ELSEVIER

Analytica Chimica Acta 444 (2001) 169–178

ANALYTICA CHIMICA ACTA

www.elsevier.com/locate/aca

Highly sensitive dioxin immunoassay and its application to soil and biota samples

Guomin Shan [a], Whitney R. Leeman [b], Shirley J. Gee [a], James R. Sanborn [a], A. Daniel Jones [c], Daniel P.Y. Chang [b], Bruce D. Hammock [a,*]

[a] *UCD Cancer Research Center, Department of Entomology, University of California, 1 Shields Avenue, 303 Briggs Hall, Davis, CA 95616, USA*

[b] *Department of Civil and Environmental Engineering, University of California, Davis, CA 95616, USA*

[c] *Department of Chemistry, Penn State Intercollegiate Center for Mass Spectrometry, The Pennsylvania State University, University Park, PA 16802, USA*

Received 22 March 2000; received in revised form 12 January 2001; accepted 12 January 2001

Abstract

Tetrachlorodibenzo-*p*-dioxin (TCDD) is a well-known highly toxic compound that is present in nearly all components of the global ecosystem, including air, soil, sediment, fish and humans. Dioxin analysis is equipment intensive and expensive requiring low ppt or even ppq levels of detection. A simple, rapid, cost-effective method of analysis is desired to enable researchers to explore issues involving dioxin more quickly and to make more rational regulatory decisions. A sensitive immunoassay for TCDD has been developed in this laboratory. In the present study, this assay is further optimized using a new coating antigen system and validated with biota samples by HRGC-HRMS. The I_{50} of current assay to 2,3,7-trichloro, 8-methyldibenzo-*p*-dioxin (TMDD), a surrogate standard for TCDD, is 36 ± 6.0 ppt, and the lower detection limit (LDL) is 4 ppt in the buffer. A good agreement between immunoassay and GC/MS was achieved for fish and egg samples. To interface with the immunoassay, a rapid sample preparation method was developed for soil samples. Without any further cleanup, the soil extracts can be directly used in the ELISA with a detection limit at the low ppt level. It can be used as an on-site tool for environmental monitoring. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Dioxins; Immunoassay; TEF; Soils; Validation

1. Introduction

Polyhalogenated dibenzo-*p*-dioxins (PHDDs) are a well-known group of highly toxic chemicals present in nearly all components of the global ecosystem, including air, soil, sediment, fish and humans [1,2]. Within the last two decades, tremendous efforts have been made to investigate the potential risks to human health associated with exposure to these compounds, especially 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). No clear conclusion has been reached, however, it has been recently agreed that TCDD can be listed as a human carcinogen [3]. In spite of the large need for monitoring PCDDs, the only analytical method with sufficient sensitivity and selectivity for detection of PCDDs, including TCDD, is a high resolution gas chromatography and mass spectrometry (HRGC-MS) in conjunction with a multi-column cleanup [4]. This

* Corresponding author. Tel.: +1-530-7527-519; fax: +1-530-7521-537.
E-mail address: bdhammock@ucdavis.edu (B.D. Hammock).

0003-2670/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0003-2670(01)01159-X

method is costly and time consuming, and worldwide there are few qualified laboratories that can perform these analyses [5]. A simple, rapid, cost-effective method to aid in screening and analysis of samples is greatly needed for TCDD environmental and toxicological monitoring.

Immunoassay may be an ideal screening and semi-quantitative technique to fill such a need. However, due to the lipophilic properties and the difficulty of the chemistry of PCDDs, only a few attempts to detect TCDD by immunoassay have been reported [6–9], and the sensitivity and tolerance to sample matrices for these assays are still far below satisfaction. Based on careful hapten design and synthesis, a sensitive polyclonal antibody-based ELISA was developed in this laboratory [10]. In this study, this assay was further optimized with a new coating antigen, and validated by GC-MS with biota samples. A simple cleanup method was also developed for soil sample monitoring.

2. Experimental

2.1. Chemicals and immunoreagents

The standards, 2,3,7,8-TCDD, 1,2,3,4-TCDD, 1,3,7,8-TCDD, 1-chlorodibenzo-*p*-dioxin (1-CDD), 2,7-dichlorodibenzo-*p*-dioxin (2,7-diCDD), 3,3′,4,4′-tetrachlorobiphenyl (TCB) were purchased from Chem Service (West Chester, PA). 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-triCDD), 1,2,3,4,7,8-HexaCDD, 1,2,3,7,8-PentaCDD, 2,3,4,7,8-PentaCDD, 1,2,3,4,5,6,7,8-octachlorodibenzodioxin (OCDD), 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TCDF), 2,3,4,7,8-PentaCDF, and ^{14}C-TCDD (122 mCi/mmol) were purchased from Cambridge Iostope Laboratories (Andover, MA). 3,3′,4,4′,5-Pentachlorobiphenyl, 3,3′,4,4′,5,5′-hexachlorobiphenyl, 1,2,3,4,7,8-hexachlorodibenzofuran, 1,2,3,7,8-pentachlorodibenzofuran, 1,2,3,4,5,6,7,8-octachlorodibenzofuran (OCDF), 2-bromo-3,7,8-trichlorodibenzo-*p*-dioxin, and 2,3-dibromo-7,8-dichlorodibenzo-*p*-dioxin were from AccuStandard Inc. (New Haven, CT). 1,2,3,4,6,7,8-HeptaCDD, 1,2,3,4,6,7,8-HeptaCDF, and 1,2,3,6,7,8/1,2,3,7,8,9-HexaCDF were kindly provided by Dr. Stephen Safe (Texas A&M University). Surrogate standard 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was synthesized in this laboratory [11]. Organic starting materials for hapten synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F_{254} on glass plates from E. Merck (Darmstadt, Germany), and detection was made by ultraviolet light or iodine vapor stain. Flash chromatographic separations were carried out on 40 μm average particle size Baker silica gel.

The coupling reagents were purchased from Aldrich. Goat anti-rabbit (GAR) immunoglobulin conjugated to horseradish-peroxidase (HRP), bovine serum albumin (BSA), Tween 20 and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma Chemical Co. (St. Louis, MO).

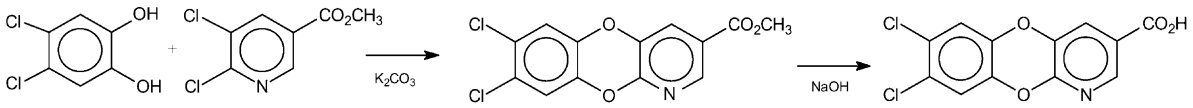
2.2. Instruments

NMR spectra were obtained using a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA). Chemical shift values are given in ppm downfield from internal tetramethylsilane. The GC/MS data were obtained using a Trio-2 GC/MS system (VG Masslab, Atrincham, UK) using 70 eV electron ionization (EI). A DB1 column (30 m, 0.25 mm i.d., 0.25 μm film; J&W Scientific, Sacramento, CA) was used with a helium flow rate of 30 cm/s. Samples were dissolved in tetrahydrofuran (THF) and splitless injections of 1 μl were made. The column was programmed from 80 (1 min hold) to 150°C at 20°C/min followed by an increase to 300°C at 10°C/min. ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark) and the absorbances were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450–650 nm).

2.3. Hapten synthesis

2.3.1. Methyl 7,8-dichlorobenzo[5,6][1,4]dioxino[2,3-b]pyridine-3-carboxylate

The synthesis route is showed in Scheme 1. In 5 ml of tetramethylene sulfone were placed under nitrogen 0.20 g (1.1 mmol) 4,5-dichlorocatechol [12] and 0.21 g (1 mmol) methyl 5,6-dichloronicotinate [13], 0.28 g (0.2 mmol) powdered potassium carbonate and 25 mg dibenzo 18-crown-6. The mixture was heated to 135–140°C and then allowed to cool to room temperature. After addition of water, the crude aqueous



Scheme 1.

mixture was extracted with ethyl acetate. The ethyl acetate solution was dried over sodium sulfate and the solvent removed under vacuum. Chromatography over silica gel with 4:1 hexane–ethyl acetate provided 71 mg (23%) of a white solid. mp: 196–200°C. ^{1}H NMR (CH_2Cl_2) δ 3.91 (OCH_3, s, 3 H), 7.07 (Ar, s, 1 H), 7.14 (Ar, s, 1 H), 7.75 (Ar, d, $J = 1.91$, 1 H), 8.47 (Ar, d, $J = 1.91$, 1 H). MS: Calculated for $C_7H_{13}Cl_2NO_4$, 311 [M^+]; 280 [$M - OCH_3$]$^+$; 252 [$M - CO_2CH_3$]$^+$.

2.3.2. 7,8-Dichlorobenzo[5,6][1,4]dioxino[2,3-b] pyridine-3-carboxylic acid (hapten III)

In 1 ml of ethanol were placed 13 mg (0.042 mmol) methyl 7,8-dichlorobenzo [5,6][1,4]dioxino[2,3-b]-pyridine-3-carboxylate and 8 mg NaOH (0.2 mmol). The mixture was heated to reflux and then allowed to cool to room temperature. Addition of 3N HCl precipitated a white solid (10 mg, 79%). mp > 280°C; NMR: attempts to dissolve the product in conventional NMR solvents (CH_2Cl_2, $HCCl_3$, CF_3CO_2H, etc.) were unsuccessful so structure was determined by mass spectrometry. MS: calculated for $C_6H_{11}Cl_2NO_4$, 297 [M^+], 280 [$M - OH$]$^+$; 252 [$M - CO_2H$]$^+$.

2.4. Conjugation of hapten III with BSA

Hapten (0.025 mmol) was dissolved in 2 ml of dry *N,N*-dimethylformamide (DMF) and then 6 mg (0.05 mmol) of *N*-hydroxysuccinimide (NHS) and 5.8 mg (0.03 mmol) of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC) were added. The reaction mixture was stirred overnight at room temperature. Forty-five milligrams of BSA was dissolved in 8 ml of PBS (PBS: 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KCl) and 2 ml of carbonate buffer (pH 9). The activated hapten was added dropwise to the protein solution. The mixture was stirred for 30 min at room temperature, and 6 h at 4°C. The solution was then dialyzed against PBS over 72 h at 4°C and stored at −20°C.

2.5. ELISA

The method was similar to that previously described by Shan et al. [14]. Microplates were coated overnight at 4°C with 100 μl per well of the appropriate coating antigen concentration in 0.1 M carbonate–bicarbonate buffer (pH 9.6). After the coated plates were washed with PBST (PBS plus Tween 20: 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KCl and 0.05% Tween, v/v), 200 μl of blocking solution (0.5% BSA in PBS) was added and incubated for 30 min at room temperature. After washing with PBST (three times), 50 μl per well antiserum diluted in PBS with 0.2% BSA and 50 μl per well of inhibitor solution (containing 50% DMSO) were added. The plate was incubated for 90 min and then was washed 8–10 times. IgG-HRP (diluted 1:3000 in PBST, 100 μl per well) was added and incubated for 60 min at room temperature. Following another washing step (PBST, 8–10 times), tetramethylbenzidine (TMB) substrate solution (100 μl per well; 3.3 μl of 30% H_2O_2, 400 μl of 0.6% TMB in DMSO per 25 ml of acetate buffer, pH 5.5) was added. The color development was stopped after 10–20 min with 2 M H_2SO_4 (50 μl per well), and absorbances were measured at 450–650 nm. All experiments were conducted in triplicate or quadruplicate. Standard curves were obtained by plotting absorbance against the logarithm of analyte 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 (I_{50}), and D is the minimum absorbance at infinite concentration.

2.6. Assay optimization

The TCDD assay was modified by using a new coating antigen. A two-dimensional titration was applied to find the best dilution of coating antigen and antiserum [15]. The effects of solvents were tested by dissolving

the analyte in PBS buffers containing various proportions of DMSO (0, 25, 50, and 75%) and incubating these with antibody in PBSB on the coated plate.

2.7. Cross-reactivity

The compounds listed in Table 3 were tested for cross-reactivity by preparing each compound in 50% DMSO-PBS, and determining the I_{50} in the ELISA. Cross-reactivity values were calculated as follows: $CR\% = (I_{50}\text{ of TMDD}/I_{50}\text{ of tested compound}) \times 100$.

2.8. Validation using biota samples

Extracts of fish and egg samples (from US Geological Survey, Columbia Environmental Research Center at Columbia, MO) were tested by our optimized enzyme immunoassay. These samples were prepared by multiple column cleanup steps, which is modified from US EPA method. The results were compared to GC/MS data provided by the USGS.

2.9. Florisil column preparation

Florisil columns were prepared as per US EPA Method 1613 [4]. Graduated serological pipettes and glass wool were rinsed with CH_2Cl_2 and dried. Sodium sulfate was baked at 400°C for 2 h, cooled in a dessicator and stored in a pre-cleaned glass bottle. To prepare column, a glass wool plug was inserted into the tapered end of a pipet, and the column was packed with 1.5 g of Florisil (60–100 mesh) topped with approximate 1 ml of sodium sulfate and a glass wool plug. This column was activated in an oven at 130–150°C for a minimum of 24 h and cooled for 30 min. It was used within 90 min of cooling.

2.10. Soil matrix effects and sample cleanup

Soil samples were from Davis, CA, and the Environmental Engineering Branch, US Army Engineer District, Sacramento, CA. Five grams of soil sample was placed in a 50 ml glass flask, and spiked with ^{14}C-TCDD (3 nmol, 1050 dpm) and mixed well before extraction for recovery studies. Extraction solvent (15 ml) was added into each flask and shaken for 1 h. After separation of solvent, another 15 ml of solvent was added and extracted for 30 min. The soil was filtered, and the combined solvent was then evaporated to a volume of 1 ml. This sample was split into two aliquots. The first aliquot was directly measured by liquid scintillation counting (LSC) and immunoassay. The second was subjected to column cleanup. The activated Florisil column was pre-eluted with 10 ml of CH_2Cl_2 and followed by 10 ml of hexane/CH_2Cl_2 (98:2, v/v) and the solvents were discarded. When the solvent was within 1 mm of 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/CH_2Cl_2 (98:2, v/v) and the eluent was discarded. The dioxins were then eluted with 35 ml of CH_2Cl_2 and collected. These eluents were evaporated to dryness under nitrogen and then redissolved into 0.5 ml of DMSO for ELISA measurement or liquid scintillation counting (LSC).

2.11. Safety considerations

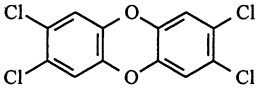
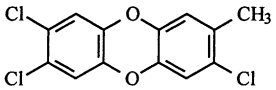
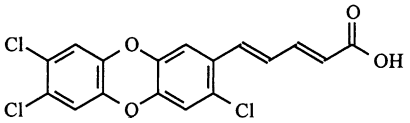
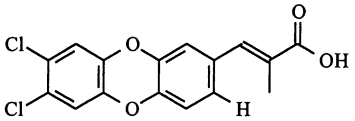
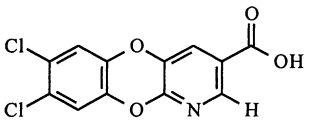
Although every effort was made to limit the use of TCDD in these assays, extreme caution is necessary because the toxicity of the surrogate TMDD is unclear. When dioxins and related compounds are handled, two pairs of protective gloves with some water between the layers, laboratory coat, and a pair of safety glasses are recommended [10].

3. Results and discussion

3.1. New antigen hapten design and synthesis

To develop a sensitive and specific assay, rational design of a coating antigen hapten is very important [16]. In the competitive ELISA format, assay sensitivity is determined by the difference in affinity between two competitive components (coating antigen and analyte of interest) with antibody. Heterologous assays usually have better sensitivity than homologous ones [14]. If the antibody has a much lower binding affinity for the coating antigen than for the target analyte, the binding of coating antigen to antibody can be easily displaced by a very low amount of analyte. Thus, a better sensitivity can be achieved with this antigen. Theoretically, an ELISA sensitivity is determined by antibody affinity. The ultimate detection limit of an

Table 1
Structures of TCDD, surrogate standard TMDD and dioxin haptens

Compound	Structure
TCDD	
TMDD	
Hapten I (immunogen)	
Hapten II	
Hapten III	

assay is approximately 10–100 times lower than the K_d of antibody [17]. The K_d of Ab7598 used in this study was measured by accelerator mass spectrometry [18] and found to be 0.1 nM. The I_{50} of a sensitive immunoassay based upon this antibody by Sugawara et al. [10] was 0.75 nM (detection limit 0.1 nM), which is far below the theoretical capability of this antibody. Thus, one of our objectives was to improve the assay sensitivity by the design of the antigen hapten.

The best antigen hapten selected in the previous report [10] was hapten II, which has similar structure to immunogen hapten I except for the replacement of Cl with H at position 8 (Table 1). We designed and synthesized hapten III as a new coating antigen hapten, in which the second benzene ring was replaced with a pyridine ring. Due to the contribution of the extra electron cloud of N in the ring, it is more structurally different from the immunogen hapten and target analyte TCDD, and thus has lower affinity to Ab7598.

3.2. Assay optimization

The optimal assay conditions for the new antigen system was obtained from antibody titration and solvent effect studies. Based on two-dimensional titration the best concentration for III-BSA with Ab7598 was 0.5 μg/ml, which is about five times higher than II-BSA in previous system. The final concentration for Ab7598 used in subsequent study is 1:7000 dilution.

Dioxin is highly lipophilic and will adhere to glass, plastic or other particle surfaces, higher concentration of co-solvent is very important for consistent assay performance and sensitivity [14]. In this system, various ratios of DMSO/PBS buffers were evaluated to prepare the analyte solutions (Table 2). The optimal DMSO concentration was selected on the basis of I_{50} values and ratios of the maximum and minimum absorbances for the TMDD sigmoidal standard curves (A/D). The lowest I_{50} values were at 25 and 50% DMSO (76 and 35 ng/l, respectively). Because of a low A/D ratio for 25% DMSO, a DMSO concentration of 50% was selected for subsequent experiments (Fig. 1).

Thus, the optimized TMDD ELISA used 0.5 μg/ml of coating antigen III-BSA, antibody 7598 at a dilution of 1:7000, and analyte in DMSO/PBS buffer (1:1). The I_{50} of this heterologous assay was 36±6.0 ng/l (Fig. 2) with a lower detection limit (LDL) of 4.0 ± 1.7 ng/l (0.012 nM). The LDL was estimated as the concentration of the absorbance value equal to the absorbance at zero concentration minus three times the standard

Table 2
Effects of DMSO concentration[a]

DMSO[b] (%)	ABS_{max} (A)	Slope (B)	I_{50} (ppt) (C)	ABS_{min} (D)	A/D	R^2
0	0.81 ± 0.10[c]	0.56	320 ± 29[c]	0.17	4.7	0.99
25	0.85 ± 0.09	1.15	76 ± 8.5	0.14	6.1	0.99
50	0.86 ± 0.11	1.05	35 ± 6.5	0.08	10.7	0.99
75	0.92 ± 0.06	0.76	86 ± 9.5	0.25	4.0	0.99

[a] ELISA conditions: coating antigen III-BSA (0.5 μg/ml); antiserum 7598 (1/6000); goat anti-rabbit IgG-HRP (1/3000).
[b] Concentration of DMSO in dioxin standard solution (PBS-DMSO).
[c] Mean value ± S.D. Each set of data represents the average of three experiments.

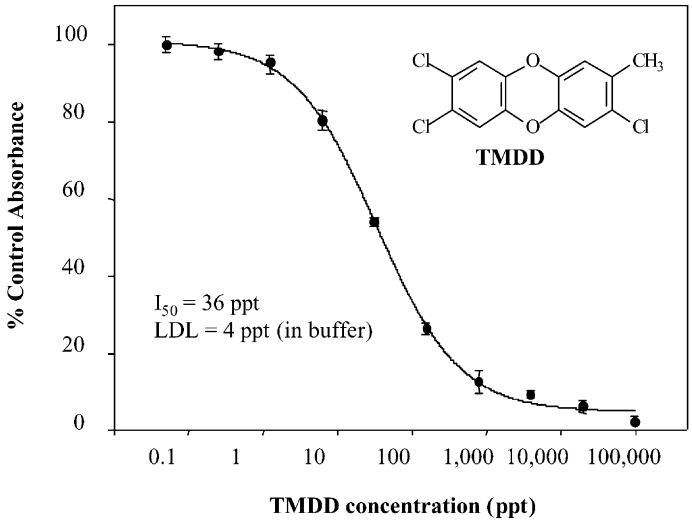


Fig. 1. ELISA inhibition curve for TMDD. Reagent concentration: antiserum 7598 (1:7000, final dilution in wells), and coating antigen III-BSA (0.5 μg/ml). Bars represent standard error. The standard curve represents the average of 14 curves.

deviation of the absorbance at zero concentration [19]. In the previous system, Sugawara et al. [10] reported 240 ng/l TMDD as the I_{50} and 40 ng/l as LDL with same antibody. Approximately 10 times better sensitivity was achieved by this new system. The ultimate sensitivity of a competitive ELISA is limited by the antibody affinity constant, the random experimental error, and precision of detection system [20]. The lowest detection limit possible for a competitive immunoassay would be 10^{-10} M with a $K_d = 10^{-8}$ M, a 1% coefficient of variation for the response at zero dose. In this new system, the assay detection limit of

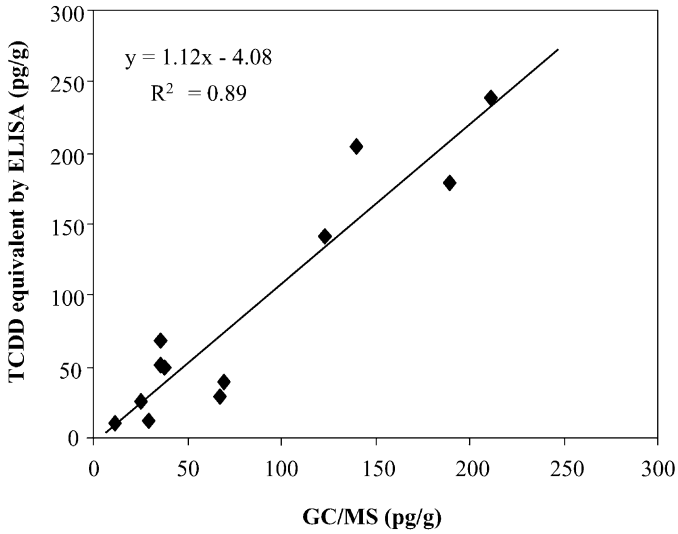


Fig. 2. Relationship between dioxins (TMDD equivalent) measured by GC–MS and ELISA. The symbol ◆ represents the fish and the egg sample extracts from USGS, Columbia, MO. $y = 1.12x - 4.08$; $R^2 = 0.89$; $n = 12$.

TMDD is 1.2×10^{-11} M, which is about 10 times lower than the antibody K_d (1.0×10^{-10} M). Therefore, rational hapten design can be a useful approach to obtain a sensitive assay near the theoretical limit.

3.3. Cross-reactivity

Various dioxin congeners, dibenzofurans, and polychlorinated biphenyls were evaluated for the cross-reactivity of this ELISA (Table 3). To determine cross-reactivity (CR), the I_{50} of surrogate standard TMDD was assigned as 100%, and the CR of other compounds were calculated according to their I_{50} relative to this value. Among the chlorinated dioxins, TCDD has the highest cross-reactivity with this assay, followed by 1,2,3,7,8-pentaCDD (72%) and 1,3,7,8-TCDD (43%). Interestingly, brominated dioxins 2-Br,3,7,8-triCDD and 2,3-diBr, 7,8-diCDD were fully recognized by this antibody, with CR values of 110 and 125%, respectively. Little or no cross-reactivity was observed for TCBs and other chlorinated dioxins. A separate experiment showed that this antibody has no cross-reactivity with herbicides 2,4-D and 2,4,5-T. Among the tested PCDF compounds, 2,3,7,8-TCDF, 2,3,4,7,8-pentaCDF, 1,2,3,7,8-PCDF had moderate cross-reactivities, while little or no cross-reactivities were found for other congeners. According to the TEF values in Table 3, most of the dioxins and PCDFs with a high TEF value (>0.1) have strong or moderate cross-reactivities in this assay, which suggests that this assay might be a good indicator of toxicity of PCDDs and PCDFs in the test samples.

3.4. Assay validation

Extracts from fish and egg samples were analyzed by both GC-MS and ELISA in a blind fashion (Fig. 2). A good agreement between GC-MS and ELISA measured TMDD equivalent was obtained from linear

Table 3
Comparison of TEF[a] value and immunoassay cross-reactivity for CDDs, PCBs and CDFs compounds

Surrogate standard	Congener	Cross-reactivity (%)	TEF value
	TMDD	100	
PCDDs	1-CDD	<0.01	<0.001
	2,7-DiCDD	0.19	<0.001
	2,3,7-TriDD	6.7	<0.001
	1,3,7,8-TCDD	43	0.1
	1,2,3,4,-TCDD	0.01	<0.001
	2,3,7,8-TCDD	129	1.0
	1,2,3,7,8-PentaCDD	72.9	1.0
	1,2,3,4,7,8-HexaCDD	1	0.1
	1,2,3,4,6,7,8-HeptaCDD	0.3	0.01
	OCDD	<0.01	0.001
	2-Br,3,7,8-TriCDD	110	1.0
	2,3-DiBr,7,8-DiCDD	115	1.0
PCBs	3,3′,4,4′-TCB	0.10	0.0001
	3,3′,4,4′,5-PCB	<0.01	0.1
	3,3′,4,4′,5,5′-HCB	<0.01	0.01
PCDFs	2,3,7,8-TCDF	26	0.1
	2,3,4,7,8-PentaCDF	9.0	0.5
	1,2,3,7,8-PentCDF	0.1	0.05
	1,2,3,6,7,8/1,2,3,7,8,9-HCDF	5.4	0.1
	1,2,3,4,7,8-HCDF	<0.01	0.1
	1,2,3,4,6,7,8-HeptaCDF	0.06	0.01
	OCDF	<0.01	0.0001

[a] TEF: toxic equivalency factor [23].

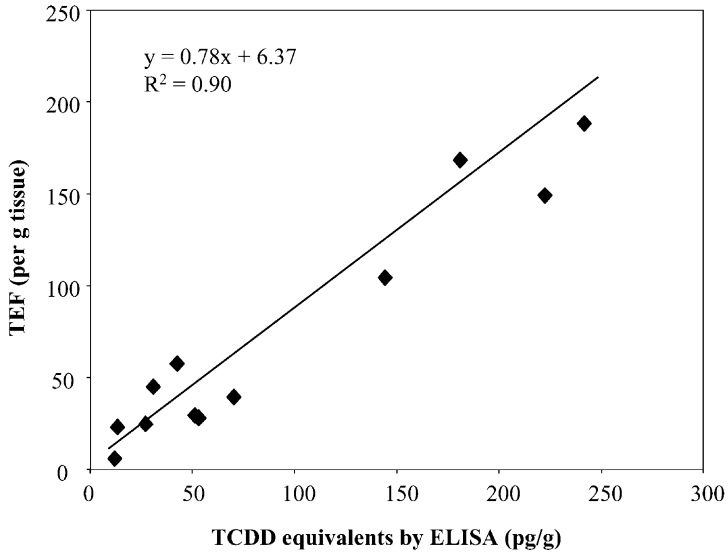


Fig. 3. Relationship between TMDD equivalents by ELISA and TEF values calculated upon GC–MS and immunoassay cross reactivity results. The symbol ◆ represents the fish and the egg sample extracts from USGS, Columbia, MO. $y = 0.78x + 6.37$; $R^2 = 0.90$.

regression analysis ($y = 1.12x - 4.08$, $R^2 = 0.89$, $n = 12$). No matrix effects were found for these extracts as prepared. A fairly good correlation between ELISA and TEF values was also observed with these samples (Fig. 3). The slope value of the linear regression equation is less then 1, which means an overestimation by ELISA in comparison to TEF values. However, a strong correlation ($R^2 = 0.90$) between ELISA and TEF suggests that this ELISA is useful for TEF screening of dioxins in these samples.

3.5. Soil matrix effects and sample cleanup

To find a good solvent for efficient extraction, seven different solvents or combinations were screened in spiked soil samples (Table 4). Three solvents (hexane, CH_2Cl_2, and MeOH/CH_2Cl_2) can efficiently extract TCDD from soil. These solvents have an extraction recovery >86%. With a Florisil column cleanup step, the total recoveries of ^{14}C-TCDD for these three solvents were from 78.3 to 80.3%. MeOH, DMSO, hexane/MeOH and CH_2Cl_2/MeOH (1:1) gave poor recoveries from extraction (<70%), the extracts from these four solvents were not tested for column cleanup studies. The soil matrix effects differed with the extraction solvents used. The hexane method gave the least interference for the ELISA and a 128 times dilution eliminated the matrix effects (Table 5). Furthermore, about 4–8-fold of this interference can be effectively removed by Florisil column cleanup. The extracts from the MeOH/CH_2Cl_2 method had the strongest matrix effects in the immunoassay. Even with a Florisil column cleanup step, a 100–200 times dilution is still needed to minimize the matrix interference. Similar to hexane, the CH_2Cl_2 method had good extraction recovery and slightly higher matrix effects than hexane. Due to public concerns about the potential problem with chlorinated solvents, hexane

Table 4
^{14}C-TCDD spiked recovery of soil sample extraction and cleanup

Solvent	^{14}C-TCDD recovery (%)	
	Extraction	After column cleanup
Hexane	88.2 ± 4.0	79.9 ± 3.0
CH_2Cl_2	88.5 ± 3.5	80.3 ± 3.3
MeOH	68.3 ± 2.8	nt[a]
Hexane/MeOH (1:1)	68.2 ± 4.2	nt
CH_2Cl_2/MeOH (1:1)	66.4 ± 2.0	nt
CH_2Cl_2/MeOH (1:2)	86.4 ± 3.0	78.4 ± 4.1
DMSO	46.2 ± 3.1	nt

[a] Not tested.

Table 5
Matrix interference to ELISA before and after column cleanup

Solvent	Sample	Dilutions to minimize matrix effects in ELISA[a]	
		Without column cleanup	With column cleanup
Hexane	Soil A	128	32
	Soil B	64	8
CH_2Cl_2	Soil A	256	64
	Soil B	128	32
CH_2Cl_2/MeOH (1:2)	Soil A	2000	200
	Soil B	1000	100

[a] Minimal effects refer to less than 10% inhibition compared with zero concentration control.

Table 6
Comparison of soil sample TMDD equivalent between ELISA and GC/MS

Soil sample	Column cleanup	ELISA (pg/g)	GC/MS[a] (pg/g)
1	Yes	54 ± 8.2	43
	No	65 ± 12	
2	Yes	1700 ± 105	1370
	No	1620 ± 120	
3	Yes	nd	nd[b]
	No	nd	

[a] The TMDD equivalent in this column is converted based on cross-reactivity of congeners in each sample. The samples used for GC/MS analysis is not the exact same as used here, both ELISA and GC samples were collected from the same location at different times (about 1 year apart).

[b] Not detected.

was chosen in this study for soil sample preparation for dioxin immunoassay. With such a nonpolar solvent one must be careful to insure that the solvent fully permeates the sample. Three field samples were tested for method validation (Table 6). Either with or without column cleanup, ELISA data showed good agreement with GC/MS results for all three samples. These data suggest that this ELISA can be used as a rapid screen and predict dioxins in the soil samples without additional cleanup steps.

4. Conclusions

Rational hapten design and preparation, and further optimization of an ELISA resulted in a highly sensitive dioxin immunoassay which could detect low ppt levels of dioxin. A high concentration of co-solvent (DMSO) in this ELISA system (50% in analyte and standard solution) is extremely important for the accurate performance of the ELISA for the highly lipophilic dioxins and PCDFs. A good correlation between this ELISA and TEF values for biota sample extracts indicates that this assay can be used as a TEF screening method for dioxins and PCDFs on its own or sequentially to a more general screen based on the Ah receptor [21,22]. Finally, a simple and rapid sample extraction and preparation method was developed with reasonable recovery. By combining this extraction method with the ELISA reported here, one can carry out effective dioxin screens in a fields setting.

Acknowledgements

We wish to thank the Department of Chemistry (UCD) for the use of their NMR instrument, and Drs. D.E. Tillitt and J. Zajicek, US Geological Survey, Columbia Environmental Research Center at Columbia, MO for biota sample extracts and the GC/MS data. We also give our thanks to Mr. Brad Call, Environmental Engineering Branch, US Army Engineer District, Sacramento, CA for providing field soil samples and corresponding GC/MS data. This project was funded by National Institute of Environmental Health Sciences (NIEHS) Superfund Research program, 5 P42 ES04699-08, the NIEHS Center for Environmental Health Sciences and Environmental Protection Agency Center for Ecological Health Research, CR819658.

References

[1] S.H. Safe, J. Anim. Sci. 76 (1998) 134–141.

[2] J.A. Grassman, S.A. Masten, N.J. Walker, G.W. Lucier, Environ. Health Perspect. 106 (Suppl. 2) (1998) 761–775.

[3] H. Becher, D. Flesch-Janys, Environ. Health Perspect. 106 (Suppl. 2) (1998) 623–624.

[4] US EPA, Method 1613, Tetra-through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS, US Environmental Protection Agency, Office of Water, Engineering and Analysis, Washington, DC, October 1994.

[5] A. Schecter, Environ. Health Perspect. 106 (Suppl. 2) (1998) 737–742.

[6] L.H. Stanker, B. Watkins, N. Rogers, M. Vanderlaan, Toxicology 45 (1987) 229–243.

[7] M. Vanderlaan, L.H. Stanker, B.E. Watkins, P. Petrovic, S. Gorbach, Environ. Toxicol. Chem. 7 (1988) 859–870.

[8] M.N. Langley, R.K. Chopra, C.S. Creaser, R.J.K. Taylor, M.D. Rose, J.R. Startin, H.A. Lee, M.R.A. Morgan, Food Agric. Immunol. 4 (1992) 143–151.

[9] R.O. Harrison, R.E. Carlson, Chemosphere 334 (1997) 915–928.

[10] Y. Sugawara, S.J. Gee, J.R. Sanborn, S.D. Gilman, B.D. Hammock, Anal. Chem. 70 (1998) 1092–1099.

[11] J.R. Sanborn, S.J. Gee, S.D. Gilman, Y. Sugawara, A.D. Jones, J. Rogers, F. Szurdoki, L.H. Stanker, D.W. Stoutamire, B.D. Hammock, J. Agric. Food Chem. 46 (1998) 2407–2416.

[12] A.P. Gray, S.P. Cepa, I.J. Solomon, O. Aniline, J. Org. Chem. 41 (1976) 2425–2437.

[13] H. Meyer, R. Graf, Chem. Ber. 61 (1928) 2112–2115.

[14] G.-M. Shan, D.W. Stoutamire, I. Wengatz, S.J. Gee, B.D. Hammock, J. Agric. Food Chem. 47 (1999) 2145–2155.

[15] S.J. Gee, B.D. Hammock, J.M. Van Emon, A User's Guide to Environmental Immunochemical Analysis, EPA/540/R-94/509 US EPA, Office of Research and Development, Environmental Monitoring Systems Laboratory, Las Vegas, NV, 1994.

[16] M.H. Goodrow, J.R. Sanborn, D.W. Stoutamire, S.J. Gee, B.D. Hammock, in: J.O. Nelson, A.E. Karu, R.B. Wong (Eds.), Immunoanalysis of Agrochemicals: Emerging Technologies, American Chemical Society Symposium no. 586, American Chemical Society, Washington, DC, 1995, pp. 1–19.

[17] T.M. Jackson, R.P. Ekins, J. Immunol. Methods 87 (1986) 13–20.

[18] G.M. Shan, W. Huang, S.J. Gee, B.D. Buchholz, J.S. Vogel, B.D. Hammock, PNAS 97 (2000) 2445–2449.

[19] H.E. Grotjan, B.A. Keel, in: E. Diamandis, T. Christopoulos (Eds.), Immunoassay, Academic Press, San Diego, 1996, pp. 51–93.

[20] T.M. Jackson, R.P. Ekins, J. Immunol. Methods 87 (1986) 13–20.

[21] P.M. Garrison, J.M.M.J.G. Aarts, A. Brouwer, J.P. Giesy, M.S. Denison, Fundam. Appl. Toxicol. 30 (1996) 194–203.

[22] A.J. Murk, J. Legler, M.S. Denison, C.J. van der Guchte, A. Brouwer, Fundam. Appl. Toxicol. 33 (1996) 149–160.

[23] M. Van den Berg, L. Birnbaum, A.T.C. Bosveld, B. Brunstrom, P. Cook, M. Feeley, J.P. Giesy, A. Hanberg, R. Hasegawa, S.W. Kennedy, T. Kubiak, J.C. Larsen, F.X.R. van Leeuwen, A.K.D. Liem, C. Nolt, R.E. Peterson, L. Poellinger, S. Safe, D. Schrenk, D. Tillitt, M. Tysklind, M. Younes, F. Warn, T. Zacharewski, Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife, Environ. Health Perspect. 106 (1998) 775–792.