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Immunochemical determination of dioxins in sediment and serum samples

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Abstract

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are considered highly toxic contaminants and the environmental and biological monitoring of these compounds is of great concern. Immunoassays may be used as screening methods to satisfy the growing demand for rapid and low cost analysis. In this work, we describe the application of an immunoassay that uses 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) as a surrogate standard for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to sediment and human serum samples. Sample extraction and preparation methods were developed with the aim to establish the simplest, cost-effective and efficient removal of the matrix interferences in the enzyme-linked immunosorbent assay (ELISA). The overall method for sediments is based on a hexane extraction; clean up by a multilayered silica gel column and an activated carbon column; an organic solvent exchange with DMSO–Triton X-100 and ELISA measurement. The gas chromatography–high resolution mass spectrometry (GC–HRMS) validation studies ($n = 13$) revealed that the method is suitable for the toxic equivalents (TEQ) screening of dioxin in sediments with a method detection limit of about 100 pg g^{-1} dry sediment with a precision of 13–33% R.S.D. The analysis of a large number of samples originating from different sources would be required to establish more precisely the screening level, as well as the number of false positives and negatives of dioxin TEQ by the immunoassay for sediments. The immunoassay method for sediment analysis offers improvement in speed, sample throughput, and cost in comparison to GC–HRMS. Dioxins were determined in serum samples after a simple liquid–liquid extraction and solvent exchange into DMSO–Triton X-100 without further dilution. The current method (approximate method LOQ of 200 pg ml^{-1} serum) is not sufficiently sensitive for the determination of dioxins in serum to measure acceptable exposure limit.
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Keywords: Dioxin; Microwave extraction; Immunoassay; Sediment; Serum

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), commonly known as dioxins, are highly toxic [1] ubiquitous environmental contaminants found in soil [2,3], sediment [4–6], air [7,8] and food (fish, meat, cow milk) [9]. Numerous epidemiological studies have demonstrated human exposure to dioxins in the low ppt range levels in adipose tissue [10], milk [11] and serum [12–14]. Due to the considerable public, regulatory, and scientific concern regarding potential human health effects of these compounds [15,16] sensitive analytical methods have been developed for monitoring dioxins at low levels. Multiple-step isolation and clean up procedures are necessary to determine trace levels of these analytes in complex environmental and biological samples. Due to its specificity and sensitivity, gas chromatography coupled with high resolution mass spectrometry (GC–HRMS) is considered the reference method in these analyses [17]. However, this analytical approach requires a time-consuming complicated sample clean up, a large equipment investment with associated maintenance costs, and highly trained analysts. Alternative bioanalytical methods have been evaluated either to replace the reference method or at least to alleviate analysis

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costs by their use in preliminary screening [18–20]. Several attempts have been made to develop sensitive and selective immunochemical techniques for dioxin analysis using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as the analyte because of its recognized higher potential toxicity [21–24]. In order to avoid using the toxic congener on a routine basis highly sensitive enzyme-linked immunosorbent assays (ELISAs) using 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) as an analytical surrogate standard have been developed [25,26]. Furthermore, it has been demonstrated that congener immunochemical recognition can be used as an estimate of the Toxic Equivalents (TEQs) for a variety of real samples [24,27–30]. Due to the intrinsic chemical properties of the dioxins, there is a general recognition of the need for sample clean up with respect to enzyme immunoassay analysis of PCDD/Fs in environmental media such as soil, sediments, fly ash, fish tissue, and milk [22,24,27–31]. However, for screening purposes, the immunoassay sample preparation needs to be less exhaustive than the multi-column clean up required for GC–HRMS.

Based on careful hapten design and synthesis a highly sensitive polyclonal antibody-based ELISA was previously developed in our laboratory [29]. The immunoassay used TMDD as a surrogate for 2,3,7,8-TCDD, had a 50% inhibition (IC_{50}) value approximately of 100 pg ml^{-1} TMDD and a detection limit of 30 pg ml^{-1} TMDD in buffer (50% DMSO–Triton X). Although a rapid sample extraction and preparation method using a Florisil column has been evaluated for the immunoassay analysis of dioxins in soil samples, further optimization and validation studies in other matrices were required. In the present work, we describe the application of the previously developed immunoassay to the determination of PCDD/Fs in sediment and serum samples. The objective of this work was to develop extraction and clean up procedures for sediment and serum samples prior to ELISA analysis according to the following criteria: quantitative dioxin extraction, efficient and simple clean up resulting in dioxin extracts that do not interfere with the immunoassay quantification; and applicability to sediments with different organic matter content.

2. Experimental

2.1. Chemicals and instruments

The surrogate standard, 2,3,7-trichloro-8-methyl-dibenzo-*p*-dioxin, used in the ELISA was synthesized according to [25,30]. The coating antigen (III-BSA) and the antibody (7598) used in the immunoassay were previously reported [29]. Tween 20, 3,3′5,5′-tetramethylbenzidine, goat anti-rabbit IgG conjugated to horseradish peroxidase, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). PBS (pH 7.5) is a phosphate buffered saline solution (8 g l^{-1} NaCl, 1.15 g l^{-1} Na_2HPO_4, 0.16 g l^{-1} KH_2PO_4, 0.2 g l^{-1} KCl). PBST is PBS with 0.05% Tween 20 (v/v). Coating buffer is 100 mM carbonate–bicarbonate buffer, pH 9.6. Citrate buffer is a 100 mM citrate–acetate buffer, pH 5.5. Human pooled serum was purchased from Valley Biomedical Inc. (Winchester, VA, USA). ^{14}C-2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (^{14}C-TCDD) was purchased from Cambridge Isotope Laboratories (Andover, MA) and native 2,3,7,8-TCDD was purchased from Cambridge Isotope Laboratories. CytoScint liquid scintillation cocktail was obtained from Fisher Scientific (Pittsburgh, PA). Solvents (hexane, dichloromethane, toluene) used in the clean up procedures were HPLC grade. All other reagents were prepared from reagent grade chemicals obtained from Fisher Scientific. Microtiter plates used for preparing standard and sample dilutions were from Dynex Technologies (Chantilly, VA). ELISA experiments were performed in high-binding 96-well microtiter plates (Nunc, Roskilde, Denmark). Absorbances were read with a SpectramaxPlus microplate reader (Molecular Devices, Sunnyvale, CA) in dual-wavelength mode (450–650 nm). Radiolabeled TCDD was counted in a Wallac Model 1409 liquid scintillation counter (Perkin-Elmer Life Sciences, Downers Grove, IL).

2.2. Sediment sample preparation

Sediment samples ($n = 12$) were from a US EPA Superfund Site. The control sediment sample was Standard Reference Material 1944, New York/New Jersey Waterway Sediment (National Institute of Standards & Technology, Gaithersburg, MD). In order to obtain efficient and reproducible extraction of the dioxins, the sediment samples were dried and homogenized. The samples were thoroughly mixed (homogenized) on a piece of aluminum foil and dried in a drying oven at 70 °C under vacuum (25 mmHg) for 24 h. Dried samples were ground in a mortar using a pestle that was covered with four layers of aluminum foil and subsequently sieved through a #10 (2 mm^2 mesh) sieve, in order to remove large stones and organic matter (e.g., wood and fibrous material) that could not be easily ground. The sieve was rinsed in hexane between samples to prevent cross contamination. Following drying and grinding, the samples were stored in glass jars with Teflon-lined lids at room temperature under desiccation. Portions of sediments were analyzed for moisture content and total organic matter (TOM). The water contents of the original wet and air-dry sediments were measured using a standard protocol (dried at 105 °C overnight in a vacuum oven). The TOM of the air-dry and sieved sediments was determined by loss on ignition (400 °C, 4 h).

2.2.1. Sample extraction (shaking, sonication and microwave extraction)

One gram of dry sediment sample (non-spiked or spiked with ^{14}C-TCDD) was extracted with 10 ml of hexane either by shaking (275 rpm, Environ shaker, Lab-line), or by

sonication in an ultrasonic water bath (Ultrasonic FS-14, Fischer Scientific), or in a microwave (Milestone Inc., Monroe, CT), then centrifuged. The hexane layer was collected by pipette, and the sediment was re-extracted twice with additional 10 ml portions of hexane. The combined solvent was then evaporated under nitrogen to a volume of 1 ml, subjected to a column clean up, then solvent exchanged with 0.5 ml DMSO containing 0.01% Triton X-100 (DMSOT). Extracts were analyzed by ELISA or liquid scintillation counting (LSC). The shaking extraction procedure was performed repeatedly (first extraction for 1 h, second extraction for 30 min and the third for 15 min). The sonication extraction was performed three times (10–15 min each) with periodic mixing of the sample by a vortex mixer. Microwave extraction was performed by the closed-vessel method using hexane as solvent and Weflon™ magnetic stir bars, which absorbed microwave energy and transferred heat to the surrounding medium. Each sediment sample was extracted with 10 ml of hexane at maximum power (1200 W). Several extraction times (6, 10, 30 min) and extraction temperatures (100, 110, and 130 °C) were studied in order to optimize conditions.

2.2.2. Sample clean up

2.2.2.1. Florisil column. Column clean up of the samples using activated Florisil columns was performed according to a procedure similar to that described in [29] with commercial Supelclean solid phase extraction (SPE) tubes filled with LC-Florisil (1 g, 100–120 mesh, Supelco, Bellefonte, PA). The Florisil column was conditioned with 10 ml of dichloromethane followed by 10 ml of hexane/dichloromethane (98:2, v/v) and the solvents were discarded. When the solvent was within 1 mm of the packing, the sample extract (in hexane) was applied to the column. The sample container was rinsed twice with 1 ml portions of hexane and applied to the column. The column was eluted with 20 ml of hexane/dichloromethane (98:2, v/v) and the eluent was discarded. The dioxins were then eluted with 10 ml of dichloromethane and collected. These eluents were evaporated to dryness under nitrogen and then redissolved into 0.5 ml of DMSOT for immunoassay measurement.

2.2.2.2. Concentrated sulfuric acid treatment. Hexane extracts obtained from sediments (non-spiked or spiked with ^{14}C-TCDD) were treated with concentrated sulfuric acid for 12 h (5 ml concentrated H_2SO_4 was added to 5 ml hexane extract and shaken at 2000 rpm). Both layers were separated by centrifugation and the colorless hexane layer was collected by glass pipette. The hexane was evaporated under nitrogen and then redissolved into 0.5 ml of DMSOT for LSC and/or immunoassay measurements.

2.2.2.3. Multilayered silica gel column. The multilayered silica gel column consisted of acid and basic silica layers. Acid/basic silica gels were prepared in a manner similar to that described in EPA Method 1613 [17]. Silica gel (100–200 mesh, Supelco) was washed with dichloromethane and then activated at 180 °C for at least one hour. Forty-four percent-sulfuric acid silica was prepared by mixing 4.3 ml conc. H_2SO_4 with 10 g of the activated silica gel. Basic (2% NaOH) silica gel was obtained after treating 10 g of the activated silica gel with 4.9 ml 1 N NaOH. The multilayered silica gel column was packed in a polypropylene filtration column (inside diameter 20 mm × length 85 mm, Supelco) loaded with the following material: 0.5 g of activated silica, 1 g of basic silica, 0.5 g of activated silica, 2 g of acid silica, 0.5 g of activated silica, and 1 g of anhydrous sodium sulfate. The silica gel bed was washed with 30 ml of hexane prior to loading of the sample extract. When the last solvent was within 1 mm of the packing, the sample extract (1 ml hexane) was added to the column followed by the hexane rinses. Dioxins were eluted with 30 ml hexane. The hexane was evaporated under nitrogen and then redissolved into 0.5 ml of DMSOT for LSC and/or ELISA measurements.

2.2.2.4. Activated carbon column. A CarboPrep™ 90 SPE column (graphitized carbon, 250 mg, Restek Corp, Bellefonte, PA) was pre-treated with 5 ml of toluene, 2.5 ml of dichloromethane/hexane (20:80, v/v), and 5 ml of hexane. The sample extract obtained after the multilayered silica gel column (1 ml hexane) was applied to the carbon column followed by the hexane rinses of the glass tube (2 × 1 ml). The carbon column was rinsed with 5 ml of hexane and 10 ml of dichloromethane/hexane (20:80, v/v). The dioxins were eluted with 40 ml of toluene. The solvent was evaporated to approximately 1 ml in the presence of 0.1 ml DMSOT as a trap solvent. When the toluene was completely evaporated, 0.4 ml of DMSOT was added and the extract analyzed by ELISA.

2.3. Serum Sample Preparation (liquid–liquid extraction)

Human serum (500 μl) (non-spiked or spiked with TCDD) was incubated for 30 min at room temperature in a glass centrifuge tube and then sonicated for 40 min. Proteins were precipitated by addition of 500 μl absolute ethanol. The mixtures were extracted three times with 4 ml of hexane with vigorous shaking for 1 min, then vortexing and centrifugation (3000 rpm for 3 min) after each extraction, using a total of about 15 ml of hexane. After each extraction the hexane was transferred to a clean tube. The combined extract was evaporated under nitrogen to 3–4 ml. To remove lipids 1 ml of sulfuric acid was added to the extract and then the extract was further washed with four approximate 4 ml portions of hexane that was added to new tubes. The transferred hexane was evaporated to complete dryness under nitrogen. The sample was reconstituted with 500 μl DMSOT and kept at room temperature in the dark. Before ELISA analysis, the samples were further diluted with PBS to make a 1:1 ratio of DMSOT and PBS.

2.4. ELISA

The method was similar to that reported in [29]. High binding microtiter plates were coated overnight at 4 °C with 100 μl per well of III-BSA coating antigen (0.05 μg ml^{-1} in coating buffer). After the coated plates were washed with PBST, 200 μl of blocking solution (0.5% BSA in PBS) was added and the plates were incubated for 30 min at room temperature. After another washing with PBST (three times), 50 μl per well of antibody 7598 diluted 1/3500 in PBS with 0.2% BSA and 50 μl per well of sample or standard (either TMDD or TCDD) (prepared in DMSO containing 0.01% Triton X-100:PBS, 1:1, v/v) were added. The plates were incubated for 90 min and then washed five times with PBST. Goat anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1:3000 in PBST, 100 μl per well) was added and the plates were incubated for 60 min at room temperature. Following another washing step with PBST (five times), the substrate solution (100 μl per well; 3.3 μl of 30% H_2O_2, 400 μl of 0.6% tetramethylbenzidine in DMSO per 25 ml of citrate–acetate buffer, pH 5.5) was added. The blue color development was stopped after 10–20 min with 2 M sulfuric acid (50 μl per well) and absorbances were measured at 450–650 nm. All samples and standards were analyzed in triplicate. Standard curves were obtained by plotting absorbance against the logarithm of TMDD or TCDD concentration, which were fitted to a four-parameter logistic equation: $y = \{(A–D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at no analyte, B the curve slope at the inflection point, C the concentration of analyte giving 50% inhibition (IC50), and D the minimum absorbance at infinite concentration. For the immunoassay, a surrogate standard, TMDD was used. TMDD provides a nearly identical calibration curve to 2,3,7,8-TCDD. The cross reactivity of TMDD/TCDD is 130%, but TMDD is less toxic to handle [25]. Sediment samples were analyzed by ELISA using TMDD standard curve. Only the accuracy and precision evaluation of the overall method for detection of dioxins in sediments was performed with sediments spiked with TCDD and quantified by ELISA using TCDD standard curve.

2.5. Gas Chromatography–High Resolution Mass Spectrometry (GC–HRMS)

Samples analyzed by GC/HRMS follow general guidelines described in EPA Methods 8290 and 1613, with some minor modifications/improvements [17,32]. A Hewlett-Packard 5890 GC equipped with a Micromass Autospec Ultima HRMS was used. Analyses were performed in the selected ion-monitoring mode at a resolution of 10,000 or greater. A DB5 column was used for initial analysis of the seventeen 2,3,7,8-PCDD/PCDF congeners. Since a DB5 column does not completely resolve 2,3,7,8-TCDF from all other tetra-chlorinated dibenzofurans, a second column (DB-225) confirmation was conducted as needed. Calculations were performed using Opusquan, a software program designed for dioxin/furan analysis by VG Co. Ltd.

3. Results and discussion

3.1. Sediments

3.1.1. Extraction of dioxins from sediments (shaking, sonication, microwave extraction)

Sample preparation for conventional GC–HRMS analysis of dioxins in soil and sediments is generally conducted with organic solvents such as hexane, toluene, dichloromethane, or hexane/acetone [33]. Enzyme immunoassays are usually performed in predominantly aqueous media (tolerating 5–20% organic solvent). However, dioxins have extremely low solubility in water miscible solvents of intermediate polarity (such as methanol) and these solvents are unlikely to be acceptable for extraction of low concentrations of dioxins from soil and sediments. Aprotic solvents such as dimethylformamide (DMF) [28] and dimethylsulfoxide (DMSO) [29] have also been tested as extraction solvents for dioxins from solid matrices for enzyme immunoassays. Our previous studies on the application of the dioxin ELISA to soil analysis demonstrated that hexane extraction resulted in the highest recoveries and the least interferences in the immunoassay [29]. Polar co-solvents used in the extraction step increased the matrix effect in the ELISA. Therefore, in the present study we focused on the use of hexane as an extraction solvent for dioxins from sediments.

Currently, the extraction of organic pollutants from solid environmental matrixes can be performed by a variety of extraction techniques each of which has some advantages and drawbacks [34,35]. Soxhlet extraction is the conventional method typically employed for extracting dioxins from soil, sediment and fly ash. However, Soxhlet extraction can be time-consuming and uses copious amounts of organic solvent [6,17,36]. US EPA Method SW-846-3550 [37] specifies sonication as one method for the extraction of non-volatile and semivolatile organic compounds from solids such as soil, sludge, and solid waste. In addition, US EPA Method 3546 [38] defines a microwave extraction procedure for semivolatile organic compounds (pesticides, herbicides, PCBs and PCDDs/PCDFs) from soil, clay, sediment, sludge, and solid waste. Microwave assisted extraction (MAE) has also been applied to the extraction of dioxin from sediment, sewage sludge, and fly ash [39–42]. We studied the efficiency of the dioxin extraction from dry sediment samples using two traditional methods (shaking and sonication) and one more modern extraction technique, MAE [43]. The recoveries obtained from ^{14}C-TCDD spiked sediments with different TOM content for shaking and sonication extraction are compared in Table 1. Repeated extractions (three times) and 30 ml of solvent were required for quantitative TCDD extraction by these two methods. The recoveries obtained for both procedures (shaking and sonication) were very similar

Table 1
Recoveries of ^{14}C-TCDD from spiked sediments by hexane extraction (shaking and sonication) and SPE

Sediment	TOM[a] (%)	Spiked amount ($pg\,g^{-1}$)	Recovery (%) (average ± S.D.)		
			Hexane extraction		
			Shaking[c]	Sonication[c]	
1	22.5	3000	78 ± 1	93 ± 9	
3	10.3		87 ± 4	86 ± 5	
SRM[b]	9		84 ± 3	81 ± 5	
4	1.7		95 ± 4	n.t.[e]	
3	10.3	600	83 ± 6	90 ± 11	
SRM	9		92 ± 8	89 ± 9	
5	4.2		89 ± 15	71 ± 5	
			SPE		
			Carbon[d]	H_2SO_4 washing[d]	Multilayered silica[d]
1	22.5	3000	83 ± 2	86 ± 6	85 ± 6
3	10.3		77 ± 11	88 ± 4	92 ± 3
SRM	9		84 ± 2	83 ± 3	90 ± 4
1	22.5	600	88 ± 9	86 ± 5	93 ± 8
SRM	9		90 ± 7	n.t.	84 ± 2
5	4.2		n.t.	n.t.	87 ± 13

[a] TOM (%)—total organic matter content of the sediment.
[b] SRM is the Standard Reference Sediment 1944.
[c] The data correspond to the average of three replicates.
[d] The data correspond to the average of two replicates.
[e] n.t.—not tested.

(majority higher than 80%). Both methods were very reproducible with slightly higher R.S.D. (8–17% R.S.D.) for the low spike level (600 $pg\,g^{-1}$).

MAE was evaluated as an attractive alternative to the above conventional techniques with the goal to minimize solvent consumption and extraction time and to improve extraction efficiency. As hexane was the solvent of choice, the MAE was performed in the presence of Weflon™ bars, which absorb microwave energy and are used to heat non-polar solvents [44]. The effect of several parameters (number of extractions, temperature, and time) on the extraction efficiency for SRM sediment spiked with ^{14}C-TCDD was studied. Two consecutive extractions (2 × 10 ml) were needed for complete dioxin extraction from the SRM sediment. Most of the analyte was recovered in the first extraction, with the second extraction yielding between 4.9% and 7.7% of the initially spiked amount. The total recoveries (first and second extraction, 6 min extraction time each) determined for the ^{14}C-TCDD spiked SRM sediment were 88.7 ± 10.5% ($n = 3$) for 3000 $pg\,g^{-1}$ spiked level and 82.1 ± 7.5% ($n = 3$) for 600 $pg\,g^{-1}$ spiked level. There was no significant difference between the recovery values obtained at different extraction times (6, 10 and 30 min). Increasing the temperature from 100 to 130 °C did not improve the extraction efficiency either. According to the literature [39,40] and US EPA Method 3546 [38], PCDDs/PCDFs can be extracted from sediments by MAE at 100–115 °C for 10–20 min with recoveries higher than 90% when hexane:acetone (1:1) was used as solvent. Our recoveries for the radioactive standard ^{14}C-TCDD under similar conditions were much lower (53.4 ± 3.8%, $n = 5$).

Summarizing, the solvent usage and microwave extraction recoveries of ^{14}C-TCDD from dry sediments were very similar to those obtained by the shaking and sonication methods. The time of the extraction procedure was significantly reduced by MAE (15 min for heating the solvent to 120 °C at maximum power plus the extraction time of 6–10 min) in relation to the shaking method (105 min); however, it was similar in time to the sonication procedure (30 min). Another aspect to be considered when choosing the extraction procedure is the effect of the interferences extracted by the three methods on the ELISA performance. No difference was found among the ELISA matrix effects of hexane extracts obtained by sonication, shaking or microwave extraction for three different sediments (2, 3, SRM) tested after their purification by the multilayered silica column. The matrix interferences extracted by the three methods were eliminated to the same extent in the clean up step. Based on this, we decided to use the sonication method, which is the simplest, easiest to perform, fastest and lowest cost procedure for dioxin extraction from sediments that we evaluated. Although not evaluated in this study, accelerated solvent extraction (Dionex) and/or pressurized solvent extraction could be another relatively simple extraction method compatible with ELISA detection.

In addition, it should be noted that the water content in the samples greatly influenced the dioxin recovery. We determined the extraction recoveries (by shaking and sonication) for the original wet sediment samples spiked with ^{14}C-TCDD after drying with anhydrous sodium sulfate. For wet samples with low water content (<20%) the recoveries were the same as for the respective dried sediment samples. However, low recoveries (20–40%) were encountered for sediments with higher water content (>50%) (data not shown). The results demonstrated that it is crucial to standardize the moisture content in all samples and that dried sediment samples are required to achieve efficient and reproducible dioxin extraction with hexane.

Decreasing recoveries resulting from aging of matrices is a well-known phenomenon and can be explained by native analytes being more strongly bound to the matrix due to weathering and longer contact time than spiked analytes [45]. We tested the possible aging effects on the dioxin extraction recovery. Dried sediments that had been spiked with ^{14}C-TCDD were kept at room temperature for three months before hexane extractions were carried out. No significant difference was observed when compared to freshly spiked samples (data not shown).

3.1.2. Clean up and ELISA matrix effect

As stated above, a Florisil column clean up has been used to minimize ELISA matrix effects from hexane soil

extracts [29]. Initially, we applied the same method for dioxin detection to several sediment samples and for most of them we observed significant overestimation of the levels determined by GC–HRMS (data not shown). In general, sediments have a higher organic matter content than soils, and this may result in higher matrix effects in the ELISA. Therefore, a more efficient clean up procedure was needed in order to accurately determine dioxin concentrations in sediment samples by the present immunoassay. Note that the critical steps in an immunoassay clean up involve removal of the high levels of polar compounds such as phthalates, phenols and carboxylic acids, as well as hydrocarbons [18]. Most current sample clean ups for the purpose of PCDD/F immunoassay employ some variation of the standard GC–HRMS clean up methods such as sulfuric acid treatment, multilayered silica gel columns, activated carbon, etc. [24,27,28,30,36,46–48]. Our main objective was to find a simple, rapid, low cost and efficient clean up method that could allow the immunoassay detection of dioxins in sediments of varying properties. Thus, we studied graphitized carbon and sulfuric acid treatments as possible methods to eliminate immunoassay interferences present in the hexane sediment extracts. CarboPrep^{TM} 90 SPE columns formed by a nonporous, chromatographic grade-graphitized carbon were used. These columns have been shown to remove sample matrix interferences such as hydrocarbons and humic acids that are common matrix interferences found in soil and sediment samples [49]. Alternatively, the strong acidic environment of the sulfuric acid treatment would likely remove acidic polar interferences, polyaromatic hydrocarbons and other contaminants. The sulfuric acid treatment of the hexane extracts was performed in two ways. The first was simple washing (shaking) of the hexane phase with concentrated sulfuric acid followed by centrifugation and collection of the hexane layer. This procedure was compared to a multilayered silica gel column (44%-H_2SO_4-silica/2%NaOH-silica) clean up. The column procedure allowed higher throughput, was more convenient to perform and avoided the use of large volumes of concentrated sulfuric acid. Initially, we evaluated the recovery of these clean up procedures by LSC for sediment samples spiked with ^{14}C-TCDD (see Table 1). The average recovery obtained for the sediments with different TOM was about 86% (with average %R.S.D. of 6%) for the three sample treatment procedures. For both high and low spiked levels the recoveries were similar. The three clean up methods were reproducible with the highest %R.S.D. observed of 14.9%. Based on the ^{14}C-TCDD recovery all three procedures could be considered suitable for clean up of the hexane extracts. The most appropriate clean up method was chosen considering the most efficient removal of the immunoassay interferences.

In sediment matrix effect studies it is very important to consider the variety of the matrix effects that could be observed for different sediment samples. The sediments (n = 13) available for the present work have TOM contents varying in the range 0.6–22.5%. As representative samples we chose sediments with different TOM and those that contained the lowest amount of dioxins according to GC–HRMS data. Thus, sediment 2 (3% TOM, 10 TEQ pg g^{-1}), sediment 3 (10.3% TOM, 3.4 TEQ pg g^{-1}), sediment 4 (1.7% TOM, 21.3 TEQ pg g^{-1}), sediment 5 (4.2% TOM, 80.9 TEQ pg g^{-1}) and sediment 6 (2.8% TOM, 20.6 TEQ pg g^{-1}) were chosen. We also used the SRM 1944 sediment (9% TOM) assuming 250 pg g^{-1} TMDD equivalents based on the GC–HRMS data and immunoassay cross-reactivity data. As no blank matrix was available, the presence of a matrix effect was evaluated using the method of standard addition, where a non-spiked sample was subjected to the hexane extraction and clean up procedure. The DMSOT extract (0.5 ml) was then further diluted in DMSOT. Each dilution was divided into two portions, with one portion fortified with a known concentration of TMDD (post-treatment spike). Both portions were measured by ELISA and the difference between them indicated the fortification level in the absence of a matrix effect. This procedure was applied to the above selected sediment samples in the evaluation of various sample treatment methods (Florisil, carbon column, sulfuric acid washing, multilayered silica gel column, multilayered silica gel column followed by carbon column). The results of the standard addition method are summarized in Table 2. As can be seen, the Florisil and carbon columns were not able to remove the immunoassay interferences; and more than 100-fold dilutions were needed to eliminate the matrix effect observed. In contrast, the sulfuric acid treatment resulted in a more effective clean up and less dilution was required to perform accurate ELISA quantification. Both methods of sulfuric acid treatment (multilayered silica gel column and sulfuric acid washing of the hexane extracts) were very similar regarding their ability to remove immunoassay matrix interferences. The effective improvement in the clean up achieved with the multilayered silica gel column is illustrated by comparing a buffer standard curve to standard curves of DMSOT extracts that underwent no clean up or clean up on either a Florisil or multilayered silica gel column (Fig. 1). It can be observed that the ELISA is strongly inhibited even after a 225-fold dilution of the extract (0.5 ml DMSOT) when no clean up is performed (Fig. 1A). This matrix effect of the sediment extract can be partially reduced by the use of a Florisil column. As seen in Fig. 1B, a 75-fold dilution corresponds to 50% signal inhibition. However, the multilayered silica column treatment is even more efficient. As seen in Fig. 1C, the standard curve performed in the 75-fold diluted extract is almost identical to the curve generated in the assay buffer. Therefore, the inhibition of the immunoassay observed for neat extracts can be significantly eliminated by the multilayered silica gel column. Furthermore, for some sediments with low TOM (e.g. 5, 6) only a two-fold dilution of the DMSOT extract was needed for accurate ELISA measurements (see Table 2). However, for other sediments (4, SRM) a matrix effect was still present and higher dilutions (8–80-fold) were required, raising the detection limit of the overall method. This fact suggested

Table 2
Matrix effect on the ELISA for various clean up methods evaluated for different sediments

Sediment	TOM[a] (%)	Dilutions to eliminate matrix effect in ELISA[b]			
		Florisil	Carbon	Multilayered silica (H_2SO_4 washing)	Multilayered silica followed by carbon column
2	3	>100	>100	5	No dilution
3	10.3	>100	>100	5	No dilution
SRM	9	>100	>100	80	10
4	1.7	n.t.	n.t.	8	2
6	2.8	n.t.	n.t.	2	n.t
5	4.2	n.t.	n.t.	2	n.t.

[a] TOM (%)—total organic matter of the sediment.
[b] The dilution factor reported is the minimum dilution of the DMSOT extract (0.5 ml) required to obtain 100 ± 15% recovery of the fortified TMDD (50 pg ml^{-1} post-treatment spike) using the standard addition method.

the necessity of an additional carbon column clean up. Although this additional clean up step made the sample preparation more time consuming and increased solvent usage, it resulted in about a 4–5-fold improvement in the limit of detection allowing most of the sediment extracts to be analyzed directly. The analysis of 10 sediment samples can be performed in 15 h requiring 60 ml of hexane for the extraction and multilayered silica column clean up and 40 ml of toluene for the carbon column clean up. Finally, it is important to note that during method development clean up blanks for the multilayered silica gel and carbon columns were tested in the ELISA. The use of high purity (HPLC grade) solvents and the complete evaporation of the organic solvent extracts in the presence of Triton X-100 as a solvent trap were crucial factors for obtaining reliable results.

3.1.3. Accuracy and precision of the method

The accuracy and precision of the method were evaluated with various sediment samples spiked with TCDD levels at least three times the ambient TCDD amount determined in non-spiked samples. Spiked and non-spiked samples were subject to hexane extraction and further clean up by multilayered silica and carbon columns, solvent exchange to DMSOT and finally quantification by ELISA using a TCDD standard curve. Quality control procedures included the analyses of standards, controls, method blanks, matrix spike and replicate analyses. The accuracy was evaluated based on the recoveries obtained from the spiked samples and the precision as the percent relative standard deviation (%R.S.D.) between replicate sample analyses (see Table 3). The results showed that the method developed was consistent and reliable and can be applied to the analysis of sediments with different TOM contents. The recoveries were in the range 70–113% with the exception of two samples spiked at a low level where 60% of the spiked amount was detected. The %R.S.D. was in the range 5–20%. These accuracy and precision parameters are acceptable according the European Commission (EC) guidance on analytical methods for residues in soil [50] and fulfill the EC requirements for the official control of dioxins by screening methods, which suggest recoveries in the range of 30%–140% and a coefficient of variation below 30% [51]. Considering the ELISA LOD (12 ± 3.7 TMDD equivalents pg per g dry sediment, $n = 23$) and the overall method accuracy we estimated that the LOD for the determination of PCDDs/PCDFs in sediments was about 20 TMDD equivalents pg per g dry sediment. This was based on 1 g of sediment extracted into 0.5 ml of DMSOT (without further dilution) after clean up on multilayered silica gel and carbon

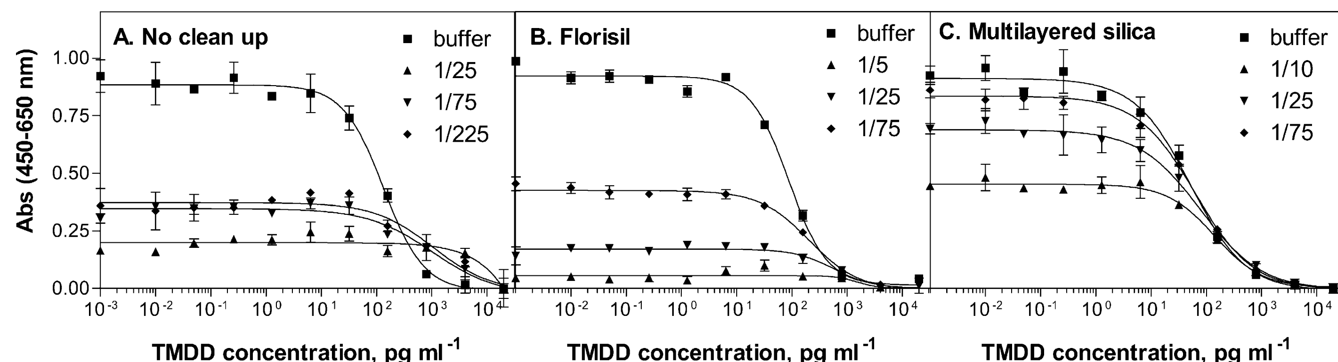

Fig. 1. Matrix effects caused by the sediment extract after different sample treatment. (A) No clean up. (One g of sediment was extracted with hexane, and solvent exchanged into DMSOT.); (B) florisil column clean up (the dichloromethane extract from the Florisil column was solvent exchanged into DMSOT); (C) multilayered silica gel column clean up. (The hexane extract from the multilayered silica column was solvent exchanged into DMSOT.) The DMSOT extract in all cases was further diluted in DMSOT (1/5 to 1/225 dilutions). The TMDD standards were prepared in DMSOT and further diluted in PBS (1:1).

Table 3
Accuracy and precision of the dioxin analysis of sediments by ELISA

Sediment	TOM[a] (%)	Spiked amount[d] ($pg\,g^{-1}$)									
		50		200		600		2500		7500	
		Recovery[b]	R.S.D.[c]	Recovery	R.S.D.	Recovery	R.S.D.	Recovery	R.S.D.	Recovery	R.S.D.
1	22.5	–	–	–	–	71	19	98	30	88	11
2	3	76	19	87	30	86	5	88	13	102	17
3	10.3	63	10	60	5	76	34	96	14	110	22
SRM	9	–	–	–	–	113	14	92	8	107	18
7	19.5	–	–	–	–	–	–	–	–	95	20

[a] TOM (%)—total organic matter of the sediment.
[b] The recovery (%) is calculated as the ratio of the difference between the concentration of TCDD measured by ELISA in the spiked and non-spiked samples vs. the spiked concentration. Sediments were subjected to hexane extraction, a multilayered silica column and a carbon column clean up.
[c] R.S.D.—% Relative standard deviation ($n = 3$).
[d] The samples were spiked with TCDD levels at least three times the ambient amount determined in non-spiked samples.

columns. For fast screening purposes simpler sample preparation methods using only multilayered silica gel (or sulfuric acid washing of the hexane extract) and 10-fold dilution of the DMSOT extract can be performed with an LOD of approximately 100 TMDD equivalents pg per g sediment. The LOD of the method presented in this work is similar to the theoretical sensitivity of 100 $pg\,g^{-1}$ soil reported for the test tube version [24] and 25 $pg\,g^{-1}$ soil for the microplate version [27] of the PCDD/Fs commercial immunoassay system of TEQ screening.

3.1.4. ELISA/GC–HRMS validation

Most of the PCDDs and PCDFs with high TEF (Toxic Equivalent Factor) values (>0.1) have strong or moderate cross reactivity in the ELISA used in the present study, which suggested that the immunoassay might be a good indicator of dioxin toxicity [29]. It has been demonstrated that this immunoassay can be used as a TEQ screening method for dioxins in biota (fish and egg) [29] and in milk samples [30]. We tested this correlation by comparison of the expected ELISA response to the TEQ values for a set of 13 sediment samples. All samples had been analyzed by GC–HRMS and concentration values had been determined for each of the 17 toxic congeners. The predicted ELISA response for each sample was calculated by multiplying the GC–HRMS concentration for each of the toxic congeners by the corresponding ELISA cross-reactivity values [29], then summing each value. The TEQ value for each sample was calculated using the GC–HRMS data and based on WHO-TEFs [52]. The relationship between the predicted ELISA value and GC–HRMS TEQs is plotted in Fig. 2. A strong correlation was observed with a slightly higher deviation at low TEQ levels. It is important to note that the theoretical LOD of the enzyme immunoassay was 12 ± 3.7 TMDD equiv pg per g sediment. Fig. 2 also presents the results of the dioxin analysis of the sediments by the ELISA after the hexane extraction and clean up (multilayered silica gel and carbon columns) procedures described above. The ELISA data corresponded to the mean value of two or three replicate analyses of each sediment sample. The %R.S.D. was in the range 13–33% with average %R.S.D. of 21%. For levels higher than 100 $pg\,g^{-1}$ a very good correlation between predicted and actual (measured) ELISA response demonstrated that immunoassay interferences had been removed. Furthermore, there was good correlation between the measured TMDD equivalents and the GC–HRMS TEQs (correlation coefficient of 0.974, slope 1.06) suggesting that the immunoassay method can be applied for screening of dioxin levels in sediment samples. No false positive values were obtained for levels higher than 100 $pg\,g^{-1}$. However, the data did indicate some false positive interference for the lower concentration samples (the estimated method detection limit is about 20 pg TMDD per g sediment). This could be attributed to the presence

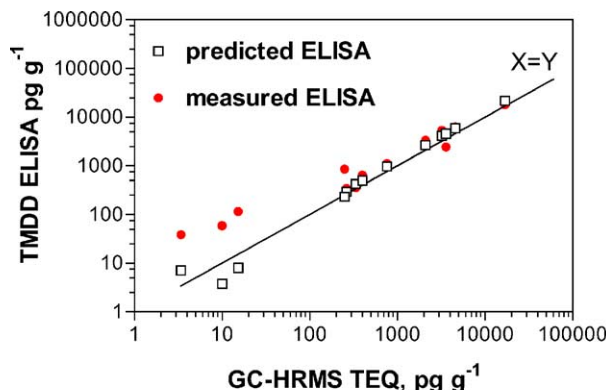

Fig. 2. Relationship between TMDD equivalents (predicted and measured by ELISA) and TEQs determined by GC–HRMS for 13 sediment samples. Each mass concentration value (each of the 17 toxic PCDD/Fs congeners for each sample) was multiplied by the corresponding ELISA cross-reactivity value [29] to obtain the predicted ELISA response. Predicted ELISA responses (open symbols) for the individual congeners were summed for each value to give the predicted ELISA response for the sample. GC–HRMS TEQ values were calculated based on WHO-TEFs [52]. The measured ELISA response (filled symbols) was the mean value of two or three replicates of the immunoassay analysis of sediments extracted with hexane, cleaned up by multilayered silica gel and carbon columns and solvent exchanged to DMSOT (see Section 2). The %R.S.D. was in the range 13–33% with average %R.S.D. of 21%. The correlation coefficient r^2 was 0.974 with a slope of 1.06.

Table 4
Recoveries obtained by the standard addition method as applied to serum samples ($n = 2$)

Spiked concentration ($pg\,ml^{-1}$)	Detected concentration ($pg\,ml^{-1}$) (average ± S.D.)	Recovery (%)
50	55 ± 15	111 ± 29
100	85 ± 7	85 ± 7
200	192 ± 10	96 ± 5

Human serum samples were subjected to liquid–liquid extraction, followed by a sulfuric acid treatment, spiked with TMDD, and measured by the ELISA.

Table 5
Accuracy and precision of the dioxin analysis of serum by ELISA ($n = 2$)

Spiked concentration ($pg\,ml^{-1}$)	Detected concentration ($pg\,ml^{-1}$) (average ± S.D.)	Recovery (%)
200	124 ± 18	62 ± 9
500	345 ± 22	69 ± 4
1000	1010 ± 79	101 ± 8
2000	2313 ± 386	116 ± 19

Human serum samples were spiked with TCDD, subjected to liquid–liquid extraction, followed by a sulfuric acid treatment, and ELISA analysis. The recovery is calculated with respect to the nominal spiked concentration.

of high levels of congeners that have significant ELISA cross-reactivity but are not determined by the conventional analysis because they contribute minimal toxicity. Such compounds are 2-Br-3,7,8-TriCDD, 2,3-DiBr-7,8-DiCDD, 1,3,7,8-TCDD, 2,3,7-TriDD, 1,2,3,4,7,8-HxCDD with immunoassay cross-reactivities of 110%, 115%, 43%, 6.7% and 1%, respectively [29]. In conclusion, the validation studies performed with 13 samples indicate that the method is suitable for screening samples prior to GC–HRMS with a screening level of $100\,pg\,g^{-1}$ sediment. This screening level is lower than the "action values" set for residential soils in Germany, The Netherlands and Finland [53] and lower than the preliminary remediation goal of $1000\,pg$ TEQ g^{-1} adopted by US EPA [54]. The analysis of a large number of samples originating from different sources would be required to establish more precisely the screening level, as well as the number of false positive and negative values of dioxin TEQs by the immunoassay.

3.2. Serum samples

3.2.1. ELISA matrix effect

Initially, we evaluated the matrix effect of non-treated serum on the immunoassay. Standard curves of TMDD were prepared in serum that was directly diluted (10-, 100-, 200- and 1000-fold) with the immunoassay buffer (DMSOT:PBS, 1:1, v/v) and compared to the buffer calibration curve. A 1000-fold dilution of the serum was necessary to remove matrix interferences (data not shown). This result suggested the application of a clean up step prior to ELISA analysis. Conventional dioxin analysis in biological samples relies on lipid extraction, either by liquid–liquid extraction or by solid–liquid extraction, and clean up based on solid-phase extraction [55–58]. We tested Florisil, C18 and activated carbon solid phases, but they were not able to efficiently remove the immunoassay interferences (data not shown). The most effective treatment of the serum was liquid–liquid extraction followed by a sulfuric acid treatment. Using the method of standard addition the ELISA matrix effect was evaluated for serum samples spiked with three different concentrations of TMDD after the sample treatment (post-treatment spike) (see Table 4). Treated serum samples were measured by ELISA without further dilution. The average recovery was 97.3%, indicating little or no matrix effect. This method was simple and required less organic solvents than reported solid-phase extraction methods and was not as time consuming [59,60].

3.2.2. Accuracy and precision of the method

The accuracy and precision of the dioxin determination in serum samples by ELISA after liquid–liquid extraction was evaluated with serum spiked with different concentrations of TCDD. Table 5 shows the overall mean recovery of TCDD from spiked serum was 86.9%. However, samples spiked at lower levels ($<500\,pg\,ml^{-1}$) had generally lower overall mean recoveries. Since the recovery decreased with decreasing concentration, lower concentrations of dioxin were not tested. Based on the data reported here the limit of quantitation was approximately $200\,pg\,ml^{-1}$. In order to reach the levels of dioxins found in human serum [61] a large volume (>100 ml) of serum would need to be extracted. Thus, the current ELISA does not seem to be a suitable screening method for this sample type.

4. Conclusions

Our studies demonstrated that the original method developed for soil extraction [29], was not completely suitable for the analysis of dioxins in sediment samples. Thus, we developed a new, relatively simple procedure for PCDD/F extraction using sonication coupled to a multilayered silica gel column clean up that allowed accurate immunoassay analysis. The hexane extraction of TCDD from dry sediments was similar in efficiency for the three methods tested (shaking, sonication and MAE). The sonication method was the simplest and easiest to perform. The multilayered silica gel column clean up provided sufficient removal of the ELISA interferences for most sediment samples (0.6–23% TOM), but for some samples an additional clean up step by activated carbon adsorption was required. Even in this case, the multi-step sample preparation used in conventional GC–HRMS analysis was significantly reduced. The accuracy (60–113%) and the precision (13–33% R.S.D.) of the immunoanalytical method are suitable for the environmental analysis of solid samples. The GC–HRMS validation studies performed with 13 samples revealed that the method is suitable for the TEQs screening of dioxin in sediments with a

method detection limit approximately 100 pg g^{-1} sediment. Further validation with a large number of sediment samples from different sites is necessary to assess more precisely the screening level, as well as the number of false positive and negative values of dioxin TEQs by the immunoassay. Similar to the sediment samples, liquid extraction followed by an acid wash of the extract was the most effective clean up method for serum samples. However, the limit of detection of the immunoassay is too high to make it practical for measuring ambient TCDD serum levels but it may be useful for determining acute exposure.

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