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Comparison of immunoaffinity column recovery patterns of polychlorinated dibenzo-*p*-dioxins/polychlorinated dibenzofurans on columns generated with different monoclonal antibody clones and polyclonal antibodies

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Abstract

Evaluation of immunoaffinity columns (IACs) for dioxin serum sample clean-up requires a determination of the recovery of various dioxin congeners. We compared the IAC performance of different monoclonal and polyclonal anti-dioxin antibodies, measuring the recovery of congeners of polychlorinated dibenzo-*p*-dioxins (PCDDs). In addition, we measured the recovery of congeners of the structurally related polychlorinated dibenzofurans (PCDFs).

The polyclonal antibody based IACs evaluated had lower recovery for highly chlorinated dioxin congeners, but were more specific toward 2,3,7,8-TCDD. The resemblance of the hapten to 2,3,7,8-TCDD appeared to play a clear role, but chlorines in the 2-, 3- and 7-positions of the hapten were essential. Recovery of 2,3,7,8-TCDD from the IAC showed some relation to the affinity for the antibody measured by either the K_a from accelerator mass spectrometry (AMS) or with 50% inhibition of color activity (IC_{50}) determined from an ELISA analysis.

The IACs prepared from four monoclonal antibodies (Mabs) derived from a common hapten showed differences in their retention patterns of PCDDs/PCDFs. Comparison of IC_{50} from ELISA with recovery from the IACs indicated that a minimum IC_{50} of 100 ppb was required for satisfactory recovery from the IAC, but the correlation was poor, indicating other factors were involved. Mab DD3 showed the broadest spectrum of the Mabs and showed satisfactory recoveries of all of the dioxin congeners, except OCDD. In addition, DD3 showed good recovery toward 2,3,4,8-TCDF, 2,3,4,7,8-PeCDF and 2,3,4,6,7,8-HxCDF but has poor recovery when PCDFs have a chlorine substitution in the 1-position.

Keywords: Dioxins; Furans; IAC; Residues; Isolation

1. Introduction

Polychlorinated dibenzo-*p*-dioxins/polychlorinated dibenzofurans (PCDDs/PCDFs) are persistent environmental contaminants that pose a health concern

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due to their toxicities (immuno-, hepato-, reproductive and developmental toxicities) as well as their carcino- and teratogenicity [1]. Because PCDDs/PCDFs are biomagnified through the food chain, it is important to survey for these chemicals in order to ensure that levels are minimal in foods. Implementation of a broad survey, however, is limited by the high cost of sample analysis for these chemicals (US$1000–2000 per sample). The complexity of the matrix, coupled with trace amounts of the analytes, requires a complicated, labor-intensive clean-up procedure involving a lengthy extraction and a multiple column fractionation procedure. These steps generate large quantities of toxic solvent waste (US EPA method 1613) [2]. New methods incorporating quicker and simpler clean-up, fewer analysis steps, reduced organic waste production and lower cost are critically needed.

Immunoaffinity columns (IACs) have been utilized to eliminate matrix interferences and concentrate the sample. This technique has proven useful in a variety of xenobiotic analyses in a variety of complex matrices [3–9]. The usefulness of IACs depends on the ability to couple an antibody to the column while maintaining the ability of the antibody to bind the analyte during column loading and washing procedures and release the substrate under elution conditions. This is measured by determining the recovery of the analyte from the column after loading the sample, washing the column and eluting the analyte.

In order to develop a successful IAC, an antibody with an appropriate affinity is essential. An antibody suitable for IAC requires an affinity sufficient to bind analytes to the IAC, but also low enough to allow complete elution from the column. Affinity may be measured from 50% inhibition of color activity (IC_{50}) or more precisely by determining the K_a of the antibody [10–12].

IACs can achieve multi-analyte isolation through the selection of a broad-spectrum antibody to retain structurally related compounds [5,13]. In general, antibodies recognize a variety of compounds, providing their structure contains the appropriate binding epitope. Since different antibodies recognize different epitopes, the spectrum of compounds recognized will be different and must be determined for each antibody. Seventeen PCDDs/PCDFs have been assigned toxic equivalent factors (TEFs) and are determined in routine dioxin analysis. The ideal IAC would bind these congeners while interferences were washed off and allow recovery of the congeners in the elution step. Consequently, it is necessary to determine the recovery of all 17 congeners for each different antibody used in an IAC.

Previously, we have reported the utilization of a polyclonal antibody (from chicken) as well as a monoclonal antibody (Mab) based IAC for the isolation of PCDDs/PCDFs from serum [14,15] and milk [16]. This study focused on discovering the variation of analyte recovery on a variety of monoclonal and polyclonal antibody based affinity columns. Multiple analytes were examined to explore the relationship between analyte structure and recovery. The relationship between an analyte's affinity for an antibody and its recovery from the corresponding IAC was investigated. Because of its usefulness in calculating dioxin body burden, this study focused on recovery from serum samples. Our ultimate goal is the rational construction of an IAC that shows good recovery of all the PCDDs/PCDFs congeners that are currently calculated for the toxicity equivalents (TEQs) based on US EPA methods. This achievement would decrease the complexity and solvent usage of current dioxin analysis and utilize the sensitivity and resolution of high resolution gas chromatograph–high resolution mass spectrometer (HRGC–HRMS).

2. Materials and methods

2.1. Reagents

Solvents (acetone, dichloromethane, hexane and toluene) were HPLC grade and were obtained from Burdick and Jackson, Muskegon, MI, USA. The solvents were confirmed to be free of PCDDs/PCDFs contamination by US EPA method 1613. All PCDDs/PCDFs standards [calibration and verification solutions (CVS and CS 1–5), standard spiking solution, labeled compound stock solution (LCS) and precision and recovery standards (PARs)] were obtained from TerraChem, Kansas City, MO, USA. CNBr-Sepharose 4B was obtained from Pharmacia Biotech, Uppsala, Sweden. Carbograph 1, 120/400 mesh was purchased from Alltech Associates Inc., Deerfield, IL, USA. Pristane® and bovine serum were obtained from Sigma, St. Louis, MO, USA. The rabbit

immunoglobulin G (IgG) assay kit was obtained from Pierce, Rockford, IL, USA.

2.2. Mab generation

A set of Mabs against dioxins have been generated by Stanker et al. [17] and deposited in the American-type culture collection (Manassas, VA, USA). The clones utilized for this study were DD1, DD3, DD4 and DD5. Ascites were generated from these clones using either BALB/c (DD3 and DD4) or nude mice (DD1 and DD5). The DD1 and DD5 ascites were obtained via a contract with Harlan Bioproducts for Science Inc. (Indianapolis, IN, USA), the DD3 and DD4 ascites were generated in-house. The mice were primed with Pristane® 2 weeks prior to inoculation with 1×10^6 hybridoma cells through i.p. injection. Ascites fluid was tapped from each mouse, pooled and stored at $-80\,^{\circ}C$ until used. The IgG was isolated using a protein G column and quantified by the method of Bradford [18] using γ-globulin as a standard. The protein purity was checked by 10% SDS-PAGE.

2.3. Monoclonal IAC application

Five milligrams of purified IgG was conjugated with 1 ml of CNBr-Sepharose 4B to generate the immunoaffinity gel beads according to the manufacturer's instruction. Briefly, purified IgG was mixed with CNBr-Sepharose 4B (activated by 1 mM HCl) in the presence of 0.1 M $NaHCO_3$ (pH 8.3)/0.5 M NaCl. The excess active groups were capped with 0.1 M Tris (pH 8). Three cycles of alternative washes with 0.1 M acetate buffer (pH 4)/0.5 M NaCl and 0.1 M Tris (pH 8)/0.5 M NaCl removed non-specific material that had been absorbed by the column. Washed supernatants were collected to measure the protein concentration to determine the conjugation efficiencies. The immunoaffinity gel beads were stored in phosphate-buffered saline (PBS)/0.02% sodium azide at $4\,^{\circ}C$ until utilized. PCDDs/PCDFs standards (PAR and LCS) were solvent exchanged from dodecane into acetone and spiked into bovine serum (10 ml) to perform the multiple congener recovery study. The PCDDs/PCDFs spiking levels in the serum are shown in Table 1. The spiked serum was applied to a 0.5 ml IAC, the non-bound material was removed by washing with 10 column volumes of 10% acetone and the analytes were eluted with 100% acetone. After evaporation of the acetone, the analytes were extracted into dichloromethane, dried with sodium sulfate, the dichloromethane evaporated with a dodecane keeper (15 μl), external standard (5 μl) added, and the solution was analyzed by HRGC–HRMS. The US EPA method 1613 considers an overall recovery of 25–150% satisfactory.

2.4. Alternative post-monoclonal IAC treatment

Instead of extracting PCDDs/PCDFs from the acetone eluant as described above, the acetone eluent was passed through a 250 mg Carbograph column and the column washed with 10 ml each of acetone, dichloromethane:hexane (1:1), hexane and the analytes eluted with toluene. Toluene was evaporated with a dodecane keeper, external standard was added and the sample was analyzed by HRGC–HRMS.

2.5. Polyclonal antibody based IAC generation and application

Rabbit sera were characterized by protein measurement using the Bradford method [18] and estimating IgG by 10% SDS-PAGE. The IgG was further quantified by an anti-rabbit IgG assay kit. The results showed good correlation between the commercial kit method and the combination of Bradford–SDS-PAGE methods. The calculations of specific IgGs were based on the estimation that 10% of the total IgG in the serum was specific toward PCDDs/PCDFs. The average gel conjugation efficiencies were $93.5 \pm 4\%$ ($n = 9$) and ranged from 84.8 to 96.9%. Thirty milligrams of total protein (containing an estimated 10% total IgG) was conjugated with 1 ml of CNBr-Sepharose 4B beads according to the manufacture's instructions. Based on the molar ratio between the immobilized specific IgG to the spiked level of PCDDs/PCDFs, the IgG has excess binding sites for spiked PCDDs/PCDFs. A 2 ml column size was utilized to test for the congeners' retention patterns for PCDDs and PCDFs. PAR and LCS standards were solvent exchanged into acetone and spiked into 10 ml of bovine serum. The spiked samples were applied to the IAC and non-specific interferences were washed off with 10% of acetone in water. The PCDDs/PCDFs were then eluted from the

Table 1
Recoveries from IACs generated from four monoclonal anti-dioxin antibodies

Congener	TEF[a]	Spike level (pg)	Percentage recovery				IC_{50} values from ELISA (ppb)[b]			
			DD1	DD3	DD4	DD5	DD1	DD3	DD4	DD5
Furans ($n = 3$)										
2,3,7,8-TCDF	0.1	2	33.4 ± 3.3	52.8 ± 4.4	10.1 ± 1.6	36.2 ± 3.6	8	7	65	9
1,2,3,7,8-PeCDF	0.05	10	19.1 ± 3.1	14.5 ± 0.9	1.7 ± 0.4	6.0 ± 1.7				
2,3,4,7,8-PeCDF	0.5	10	39.5 ± 5.4	83.2 ± 5.9	28.6 ± 4.8	30.4 ± 2.6	50	3	5	15
1,2,3,4,7,8-HxCDF	0.1	10	12.4 ± 0.8	7.0 ± 0.3	3.5 ± 0.2	5.5 ± 1.2				
1,2,3,6,7,8-HxCDF	0.1	10	27.2 ± 1.7	7.3 ± 0.6	8.6 ± 0.2	0.9 ± 0.2				
2,3,4,6,7,8-HxCDF	0.1	10	9.0 ± 0.6	54.5 ± 2.7	1.0 ± 0.1	0.5 ± 0.1				
1,2,3,7,8,9-HxCDF	0.1	10	2.6 ± 0.2	0.5 ± 0.0	0.6 ± 0.1	0.4 ± 0.1				
1,2,3,4,6,7,8-HpCDF	0.01	10	1.3 ± 0.2	1.1 ± 0.1	0.5 ± 0.1	0.5 ± 0.3				
1,2,3,4,7,8,9-HpCDF	0.01	10	15.2 ± 1.1	2.2 ± 0.1	1.4 ± 0.1	0.6 ± 0.3				
Dioxins (n=3)										
2,3,7,8-TCDD	1.0	2	54.8 ± 0.7	80.5 ± 7.4	52.5 ± 3.5	29.5 ± 2.8	20	25	29	90
1,2,3,7,8-PeCDD	1.0	10	64.5 ± 7.6	88.5 ± 4.0	55.5 ± 7.0	29.6 ± 2.5	3.2	8	1.4	30
1,2,3,4,7,8-HxCDD	0.1	10	20.4 ± 1.4	39.1 ± 4.1	2.2 ± 0.3	5.0 ± 1.0	200	200	1000	1000
1,2,3,6,7,8-HxCDD	0.1	10	37.7 ± 2.4	54.0 ± 4.1	18.5 ± 0.8	5.5 ± 1.0	15	>2000	>2000	>2000
1,2,3,4,6,7,8-HpCDD	0.01	10	9.4 ± 0.9	25.5 ± 1.9	1.2 ± 0.2	1.0 ± 0.6				
OCDD	0.0001	20	2.1 ± 1.0	1.2 ± 0.3	0.0 ± 0.1	2.0 ± 1.5	>2000	>2000	>2000	>2000

[a] Data obtained from WHO.
[b] Data obtained from [17].

column using 100% acetone. The acetone elute was passed through a 250 mg Carbograph column and the bound PCDDs/PCDFs were then eluted with toluene (10 ml). After addition of the external standard the samples were analyzed by HRGC–HRMS.

2.6. HRGC–HRMS analysis

The analyses of PCDDs/PCDFs individual congener recovery were performed with HRGC–HRMS using an isotope dilution method according to US EPA method 1613. The HRMS was operated using the electron impact ionization mode with selected ion monitoring. The resolution was set at 10,000–11,000 using an acceleration voltage of 8000 V. Response factors were calculated from five-point calibration curves of the PCDD/PCDF congeners and two internal standards. All conditions were similar to those previously reported [19], except the following changes: the GC was an Agilent 6890 using a splitless mode for the injector; the initial column temperature was 140 °C held for 1 min, ramped at 50 °C/min to 190 °C, held for 1 min and the temperature was increased at a rate of 2 °C/min to 290 °C then ramped at 20 °C/min to 320 °C.

2.7. Measurements of affinity constants using accelerator mass spectrometry (AMS)

The measurement of antibody affinity constants by AMS followed the method described in the previous studies [12]. In brief, 50 μl of antibody (1.0 pmol serum 99, 2114, 2492 or 7600) in PBS with 0.2% BSA was mixed with 50 μl of different dilutions of ^{14}C-TCDD (1–200 fmol in 50% DMSO–PBS) in test tubes and incubated for 15 min. A 200 μl aliquot of magnetic particles which contained anti-rabbit IgG was then added and incubated for 30 min with shaking. After separation and washing steps, the ^{14}C content bound onto the particles was determined with AMS.

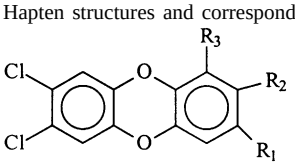
3. Results and discussion

3.1. Polyclonal antibody IAC

The ability of an IAC to serve as a clean-up column relies on the specific retention of the analytes during the application and washing procedure and their recovery during the elution process. For pollutants, such as PCDDs and PCDFs with multiple congeners, the evaluation of antibodies for IACs includes measurement of the recovery of multiple congeners, with particular emphasis on the most toxic congeners. In this paper, the performances of different Mabs used as reagents for IACs are compared with each other and with a collection of polyclonal antibodies. The polyclonal antibodies were derived by immunizing with four different haptens and include the antibodies recovered from several different animals. This made it possible to assess animal-to-animal variation for a specific immunogen and the effect of changes in hapten structure on antibody performance. The Mabs were derived using a single hapten, but each was the result of a different cell fusion experiment, thus it was possible to evaluate the performance of independently derived Mabs.

Polyclonal antisera were derived from nine different rabbit sera that were generated using four different dioxin haptens. The individual sera (designated 69, 89, 99, 2114, 2492, 2493, 2494, 7599 and 7600) and the haptens (designated IV, X, XII and XIII) of origin are summarized in Table 2. Previous studies demonstrated that these sera contained antibodies capable of binding 2,3,7-trichloro-8-methyl-dibenzo-*p*-dioxin (TMDD), a surrogate of 2,3,7,8-TCDD [20,21]. The affinity constants of four antibodies (which originated from four different immunogen haptens) for ^{14}C-2,3,7,8-TCDD were measured using AMS. The results of affinity constant determination, congener recoveries from IAC and the concentration of analyte resulting in IC_{50} in a competition ELISA are listed in Table 3. The correspondence between the IC_{50} from ELISA and the K_a from AMS measurements are good. The serum with the lowest K_a has an undetectable IC_{50} and the sera with the two highest K_a have the lowest IC_{50}. However, the correlation of K_a or IC_{50} and recovery was modest. Serum 2114 had the lowest K_a (4×10^8) and it did not retain 2,3,7,8-TCDD. Serum 7600 with a somewhat stronger affinity ($K_a = 1 \times 10^{10}$), had the best recovery of 2,3,7,8-TCDD (74%) from the IAC. Sera 99 ($K_a = 6.2 \times 10^{10}$) and 2492 ($K_a = 8.0 \times 10^{10}$) had the highest K_a, but only modest recovery of 2,3,7,8-TCDD (38 and 37%) from the IAC was observed. These results suggest that an intermediate K_a is likely to result in good IAC

Table 2
Hapten structures and corresponding serum/antibody designations



Antibody or serum	Hapten[a]	Substituant		
		R1	R2	R3
Mab DD1, DD3, DD4, DD5[b]		Cl	H	N–CO–$(CH_2)_4$–COOH
Sera 69, 89, 99[a]	IV	Cl	Cl	CH=CHCOOH
Sera 7599, 7600[a]	X	Cl	$(CH=CH)_2COOH$	H
Sera 2114[a]	XII	H	CH=CHCOOH	H
Sera 2492, 2493, 2494[a]	XIII	Cl	$CH=CHC_6H_4COOH$	H

[a] Polyclonal serum and hapten designation was obtained from [21].
[b] Mab designation was obtained from [17].

performance. An antibody with a very low K_a, such as serum 2114, certainly is not useful for an IAC because of its poor capability to bind analytes. Those antibodies with the highest affinities, however, gave only modest analyte recovery. This could be caused by their tighter binding, which makes elution difficult or simply because of the difference in operating conditions between the IAC and determination of K_a.

Table 3
Recoveries from IAC of PCDDs/PCDFs using nine rabbit sera containing polyclonal antibodies[a]

Congener	Serum								
	69	89	99	2114	2492	2493	2494	7599	7600
Percentage recovery of furans ($n = 3$)									
2,3,7,8-TCDF	13 ± 0.4	5.5 ± 0.1	16 ± 1.0	2.4 ± 0.2	4.7 ± 0.0	2.4 ± 0.1	4.2 ± 0.2	5.5 ± 0.2	13 ± 0.3
1,2,3,7,8-PeCDF	13 ± 0.3	4.4 ± 0.1	9.2 ± 0.1	5.6 ± 0.9	7.4 ± 0.7	6.3 ± 0.5	3.9 ± 0.2	3.8 ± 0.2	11 ± 1.3
2,3,4,7,8-PeCDF	9.8 ± 0.3	11 ± 0.4	7.6 ± 0.2	3.7 ± 0.6	4.8 ± 0.4	3.7 ± 0.4	2.3 ± 0.1	3.4 ± 0.4	6.1 ± 0.2
1,2,3,4,7,8-HxCDF	21 ± 0.2	9.8 ± 0.2	16 ± 0.9	18 ± 2.9	17 ± 2.7	18 ± 1.1	12 ± 0.7	8.3 ± 0.4	12 ± 0.9
1,2,3,6,7,8-HxCDF	10 ± 0.0	5.3 ± 0.2	9.2 ± 4.3	17 ± 2.7	15 ± 2.5	8.0 ± 0.7	4.8 ± 0.2	6.5 ± 2.3	6.5 ± 0.5
2,3,4,6,7,8-HxCDF	10 ± 0.2	6.0 ± 0.1	7.1 ± 0.9	7.1 ± 0.7	7.5 ± 1.0	8.1 ± 0.6	4.7 ± 0.2	4.2 ± 0.1	5.8 ± 0.4
1,2,3,7,8,9-HxCDF	7.6 ± 0.5	4.3 ± 0.1	4.4 ± 0.2	3.3 ± 0.3	5.1 ± 0.4	3.5 ± 0.3	2.6 ± 0.2	2.2 ± 0.1	10 ± 0.7
1,2,3,4,6,7,8-HpCDF	15 ± 0.8	7.3 ± 0.4	14 ± 1.1	17 ± 1.7	15 ± 1.0	16 ± 1.1	8.8 ± 0.2	9.7 ± 0.3	11 ± 1.0
1,2,3,4,7,8,9-HpCDF	5.0 ± 0.5	3.6 ± 0.2	3.9 ± 0.3	5.4 ± 0.8	5.2 ± 1.2	3.9 ± 0.3	2.2 ± 0.1	3.3 ± 0.2	3.8 ± 0.1
Percentage recovery of dioxins ($n = 3$)									
2,3,7,8-TCDD	21 ± 1.8	27 ± 0.3	38 ± 0.5	2.0 ± 0.2	37 ± 1.2	9.8 ± 0.6	15 ± 0.6	17 ± 0.9	74 ± 1.6
1,2,3,7,8-PeCDD	14 ± 0.4	23 ± 0.4	18 ± 0.1	2.6 ± 0.5	17 ± 1.2	5.1 ± 0.2	5.5 ± 0.4	6.9 ± 0.8	27 ± 0.9
1,2,3,4,7,8-HxCDD	7.5 ± 0.3	14 ± 0.4	6.7 ± 0.2	3.8 ± 0.7	4.3 ± 0.5	3.8 ± 0.3	3.4 ± 0.3	3.4 ± 0.2	3.9 ± 0.3
1,2,3,6,7,8-HxCDD	5.2 ± 0.3	8.0 ± 0.2	3.7 ± 0.3	2.9 ± 0.5	3.3 ± 0.4	3.3 ± 0.4	2.7 ± 0.1	2.6 ± 0.2	3.2 ± 0.2
1,2,3,4,6,7,8-HpCDD	10 ± 0.4	7.2 ± 0.2	10 ± 1.6	12 ± 2.0	11 ± 2.0	10 ± 0.7	5.8 ± 0.3	7.0 ± 0.4	6.8 ± 0.4
OCDD	4.7 ± 2.1	3.7 ± 0.2	5.6 ± 0.7	11 ± 1.8	12 ± 3.5	5.8 ± 0.4	3.8 ± 0.3	5.7 ± 0.1	4.1 ± 0.4
K_a (M^{-1})[b]	Nd[c]	Nd	6.2×10^{10}	4.0×10^{8}	8.0×10^{10}	Nd	Nd	Nd	1.0×10^{10}
IC_{50} (ppb)[d]	Nd	Nd	0.04	Below det.	0.08	Nd	Nd	Nd	0.3

[a] Haptens used to produce the antisera are described in Table 2.
[b] K_a was determined using AMS.
[c] Nd: not determined.
[d] IC_{50} (ppb) were determined using indirect competition ELISA with TMDD as the competitor.

Clearly, K_a or IC_{50} provide guidance for the suitability for IACs, but unfortunately, optimization of IAC must be carried out for multiple antibodies, not just the one with the highest K_a. Even differences between antibodies from a common hapten cause significant variation in IAC performance.

All columns generated from the polyclonal sera had relatively low recoveries of the congeners, except the IAC generated from serum 7600. Even immunized with the same antigen, different rabbits produced sera that resulted in different recovery patterns. Although the patterns are similar, important differences do exist. The hapten with the least number of chlorines (XII, serum 2114) produced a column with the poorest recovery of the most toxic congener (2,3,7,8-TCDD) consistent with the low K_a of this product. The poor recovery of 2,3,7,8-TCDD and the penta- and hexa- congeners might be expected based on the structure of hapten XII, which lacks chlorine in the second ring and the resulting antibody would not be expected to bind congeners having chlorine in this ring. Surprisingly, better recovery was observed for hepta- and octa-congeners having multiple chlorines in both rings. Serum 2114 binds these heavily substituted congeners the best (serum 2492 is approximately equal) of all the antibodies tested. This clearly indicates that the structure of the hapten, although of great importance, does not completely define the behavior of the antibody.

The hapten with the most chlorines (IV, sera 69, 89 and 99) generally produced antisera that showed acceptable recovery for 2,3,7,8-TCDD. These sera, however, showed distinctive patterns demonstrating differences between the antibodies produced by different animals. For example, serum 99 showed a much better recovery for 2,3,7,8-TCDD than sera from the other two animals (69 and 89), but the decreased recovery of congeners containing higher chlorine substitutions was more severe in serum 99 than in the other sera produced with hapten IV. In contrast to serum 99, serum 89 showed almost no decrease in recovery of 1,2,3,7,8-PeCDD relative to 2,3,7,8-TCDD. A rather curious observation was that most sera contained antibodies with better retention of the 1,2,3,4,7,8-HxCDD than of 1,2,3,6,7,8-HxCDD congener. This could be the result of some sort of proximity effect or an asymmetrical binding site. The proximity effect might be supported by the increase in recovery observed in most sera for the 1,2,3,4,6,7,8-HpCDD, although both rings cannot be fully substituted, as the OCDD congener shows poor recovery for most sera. A similar phenomenon has been observed in the binding of the dibenzofurans. Good recovery was observed for the 1,2,3,4,7,8-HxCDF congener, with much lower recovery for the other hexachloro-congeners, but an increased recovery for the 1,2,3,4,6,7,8-HpCDF congener. Despite the differences in the haptens used to produce the antisera, the increased recovery of this congener holds throughout all nine tested columns.

The retention performance observed on the IACs produced from the antisera to hapten XII (sera 7599 and 7600) support the conclusion that antibody quality varies greatly between animals even when the same immunizing hapten was used. Antiserum 7600 shows the highest recovery of the target 2,3,7,8-TCDD and although the recovery of 1,2,3,7,8-PeCDD is approximately 1/3 that of TCDD, it still meets the requirement of the US EPA method 1613 (>25%). The recovery of more highly substituted congeners however, drops markedly, making this antibody rather specific.

3.2. Mab IAC

The recovery patterns observed on IACs produced with the Mabs DD1, DD3, DD4 and DD5 are summarized in Table 1. In addition, the IC_{50} from a competition ELISA analysis also are shown. Fig. 1 shows the correlation between the logarithms of the IC_{50} with the recovery from the IAC. Although the data is widely scattered, with a low correlation coefficient, two important facts are demonstrated. First, as expected, compounds that show low binding to the antibody show low recoveries. Also, three of the four compound–antibody data points with IC_{50} over 100 ppb show unacceptably low recoveries. Conversely, 16 of the 17 with IC_{50} below 100 ppb show IAC recoveries >25%. The second important conclusion from this figure is that the relatively poor correlation indicates factors other than affinity are important for good IAC performance or alternately, that affinity is altered when the antibody is immobilized on the column. The expected complex differences in the interactions of congeners with different antibodies make the correlation interesting. Good recovery of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 1,2,3,6,7,8-HxCDD and poor recovery of OCDD

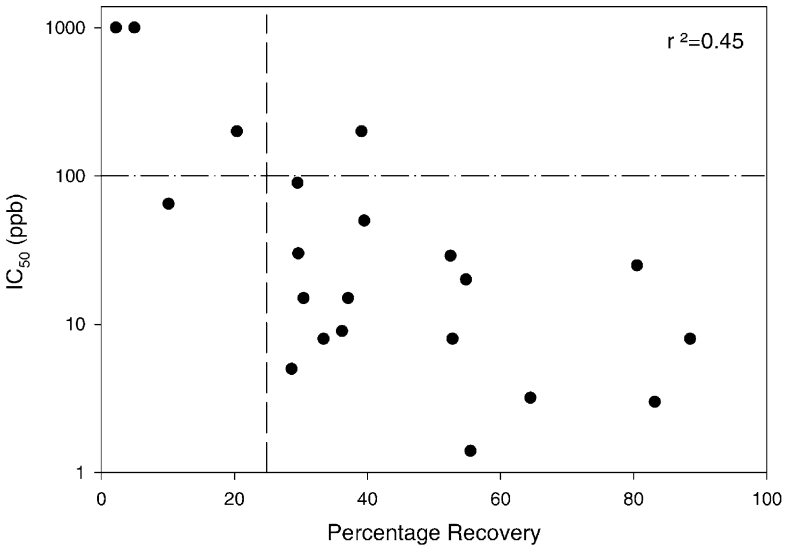


Fig. 1. The correlation between the logarithm of the ELISA IC_{50} and the percentage recovery of the IACs.

roughly correlates with the IC_{50} for the Mabs. Notable exceptions occur however. For example, the IC_{50s} for DD1 predict that 1,2,3,6,7,8-HxCDD should have a better recovery than 2,3,7,8-TCDD, whereas, the reverse was observed. Similarly, for 1,2,3,7,8-PeCDD recovery from DD1, DD3 and DD4, the IC_{50s} predict the reverse of the recoveries actually observed, while DD5 has the lowest recovery and the highest IC_{50} as expected. Finally, DD3 should show very poor recovery of 1,2,3,6,7,8-HxCDD from its very high IC_{50}, whereas, a very good recovery was observed. As observed with K_a, for the polyclonal antisera, affinity measurements by themselves are not an absolute indicator of IAC performance, but instead serve as a general guide to column performance.

Mabs DD1 and DD3 had significant differences in the primary sequences of their binding domain [22]. Molecular modeling of the antibody described previously also showed the binding sites were quite different [23]. Using 25% recovery as the criterion, DD3 has the broadest recovery spectrum, with acceptable recovery of all the dioxins tested, except OCDD. In addition, DD3 also shows over 80% recovery of 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD, the most toxic congeners, which are well above the recovery observed with any of the other antibodies. An interesting observation is that although the 1,2,3,6,7,8-HxCDD congener showed little binding in the ELISA assay it showed good recovery from our column, better in fact than the corresponding 1,2,3,4,7,8-HxCDD that showed ELISA binding at least 10-fold better than the 1,2,3,6,7,8-HxCDD. This same discrepancy in recovery of HxCDD congener was observed, less dramatically, with antibodies DD1 and DD4. This clearly demonstrates that the correlation between ELISA and column retention is dependent on other factors in addition to the affinity determined by ELISA. Thus, antibody behavior and performance in IAC is the result of a complex and as yet, incompletely defined set of parameters. Overall, excellent recovery of most of the toxic PCDDs was observed with DD3 as well as good recovery of three out of the five most toxic PCDFs. These congeners accounted for 84% of the TEQ spiked into the serum samples.

The DD1 antibody shows a similar recovery pattern to DD3, although the recovery of 1,2,3,4,7,8-HxCDD and 1,2,3,4,6,7,8-HpCDD fell below the 25% criterion. Even though the IAC performance of DD1 and DD3 are qualitatively similar, their recovery of chlorinated furans is distinctly different. Both 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF showed recoveries well above the 25% minimum, although DD3 showed a significantly higher recovery (as observed in the dioxin case). DD3 showed good recovery of 2,3,4,6,7,8-HxCDF,

but DD1 shows poor recovery of this congener. Conversely, DD1 but not DD3 demonstrated good recovery of 1,2,3,6,7,8-HxCDF. Examination of the recoveries of furan congeners by the DD3 IAC shows that a 1-chloro group on the furan produces a marked decline in recovery of these congeners. Although DD1 shows a similar effect, it is much less than is shown in DD3. DD1 shows an unusually high recovery of

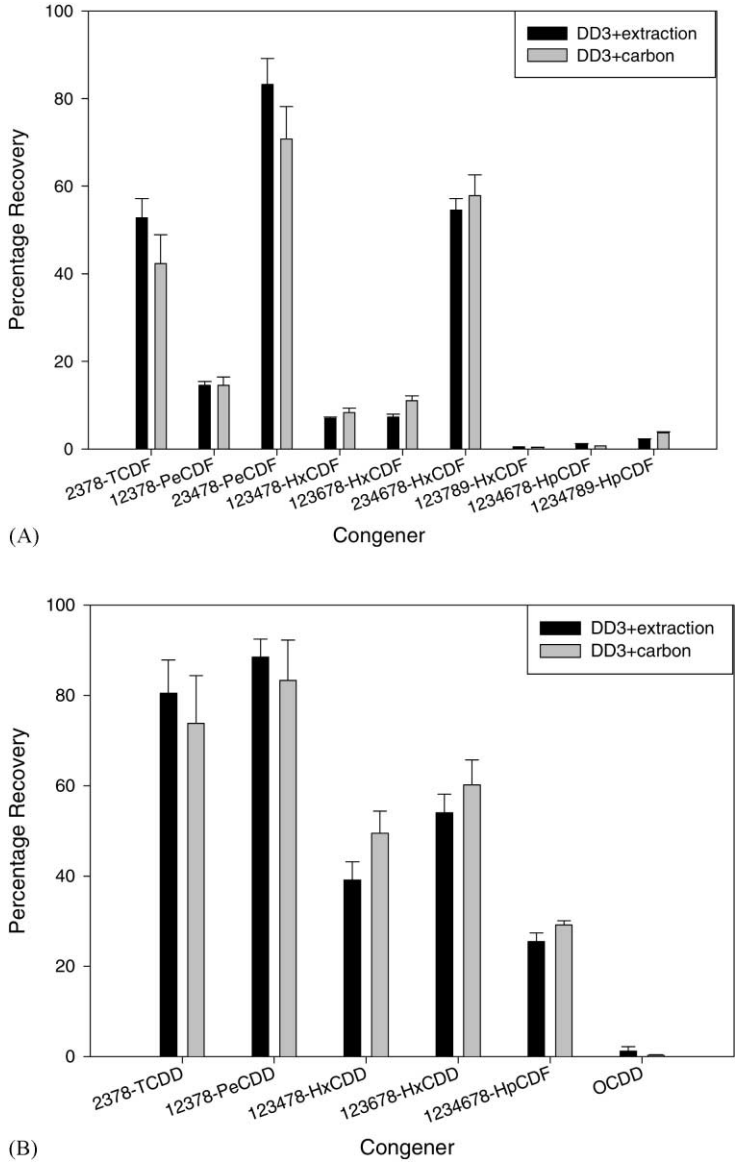


Fig. 2. Panel (A) shows a comparison of PCDF congener recovery between different post-DD3 IAC treatment: black is recoveries obtained from liquid–liquid partition between IAC eluate and dichloromethane ($n = 3$); gray is recoveries obtained from carbon column extraction of IAC eluate ($n = 3$). Panel (B) shows a comparison of PCDD congener recovery between different post-DD3 IAC treatment: black is recoveries obtained from liquid–liquid partition between IAC eluate and dichloromethane ($n = 3$); gray is recoveries obtained from carbon column extraction of IAC eluate ($n = 3$).

the heavily substituted 1,2,3,4,7,8,9-HpCDF. Clearly, the recovery characteristics of these two antibodies are qualitatively as well as quantitatively different.

DD4 shows good recovery of the 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD congeners. Similar to DD1 and DD3, the recovery of 1,2,3,6,7,8-HxCDD is much greater than 1,2,3,4,7,8-HxCDD, but the recovery is well below the 25% criterion. The difference may be due to the unequal ring substitution of the 1,2,3,4,7,8-HxCCD. Among the furan congeners, DD4 shows greatest recovery for the 2,3,4,7,8-PeCDF although it barely meets the 25% criterion. All of the antibodies showed their highest recovery for this congener. The IAC from DD4 showed relatively poor recovery for all the remaining congeners, including the 2,3,7,8-TCDF, which showed satisfactory recovery with all of the other antibodies.

DD5 showed the poorest recovery for the 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD congeners, barely above the 25% minimum. Interestingly, the furan congeners (2,3,7,8-TCDF and 2,3,4,7,8-PeCDF) showed better recoveries than the dioxin congeners.

3.3. Comparison of post-IAC treatments

DD3 was utilized to compare different post-IAC treatments in an attempt to develop a cleaner assay. The use of a graphitic carbon column has been previously demonstrated to assist in separation of coplanar aromatic compounds from lipids [24], so we compared the use of Carbograph columns with the solvent extraction/evaporation procedure. Comparison of the conventional extraction step with the carbon column procedure revealed no statistically significant differences in recovery (Fig. 2). This clearly indicates the carbon column does not affect recovery, regardless of congener. The carbon column technique can be easily incorporated into currently available commercial automation systems, allowing greater throughput in the analysis.

4. Conclusions

Each of the antibodies tested had a distinct pattern of recovery from the IAC, just as they show different binding pathways in ELISA experiments. The recovery obtained from an IAC is dependent upon the affinity of the antibody for the congener and a minimum affinity is required for a successful IAC recovery. Although the forces binding the congener to the antibody are similar for the IAC and other affinity measurements because the antibody is the same in both cases, the conditions of the assays are quite different. For example, most determinations of K_a or IC_{50} are conducted under equilibrium conditions, whereas, IACs operate under conditions where the kinetics of the binding process is more important. In addition, the process by which the antibody is immobilized is different for each assay and the binding forces could be affected by allosteric interactions caused by the immobilization procedure. Consequently, it is necessary to screen a number of antibodies to find the most suitable candidate for IAC use. Cattle, sheep or goats can be immunized to produce large batches of polyclonal antibodies. The nature of Mabs allows the continuous production of homogenous antibodies, whereas, polyclonal antibodies show considerable batch-to-batch variation. The production of a homogeneous antibody is critical for IACs because any variation would require different operating parameters for the column. Of the presently available antibodies, the monoclonal DD3 appears to be the most suitable for IAC retention of multiple PCDD/PCDF congeners.

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