CAPE Technologies

High Performance Dioxin/Furan Immunoassay Kit

Application Note AN-008

Immunoassay analysis of ppt (pg/g) range PCDD/Fs in soil and sediment using rapid extraction and rapid cleanup (modified EPA Method 4025 or Method 4025m)

Contents of this Application Note

A. Introduction

This Application Note describes a rapid extraction and rapid extract cleanup to prepare soil or sediment samples for ppt (pg/g) range analysis using the CAPE Technologies High Performance Dioxin/Furan Immunoassay Kit (referred to as modified EPA Method 4025 or Method 4025m). This sample preparation method is more rigorous than the one described in US EPA Method 4025, but the analysis of the prepared sample extracts is still done with the same immunoassay validated for Method 4025. This modified extraction and cleanup are much faster and simpler than typically required for GC-MS analysis (Method 8290/1613B). Using modified Method 4025, one person can analyze 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 SP3 Sample Preparation Kit. Please read this Application Note carefully as part of planning your sample preparation and analysis. The documents cited here are available by email from CAPE Technologies or at the CAPE Technologies web site (www.cape-tech.com).

B. Summary of Procedure

1. Weigh soil or sediment sample. Add sodium sulfate and mix. Add 1:1 hexane:acetone and extract sample by shaking 2-6 hours. Remove the supernatant hexane:acetone extract.
2. Evaporate an aliquot of the supernatant hexane:acetone extract using a hydrocarbon keeper such as tetradecane, then redissolve in hexane. Pretreat sample with acid silica, then load onto coupled acid-silica:activated carbon mini-column. Wash acid silica with hexane and add washes to column system.
3. Force hexane through the system until the sample & washes pass through carbon mini-column.
4. Transfer carbon mini-column to empty reservoir and wash with 1:1 toluene:hexane.
5. Reverse carbon mini-column on reservoir and elute with toluene.
6. Evaporate the toluene to exchange sample into water-miscible keeper solution.
7. Perform the immunoassay procedure as described in the kit insert IN-DF1.
8. Interpret the immunoassay results as described in section H of this Application Note.

C. Reagents Required (not provided by CAPE Technologies)

1. Anhydrous sodium sulfate, approx. 20 g per sample; reagent grade.
2. Acid washed quartz sand, approx. 10 g per sample; reagent grade.
3. Hexane, approx. 60 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; n-hexane (CASRN 110-54-3) could also be used, but is generally more expensive).
4. Acetone, approx. 10 mL per sample; HPLC grade or better.
5. Toluene, ultra-pure grade intended for the most demanding residue analysis, approx. 15 mL per sample. All development work for this application has been done with the best grade available (Burdick & Jackson), which has a **specification for residue after evaporation of <1 mg/L**. **All non-volatile residue (oil) in the toluene will end up in the EIA sample, so maximum purity at this step is crucial.**
6. Methanol, approx. 1 mL per sample; HPLC grade or better.
7. Tetradecane or similar high boiling aliphatic hydrocarbon keeper, approx. 0.25 mL per sample.

D. Supplies Required (not provided by CAPE Technologies)

1. Pipet bulbs and glass Pasteur pipets for transfer of extracts.
2. Pipettors and glass pipets, graduated cylinders, and/or glass syringes for measuring approx. 5 to 15 mL.
3. Glass vials (2 to 12 mL) with Teflon lined caps for storage of sample extracts (only if storing extracts).
4. Disposable glass test tubes (15-20 mL; typical is 16x125 mm) for evaporation of carbon column eluates.
5. Basin or other receptacle(s) to catch waste from column cleanup procedure.

E. Supplies Required (provided by CAPE Technologies)

1. DF1 Immunoassay Kit and supplies specified in section G of the kit insert (IN-DF1).
2. SP3 Sample Preparation Kit (SP3-12 or SP3-60) containing materials for sample extraction, prepacked disposable acid silica columns, and prepacked disposable carbon mini-columns.
3. SP2-ST or SP3-ST Starter Sample Preparation Kit containing reusable hardware for manual execution of the column cleanup procedure described in Section G below.
4. SP2-RK rack for holding SP3 acid silica columns and SP2/3-ST reservoirs (glass columns like 25 mL pipets; 16 mm dia. by 30 cm long); a second SP2-RK rack can be used for holding eluate collection tubes aligned under columns.

F. Equipment Required (see Equipment Lists EL-001 and EL-002 for details)

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)
3. Orbital platform shaker for mixing during extraction
4. Centrifuge with capacity for holding 40 mL vials (28 mm outside diameter x 98 mm high, flat bottoms)
5. Fume hood and solvent exchange system for samples of ≤ 20 mL in glass tubes (see IN-DF1, section I3)
6. Small vacuum pump or other vacuum source for starting flow through coupled columns (preferred because it provides best performance, but is not absolutely essential; see section F5a/5b)
7. Compressed gas source (such as nitrogen cylinder or small oil-free air compressor) for column pressurization (only if using SP3-ST Kit hardware; not needed if using SP2-ST Kit hardware)
8. Computer with Microsoft Excel for data analysis; **Solver** Add-In must be installed and available (look under **Tools** menu; may need to reload Add-Ins from original disk)

G. Detailed Sample Preparation Procedure

Before beginning this procedure, please read section G carefully, especially step G12. 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 G11 below. Steps J3-6 of the EIA procedure should be done at the end of step G11 and before starting step G12 below.

1. **Weigh sample**: Use CAPE Technologies SP3 Sample Preparation Kit. 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 (such as unspiked and spiked method blanks and reference soils) should be included at this point. If samples have standing water, it must be removed before extraction by pouring off and wicking the remainder away with a clean paper towel. Any samples this wet will probably also require additional drying before extraction. Some samples even without standing water may require similar treatment. Examples of appropriate treatment include pressing a portion of sample between layers of a baby diaper or leaving an aliquot overnight on a clean paper towel. Very wet samples may also require gentle overnight heating.
2. **Extract sample**: Add 10-20 g anhydrous sodium sulfate to extraction vial. Add 3 steel mixing balls from the SP3 Kit, then 20 mL of 1:1 hexane:acetone. Read and follow precautions and other instructions in SP3 Kit insert (IN-SP3). Cap vials tightly and extract by shaking 2 to 4 hours at 350 rpm on orbital platform shaker. Extraction vials should lie flat on their sides for maximum agitation. If the sample-sodium sulfate mixture is not completely homogeneous and free flowing within the solvent during extraction, then additional drying is required. Either a new aliquot can be extracted or more sodium sulfate can be added to the first attempt. In the latter case, the sample should be shaken for 2 to 4 hours after the sample-sodium sulfate mixture becomes homogeneous and free flowing within the solvent.
3. **Spin extract and store**: Centrifuge extraction vial for 10 to 15 minutes at 1000 x g or less. **Caution**: Exceeding this force during centrifugation can cause breakage of glass vials. Remove a portion of the supernatant hexane:acetone extract to a clean glass vial with Teflon lined cap for storage. The concentration of soil matrix in the extract will be 0.25 mg soil equivalent per µL.
4. **Choose sample load and evaporate aliquot of extract**: This extract cleanup protocol is designed for processing only a portion of the sample extract. You must first choose your target sensitivity, which will determine the sample load and the volume of extract used. Different sensitivities can be achieved by using different volumes of extract in this step to set up different sample loads. **It is critical that you first read Sections I2 and I3 of the DF1 Kit Insert (IN-DF1).** The procedure described in step G11/12 below is based on Evaporation Protocol Option B (IN-DF1 Table 3) and allows recovery of 80% of the prepared sample for introduction to the EIA tube. If you wish to use a different Evaporation Protocol Option, you must adjust the amount of sample extract applied to the cleanup procedure. Nominal sensitivity in the low pg/g range will require cleanup sample sizes in the gram range. Exact amounts must be determined using Sections I2 and I3 of the Kit Insert IN-DF1. A reasonable working assumption for combined extraction efficiency and cleanup recovery would be 50%. Using glass capillary micropipettor, add the chosen amount of hexane:acetone extract and 250 µL of tetradecane or similar hydrocarbon keeper to a glass tube or vial and evaporate. **Note on sample load, target sensitivity, and quality assurance**: The analyst is responsible for quality assurance and should consider significantly different sample loads to be separate methods with respect to blanks, spikes, and other quality assurance samples.

5a. **Prepare coupled carbon-acid silica column system using vacuum**: If you do not have a vacuum source as noted in F6 above, go to step G5b below. If you have a vacuum source such as a pump, set it up outside fume hood so that flexible tubing can be run easily from intake into hood, with exhaust vented to hood. Remove endcaps from acid silica column of SP3 Kit. Remove a carbon mini-column (SP3 Kit) from its pouch and twist square cut end firmly onto tip of acid silica column (gloves may be needed for gripping the Teflon). Insert Luer plug (SP2/3-ST Kit) into slant cut end of carbon column and place in SP2-RK rack. Add 10 mL of hexane to acid silica column and attach pressurization hardware (SP2/3-ST Kit) firmly to top of column. Connect to vacuum source and depressurize

headspace above hexane for 15-30 seconds. Bubbles should stream up through hexane, rapidly at first, then more slowly. Disconnect vacuum and let solvent flow downward into acid silica. When solvent flow has nearly stopped, reconnect vacuum, briefly repeat depressurization, then disconnect vacuum again. This time hexane should flow all the way through the acid silica column and into the carbon column. The acid silica column should appear translucent and should not have any air bubbles visible. The carbon column should appear uniformly black. With the SP3-ST kit manifold system, these steps consist of simply opening the stopcock to vent a column to the atmosphere, then closing it again to reapply the vacuum. Remove Luer plug from tip of carbon column. A few mL of hexane should remain in reservoir above acid silica. The reservoir and column assemblies can be left unpressurized at this point until all are assembled and ready for step G6. **DO NOT ALLOW TOP OF ACID SILICA COLUMN TO GO DRY.**

5b. **Prepare coupled carbon-acid silica column system without vacuum:** This step is needed only if you do not have a vacuum source for starting column flow. If you have completed step G5a, proceed directly to step G6. Otherwise, remove endcaps from acid silica column of SP3 Kit and place in SP2-RK rack. Add 10 mL of hexane to acid silica column and allow to flow by gravity until hexane begins to drip from the column tip. Remove a carbon mini-column (SP3 Kit) from its pouch and use a Pasteur pipet to fill square cut end with hexane. As hexane is dripping from the tip of the acid silica column, place mini-column firmly onto tip with a twisting action (gloves are essential for grip as well as skin protection). Be sure top of mini-column is full of hexane so that it can be attached without air bubbles. Flow will nearly stop, with solvent front advancing very slowly through carbon mini-column. Add more hexane if necessary to keep a few mL in reservoir. Attach pressurization hardware (SP2/3-ST Kit) firmly to top of reservoir. The reservoir and column assemblies can be left alone at this point until all are assembled and ready for step G6. **DO NOT ALLOW TOP OF ACID SILICA COLUMN TO GO DRY.**

6. **Complete prewash:** A few mL of hexane should remain above the acid silica column. If not, then add hexane as needed. Using SP2/3-ST Kit hardware, pressurize the reservoir (approx. 0.5 to 0.7 bar, or 7 to 10 psi) The hexane should flow immediately through the column at 0.5 to 2.0 mL/min. Catch solvent in waste basin. Stop prewash with 0.5-2 mL (2-5 mm height) of hexane remaining above bed. This procedure should be done carefully to avoid drying of columns. **DO NOT ALLOW TOP OF COLUMN TO GO DRY; IT IS CRITICAL TO AVOID AIR BUBBLES IN ACID SILICA COLUMN.**

7. **Load sample**: If hexane level drops to the top of the bed before loading sample, add more. Using a glass Pasteur pipet, add sample in tetradecane or other hydrocarbon keeper from step G4 to the top of the acid silica column. Include all of pretreatment acid silica, adding slurry to top of acid silica column. For SP2-ST kits, remove stopper/stopcock during addition. For SP3-ST kits, remove pressure caps first or add slurry carefully through stopcock. Gently rinse the sides of the sample tube with approx. 2 mL of hexane, then add to acid silica column, rinsing the sides thoroughly. Repeat wash of sample tube with another 2 mL of hexane and add to acid silica column. Rinse stopcock with hexane if necessary to remove residual acid silica from oxidized sample. Pressurize as before to push sample and washes into acid silica bed. Catch solvent in waste basin. Stop flow by opening stopcock just before solvent level reaches top of bed. **DO NOT ALLOW TOP OF ACID SILICA BED TO GO DRY.**

8. **Wash acid silica column**: Remove stopper/stopcock assembly and add 10 mL of hexane to acid silica column. Replace stopper/stopcock assembly and pressurize as before. Catch solvent in waste basin. Maintain pressure to keep hexane flowing at 0.5 to 2.0 mL/min. Stop flow by opening stopcock just before solvent level reaches top of bed. Repeat twice for a total wash volume of 30 mL of hexane. On final wash only, maintain pressure to keep hexane flowing all the way through the acid silica column. When air penetrates the neutral silica layer (bottom 1-2 cm layer at bottom of column, just above tip of column), release pressure to stop flow. **IT IS NECESSARY TO RUN THE SOLVENT ALL THE WAY THROUGH THE ACID SILICA BED, BUT DO NOT ALLOW TOP OF CARBON MINI-COLUMN TO GO DRY.**

9. **Remove carbon mini-column and wash**: Remove carbon mini-column from the acid silica column, attach square cut end to a clean and empty reservoir (SP2/3-ST Kit), and place in rack over waste capture basin. Add 6 mL of 1:1 toluene:hexane and pressurize as before. Catch solvent in waste

basin or catch each sample in tubes for evaporation and analysis (dlPCBs). When solvent level reaches tip of reservoir, release pressure to stop flow. **DO NOT ALLOW TOP OF CARBON MINI-COLUMN TO GO DRY.**

10. **Elute sample**: Remove carbon mini-column from tip of reservoir and replace on same reservoir in reverse orientation, slant cut end first. Add 12 mL of toluene and pressurize as before. Capture eluate in clean 16 x 125 mm borosilicate glass tube, allowing air to drive last of toluene through carbon mini-column.
11. **Add keeper and evaporate solvent**: Keeper solution (80:20 methanol:polyethylene glycol [PEG] + 100 ppm Triton X-100) is made by adding methanol to a stock vial which is part of the DF1 kit (see the DF1 Kit Insert IN-DF1 section I1 for instructions). Add 62.5 µL of PEG-Triton-methanol keeper solution to each evaporation tube containing a toluene eluate. **Note**: This procedure recovers 80% of the prepared sample for introduction to the EIA tube (this is Option B from Table 3 in Section I2 of IN-DF1; for other options, review Table 3 carefully before starting). Evaporate the toluene at 70-90°C under a gentle stream of nitrogen as described in the immunoassay kit insert IN-DF1, section I3. When only keeper remains, centrifuge at 1-2000 x g for 2 minutes to concentrate all of the sample at the bottom of the tube.
12. **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 50 µL of methanol (setting 2.5 of Repeater Plus pipettor with 1.0 mL tip) to each evaporation tube and mix vigorously for 15 seconds. Let stand for 15-30 seconds to allow liquid to flow back to bottom of tube, then remove 50 µL for EIA analysis. **Note**: This procedure recovers 80% of the prepared sample for introduction to the EIA tube (this is Option B from Table 3 in Section I2 of IN-DF1; for other options, review Table 3 carefully before starting). 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 the water or onto side of EIA tube. Mix each tube individually as soon as sample is added.
13. **Run EIA**: Perform remainder of EIA as described in section J of DF1 Immunoassay Kit Insert (IN-DF1).

H. 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 from CAPE Technologies). Select “Introduction” worksheet and read the information on background and procedure, then select the “4025m for low-mid ppt soil+sed” 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. Enter sample load (e.g. 500 mg sample equivalent per EIA tube) and other required information.
5. Revise calibration adjustment if necessary and read original sample ppt values for each sample in designated row.
6. Final interpretation of data will be based also on analyst experience, knowledge of samples, and especially on results for quality assurance samples.

I. Validation Data Supporting this Method

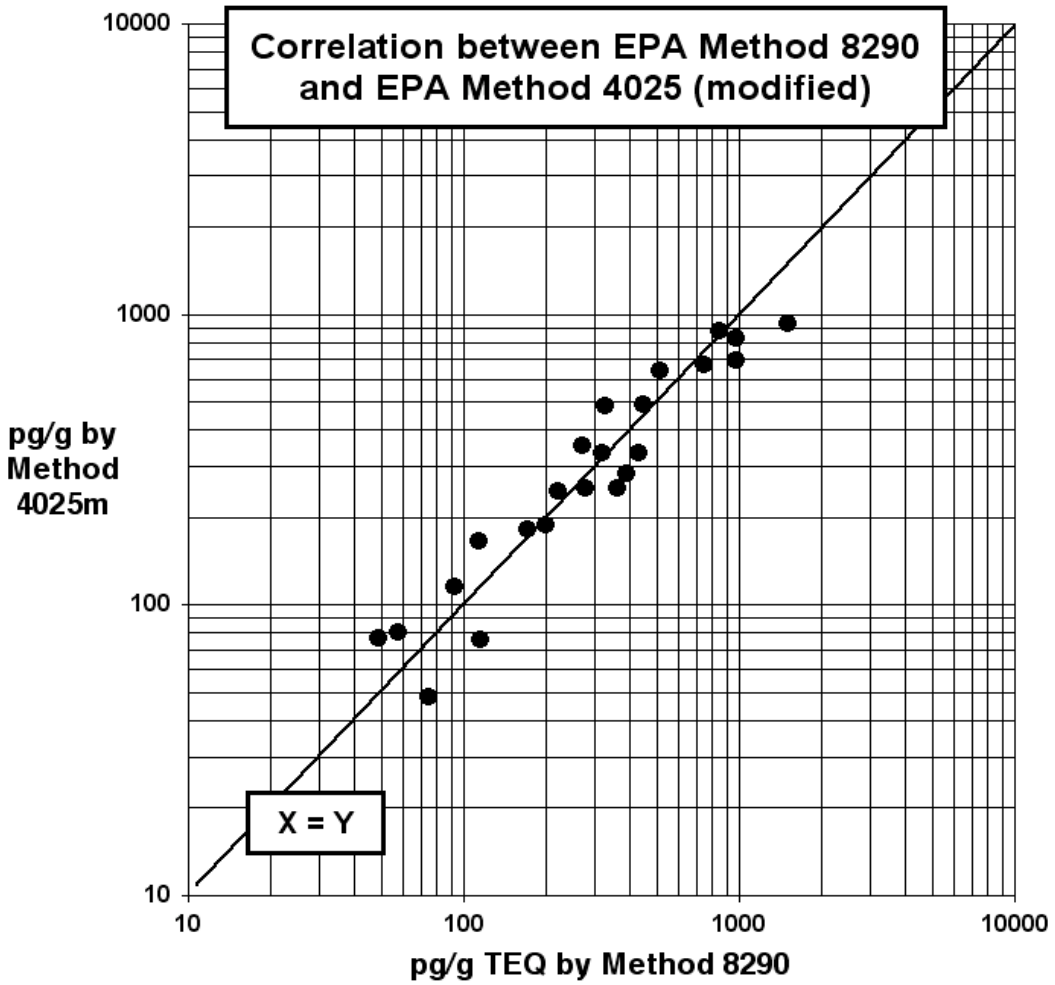
Data from two separate studies are provided below. In the first study, more than 200 samples from a sewage treatment plant were extracted and analyzed by Method 4025m. Numerous quality assurance samples were run within Method 4025m, including method blanks, spiked method blanks, duplicates, spiked samples, and controls on different parts of the sample preparation procedure. Results for these QA samples are summarized in Table 1. Selected field samples were also analyzed by Method 8290 to validate Method 4025m performance and to calibrate the immunoassay response. Correlation data between Method 4025m and Method 8290 are shown in Figure 1.

Table 1. Quality assurance data within modified EPA Method 4025

QA sample	n	mean±SD	units (comment)
Solvent exchange negative controls	24	1.9±1.0	pg (lowest standard = 3.2 pg)
Unspiked method blanks	22	2.9±1.7	pg (lowest standard = 3.2 pg)
Solvent exchange positive controls	22	102±20	% of nominal pg (generally 50 pg)
Spikes into method blank extracts	14	79±27	% of nominal pg/g (30 to 195 pg/g)
Spikes into sample extracts	32	67±28	% of nominal pg/g (30 to 390 pg/g)
Duplicate precision (two aliquots of one extract cleaned and analyzed in parallel)			
	23	13±14	% cv (range 5 to 750 pg/g)

Figure 1. Correlation between modified EPA Method 4025 and EPA Method 8290

A set of 23 soil samples from a sewage treatment facility were prepared and analyzed as described in Section G above. Each EIA tube received prepared extract equivalent to 500 to 640 mg of original sample. Samples which gave off-scale high results were diluted and the EIA was repeated so that each EIA tube received the equivalent of 50 to 100 mg of prepared sample. Subsamples of each sample were analyzed separately by HRGC-HRMS. The TEQ values were calculated from TEF values and individual congener concentrations as measured by Method 8290. The line represents x = y. The correlation coefficient was 0.95. The mean relative percent difference value for the samples plotted below was 24±15% (±SD), with a maximum of 47%.



The second set of validation data is from Phase 2 of a US EPA field demonstration, performed under the Superfund Innovative Technology Evaluation (SITE) Program. Complete reports describing the study design and results can be found on the CAPE Technologies web site, labeled as Technical References TR-007 (Phase 1) and TR-008 (Phase 2). A concise description of the Phase 2 design is given with the data in Figure 2 below.

Figure 2. Correlation between modified EPA Method 4025 and EPA Method 1613B in EPA Field Demo

Samples from 5 diverse sites were analyzed by Method 4025m and Method 1613B. The total number of samples analyzed was 112, representing blind quadruplicates of 28 unique samples. Data points plotted below represent the means of those quadruplicate sets. Samples for each site included some previously analyzed in Phase 1 and some not yet analyzed. All samples were dried, sieved, and homogenized by EPA before analysis. The site of origin was identified for each sample to allow use of a site-specific calibration, technique, incorporating knowledge regarding typical congener patterns at a particular site. Samples were prepared and analyzed as described in Section G above. Each EIA tube received prepared extract equivalent to 0.5 to 3.0 g of original sample. Samples which gave high results were diluted ten-fold serially and the EIA was repeated. Data from Method 1613B were obtained independently by EPA. The TEQ values were calculated from TEF values and individual congener concentrations as measured by Method 1613B. The line represents x = y. The correlation coefficient was 0.94. The mean relative standard deviation for the quadruplicates plotted below was 26% (range 17-116%).