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Development of a Multi-Analyte ELISA for Permethrin and Aroclors and its Implementation for Analysis of Soil/Sediment and House Dust Extracts

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Abstract Development of a multi-analyte enzyme linked immunosorbent assay (ELISA) for detection of permethrin and aroclors 1248 or 1254, and implementation of the assay for analysis of soil/sediment samples is described. The feasibility of using the multi-analyte ELISA to monitor aroclors 1254 and permethrin simultaneously was tested with permethrin and aroclor standards, and with aroclor- and permethrin-containing soil/sediment and house dust samples. Comparison of the I_{50} and I_{20} values of the multi-analyte with those of a single-analyte assay revealed similar results, and multi-analyte ELISA determination of analyte amounts in soil/sediment dust samples yielded similar results to those of a single-analyte assay. A single-analyte assay of permethrin content in permethrin-containing dust samples showed that the ELISA can determine the analyte accurately in samples with dust matrix content ranging from 1.2 to 20 mg as indicated by the good correlation between the results of the immunoassay and those of the GC analysis.

Keywords: Aroclors, permethrin, multi-analyte ELISA, residue analysis, soil/sediment residue analysis.

Introduction

Polychlorinated biphenyls (PCBs) comprise 209 different compounds that share a common structure but vary in the number and position(s) of attached chlorine atoms. Aroclors are specific mixtures of PCB congeners. PCBs were widely used in many applications, especially as dielectric fluids in transformers and capacitors, and as coolants, but their manufacture was banned in the U.S. in the late 1970s and by the Stockholm Convention on Persistent Organic Pollutants in 2001, because of their persistent accumulation in the environment, and harmful effects on humans. Although the manufacture of PCBs was stopped over 30 years ago, they are still being detected in various environments (e.g., air, soil, dust, sediments, food).[1] Because of the ubiquitous presence of PCBs in the environment, humans can be exposed to PCBs through several routes: inhalation of contaminated air (both outdoors and indoors); ingestion of contaminated food; and dermal contact with contaminated surfaces. Studies have shown that dietary ingestion, e.g., through consumption of contaminated fish or oil, is the primary route of exposure to PCBs, and adverse health effects in both children and adults have been linked to PCB exposure. In addition, PCBs have been found in structural caulking materials used in schools and other public buildings, where they present widespread exposure hazards.[2]

Synthetic pyrethroid insecticides have been used in agricultural, domestic and veterinary applications for more than four decades, and they account for approximately 25% of the worldwide insecticide market.[3] The growth in use of synthetic pyrethroids, relative to other classes of insecticides, is attributed to their remarkably high insecticidal activity and their generally assumed low acute toxicity to mammals. Although these compounds are widely considered safe for mammals, recent studies have shown that short-term and long-term neonatal and subsequent adult exposure to synthetic pyrethroids may cause developmental neurotoxic and immunotoxic effects that may lead to spontaneous behavioral aberrations; exposure may also cause changes in the muscarinic cholinergic

system, impairment of memory and learning, lymph node and spleen damage, and carcinogenesis.[4] The widespread use of pyrethroids in agriculture, horticulture and forestry increases human exposure via the diet, and via occupational and domestic routes, and presents potential risks to mammals, non-target invertebrates and aquatic organisms[5] that may be exposed to field runoff or drift from aerial and ground-based spraying. All of the above raise an urgent need for large-scale monitoring of PCBs and pyrethroids in agricultural produce, and in environmental, domestic and biological samples.

Currently, high-resolution gas chromatography/mass spectrometry (GC-MS) is the widely accepted and reliable technique for quantitation of PCB contaminants. Receptor gene assays such as the chemically activated luciferase gene expression assay (termed CALUX,[6]), and a wide variety of other bioanalytical screening methods are also used for monitoring PCBs and other dioxin-like compounds. (For example see [7]). Many methods have also been developed and employed for the detection of pyrethroids. (For example see [8,9]). Although the above methods are reliable, sensitive, precise and reproducible, they are time consuming and expensive, must be performed by highly trained personnel, involve the use of large volumes of toxic solvents and typically cannot be applied on-site. These methods are, therefore, unsuitable for quick or high throughput screening tests. In attempts to develop highly sensitive methods and to meet the increasingly stringent regulations and demands for continuous monitoring of PCB and pyrethroid residues in food and in the environment, alternative methods were sought among them immunoassays. (For example see [10-16]).

Although application of immunoassays for individual compound screening is now well established, and despite the relatively large number of immunoassays that have been developed for residue monitoring, high-volume application of these methods is still limited. One major reason is the inability of the immunoassay to detect several analytes in a given sample, unlike chemical analytical methods, which can accommodate multi-residue samples. As a result, a main trend that focuses on

development of multi-analyte immunoassays has emerged recently. Indeed, in the past few years multi-residue immunoassays have been developed for a variety of agricultural, industrial, environmental, and medical applications in many different formats.[17,18]

In the present study we developed a multi-analyte enzyme linked immunosorbent assay (ELISA) for detection of permethrin and aroclors 1248 or 1254 simultaneously, and implemented the assay for analysis of soil/sediment and house dust samples. The feasibility of using the multi-analyte ELISA to monitor aroclor 1254 and permethrin simultaneously was tested with permethrin and aroclor standards as well as with aroclor- and permethrin-containing soil/sediment samples.

Materials and Methods

Materials

Aroclors 1016, 1221, 1242, 1254, 1262 and 1268, and PCBs 77, 126 and 169 were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Aroclors 1232, 1248 and 1260 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Aroclors 1232, 1248 and 1260, and all PCB stock solutions were prepared in Ultra Resi-Analyzed absolute methanol (J.T. Backer, Phillipsburg, NJ, USA). Aroclors 1016, 1221, 1242, 1254 and 1262 were provided in cyclohexane and aroclor 1268 in iso-octane.

Pyrethroids: permethrin, tetramethrin, imiprothrin, allethrin, prallethrin, cyphenothrin, cyfluthrin, phenothrin (summithrin), deltamethrin, esfenvalerate, bifenthrin, λ-(lambda) cyhalothrin, resmethrin, tralomethrin, γ-(gamma) cyhalothrin, tefluthrin, σ-(tau)-fluvalinate, and fenvalerate, were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Cypermethrin and fenpropathrin were purchased from Riedel-de-Haen (Buchs SG, Switzerland). All pyrethroid stock solutions were prepared in absolute methanol (J.T. Backer, Phillipsburg, NJ, USA).

115 Immunochemical methods

116 *Antisera and coating antigens*

117 Anti-PCB polyclonal antiserum was produced against a 4'-hydroxy analog of 2,2',4,5-
118 tetrachlorobiphenyl, namely, 6-[(2,2',4',5'-tetrachloro-4-biphenylyl)-oxy]hexanoic acid, which was
119 linked covalently to keyhole limpet hemocyanin (KLH) (Sigma, Rehovot, Israel). The PCB-BSA
120 (Bovine serum albumin) coating conjugate (CoAg 560-52) was generated by using 4-(2,4,5,-
121 trichlorophenoxy)butyric acid conjugated to BSA as previously described.[15] The protein content of
122 the conjugate was determined with Bradford (Bio-Rad Laboratories GmbH, Munich, Germany) by
123 comparison with a BSA standard curve and was found to be 4 mg/mL. Anti-permethrin polyclonal
124 antiserum, generated in rabbits (termed 549) and a coating antigen, permethrin-BSA conjugate
125 (termed cis-4-BSA) (10.3 mg/mL in double-distilled water) were generated as previously described
126 [19] and kindly provided by Prof. Bruce Hammock of University of California, Davis, USA.

127

128 *Single-analyte assays*

129 *Aroclor 1248 and 1254 (**Figure 1A & B**) ELISA*: The assay was used to determine both the cross
130 reactivity of anti-aroclor antiserum with a variety of aroclors, PCBs and pyrethroids and the content
131 of aroclor in soil/sediment samples (see below: Determination of aroclor 1254 in soil/sediment
132 samples) and served as a basis for the development of the multi-analyte ELISA. MaxiSorp ELISA
133 plates (NUNC Roskilde, Denmark) were coated with 100 µl of PCB-BSA conjugate, diluted
134 1:40,000 (containing 40 ng per 100 µl) in 0.5 M carbonate buffer, pH 9.6. After an overnight
135 incubation at 4 °C, the wells were washed three times with phosphate-buffered saline (PBS) that
136 comprised 0.15 M NaCl in 50 mM sodium phosphate, pH 7.2, containing 0.1% (v/v) Tween-20
137 (PBST) (Sigma, Rehovot, Israel), and 50 µl of aroclor standards (1248 or 1254, in 12 serial dilutions
138 ranging from 0.0048 to 10 ng per 50 µl of PBST containing 40% methanol (hereafter:
139 "PBST/methanol") were added to the wells in duplicate, together with 50 µl of anti-PCB antiserum

diluted 1:3,000 in PBST (final dilution 1:6,000). Four wells received a tenfold excess of each aroclor (i.e., 100 ng per 50 µl of PBST/methanol) and served to determine the background of the reaction, and four additional wells received just PBST/methanol, instead of the aroclors, and served to determine maximal binding in the absence of competing analyte, which was designated as 100%. The plates were incubated for 3 h at room temperature and washed as above with PBST, and 100 µl of secondary antibody conjugated to horseradish peroxidase (HRP) (anti-rabbit HRP conjugated, Sigma), diluted 1:30,000 in PBST were added to the plates. The plates were incubated for 2 h at room temperature, rinsed with PBST, and tested for HRP activity by addition of 100 µl of substrate solution – 3,3',5,5' tetramethyl benzidine (TMB) (Thermo Scientific, Rockford, IL, USA). The reaction was stopped after 10 min by addition of 50 µl of 4 N sulfuric acid, and the absorbance at 450 nm was measured with a Lucy 2 microplate reader (Anthos, Eugendorf, Austria).

The cross reactivity of the antibodies was tested with a variety of aroclors, PCBs and pyrethroids. The reaction was carried out as described above (Single-analyte assays) by adding these compounds (instead of aroclors 1248 or 1254 in the respective assays) at 12 serial dilutions, using the same concentration range (0.0048 to 10 ng per 50 µl), and testing their ability to compete with the PCB-BSA conjugate coated on the microplate in binding the anti-PCB antiserum.

*Permethrin (**Figure 1C**) ELISA*: The permethrin competitive ELISA assay was carried out as described above for aroclor 1248 or 1254, except for the following modifications: microtiter plate wells were coated with 100 µl of permethrin-BSA conjugate, diluted 1:2,000 (containing 514 ng per 100 µl of protein) in 0.5 M carbonate buffer, pH 9.6, and 50 µl of each of 12 serial dilutions of permethrin standard (a 58/42% mixture of *trans* and *cis*, respectively) were added, together with 50 µl of anti-permethrin antiserum diluted 1:1,250 in PBST (final dilution 1:2,500). All other details were identical to those described above.

The cross reactivity of the antibodies was tested with a variety of aroclors, PCBs and pyrethroids. The reaction was carried out as indicated above for permethrin competitive ELISA, but with these compounds added instead of permethrin, at 12 serial dilutions, covering the same concentration range (0.0048 to 10 ng per 50 µl), and testing their ability to compete with the permethrin-BSA conjugate coated on the microplate in binding the anti-permethrin antiserum.

Multi-analyte assay

MaxiSorp ELISA plates (NUNC, Roskilde, Denmark) were coated with either 100 µl of PCB-BSA conjugate, diluted 1:40,000 (containing 40 ng per 100 µl) or with permethrin-BSA conjugate, diluted 1:2,000 (containing conjugate at 514 ng per 100 µl) in 0.5 M carbonate buffer, pH 9.6. After incubation overnight at 4 °C, the wells were washed three times with PBST, and 50 µl of a mixture of aroclor (1248 or 1254) and permethrin standard (12 serial dilutions of each standard compound, ranging from 0.0048 to 10 ng per 50 µl of PBST/methanol) were added, in duplicate, to the wells, together with 50 µl of a mixture of anti-PCB and anti-permethrin antiserum diluted 1:3,000 and 1:1,250, respectively, in PBST; (i.e., final dilutions 1:6,000 and 1:2,500, respectively). Four wells received a tenfold excess of a mixture of each aroclor with permethrin (i.e., each at 100 ng per 50 µl of PBST/methanol) and served to determine the background of the reaction, and four additional wells received just PBST/methanol, and served to determine maximal binding in the absence of competing analyte (designated as 100%). The plates were incubated for 3 h at room temperature, washed as above with PBST, and 100 µl of secondary Ab conjugated to HRP, diluted 1:30,000 in PBST, were added to the plates. The plates were incubated for 2 h at room temperature, rinsed with PBST, and tested for HRP activity by the addition of 100 µl of TMB substrate solution. The reaction was stopped after 10 min by addition of 50 µl of 4 N sulfuric acid, and the absorbance at 450 nm was measured with a Lucy 2 microplate reader (Anthos, Eugendorf, Austria).

Analysis of analytes in soil/sediment samples

Determination of aroclor 1254 in soil/sediment samples

Soil/sediment samples ("aroclor- containing soil/sediment samples") were prepared as follows: The procedure employed was based on the extraction method developed for dioxins and furans in sediment/soil.[20] In brief, an aliquot (4 g) of sediment/soil sample was mixed with Hydromatrix (3 g), alumina (3 g), 10% $AgNO_3$ in silica (1 g), and acid silica (6 g) and extracted with dichloromethane using Dionex Accelerated Solvent Extraction 200 system (Sunnyvale, CA). The extraction was carried out at 100ºC, with a purge time of 60 s, a flush volume of 100%, and an extraction time of 10 min and 3 cycles. The resulting extracts were concentrated and divided into two portions: one for GC and one for ELISA. The sample extract for ELISA portion was solvent exchanged to methanol for subsequent analysis.

GC analysis

The samples and standard solutions were analyzed by GC with electron capture detection for aroclor concentrations based on EPA Method 8082 (US EPA, 1996).[21] The GC column used was a DB-5 fused silica capillary column (60 m x 0.25 mm i.d., 0.25 µm film thickness), and hydrogen was used as the carrier gas. Identification and quantitation were accomplished by integrating representative major peaks in the aroclor standard, and identifying and integrating those same peaks (by retention time and pattern matching) in the samples.

ELISA analysis

Single- and multi-analyte aroclor ELISA was used to analyze aroclor 1254 content in soil/sediment samples as described above. The soil/sediment samples were diluted 1:2.5 with PBST to bring the methanol concentration in the sample to 40%. Samples 1 and 2 were then diluted 1:30 and samples 3, 4 and 5 diluted 1:150, all in PBST/methanol.

In the single-analyte aroclor ELISA soil/sediment samples were added (in a volume of 25 µL) together with 25 µl of PBST/methanol to wells coated with PCB-BSA to reach final dilution ranges of 1:150 to 1:2,400 for samples 1 and 2, (equivalent to 6.7 to 0.42 mg of sediment/soil) and 1:750 to 1:12,000 for samples 3, 4 and 5, (equivalent to 1.3 to 0.083 mg of sediment/soil). Fifty microliters of PCB antiserum that had been diluted 1:3,000 in PBST, (final dilution of 1:6,000), were added to the plates and the reaction mixture was incubated for 3 h at room temperature and processed as above (see Single-analyte assays).

In the multi-analyte ELISA format 'aroclor' soil/sediment samples at the same initial dilution (1:30 for samples 1 and 2; 1:150 for samples 3, 4 and 5), together with 25 µl of standard permethrin (at 2.5 ng per 25 µl) instead of the PBST/methanol were added to wells coated with either PCB-BSA or permethrin-BSA. Fifty microliters of a mixture of anti-PCB and anti-permethrin antiserum diluted 1:3,000 and 1:1,250 in PBST (final dilutions 1:6,000 and 1:2,500, respectively), were added to the PCB-BSA- and permethrin-BSA-coated wells and the reaction mixture was incubated as indicated above.

In order to exclude the possibility that the 'aroclor' soil/sediment samples interfered with the permethrin ELISA and might thereby have affected the values obtained in the multi-analyte assay, aroclor soil/sediment samples were tested for their interference with a single-analyte permethrin assay. Twenty-five-microliter samples, (diluted 1:2.5 with PBST as above), together with 25 µl of PBST/methanol were added to wells coated with permethrin-BSA conjugate (diluted 1:2,000) to reach final sample dilution ranges of 1:150 to 1:2,400 (samples 1 and 2) or 1:750 to 1:12,000 (samples 3, 4 and 5). Fifty-microliter aliquots of permethrin antiserum, diluted 1:1,250 in PBST (to a final dilution of 1:2,500) were added to the wells and the reaction mixture was incubated for 3 h at room temperature and processed as above.

Five serial dilutions (ranging from 5.0 to 0.312 ng per 50 μl) of standard aroclor 1254 in PBST/methanol served as a quality control to determine the assay accuracy. Aroclor content in the single-aroclor format was calculated from a calibration curve of aroclor 1254, and that in the multi-aroclor format from a calibration curve of a mixture of aroclor 1254 and permethrin diluted in PBST/methanol (each analyte in the range of 0.0048 to 10 ng/50 μL, diluted in PBST/methanol), after linearization of the data by transformation to a logit-log plot by means of Microcal Origin software, Version 7.5 (Microcal Software, Northampton, MA, USA). All 'aroclor' soil/sediment samples were tested in duplicate at dilutions within the range of the standard curve. Slopes of all samples were tested for parallelism with the standard curve, by examining the homogeneity of regression slopes, and only samples whose regression lines were parallel to the standard curve were considered.

Determination of permethrin in house dust samples

House dust samples (‘permethrin’-containing dust samples) were prepared for analysis as described previously.[9] In brief, the dust sample was extracted with dichloromethane using sonication and solvent exchanged into acetonitrile for subsequent solid phase extraction (SPE) cleanup prior to GC-MS analysis. For ELISA analysis, the dust sample (0.5 g) together with neutral silica (5 g), acid silica (0.5 g), and Hydromatrix (1 g) were extracted with dichloromethane. The extraction was performed at 2000 psi and 100ºC for 3 cycles of 5 min. The dichloromethane extract was concentrated and solvent exchanged into methanol prior to ELISA.

GC-MS analysis

GC-MS procedure for analyzing the 'permethrin'-containing dust sample extracts was described previously.[9] Briefly, a Hewlett Packard GC-MS was operated in the SIM mode. The GC column was a DB35MS fused silica capillary (30 m x 0.25 mm i.d., 0.25 μm film thickness). Helium was

used as the carrier gas. Data acquisition and processing were performed with a ChemStation data system.

ELISA analysis

Single analyte permethrin ELISA was applied to the 'permethrin'-containing dust samples as described above. The samples were diluted 1:2.5 with PBST, to bring the methanol concentration in the sample to 40%, and then serially diluted five times in PBST/methanol, to final dilutions ranging from 1:5 to 1:80 (equivalent to 20 to 1.2 mg of house dust). Fifty-microliter samples were added to permethrin-BSA conjugate-coated wells together with 50 µl of anti-permethrin antiserum diluted 1:1,250 in PBST, to a final dilution of 1:2,500. Five serial dilutions, ranging from 10 to 0.625 ng of standard permethrin per 50 µl of PBST/methanol served as a quality control to determine the assay accuracy. Permethrin concentrations were calculated from a calibration curve (in the range of 0.0048 to 10 ng per 50 µl diluted in PBST containing 40% methanol), after linearization of the data as described above. All permethrin dust samples were tested in duplicate at dilutions within the range of the standard curve. All other details were as described above for single-analyte assay.

Statistics

Results were subjected to one-way ANOVA. The significance of differences among means was evaluated with the Tukey-Kramer HSD (Honestly Significant Difference) test at $p < 0.05$.

Results and Discussion

Single-analyte ELISA

The first part of the study focused on optimization of two single-analyte ELISAs: permethrin and aroclors (1248 and 1254). Although the antisera that were used in the study have been previously described and employed to develop permethrin and aroclor ELISAs[15,19] the assay conditions in this

study had to be re-evaluated and modified in order to fulfill the basic requirement of a multi-analyte ELISA: employment the very same reaction conditions (i.e., buffers, incubation times, secondary Ab dilution, etc.) with all antibodies and analytes. As a consequence optimization of each ELISA was carried out separately for each analyte. The optimization involved two sets of experiments: the first set was intended to determine the optimal concentrations of the coating antigen conjugates permethrin-BSA or PCB-BSA, the antiserum and the secondary Ab (checkerboard tests); the second set was intended to generate a standard curve, to determine the I_{50} value and the limit of detection (LOD, I_{20}) of the assay, cross reactivity and the tolerance of the antibodies to organic solvents.

The first set of experiments revealed that for the permethrin ELISA dilutions of 1:2,000 for the permethrin-BSA conjugate and 1:2,500 (final) of the anti-permethrin antiserum resulted in high binding and a low background, i.e., non-specific binding. In the aroclor ELISA dilution of 1:40,000 for the coating antigen and 1:6,000 for the anti-PCB antiserum resulted in a good signal-to-background ratio for both aroclors (1248 and 1254; data not shown). The second set of experiments determined the working ranges of both assays (0.06 to 2.5 ng per 50 µl) (**Figures 2A and B**) and their I_{50} and I_{20} values **(Table 1**). Basically there were no marked differences in the working ranges of the two ELISAs, and the I_{50} and I_{20} values were very similar. Because of the very low solubility of permethrin and the aroclors in neutral aqueous buffers both assays were carried out in the presence of methanol at a final concentration of 20%.

Cross reactivity

Once the assays had been optimized and the sensitivities determined, the ELISAs were used to characterize the antiserum for specificity and for cross reactivity with other pyrethroids, aroclors and PCBs (**Table 2**). Although both antisera have been previously tested for their cross reactivity [15,19], they were not tested under the same reaction conditions (as specified in this study) nor were they

examined for their ability to react with analytes of the other group (namely anti-PCB antisera with pyrethroids and anti-permethrin antisera with aroclors and PCBs). Since cross reactivity may differ as a function of reaction conditions and since it was most important to prove lack of cross reactivity between the antisera and the other group of analytes, cross reactivity had to be re-evaluated under the tested experimental conditions. Cross reactivity of the PCB-antiserum was tested with aroclor and PCB compounds (**Table 2)** as well as with pyrethroid compounds (**Table 3)**. A similar analysis was carried out with the permethrin antibodies. The cross reactivity was determined by means of the single-analyte ELISA format. As indicated in **Table 2**, the PCB antiserum did cross-react with a few aroclors. When aroclor 1248 was used as a reference the reactivity of the PCB antiserum with 1254, 1016 and 1242 was higher than that with 1248, and the antiserum also showed high cross reactivity with aroclors 1262 and 1260; cross reactivity with 1232 was low, and no cross reactivity with aroclors 1221 and 1268, and the three tested PCBs could be detected. Examination of cross reactivity with 1254 as a reference revealed lower values (76, 76, 47, 41 and 35% with aroclors 1016, 1242, 1248, 1262 and 1260, respectively) and 13% with 1232. No cross reactivity was detected with any of the other aroclors or PCBs. Under the conditions of the ELISA experiments the PCB antiserum did not cross-react with any of the tested pyrethroid compounds (**Table 3**) at concentrations up to 0.2 ppm (10 ng per 50 µl) (data not shown). Cross reactivity analysis of the anti-permethrin antiserum revealed very high values toward tetramethrin, imiprothrin, allethrin and prallethrin (**Table 3**). The antiserum also recognized cypermethrin, and cyphenothrin, cyfluthrin and phenothrin to a lesser extent. No cross reactivity was monitored with the other pyrethroids or with any of the tested aroclors or PCBs (**Table 2**, data not shown). The cross reactivity of the anti-PCB antiserum with several aroclors and that of the anti-permethrin antiserum with several pyrethoids necessitates to term the assays developed herein 'single-group' and 'multi-group' ELISAs. For simplicity reasons we chose to term the assays 'single-analyte' and 'multi-analyte' ELISAs, respectively, despite the fact each assay can monitor more than one analyte of a given group.

Multi-analyte ELISA

Absence of cross reactivity of the PCB antiserum with the tested pyrethroids and of the permethrin antiserum with the aroclors and PCBs enabled us to develop a multi-analyte assay format in which the plates were coated with either PCB or permethrin-BSA conjugates and the PCB- or permethrin-antisera were tested for their ability to recognize their specific analytes (aroclors 1248 or 1254, or permethrin, respectively) in the presence of a mixture of two antibodies and two analytes (one of the aroclors and permethrin). The assay was performed in the presence of both antibodies to investigate that other components present in the reaction mixture (e.g., other antibodies or analytes) do not cause interference. Each experiment included, in addition to the above, a positive control in which the assay was carried out with a single Ab and its respective single analyte (i.e., PCB antiserum and aroclor 1248 or 1254; and permethrin antiserum and permethrin) and a negative control in which each Ab was tested for its ability to recognize the other analyte (i.e., PCB antiserum for permethrin; and permethrin antiserum for aroclor 1248 or 1254). The data presented in **Figure 2A** and **Table 1** clearly indicate that presence of the permethrin antiserum, and permethrin analyte did not interfere with the ability of the anti-aroclor antiserum to recognize the aroclors, and the I_{50} values that were obtained in their presence and absence did not differ significantly from those obtained in the single-analyte aroclor 1248 assay (16.0 ± 2.8 (n = 2) and 16.0 ± 2.8 (n = 2), respectively). Similar data were obtained with the aroclor 1254 multi-analyte ELISA format (I_{50} values of 7.5 ± 1.0 (n = 8) and 8.9 ± 2.9 (n = 7) in the single- and multi-analyte formats, respectively). PCB antiserum did not react with permethrin under the tested conditions.

The data presented in **Figure 2B** and **Table 1** indicate similar results for the permethrin antiserum. Presence of the PCB antiserum and aroclors 1248 or 1254 did not interfere with the ability of the permethrin antiserum to recognize the permethrin, and the I_{50} values that were obtained in their presence and absence did not differ significantly from those obtained in the single-analyte

365 permethrin assay (48.0 ± 29.0 (*n* = 4) and 35.8 ± 26.7 (*n* = 6), respectively). Anti-permethrin by itself
366 did not react with any of the aroclors.
367
368 Various approaches have been reported to the simultaneous determination of several analytes using
369 immunochemical assays indicating that the concept 'multi-residue' immunoassay refers to a large
370 variety of formats.[18,22] To date, the most commonly used method for multi-analyte analysis is the
371 bead-based Luminex flow cytometric system.[23] As in the case of the multi-analyte ELISA, absence
372 of cross reactivity of the antibodies is a prerequisite for development of a reliable Luminex assay.
373 The current assay format (which employs several antibodies with high affinity toward a given class
374 of analytes and no cross reactivity with the other class of compounds), enables differentiation and
375 quantitation of the concentrations of the individual tested analytes in a simple, fast and direct manner
376 using the identical assay conditions (i.e., same buffers, reporting enzyme substrates, incubation
377 times, etc.) for both analytes; it can be used for a large variety of analytes and therefore introduces
378 many advantages over current published methods. A similar approach has been reported for the
379 detection of pharmaceutical residues in porcine kidneys[24], although it is difficult to determine from
380 the report whether quantitation of a given analyte was determined in the presence of all other
381 analytes and antibodies.
382
383 *Application of single- and multi-analyte ELISA for analysis of aroclor 1254 and permethrin content*
384 *in 'aroclor' soil/sediment samples*
385 We examined the ability of the above ELISAs to determine the content of aroclor 1254 in
386 soil/sediment extracts, and compared the values obtained in single and multiple ELISAs. In the
387 single-analyte aroclor assay samples were applied onto PCB-BSA coated plates and tested with anti-
388 PCB antiserum; this assay was designed to determine the amount of aroclor 1254 in the soil/sediment
389 samples in standard aroclor assay format. In the multi-analyte assay, soil/sediment samples spiked

with permethrin at 100 ng/mL were tested in the presence of both PCB and permethrin antiserum in plates that were coated with PCB-BSA conjugate. This assay was designed to determine the amount of aroclor 1254 in the tested soil/sediment samples in an assay format in which another analyte (permethrin) and another Ab (anti-permethrin) were present in the reaction mixture. In another multi-analyte assay that was carried out in a similar manner the 'aroclor' soil/sediment samples, spiked with permethrin at 100 ng/mL, were tested with plates coated with permethrin-BSA conjugate and a mixture of the two antibodies. This assay was designed to determine whether the 'aroclor' soil/sediment samples interfered in any manner with the ability to determine permethrin in a multi-analyte ELISA format.

In parallel, a single-analyte permethrin assay was carried out on these 'aroclor' soil/sediment samples (in the absence of spiked permethrin); plates coated with permethrin-BSA were used, which were tested just with anti-permethrin antiserum, in order to determine the degree of interference of the 'aroclor' soil/sediment samples themselves with the ELISA, and to exclude the possibility that the samples interfered with the permethrin ELISA and thereby affected the values obtained in the multi-analyte assay. Comparison of the amount of aroclor 1254 in the 'aroclor' soil/sediment samples obtained in the single aroclor 1254 with that obtained in the multi-analyte ELISA format (**Figure 3**) revealed no significant differences, indicating that the presence of an unrelated analyte (permethrin) and antiserum (anti-permethrin) in the reaction mixture did not have a significant effect on the ability to evaluate aroclor 1254 content in the samples.

Application of a multi-analyte ELISA for analysis of spiked permethrin content in 'aroclor' soil/sediment samples

Examination of permethrin content in the 'aroclor' soil/sediment samples spiked with permethrin at 100 ng/mL, which were tested in a multi-analyte ELISA format, revealed that the values obtained did

not differ significantly from those expected (**Table 4**), which confirmed once again that presence of an unrelated analyte (aroclor) and its antiserum (anti-PCB) did not affect the ability to quantitate permethrin accurately. The 'aroclor' soil/sediment extracts themselves did not interfere with the permethrin ELISA, and the percentage of binding of the permethrin antiserum to the coating antigen (permethrin-BSA) in the presence of the 'aroclor' soil/sediment samples yielded binding values (84, 87, 81, 80 and 82% for samples 1 to 5, respectively) that did not differ markedly from those obtained in the absence of the soil/sediment sample (designated as 100%).

Application of a single-analyte permethrin ELISA for analysis of permethrin content in 'permethrin' dust samples

The permethrin ELISA was also used to determine the content of permethrin in the 'permethrin' dust samples, and the values obtained in the ELISA were compared with those obtained by GC-MS. The data in **Table 5** show a good correlation in six out of the 10 samples, and the values obtained in the immunoassay did not differ significantly from those obtained for *trans*-permethrin by the chemical analytical method. Four samples (Nos. 1, 3, 7 and 9) yielded significantly lower values in the immunoassay than those obtained in the GC-MS analysis. The ELISA and GC-MS values were compared only for *trans*-permethrin, because the antiserum recognizes *trans*-permethrin and has low cross reactivity (13%) toward *cis*-permethrin. It is interesting to note that lack of correlation between the values obtained by the two respective methods was evident mainly for the samples that contained high levels of *cis*-permethrin, which may have resulted from interference of the *cis*-isoform with the accurate determination in the immunochemical assay. Lack of correlation was also noticed in sample No. 1, in which the level of permethrin was not that high. It may very well be that this samples as well the other samples in which no correlation was observed contained ingredients that might have interfered with the assay. It is important to indicate that, in contrast to the 'aroclor' soil/sediment samples, which contained relatively large amounts of analyte and had to be diluted by at least 150-

fold (equivalent to 6.7 mg of sediment/soil) prior to ELISA analysis, the 'permethrin' samples were diluted only 1:5 (equivalent to 20 mg of house dust) and may, thus contain interfering components. The difference in dilutions employed in the aroclor and permethrin containing samples is mostly due to the difference in sample size, sample matrix, and analyte concentrations. Nonetheless, it seems that in most of the samples, matrix interference did not have a major effect: the curve generated by the different sample dilutions in the ELISA fully paralleled the standard curve, and the data, in most samples correlated well with the GC-MS analysis. In the past few years we developed highly efficient sol-gel immunoaffinity purification methods for pyrethoids and PCBs which eliminate matrix interference.[10,16] Such methods can be used in further studies, in combination with ELISAs, to overcome this problem.

Most studies on presence of pyrethroids in soil and dust samples were based on chemical instrumental analysis. Only a few immunoassays were employed for analysis of pyrethroids in soil and dust samples. In a study by Nakata[13], methanol-extracted spiked soil samples were analyzed by means of an immobilized hapten-conjugate competitive ELISA. Although the recoveries obtained were above 95%, the study did not examine the correlation of the immunoassay results with those of GC-MS. High recoveries (nearly 100%) of type II synthetic pyrethroids and an excellent correlation coefficient (0.99) with an immobilized Ab competitive ELISA were reported[11], and recently, permethrin content in soil and dust samples was evaluated with a magnetic particle format immunoassay.[25] As in the present study, the ELISA-derived permethrin concentrations were highly correlated with the GC-MS-derived sums of *cis/trans* permethrin concentrations, with a high correlation coefficient.

In summary, the ELISAs that were developed in the course of the present study can serve as an excellent tool for further studies toward the development of similar ELISA formats with many more

analytes, and be easily adjusted to a high-throughput automated format, which will allow us to widen the application of immunoassays for screening of agricultural (e.g., food), environmental, and medical samples, to reduce costs, to increase precision, through elimination of variations between individual experiments, and to further shorten analysis time and thereby overcome one of the major obstacles in the way of further implementation of such simple, cost-effective and sensitive assays, and their application to analysis of additional 'real world' samples.

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555

Figure Legends

Figure 1: Structure of Aroclors 1248 (**A**), 1254 (**B**) and the Pyrethroid Permethrin (**C**).

Figure 2: Representative Standard Curves of a Single- and a Multi-Analyte Aroclor 1248 and 1254 ELISA in the Presence and Absence of Permethrin (**A**) and in the Presence and Absence of Aroclor 1248 and 1254 (**B**).

Figure 3: Aroclor 1254 Content in 'Aroclor' Soil/Sediment Samples: Comparison of Values Obtained in Single- and Multi-Analyte Aroclor 1254 ELISA Formats. Each bar represents the mean ± SEM (standard error mean) of three or four measurements. Statistical analysis compared values obtained in the single vs. multiple assay formats for each sample independently. Values of all tested samples did not differ significantly at $p < 0.05$. Sample codes are sample 1: 51733-83-02; sample 2: 51733-83-04; sample 3: 51733-83-05; sample 4: 51733-83-06; sample 5: 51733-83-07.

Table 1: Comparison of I_{50} and Limit of Detection (I_{20}) Values of Single- and Multi-Analyte ELISAs.

Analyte	**I_{50} (ng/mL)**		**I_{20} (ng/mL)**	
	Single-analyte	**Multi-analyte**	**Single-analyte**	**Multi-analyte**
Aroclor 1248	16.0 ± 2.8 (*n*=2)	16.0 ± 2.8 (*n*=2)	1.6 ± 2.0 (*n*=2)	1.6 ± 2.0 (*n*=2)
Aroclor 1254	7.5 ± 1.0 (*n*=8)	8.9 ± 2.9 (*n*=7)	1.8 ± 0.8 (*n*=8)	1.5 ± 0.7 (*n*=7)
Permethrin	35.8 ± 26.7 (*n*=6)	48.0 ± 29.0 (*n*=4)	6.2 ± 7.1 (*n*=6)	14.3± 18.1 (*n*=4)

Each value represents the mean ± SEM (standard error mean) of n measurements (as indicated in the Table). I_{50} represents the concentration of the analyte required to displace 50% of the analyte bound to the Ab. Statistical analysis compared values obtained in the single- and the multi-analyte formats for each sample independently. Values for all tested samples did not differ significantly at $p < 0.05$.

Table 2: Cross Reactivity of AC-3 Anti-Aroclor Antiserum with Various Aroclors and PCBs.

N	Compound	CR (%)	Compound	CR (%)
1	Aroclor 1248	100	Aroclor 1254	100
2	Aroclor 1254	163	Aroclor 1248	47
3	Aroclor 1016	125	Aroclor 1016	76
4	Aroclor 1242	125	Aroclor 1242	76
5	Aroclor 1262	67	Aroclor 1262	41
6	Aroclor 1260	57	Aroclor 1260	35
7	Aroclor 1232	22	Aroclor 1232	13
8	Aroclor 1221	0	Aroclor 1221	0
9	Aroclor 1268	0	Aroclor 1268	0
10	PCB-77	0	PCB-77	0
11	PCB-126	0	PCB-126	0
12	PCB-169	0	PCB-169	0

Reactivity with aroclor 1248 was designated as 100% in the left-hand column and that with aroclor 1254 as 100% in the right-hand column. Cross reactivity (CR) was calculated as the ratio (as percentage) between the I_{50} value of aroclor 1248 or 1254 and that of the tested compounds.

Table 3: Summary of the Cross Reactivity of Anti-Permethrin Antibodies with Various Pyrethroids.

N	Compound	CR (%)
1	Permethrin	100
2	Tetramethrin	5,242
3	Imiprothrin	4,325
4	Allethrin	2,883
5	Prallethrin	2,163
6	Cypermethrin	100
7	Cyphenothrin	47
8	Cyfluthrin	29
9	Phenothrin (Sumithrin)	12
10	Deltamethrin	0
11	Esfenvalerate	0
12	Bifenthrin	0
13	lambda-Cyhalothrin	0
14	Resmethrin	0
15	Tralomethrin	0
16	Fenpropathrin	0
17	gamma-Cyhalothrin	0
18	Tefluthrin	0
19	tau-Fluvalinate	0
20	Fenvalerate	0

Reactivity with permethrin was designated as 100%. Cross reactivity (CR) was calculated as the ratio (as percentage) between the I_{50} value of permethrin and that of all other tested compounds.

Table 4: Amount of Spiked Permethrin Detected in 'Aroclor' Soil/Sediment Samples in a Multi-Analyte ELISA Format

Sample	**Permethrin content (ng/mL) ± SEM**
Standard	100[a]
Sample 1	78 ± 11 (*n*=5)
Sample 2	109 ± 20 (*n*=2)
Sample 3	78 ± 16 (*n*=3)
Sample 4	130 ± 15 (*n*=3)
Sample 5	110 ± 10 (*n*=2)

Permethrin content was determined from a mixed aroclor/permethrin standard curve in wells coated with permethrin-BSA conjugate.

[a] Expected value based on the amount of spiked permethrin (100 ng/mL). Results were subjected to one-way ANOVA.

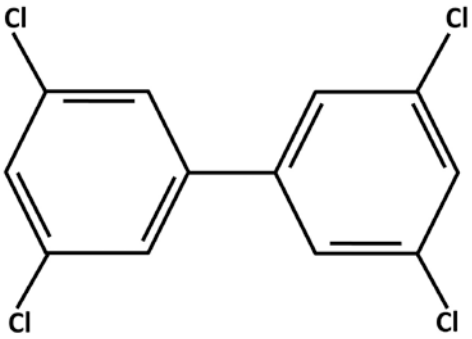
Each value represents the mean ± SEM (standard error mean) of n measurements (as indicated in the Table). n represents the number of repetitions of each sample whose results fell within the linear range of the standard curve and were used to calculate the amount of aroclor in the sample. The significance of differences among means was evaluated with the Tukey-Kramer HSD (Honestly Significant Difference) test at $p < 0.05$. No differences were observed between the permethrin contents in the presence and absence of the soil/sediment samples.

Table 5: Permethrin Content of 'Permethrin' Dust Samples

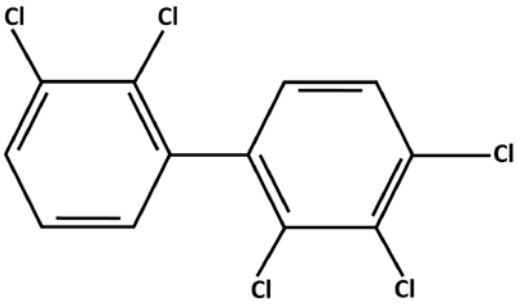
		Theoretical value (ng/mL) GC-MS		**Experimental value (ng/mL) ELISA**
Sample No.	**Sample code**	***cis*-permethrin**	***trans*-permethrin**	***trans*-permethrin**
Sample 1	16923	21	25	7 ± 2 (*n*=6)*
Sample 2	16949	26	22	22 ± 3 (*n*=9)
Sample 3	16935	95	106	33 ± 7 (*n*=12)*
Sample 4	23295	47	46	24 ± 3 (*n*=8)
Sample 5	23473	5	6	0 ± 0 (*n*=5)
Sample 6	23285	27	26	12 ± 2 (*n*=5)
Sample 7	23239	157	168	30 ± 3 (*n*=18)*
Sample 8	14787	6	6	5 ± 1 (*n*=5)
Sample 9	14709	267	243	66 ± 4 (*n*=20)*
Sample 10	14716	8	7	0 ± 0 (*n*=5)

Experimental value represents *trans*-permethrin content in 'permethrin' dust samples as obtained with a single-analyte permethrin ELISA format. The amount of *Trans*-permethrin was calculated by multiplication of the value obtained from the standard curve by 0.58 to correct for the 58% of *trans*-permethrin in the standard (which was composed of 58 and 42% *trans*- and *cis*-permethrin, respectively). Theoretical values of *cis*- and *trans*-permethrin were obtained by GC-MS analysis. Each value represents the mean ± SEM (standard error mean) of n measurements (as indicated in the Table). Statistical analysis compared the *trans*-permethrin values obtained by GC-MS with those obtained by ELISA. * An asterisk indicates significant difference at $p < 0.05$.

A.



B.



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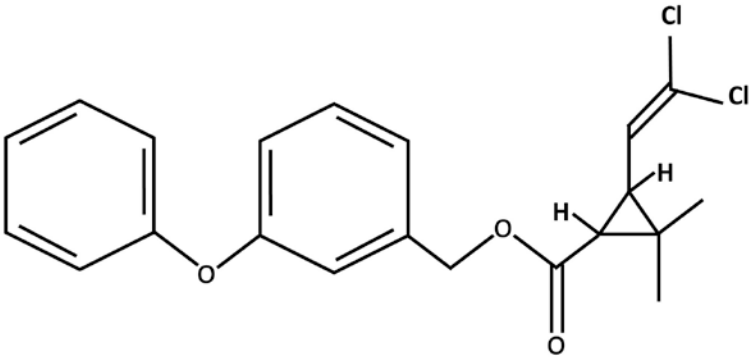
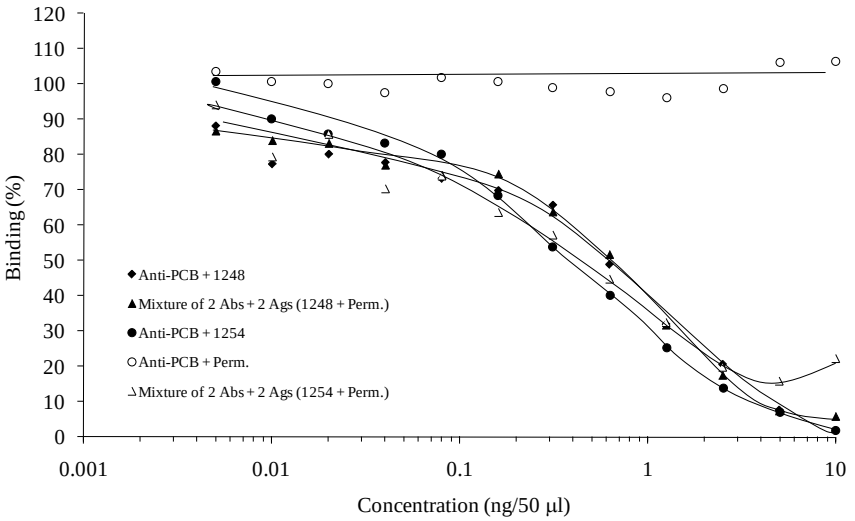


Figure 1

A.


Binding (%)
Concentration (ng/50 µl)
Anti-PCB + 1248
Mixture of 2 Abs + 2 Ags (1248 + Perm.)
Anti-PCB + 1254
Anti-PCB + Perm.
Mixture of 2 Abs + 2 Ags (1254 + Perm.)

B.

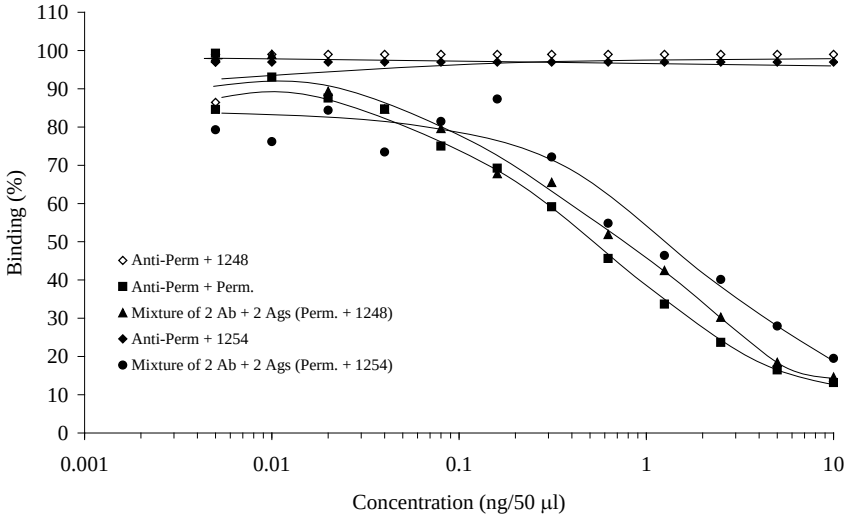

Binding (%)
Concentration (ng/50 µl)
Anti-Perm + 1248
Anti-Perm + Perm.
Mixture of 2 Ab + 2 Ags (Perm. + 1248)
Anti-Perm + 1254
Mixture of 2 Ab + 2 Ags (Perm. + 1254)

Figure 2

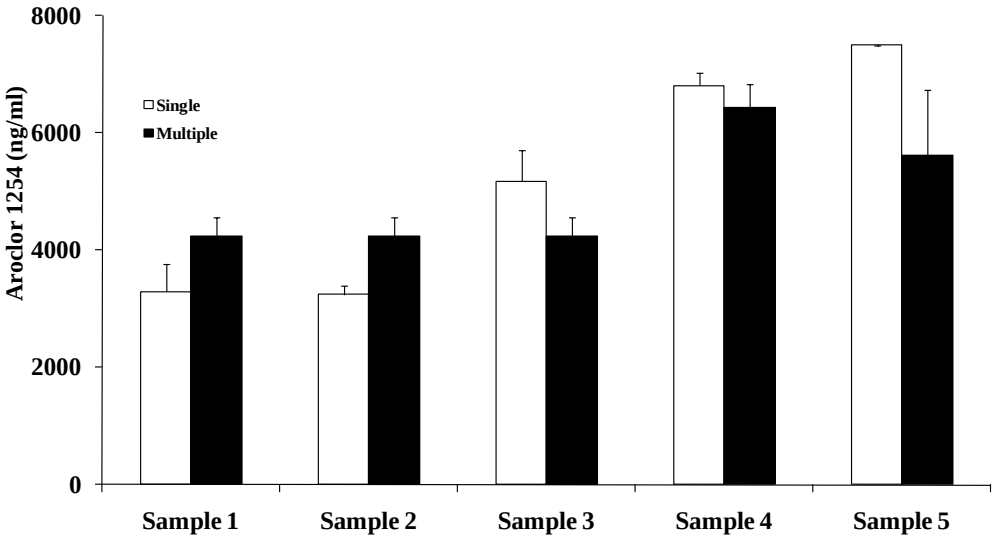

8000
6000
4000
2000
0
Aroclor 1254 (ng/ml)
Single
Multiple
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5

Figure 3

Table of Contents Graphic