using single or duplicate EIA tubes at 20 mg of sample equivalent per EIA tube.
5. **Oxidize aliquot of extract**: Add 6 mL of hexane to the oxidation vial containing the 180 µL aliquot of extract. Using a glass pipet, slowly add 1.8 mL of 15% SO_3 in concentrated H_2SO_4. **Caution**: Fuming sulfuric acid is corrosive and hygroscopic. Carefully follow handling and storage instructions. Excessive exposure to ambient air will allow absorption of water, which will decrease the SO_3 concentration and may cause invalid results due to incomplete oxidation. Cap sample oxidation vial tightly and mix for 15 minutes at 350 rpm on orbital platform shaker. Oxidation vials should lie flat on their sides for maximum agitation. At end of oxidation vials should be slightly warm, but not hot. Centrifuge oxidation vials to separate phases completely (5 minutes at 1-2000 x g). **Important Note**: The acid phase should be dark reddish brown and should separate cleanly from the hexane. DMF extracts which are extremely dark may produce a black or dark gray acid phase during oxidation. Some oxidized DMF extracts also may not yield a clean phase separation after the specified centrifugation, showing flocculation or murkiness at the interface. If any of these problems are observed, oxidation should be considered incomplete and a second treatment of the hexane supernatant with fuming sulfuric acid is required (see for example section I2 below, Table 3, sample 46). EIA data from samples which are analyzed without complete oxidation should not be considered valid. Quality assurance samples (such as spiked vs. unspiked aliquots of one extract) to verify complete oxidation are strongly recommended. Other quality assurance samples to verify method performance (unspiked and spiked oxidation blanks and reference DMF extracts) should be included at this point. See for example section I2 below, Table 3.
6. **Recover supernatant**: Remove as much hexane supernatant as possible without disturbing the lower layer. **Caution**: Do not allow the lower phase to contaminate the hexane sample. Any oxidizer which contaminates the sample at this point will be carried through into the immunoassay, leading to invalid results. Greater than 95% volume recovery should be attainable without danger of contamination from oxidizer (95% is 5.7 mL recovered and only 300 µL remaining with oxidizer). Place the recovered hexane in a small round bottom glass tube, such as 13 x 100 mm. If contamination with oxidizer occurs, repeat separation and supernatant removal with clean pipet and evaporation tube.
7. **Add keeper**: Keeper solution (80:20 methanol:tetraethylene glycol [TEG] + 100 ppm Triton X-100) is made by adding methanol to a stock vial which is part of the DF1 kit. See the immunoassay kit insert IN-DF1 section I1 for instructions. Add 150 µL of keeper solution to each evaporation tube containing an oxidized sample in hexane.
8. **Evaporate solvent**: Evaporate the hexane at 40-50°C under a gentle stream of nitrogen as described in the immunoassay kit insert IN-DF1, section I3. When the hexane is gone, the 30 µL of TEG remaining (20% of 150 µL added in last step) should be visible as a clear viscous residue on the walls and bottom of the evaporation tube. When this point is reached, centrifuge the tube at 1-2000 x g for 2 minutes to concentrate all of the sample at the bottom.
9. **Dilute sample with methanol**: Before beginning this step you should have completed steps 1 through 6 described in section J of the DF1 Immunoassay Kit Insert (IN-DF1). Once these beginning EIA steps are completed, add 120 µL of methanol (setting 6 of Repeater Plus pipettor with 1.0 mL tip) to each evaporation tube and mix vigorously for 15 sec. Remove one or two 50 µL aliquots for EIA analysis in single or duplicate EIA tubes. Dilution and EIA loading should be done in batches of 4 samples or

fewer to minimize concentration changes due to methanol evaporation before pipetting. Add the sample directly to the water in EIA tube, not above water or to side of EIA tube. Mix each tube individually as soon as sample is added. At this point, enough sample remains in the evaporation tube for preparation of a small amount of 1/5 or 1/10 dilution (e.g. 20 µL into 80 µL keeper for 1/5). If such a dilution is made, it should be done immediately to avoid concentration changes due to evaporation. The diluted sample should then be loaded into the EIA immediately.

10. Run EIA: Perform remainder of EIA as described in section J of DF1 Immunoassay Kit Insert (IN-DF1).



G. Data Reduction and Interpretation of Immunoassay Results

1. Open Calculation Module C (Microsoft Excel workbook downloadable from CAPE Technologies web site [www.cape-tech.com] or available by email at cape-tech@ceemaine.org). Select "Introduction" worksheet and read the information on background and procedure, then select the "AN-007" worksheet. Install Excel "Solver" Add-In if it is not already done.
2. Enter optical density (OD) data for standards and samples into designated spaces.
3. Perform non-linear curve fitting procedure using Excel "Solver" function.
4. Verify sample load (20 mg sample equivalent per EIA tube) and decision level (500 ppt).
5. Modify calibration adjustment factor if necessary for specific sample group.
6. Read ppt values and/or positive/negative interpretation for each sample in designated row.

H. Additional Extract Cleanup by Carbon Mini-Column

Note on the need for carbon column cleanup:

The combination of cosolvents and detergent in the sample incubation of the EIA (IN-DF1, step J7) is designed to give a substantial capacity for solubilizing non-polar molecules. If this capacity is exceeded, the sample incubation mixture separates into a biphasic system, resulting in visible heterogeneity (cloudiness). Because the dioxin stays with the non-polar portion of the biphasic system, this condition leads to false negative results and requires additional extract cleanup before valid results can be obtained. Adequate quality assurance, including extract spikes (see section I2 below), is critical to overall method performance.

The choice of DMF as an extraction solvent is intended to reduce the co-extraction of aliphatic oils from samples, while effectively extracting PCDD/Fs. Some samples which are heavily contaminated with aliphatic oils may still give DMF extracts with levels of oil beyond the capacity of the EIA to dissolve them. In such cases, the co-extracted oils are typically unaffected by fuming sulfuric acid and must be removed before EIA analysis. The simplest cleanup method is adsorption of the PCDD/Fs on activated carbon, followed by washing to remove the oils and elution to recover the PCDD/Fs. This can be done easily using the SP2 and SP2-ST Sample Preparation Kits, as described in steps H1-6 below. The sample preparation process, including the key points at which the analyst must assess the need for additional cleanup, is also summarized in the flow chart at the end of Section F above.

The need for additional carbon column cleanup can be influenced by many factors, including age and weathering of the site, plus the original source material and vehicle for the PCDD/F contamination. For the first sample set described in Section I (Table 1), only 2 of 56 samples required this additional carbon column cleanup. In the second sample set of Section I (Table 2), no cloudiness was observed for any samples and no additional carbon column cleanup was required.

Detailed procedure for carbon column cleanup:

This additional cleanup requires both SP2-ST and SP2 kits as noted in section E above, plus a rack for holding reservoirs during the procedure. The sample should be in hexane after step F6 above. Running a column positive control to use for column recovery correction is strongly recommended. Additional quality assurance samples are also encouraged.

1. **Prepare column:** Read this entire step carefully before beginning. Place 25 mL glass reservoir from the SP2-ST Kit into rack. Put a small PTFE frit from SP2 Kit into the reservoir, making sure it fits as far as possible into the reservoir tip. Rinse the sides of the reservoir with 3-4 mL hexane, then immediately add approx. 2 g of sodium sulfate. To avoid air bubbles, the sodium sulfate should always be submerged in hexane. Immediately place a carbon mini-column from the SP2 Kit firmly onto tip of reservoir, square-cut end first (to avoid air bubbles, fill the top of the column with hexane first). Rinse sides of reservoir with approx. 5 mL of hexane to wash sodium sulfate down. Place stopper/stopcock assembly from SP2-ST Kit securely into top of reservoir. Pressurize column by placing syringe from SP2-ST Kit into open stopcock and pushing 10-25 mL of air into the reservoir. Close stopcock and remove syringe. Add air as needed to maintain dropwise flow rate of 0.5 to 1.0 mL per minute. Open the stopcock to release the pressure as soon as the hexane level reaches the top of the sodium sulfate. Discard all solvent passed through column. **Important Note:** Avoid air bubbles in the sodium sulfate and in the carbon mini-column.

2. **Load sample**: Add oxidized sample (hexane supernatant from step F6 above) to reservoir. Replace stopper and pressurize as in step H1. Open the stopcock to release the pressure as soon as the hexane level reaches the top of the sodium sulfate. Discard all solvent passed through column.
3. **Wash 1**: Add 10 mL hexane to reservoir. Replace stopper and pressurize. Open the stopcock to release the pressure as soon as the hexane level reaches the top frit of the carbon mini-column. Discard all solvent passed through column.
4. **Wash 2:** Remove carbon mini-column from reservoir and place in same direction (square cut end first) on tip of clean reservoir. Add 6 mL of 1:1 toluene:hexane to reservoir and pressurize. Open the stopcock to release the pressure as soon as the solvent level reaches the top frit of the carbon mini-column. Discard all solvent passed through column.
5. **Elute column**: Remove carbon mini-column and replace on tip of same reservoir in reverse direction (slant cut end first). Add 12 mL of toluene to reservoir and pressurize until air passes through column. Capture eluate in a 16 x 125 mm or similar size glass tube.
6. **Evaporate toluene**: Add 150 µL of keeper solution as for step F7 above. Evaporate the toluene as for step F8 above, except for using 60-75°C. Continue from step F8 to complete sample preparation.

I. Validation Data Supporting this Method

1. Correlation between immunoassay screening analysis and TEQ as determined by high resolution gas chromatography-high resolution mass spectrometry (HRGC-HRMS): Two sets of soil samples from two separate sites were extracted, oxidized, and analyzed as described in Sections F and G above. Subsamples of each sample from both sites were analyzed separately by HRGC-HRMS. The TEQ values were calculated from individual congener concentrations and TEF values. Congener concentrations stated to be below the HRGC-HRMS detection limit were assumed to be 1/4 of that detection limit for calculation purposes.

Table 1. Correlation between immunoassay screening analysis and TEQ as determined by high resolution gas chromatography-high resolution mass spectrometry (HRGC-HRMS) for 56 soil samples from Site 1. Samples were prepared and analyzed as described in Sections F and G above. An empirically determined calibration factor of 1.07 was applied to all quantitative EIA results before semiquantitative scoring. Results by category are 29 correct negative, 22 correct positive, and 5 false positive (91% correct, 9% FP). Two immunoassay samples which appeared heterogeneous or contained visible precipitate during the first EIA incubation were interpreted as giving invalid results. These samples were put through an additional carbon column cleanup for oil removal, were analyzed again by EIA, and the latter results reported (*).

Sample ID	ppt TEQ	EIA Result	Sample ID	ppt TEQ	EIA Result
1	94338	CP	29	13	CN
2	1528119	CP	30	14	CN
3	234492	CP	31	11	CN
4	822885	CP	32	579	CP
5	73750	CP	33	220	CN
6	4733	CP	34	13	CN
7	39	CN	35	1501	CP
8*	6278	CP	36	3702	CP
9	276	***FP***	37	248	CN
10	2390	CP	38	216	***FP***
11	2101	CP	39	1088	CP
12	30	CN	40	1210	CP
13	14	CN	41	13	CN
14	210	CN	42	451	CN
15	5860	CP	43	16	***FP***
16	2191	CP	44	105	CN
17	343	***FP***	45	16	CN
18	18	CN	46	10	CN
19	25	CN	47	211	***FP***
20	70	CN	48	1725	CP
21	599	CP	49	41	CN
22	217	CN	50	551	CP
23	27	CN	51	622	CP
24	43	CN	52*	26856	CP
25	18	CN	53	2122	CP
26	18	CN	54	24	CN
27	14	CN	55	31	CN
28	26	CN	56	31	CN

29 CN, 22 CP, 5 FP, 0 FN from 56 samples

Table 2. Correlation between immunoassay screening analysis and TEQ as determined by high resolution gas chromatography-high resolution mass spectrometry (HRGC-HRMS) for 18 soil samples from Site 2. Samples were prepared and analyzed as described in Sections F and G above. No calibration adjustment factor was applied to EIA results before semiquantitative scoring. None of these samples required additional cleanup before EIA analysis. Several days later, aliquots of 13 of the 18 DMF extracts were oxidized and analyzed in a second EIA and the concentrations were calculated based on a new standard curve. The mean coefficient of variation for the 13 pairs of duplicate ppt values was 35%.

ppt by GC-MS	EIA run 1	EIA run 2
2730	CP	FN**
470	FP	FP
65	CN	CN
1400	CP	CP
70	CN	
4900	CP	CP
245	FP	
7300	CP	CP
365	CN	
360	CN	CN
140000	CP	CP
7000	CP	
33	CN	CN
990	CP	CP
4100	CP	CP
205	CN	
360	CN	CN
65	CN	CN

** actual calculated value with no calibration adjustment was 497 ppt (run 1 was 630 ppt)

8 CN, 7 CP, 2 FP, 1 FN (**) from 18 samples

2. Accuracy and Precision Data From EIA Analysis of Spiked Negative Soil Extracts: Fourteen extracts of the Site 1 soils presented in Table 1, ranging from 10 to 26 ppt by HRGC-HRMS, were spiked with 2378-TCDD at levels corresponding to 250 and 1000 ppt in the original sample. Extracts were oxidized and analyzed by EIA according to the method described in section F, steps 4-10. Four aliquots of each extract were spiked, two at each level. Each oxidation replicate was split for duplicate EIA analyses. All standards were unreplicated within runs. Results for each run were calculated as described in section G. Controls consisting of DMF spiked at both levels were processed with each of the 5 runs and the calculated ppt values were used to correct samples for spike recovery within each run. None of these 14 extracts required carbon column cleanup.

Table 3: False positive/false negative data for negative soil extracts spiked near the 500 ppt decision level. Samples were spiked and analyzed as described above. Individual EIA replicate ppt values were scored as positive if equal to or greater than 500 ppt and were scored as negative if less than 500 ppt. Each semiquantitative score below corresponds to one EIA tube. Sample 41A and 41B were field duplicates which appeared different based on gross color and particle size. Extract 46 was nearly black in color and totally opaque. Initial data for sample 46 (*) indicated false negatives at the high spike level (individual EIA replicates were 401, 366, 366, and 332 ppt). The sample 46 analysis was repeated using a second oxidation of the first hexane supernatant (plus a fresh aliquot of DMF). The individual EIA replicates of 1872 and 1318 ppt gave a correct positive interpretation (the corresponding twice oxidized DMF controls were 1068 and 846 ppt).

	Spike Level	**250 ppt spike**		**1000 ppt spike**	
		Oxidation Replicate		**Oxidation Replicate**	
Sample #	**EIA Replicate**	**1**	**2**	**1**	**2**
13	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
18	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
19	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
25	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
27	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
28	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
29	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
30	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
31	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
34	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
41A	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
41B	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
45	1	CN	CN	CP	CP
	2	CN	CN	CP	CP
46*	1	CN*	CN*	***FN****	***FN****
	2	CN*	CN*	***FN****	***FN****
46 (oxid. x 2)	1			CP	
	2			CP	

Summary of FN/FP results (based on repeat oxidation data for sample 46):

Correct Positives:	54	False Positives:	0
Correct Negatives:	52	False Negatives:	0

Table 4: Summary of quantitative accuracy and precision data from false positive/false negative experiment of Table 3. Data are based on four parameter curves and calculated ppt values for each of 5 runs on 5 separate days. The accuracy and precision shown in sections A and B support the semiquantitative screening method described here. These data also support quantitative use of the test in certain situations with sufficient quality assurance samples. It should be noted that the data of section B include variation among the 14 different soils, as well as the intrinsic variability of the method itself. The precision data shown in sections C and D support screening analysis based on unreplicated sample oxidation and unreplicated EIA tubes for both standards and samples.

A. Summary of quantitative ppt data from DMF controls

spike level	250 ppt spike	1000 ppt spike
number of runs (2 or 4 EIA replicates within each run)	4	5
mean±SD of within run means (ppt)	271±74	957±174
coefficient of variation of ppt	27%	18%

B. Summary of quantitative ppt data from spiked extracts (sample 46 data includes only 2x oxidation)

spike level	250 ppt spike	1000 ppt spike
number of individual EIA replicates	56	54
overall mean±SD (ppt)	266±61	984±356
coefficient of variation of ppt	23%	36%

C. Coefficients of variation for ppt within runs (all EIA replicates at each spike level for each run)

	spiked extracts	DMF controls
number of runs	9	9
mean of within run %cv values	18%	15%

D. Summary of EIA replicate precision (both spike levels combined)

	spiked extracts	DMF controls
number of pairs of EIA replicates	57	11
mean of all EIA replicate %cv values for ppt	10%	13%