Bull. Environ. Contam. Toxicol. (1999) 63:282-288

Bulletin of Environmental Contamination and Toxicology

A One-Step ASE™ Extraction Method for TCDD TEQ Determination

D. D. McCant,[1] L. S. Inouye,[2] V. A. McFarland[2]

[1] AScI Corporation, 3402 Wisconsin Avenue, Suite 5, Vicksburg, MS 39180, USA
[2] United States Army Engineer Research and Development Center, Waterways Experiment Station, 3909 Halls Ferry Road, Vicksburg, MS 39180, USA

Received: 20 May 1999/Accepted: 12 July 1999

The high cost of chemical analysis for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and TCDD-like compounds has led to the use of cell-based assays for determining TCDD toxic equivalents (TEQs). The Reuber H4IIE rat hepatoma cell line is considered a staple in in vitro cell culture screening for dioxin and dioxin-like compounds and has been demonstrated to possess good reproducibility among laboratories and repeatability within a laboratory (Tillitt et al. 1991). The assay has been employed by FDA (Casterline et al. 1983) and US Fish & Wildlife Service (Giesy et al. 1994) to determine TEQs in various biological samples, and a recent paper has also established its utility for soils, fly ash, and other environmental samples (Schwirzer et al. 1998). Binding of the Ah receptor in these cells by dioxin and related compounds causes increased expression of several genes, including CYP1A1, which results in the production of ethoxyresorufin-O-deethylase (EROD). EROD activity is easily measured as it metabolizes ethoxyresorufin to the fluorescent product, resorufin, which can be monitored spectrofluorometrically. TEQs are determined by comparing EROD activity in cells exposed to a TCDD standard to that of cells exposed to environmental samples.

In order to assay environmental samples for dioxins, they must first be extracted and treated to remove unwanted contaminants such as polycyclic aromatic hydrocarbons (PAHs). The extraction of TCDD and TCDD-like compounds, including congeners of polychlorinated dibenzodioxins (PCDDs) polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs), has long been accomplished by Soxhlet or sonication techniques. Although these techniques extract the compounds of interest with high efficiency, they require large volumes of chlorinated solvents and have extraction times of 18 to 24 hours per sample.

This investigation was aimed at producing a rapid method for sediment extract preparation for use in determining TEQs in cell-based assays. The DIONEX Accelerated Solvent Extraction (ASE™) technique employs elevated temperature and pressure to yield recoveries comparable to the traditional Soxhlet methods with a fraction of both solvent volume and extraction time (Richter et al. 1997). TEQs for Michigan's Saginaw River sediment extracts prepared by the two

Correspondence to: D. D. McCant

methods were compared using the H4IIE cell line. Extraction efficiencies were also determined for both ASE and ASE one-step methods using ^{3}H-TCDD, while the efficacy of sulfuric acid silica gel (SA/SG) procedures for removal of PAHs was determined by spiking a clean sediment with a PAH standard prior to analysis with the H4IIE cell line.

MATERIALS AND METHODS

Six 10 g replicates of air dried Saginaw River sediments were prepared by either ASE or Soxhlet techniques using 50 mL and 250 mL of dichloromethane (DCM), respectively. ASE sediments were extracted under the following programed parameters: 1500 psi, 100°C , with a static and heat time of five minutes, a flushing volume of 60 mL, 60 seconds purging, and a 60% flushing volume for two cycles. Soxhlet sediments were extracted under temperatures suitable for solvent reflux for 18 to 24 hours per sample. Both methods comply with EPA Method 3540 guidelines for chlorinated hydrocarbons and for PAH in soils and sediments. Crude extracts were concentrated in a Zymark® Turbo Vap II evaporator to five mL, 2.5 mL of which were run through an SA/SG column to remove PAH and other undesired contaminants. SA/SG was prepared by activating silica gel (60-200 Mesh) in a drying oven for at least 24 hours at 130°C, computing 2/3 of the weight of activated silica gel, and placing this amount (by weight) of concentrated sulfuric acid in the bottle with a Teflon-lined screw cap. This mixture was then shaken vigorously until there were no lumps and the contents exhibited a dry powdery appearance. SA/SG was then tumbled on a bottle roller for a minimum of two hours, given a six month expiration date, and stored at room temperature. Chromatography columns 25 cm long with 250 mL reservoirs were then packed with approximately ½ cm of glass wool, one cm of pre-dried Na_2SO_4, four cm of neutral activated silica gel, and 10 cm of SA/SG from stopcock to reservoir. Columns were then conditioned with 80 mL of pesticide grade hexane just prior to adding 2.5 mL of crude sediment extract concentrate. The sample was eluted with 135 mL of 10% DCM in hexane (v:v), which was collected in a clean evaporating tube. Clean extracts were concentrated to one mL then solvent exchanged to one mL of iso-octane.

Alternatively, an ASE method was developed which combines the extraction and SA/SG cleaning into a single step in which the sediment extract is stripped of PAHs as it passes through the cell to its collection vial. A 33 mL extraction cell was prepared by placing a cellulose filter in the capped end, then tightly packing into it two g of neutral silica gel followed by eight g of SA/SG. The cap was temporarily removed and 80 mL of hexane passed over the column in order to condition it. After replacing the cap at the end of the column, a five g aliquot of air dried sediment was thoroughly mixed with diatomaceous earth (DE) in a 4:1 weight to weight ratio (sediment:DE) and added to the top of the conditioned column/cell. Standard Ottawa sand was used as a filler before adding a second cellulose filter and capping vessel. The cell was then extracted under the same

parameters as the standard ASE method described above, using a 10% DCM in hexane mixture as the single solvent system. Clean extracts were concentrated to approximately one mL then solvent exchanged to iso-octane (1.0 mL final volume) and stored at -20 °C until used in the H4IIE assay.

TCDD extraction efficiencies of the standard ASE extraction and the one-step ASE method were determined using ^{3}H-TCDD. Three replicates of Ottawa sand were spiked with 50 µL aliquots of ^{3}H-TCDD (6.8 x 10^{-5} mCi/mL) for each method.

The cleanup efficiency of the ASE one-step method was tested by spiking 250 µL of a PAH mixture (ULTRAstandard™ catalog # PM-810, diluted such that the B[a]P concentration was 20 µg/mL) onto Ottawa sand, then using the one-step ASE extraction method described above. For comparison, Ottawa sand was spiked at the same level, then extracted and cleaned with the standard ASE method. Extracts (crude ASE, cleaned ASE, and one-step ASE) were then tested with the H4IIE assay to verify the efficiency of the ASE one-step method in removing PAHs.

In order to determine whether the SA/SG column would be overloaded by high levels of PAHs in highly contaminated sediments, 2.5 mL of crude sediment extract (New York's Arthur Kill B/A, containing -14.3 ppm total PAHs) was spiked with 250 µL of the PAH mixture. The response of the H4IIE cell line to the cleaned, PAH-spiked extract was compared to the response of the cell line to the cleaned, unspiked extract. The ability of the one-step ASE method to remove high levels of PAH contamination from actual sediment samples was also tested by extracting the New York sediment spiked with the PAH standard.

The modified TEQ assay performed in 96-well microtiter plates was performed as described by Sanderson, et al. (1996). The detailed laboratory protocol as used at the USAE Waterways Experiment Station can be found at http://www.wes.army.mil/el/dots/doer/technote.htmL (McFarland et al. 1997). Briefly, 5,000 H4IIE rat hepatoma cells (American Type Culture Collection) were added to each of 60 wells on a 96-well plate. Following a 24-hour incubation, 5 µL of different sediment extract concentrations (serial dilutions) in iso-octane were added to each well in replicates of five. Iso-octane and a series of TCDD concentrations were also employed to generate a standard dose-response curve. Plates were incubated for another 72 hours, after which the cells were rinsed, lysed, and the TEQ assay performed. Raw fluorescent values for resorufin and proteins were read on a Dynatech™ microplate fluorometer at 550 & 590 nm and 400 & 450 nm, respectively, and the values were converted to EROD activity (µg resorufin/min*mg protein). TCDD TEQ values (pg TCDD TEQ/g sediment) were calculated by converting the EROD response to pg TCDD using the TCDD standard dose-response curve. Only dilutions which were part of the linear section of the dose-response curves were used in the generation of the TCDD standard

curves and in the calculation of TEQs.

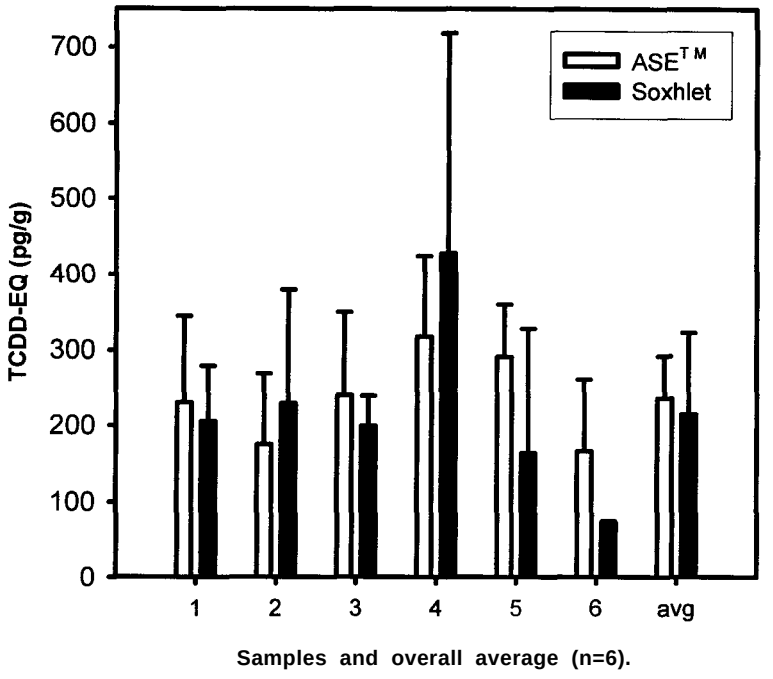

Figure 1. Comparison of TCDD TEQ (pg/g) estimates based on H4IIE assays for extracts using ASE™ and Soxhlet methods.

RESULTS AND DISCUSSION

Six samples of Saginaw River sediments were extracted with the ASE and Soxhlet methods and the TEQ determined for each replicate extraction (Figure 1). ASE and Soxhlet extraction techniques were averaged to yield 237 ± 55 and 216 ± 107 pg TCDD TEQ/g sediment, respectively. These concentrations were not significantly different (P = 0.7120) by t-test. The equal recovery efficiencies for the two methods have also been previously established by Richter et al. (1997), who reported [^{13}C] TCDD surrogate recoveries for ASE and Soxhlet were 72 and 68%, respectively. Both the reported recovery values and the determined responses of the H4IIE cell line to sediment extracts prepared by ASE and Soxhlet extraction indicate that the two methods are comparable in efficiency. However, ASE has the advantage of requiring approximately 20% less time (Table 1) and

50% less solvent (Table 2) than Soxhlet extraction. Additionally, unlike Soxhlet extraction, which requires that bulky glassware and heating mantles must be set-up in a hood and is therefore highly limited in the number of simultaneous extractions due to space requirements, the ASE extraction device is relatively compact and is capable of holding 24 cells at one time. Thus, for laboratories that have high sample throughput, 18-24 hrs may generate perhaps 12 samples using Soxhlet extractions, while 24 samples can be extracted overnight with the ASE system. The ASE one-step method eliminated a separate sample cleanup and offered a further improvement, providing a 15% reduction in time and a 52% reduction in solvent use as compared to standard ASE extraction (Tables 1 and 2). The recovery of [^{3}H] TCDD was determined to be similar for the two ASE methods (87.2% ± 1.8 for standard ASE, 80% ± 6.2 for ASE-one-step).

Table 1. Time Requirements in hours over five day period

	Soxhlet	ASE™	ASE™ One-Step
Extraction	18 to 24	0.5	0.5
*SA/SG Cleanup & #Concentrating	18	16.5	2
Total Time	36 to 42	17	2.5

* Time includes making the SA/SG and packing columns
# Includes concentration of crude and clean extracts

Table 2. Volume requirements for one sample in mL for each technique

Step (Solvent Used)	Soxhlet	ASE™	ASE™ One-Step
Extraction (DCM)	250	50	N/A
Column Conditioning Hexane (HEX)	80	80	80
SA/SG Cleanup (10% DCM in HEX)	140	140	50
Total Volume	470	270	130

When using the H4IIE cell line to determine TCDD TEQs, it is important that the extract cleanup method remove all PAHs, as small amounts of PAHs bleeding through the column would cause induction of EROD activity and falsely elevate the estimated TEQ. The standard ASE method coupled with the SA/SG column and the ASE one-step method were shown to be equally effective in removing PAHs, with no EROD response observed in H4IIE cells exposed to cleaned

extracts as compared to a high response to the uncleaned extract (Table 3).

Table 3. H4IIE maximal responses of PAH spiked sediments extracted by ASE™

Method	Response (ng resorufin / µg protein)
No Clean Up	540
Separate SA/SG cleanup	Below Limit of Detection
One-Step	Below Limit of Detection

To test whether samples containing high levels of PAHs could overload the SA/SG column and allow PAH breakthrough, a crude extract of a sediment highly contaminated with PAHs was subjected to the standard SA/SG column cleanup both with and without the addition of a PAH standard mixture, and the responses of the H4IIE cell line were compared. If the column were overloaded, the addition of the PAH standard would result in a higher TEQ for the spiked extract as compared to that of the unspiked extract due to column breakthrough. Crude extracts of the highly PAH-contaminated New York sediment resulted in an extremely high TEQ (15125 ± 2850 pg TCDD TEQ/g dry sediment, n = 5), which dropped to below detection limits upon SA-SG column cleanup. Spiking the extract with PAHs prior to column cleanup did not affect the results, indicating that the SA-SG column was not overloaded.

Aliquots of the highly PAH-contaminated New York sediment were spiked to assess effectiveness of the one-step ASE method in removing PAHs directly from sediments. No EROD induction was observed in cells exposed to these extracts, indicating that this method is as effective in removing PAHs as the more laborious column cleanup.

In conclusion, the one-step ASE extraction and cleanup procedure described in this paper results in TCDD extraction and PAH cleanup efficiencies comparable to Soxhlet extraction, but requires 33% less time and 72% less solvent than Soxhlet extraction. This rapid method represents a significant improvement for sample preparation in the cell based H4IIE assay for TCDD TEQ determination.

Acknowledgments. This research was supported by the Dredging Operations Environmental Research Program of the U.S. Army Engineers. Permission was granted by the Chief of Engineers to publish this information.

REFERENCES

Casterline JL, Bradlaw JA, Puma BJ, and KU Y (1983) Screening of fresh water fish extracts for enzyme-inducing substances by an aryl hydrocarbon hydrolase

Induction Bioassay Technique. J Assoc Off Anal Chem 66:1136-1139

Giesy JP, Verbrugge DA, Othout RA, Bowerman WW, Mora MA, Jones PD, Newsted JL, Vandervoort C, Heaton SN, Aulerich RJ, Bursian SJ, Ludwig JP, Dawson GA, Kubiak TJ, Best DA, and Tillit DE (1994) Contaminants in fishes from Great Lakes-influenced sections and above dams of three Michigan rivers. II: Implication for health of mink. Arch Environ Contam Toxicol 27:213-233

McFarland VA, McCant DD, Inouye LS, McNair EC, and Engler RM (1997) Guidance for performance of the H4IIE dioxin screening assay. DOER-C1, Dredging Operations and Environmental Research Program Technical Notes, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

Richter BE, Ezzell JL, Knowles DE, and Hoefler F (1997) Extraction of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans from environmental samples using accelerated solvent extraction (ASE). Chemosphere 34:975-987.

Sanderson JT, Aarts JMMJG, Brouwer A, Froese KL, Denison MS, and Giesy JP (1996) Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: Implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 137:316-325.

Schwirzer SMG, Hofinaier AM, Kettrup A, Nerdinger PE, Schramm KW, Thoma H, Wenege M, and Wiebel FJ (1998) Establishment of a simple cleanup procedure and bioassay for determining 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity equivalents of environmental samples. Ecotoxicol Environ Safety 41:77-82

Tillitt DE, Giesy JP, and Ankley GT (1991) Characterization of the H4IIE rat heptoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons in environmental samples. Environ Sci Technol 25:87-92.