SPECIAL GUEST EDITOR SECTION

Analysis of DDT Isomers with Enzyme-Linked Immunosorbent Assay and Optical Immunosensor Based on Rat Monoclonal Antibodies as Biological Recognition Elements

PETRA M. KRÄMER, CRISTINA M. WEBER, and STEPHAN FORSTER[1]
Institute of Ecological Chemistry, Helmholtz Center Munich–German Research Center for Environmental Health, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
PETER RAUCH
Candor Bioscience GmbH, Eggenwatt 12, 88138 Weißensberg, Germany
ELISABETH KREMMER
Institute of Molecular Immunology, Helmholtz Center Munich–German Research Center for Environmental Health, Marchioninistraße 25, 81377 Munich, Germany

New rat monoclonal antibodies (mAbs) for DDT [1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane], namely DDT 7C12, DDT 1C1, and DDT 1B2, were developed, characterized, and applied in ELISA both in coating antigen and in enzyme-tracer format. The latter used horseradish peroxidase (HRP) or glucose oxidase as enzymes. The lowest concentration of *p,p′*-DDT was determined with mAb DDT 7C12 and DDT-hapten HRP, with a test midpoint (IC_{50}) of 0.5 ± 0.2 μg/L (*n* = 10) in 40 mM PBS (phosphate-buffered saline). The mouse anti-rat immunoglobulin lambda-light chain mAb LA1B12 was used as capture mAb. The best IC_{50} for *o,p′*-DDT in 40 mM PBS was 1.0 ± 0.3 μg/L (*n* = 12) and was obtained with mAb DDT 1C1 and DDT-hapten HRP, whereas mAb DDT 1B2 was very selective for *p,p′*-DDT with an IC_{50} of 4.2 ± 1.6 μg/L (in 40 mM PBS, *n* = 9). An optical immunosensor was optimized and applied for the analysis of DDT (or DDT equivalents). This immunosensor consists of a bench-top optical readout device and disposable sensor chips, which include the fluidic system. Evanescent field excitation and emission of the fluorophore Oyster®-645 was used. An IC_{50} for *p,p′*-DDT [in 5% (v/v) isopropanol in 40 mM PBS] of 4 μg/L was obtained using DDT 7C12-Oyster-645. ELISA and immunosensor were used for the analysis of *p,p′*-DDT in unspiked and spiked surface water samples. Within the working ranges of these immunotechniques, recoveries ranged from 80 to 120%.

DDT [1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane] is one of the most famous pesticides in the world since its discovery in 1939 by Paul H. Müller as the first synthetic multipurpose insecticide with low nontarget toxicity (1). It was extensively used during the Second World War among Allied troops and certain civilian populations to control malaria, typhus, and other insect-transmitted diseases. Since 1945, DDT has been extensively used as an agricultural insecticide (2). Since the early 1970s, DDT has been banned in several countries, for example, in the United States and in Germany (3, 4). Its use is restricted under the Stockholm Convention on Persistent Organic Pollutants (5) for disease vector control (malaria and yellow fever). Lately, debates about the use of DDT have come up again. Although it is highly persistent in the environment, it might save millions of lives for an affordable price (6–10). Technical grade DDT is a mixture of up to 14 chemical compounds, of which only about 65–80% is the active ingredient *p,p′*-DDT. The other compounds include *o,p′*-DDT (approximately 15–21%) and up to 4% *p,p′*-DDD (2, 11). DDT and its primary metabolites, DDE [1,1-dichloro-2,2-bis (chlorophenyl) ethylene] and DDD [1,1-dichloro-2,2-bis (4-chlorophenyl) ethane], are very persistent and strongly adsorbed to sediments and soils that can act both as sinks and long-term sources of exposure (12).

Different methods are applied for the analysis of DDT and its isomers and metabolites. The current conventional methods are based on GC/MS or GC with an electron capture detector, and many recent investigations show that DDT and its metabolites are still present in many environmental matrixes, including soil, sediment, Alpine glacier, urban air, biota, and water (13–18). Conventional methods are sufficiently sensitive and accurate, but they are complex, expensive, time-consuming, and use high amounts of organic solvents. Complementary or alternative analytical methods are based on immunochemical techniques such as immunoassays and immunosensors (19–25). ELISAs are known as fast screening methods and/or sensitive quantitative tools for pesticide analysis (26, 27), but they are susceptible to

Guest edited as a special report on "Pesticide Immunoassay" by Uzma Maqbool and Bertold Hock.

[1] Present address: Wehrwissenschaftliches Institut für Werk-, Explosiv- und Betriebsstoffe (WIWEB), Geschäftsfeld 520 Chemie, Analytik, Institutsweg 1, 85435 Erding, Germany.
Corresponding author's e-mail: kramer@helmholtz-muenchen.de

pipetting errors and are not suitable for on-site environmental analysis (28, 29). Therefore, another driving force in the development of immunochemical techniques is the need for rapid, simple, automated, or semiautomated setups that can be carried out on-site without extensive sample preparation and sample transfer to an analytical laboratory (30, 31). Antibody-based biosensors (immunosensors), in which the antibody or antigen is immobilized to the transducer, have been constructed in a variety of ways, but they generally fall into one of three basic configurations. These formats involve direct noncompetitive assays, competitive (direct or indirect) assays, or sandwich-type assay formats (32). Applying the International Union of Pure and Applied Chemistry definition, which was recommended for electrochemical biosensors (33), the optical immunosensor described herein is a single-use biosensor. This immunosensor consists of a base unit for automation and optical readout and disposable low-cost, single-use sensor chips, which include a fluidic system.

Several immunoassays and immunosensors have been already developed for DDT, which were either based on polyclonal or monoclonal antibodies (mAb; 34–43). In addition, several commercial ELISA kits from U.S. companies are available for the detection of DDT residues in environmental matrixes (water, soil, sediment, and fish plasma); for example, from Strategic Diagnostics (Newark, DE); EnviroLogix Inc. (Westbrook, MA); and Abraxis LLC (Warminster, PA). One test kit, namely EnviroGard™ DDT in Soil Test Kit, was already transferred to U.S. Environmental Protection Agency Method 4042 (44). All test kits use polyclonal antibodies.

In spite of all of these past developments, the newly developed immunochemicals for DDT offer unique features. For example, the very high selectivity of mAb DDT 1B2 for *p,p′*-DDT was not reported in former publications. In addition, the ELISA for DDT with glucose oxidase (GOD) in the enzyme-tracer was only developed by our group; the same is true for the mAbs labeled with the fluorophore Oyster®-645. The optimized optical immunosensor (45, 46) is described for the analysis of DDT in water samples from different sources. These efforts show various opportunities of immunochemical techniques for surveillance monitoring of DDT occurrences.

Experimental

Chemicals, Reagents, Antibody Development, and Immunochemical Analyses

(**a**) *Certified analytical standards of p,p′-DDT, o,p′-DDT, p,p′-DDE, o,p′-DDE, p,p′-DDD, and o,p′-DDD*.—Institute of Organic Industrial Chemistry (Warsaw, Poland).

(**b**) *4,4′-DDA (2,2-bis(4-chlorophenyl) acetic acid)* (Pestanal®).—Riedel-de Haën (Seelze, Germany).

(**c**) *Stock solutions of standards*.—Prepared in methanol with a concentration of 1 mg/mL and stored at 4°C.

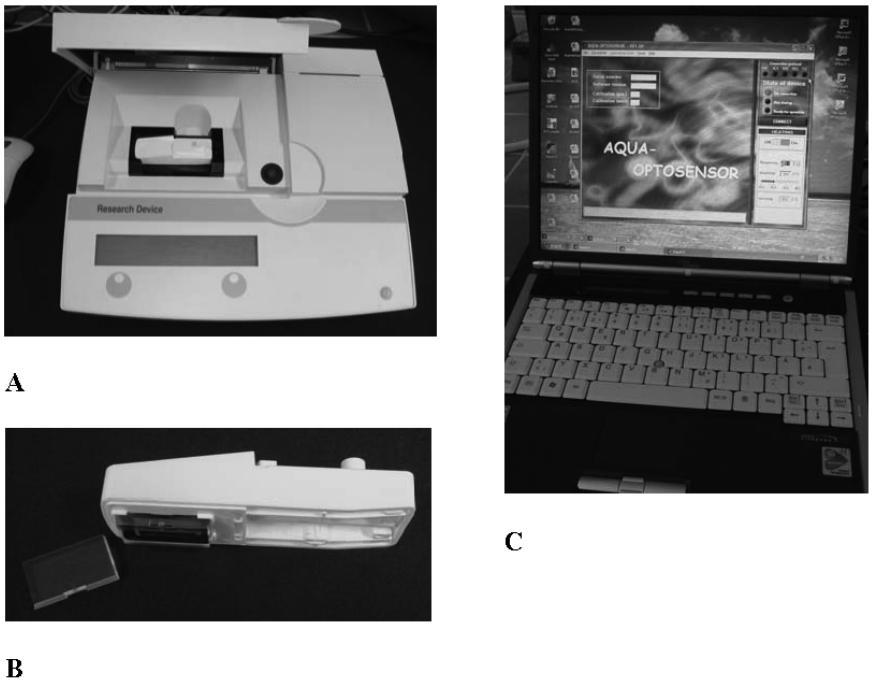

Figure 1. Optical immunosensor: (A) bench-top optical readout device; (B) PMMA prism (left side, before mounting to the sensor chip); optical sensor chip with mounted PMMA prism; (C) laptop with Aqua-Optosensor software for control of the bench-top device.

(**d**) *Working standard solutions (0.001–10 000 µg/L).*—Unless otherwise noted, prepared fresh daily in 40 mM phosphate buffered saline (PBS).

(**e**) *DDT-hapten (4-{4-[1-(4-chlorophenyl)- 2,2,2-trichloroethyl]phenyl}butanoic acid).*—Synthesized by Solvias (Basel, Switzerland).

(**f**) *Keyhole limped hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA), skim milk powder, hydrogen peroxide (H_2O_2; 30%), 3,3′,5,5′-tetramethylbenzidine (TMB), N,N′-dicyclorohexylcarbodiimide (DCC; 98%), and N-hydroxysuccinimide (NHS; 97%).*—Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany).

(**g**) *N,N-dimethylformamide (anhydrous DMF) and dimethylsulfoxide (DMSO).*—Fluka (part of Sigma-Aldrich Chemie).

(**h**) *Micro-O-protect.*—Roche Diagnostics (Mannheim, Germany).

(**i**) *Glycerol peroxidase conjugate stabilizer and Slide-A-Lyzer® dialysis cassettes, needles, and syringes.*—Pierce (Rockford, IL).

(**j**) *Goat anti-rat immunoglobulin G (IgG)+IgM mAb conjugated to horseradish peroxidase (goat anti-rat-HRP) and biotinylated rat anti-mouse mAbs.*—Dianova (Hamburg, Germany).

(**k**) *Peroxidase from horseradish [molecular weight (MW) about 40 kD, about 1000 U/mg protein].*—Serva Electrophoresis GmbH (Heidelberg, Germany).

(**l**) *GOD (Aspergillus niger, MW 160 kD, about 365 U/mg protein).*—Biozyme Laboratories Ltd. (Gwent, UK).

(**m**) *The mAb TIB172 (mouse anti-rat immunoglobulin kappa light chain) and biotinylated mAbs specific for rat IgM, IgG subclasses, and light chains (α-IgG1, α-IgG2a, α-IgG2b, and α-kappa).*—American Type Culture Collection (Manassas, VA).

(**n**) *α-IgM mAb.*—Alexis (Grünberg, Germany).

(**o**) *Anti-IgG2c.*—Ascenion (Munich, Germany).

(**p**) *CPG2006 and CPG1668.*—TIB MOLBIOL (Berlin, Germany).

(**q**) *Biotinylated, monoclonal, anti-mouse, subclass-specific mAbs.*—BD Pharmingen (Heidelberg, Germany).

(**r**) *Oyster-645 (free dye: λ_{ex} 645 nm, λ_{em} 666 nm; protein conjugate: λ_{ex} 650 nm, λ_{em} 669 nm).*—Denovo Biolabels GmbH (Münster, Germany).

(**s**) *Buffer salts (sodium hydrogen carbonate, sodium acetate, sodium phosphate), methanol p.a., isopropanol p.a., and D(+)glucose.*—Merck (Darmstadt, Germany).

(**t**) *Demineralized water.*—Prepared by a Milli-Q filtration system (Millipore, Eschborn, Germany) and used for the preparation of all buffers.

(**u**) *Microtiter plates (MaxiSorp™).*—Nunc (Wiesbaden, Germany).

(**v**) *Washing steps.*—Carried out either with the semiautomated Nunc Immunowash (Roskilde, Denmark) or with the automated microtiter plate washer ELX405R (Bio-Tek Instruments, Bad Friedrichshall, Germany).

(**w**) *Absorbance measurements.*—Made with a ThermoMax microtiter plate reader (Molecular Devices, Palo Alto, CA) at 450 nm (reference 650 nm).

(**x**) *Inhibition curves.*—Analyzed with the four-parameter logistic equation (Softmax Pro, Molecular Devices).

Preparation of Hapten-Protein Conjugates

The DDT-hapten was covalently attached to KLH, BSA, and OVA using the modified active ester method according to

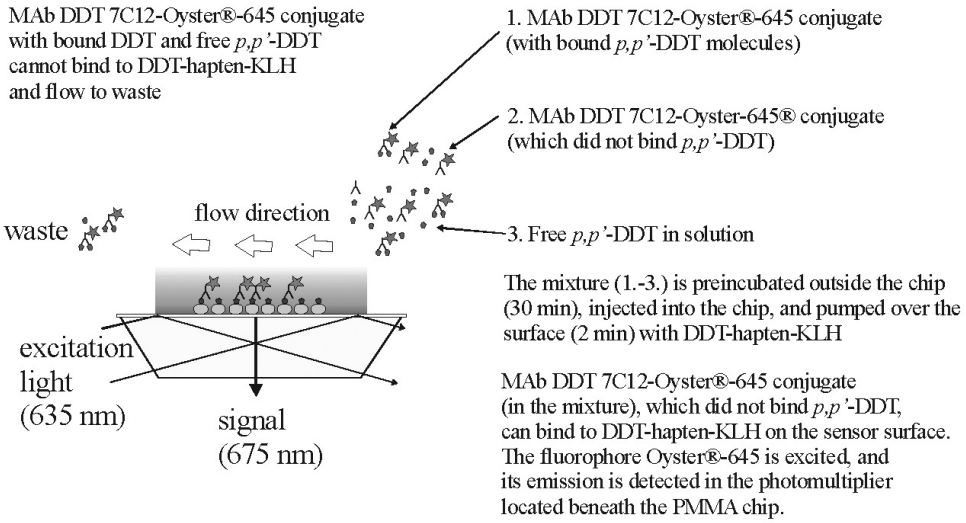

Figure 2. Measurement principle for the analysis of *p,p′*-DDT using mAb DDT 7C12-Oyster-645 conjugate and DDT hapten-KLH (surface of the PMMA prism).

Langone and Van Vunakis (47). The procedure was as follows: Approximately 0.200 mM DDT-hapten, 0.202 mM NHS, and 0.223 mM DCC in 1 mL DMF were incubated and stirred for 4 h at room temperature (RT). Then the mixture was transferred to a refrigerator and stirred for an additional 12 h at 4°C. The mixture was centrifuged (10 min/1400 rpm), and the clear supernatant was added slowly (20 μL/10 min) to KLH, BSA, or OVA solutions (all 10 mg/mL in 0.05 M borate buffer, pH 8). The mixture was stirred for an additional 16 h at 4°C. Then the conjugate solutions were centrifuged (10 min/1400 rpm), and the clear supernatants were transferred to Slide-A-Lyzer cassettes for dialysis against 0.2 M PBS, pH 7.6, for 3 days. Conjugation was confirmed by UV-Vis scans (240–450 nm; Ultraspec 3000, Amersham Pharmacia Biotech, Piscataway, NJ); the DDT-hapten-protein conjugates were measured in comparison to their corresponding parent compounds. DDT-hapten-KLH, DDT-hapten-OVA, and DDT-hapten-BSA were used as immunogens and/or as coating antigens.

In addition, DDT-hapten-OVA conjugates were prepared with reduced amounts of hapten/protein: instead of 0.200 mM of DDT-hapten, only 0.100 mM (named DDT-hapten-OVA½) and 0.050 mM (named DDT-hapten-OVA¼) were used. NHS and DCC were also reduced accordingly, and the protein concentration was kept the same (10 mg/mL). The conjugation was carried out as described above. This procedure of hapten reduction was meant to improve the sensitivity of the assays within the coating antigen format.

Preparation of Hapten-Enzyme Conjugates

DDT-hapten was conjugated to HRP and GOD according to Schneider et al. (48) using the NHS ester method via DCC. The procedure was as follows: Approximately 3 μM DDT-hapten, 15 μM NHS, and 30 μM DCC were dissolved in 130 μL anhydrous DMF. The obtained mixture was stirred for 12 h at 4°C (refrigerator). After centrifugation (10 min/1400 rpm), the clear supernatant was added slowly (10 μL/10 min) to the enzyme solution (2 mg HRP or 3 mg GOD in 3 mL 130 mM sodium hydrogen carbonate, pH 8.2). The conjugate solutions were stirred at 4°C overnight. The next day, the conjugates were centrifuged (10 min/1400 rpm), and the clear supernatants were transferred to Slide-A-Lyzer cassettes for dialysis against 130 mM sodium hydrogen carbonate, pH 8.2, for 3 days. The conjugates (DDT-hapten-HRP and DDT-hapten-GOD) were stored at –27°C (DDT-hapten-HRP in 50% glycerol solution) and used for the development of immunoassays in the enzyme-tracer format.

Production of mAbs to DDT

Approximately 50 μg DDT-hapten-KLH conjugate was injected intraperitoneally (i.p.) and subcutaneously (s.c.) into LOU/C rats using CPG2006 as adjuvant. After a 2 month interval, a boost was given i.p. and s.c. 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to standard procedure (49, 50). Hybridoma supernatants were tested in a solid-phase immunoassay using DDT-hapten-BSA conjugate adsorbed to polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound mAb were detected using peroxidase-labeled goat anti-rat IgG+IgM antibodies and *o*-phenylenediamine as chromogen in the peroxidase reaction. A hapten-BSA conjugate with a hapten that was not recognized was used as a negative control.

IgG type of the mAbs was determined using biotinylated mAbs specific for rat IgM, IgG subclasses and light chains (α-IgG1, α-IgG2a, α-IgG2b, α-IgG2c, α-IgM, α-κ, and anti-λ, the latter described in this paper). The mAbs DDT 7C12 (IgG2a,λ), DDT 1C1 (IgG2b,κ), and DDT 1B2 (IgG2a,κ) were selected for this study.

Purification of rat mAbs was performed via Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored in 1% (v/v) Micro-O-protect at 4°C.

Production of a Mouse mAb Against the Rat λ-Light Chain

Fifty micrograms of a Protein G purified rat mAb with a λ-light chain [Cyto 7H2, rat IgG1 (51)] was injected into C57BL/6 mice using CPG1668 as adjuvant. After a 2 month interval, a boost was given i.p. and s.c. 3 days before fusion. Fusion was done as described above. Screening was performed on the antibody (Cyto 7H2) adsorbed to polystyrene microtiter plates. Several rat mAbs (IgG1) with a kappa light chain were used as negative controls. Bound mouse mAbs were detected using biotinylated rat anti-mouse antibodies. The isotype of the mAb was determined using biotinylated, monoclonal, anti-mouse, subclass-specific mAbs. The established mouse mAb LA1B12 recognized the λ-light chain of the rat mAb DDT 7C12 and was used as the capture antibody.

ELISAs in Coating Antigen Format

The hapten-protein conjugates (DDT-hapten-KLH, -BSA, and -OVA) were used as coating antigens for assay optimizations with the different mAbs. The coating antigens were used with a final concentration of 2 μg/mL. During this procedure, culture supernatants were used undiluted and in serial dilutions from 1:10 to 1:1280. In addition, both *p,p′*- and *o,p′*-DDT were added (20 μg/L) as inhibitors. After all coating antigens were checked, and the final concentrations and dilutions were obtained, the procedure of the optimized coating antigen ELISA was as follows. Polystyrene ELISA plates were coated overnight at 4°C at final concentrations of DDT-hapten-OVA¼ of 0.5 μg/mL for mAb DDT 1C1, and 1 μg/mL for mAb DDT 7C12 and mAb DDT 1B2, in 0.05 M sodium carbonate buffer, pH 9.6. The next day, plates were washed three times with 4 mM PBST [PBS, pH 7.6, with 0.05% (v/v) Tween 20]. For mAbs DDT 7C12 and DDT 1B2, optimized assays needed a blocking step with milk powder [1% (w/v) in 40 mM PBS pH, 7.6, 250 μL/well] with incubation for 1 h at RT. For the optimized ELISA with mAb DDT 1C1, a blocking step was not necessary, but 0.1% (w/v) BSA was added instead in the PBS buffer of the antibody

solution. After a washing step with 4 mM PBST, 100 μL/well of DDT or DDT metabolites in 40 mM PBS, pH 7.6, and 100 μL/well of mAbs (250 ng/mL for mAb DDT 1C1, 125 ng/mL for mAb DDT 7C12, and 500 ng/mL for mAb DDT 1B2) were added successively and incubated for 2 h at RT. After another washing step with 4 mM PBST, 200 μL/well of goat anti-rat-HRP (1:20 000 in 40 mM PBST, pH 7.6) was added and incubated for 1 h at RT. After a final washing step, 200 μL/well of substrate solution (0.4 mM TMB, 1.3 mM H_2O_2 in 100 mM sodium acetate, pH 5.5) was added. Product development (color) of the enzymatic reaction was stopped after 10–15 min with 50 μL/well of 2 M H_2SO_4. The absorbance was read at 450 nm (reference 650 nm), and it was inversely proportional to the concentration of the analyte. Standard curves were set up using the four-parameter equation (*see* below). For better comparison of standard curves, they were normalized to % control according to the equation:

$$\%Control = \frac{A - A_{background}}{A_0 - A_{background}} \times 100$$

with A being the value of absorbance for each standard or sample and A_0 the value of absorbance for the zero standard (e.g., 40 mM PBS).

Curve fitting of the normalized standard curves was also carried out with the four-parameter equation (SigmaPlot):

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D$$

where A is the y-value corresponding to the asymptote at low values of the x-axis and D is the y-value corresponding to the asymptote at high values of the x-axis. The coefficient C is the x-value corresponding to the midpoint between A and D (IC_{50}, μg/L). The coefficient B describes how rapidly the curve makes its transition from the asymptotes in the center of the curve. Unless otherwise noted, the working range of the standard curves was defined from 90 to 10% control.

ELISAs in Enzyme-Tracer Format

Several dilutions of the DDT-hapten conjugated to enzymes (enzyme-tracer) and newly developed mAbs were checked during the optimizations of the assays. Two different enzymes were used in the enzyme-tracers of this format, HRP and GOD. The assay protocols of the optimized competitive ELISAs were as follows. ELISA plates were coated overnight at 4°C with 2 μg/mL mAb TIB172, 200 μL/well (for mAb DDT 1C1 and mAb DDT 1B2), or with 2 μg/mL mAb LA1B12 (for mAb DDT 7C12) in 0.05 M sodium carbonate buffer, pH 9.6. The next day, plates were washed three times with 4 mM PBST. One hundred and fifty microliters/well anti-DDT mAbs in 40 mM PBS, pH 7.6, were added, containing 1% (w/v) milk powder. The optimized concentrations used for ELISAs were 300 ng/mL and 600 ng/mL mAb DDT 1C1 with DDT-hapten-HRP and DDT-hapten-GOD, respectively, 125 ng/mL and 300 ng/mL mAb DDT 7C12 with DDT-hapten-HRP and DDT-hapten-GOD, respectively, and 250 ng/mL and 1000 ng/mL mAb DDT 1B2 with DDT-hapten-HRP and DDT-hapten-GOD, respectively. After 2 h incubation at RT, plates were washed again (4 mM PBST), followed by the addition of 100 μL/well of DDT isomer standards or metabolites in 40 mM PBS, pH 7.6, and incubation for 1 h at RT. Then, without a washing step, 50 μL/well of DDT-hapten-HRP (1:8000 for mAbs DDT 1C1 and DDT 7C12, and 1:1000 for mAb DDT 1B2) or DDT-hapten-GOD (1:1000 for mAbs DDT 1C1, DDT 7C12, and DDT 1B2) were added and incubated with shaking for another 30 min at RT. After a final washing step, 150 μL/well of substrate solution for the HRP reaction (0.4 mM TMB, 1.3 mM H_2O_2 in 100 mM sodium acetate, pH 5.5) and for the GOD reaction [0.25 M D(+)Glucose, 0.4 mM TMB, 250 nM HRP in 100 mM sodium acetate, pH 5.5] were added. The reaction was stopped after 20 (HRP) or 30 min (GOD) with 50 μL 2 M H_2SO_4/well, and the absorbance was read at 450 nm (reference 650 nm). Standard curves were processed using the 4-parameter equation, as reported earlier. The working range of the standard curves was defined from 90 to 10% control, unless otherwise noted.

Effect on the Test Midpoint of the Standard Curve

Using the enzyme-tracer format with DDT hapten-HRP (1:4000) and mAb DDT 1C1 (500 ng/mL), the effect of the time between the setup of the *o,p′*-DDT standards and the addition of the standards to the microtiter plate was investigated. For these experiments, *o,p′*-DDT standards were set up either in 10% (v/v) isopropanol in 40 mM PBS, pH 7.6; in 10% isopropanol in 40 mM PBS with 0.01% (v/v) Tween 20; or in 10% (v/v) isopropanol–DMSO (4 + 1) in 40 mM PBS. These setups of standards in different buffer-solvent-surfactant matrixes were added either directly to the microtiter plate or after 2 h.

Determination of Cross-Reactivities

To determine the selectivity of the anti-DDT mAbs for DDT isomers and metabolites, all three mAbs were tested for cross-reactivities using the optimized ELISA procedures, both in the coating antigen and in enzyme-tracer formats. Inhibition curves with cross-reactants were executed in ELISAs, and their test midpoints (IC_{50}) were compared to those from a standard curve for *p,p′*-DDT with mAb DDT 7C12 and mAb DDT 1B2, respectively, and for *o,p′*-DDT with mAb DDT 1C1 on the same microtiter plate. Percent cross-reactivity (%CR) was calculated according to the following equation:

$$\%CR = IC_{50} \text{ main analyte } (p,p'\text{-DDT or } o,p'\text{-DDT}) / IC_{50} \text{ cross-reactant} \times 100$$

Preparation of mAb-Oyster-645 Conjugates

Oyster dyes are novel members of the cyanine family of fluorophores that are chemically modified to reduce their tendency to aggregate. As a result, these dyes are less "sticky"

Table 1. Test midpoints (IC_{50}) and cross-reactivities (%CR) of DDT isomers and metabolites in coating antigen format[a]

mAb	mAb DDT 1C1 (Rat IgG2b,κ), 500 ng/mL		mAb DDT 7C12 (Rat IgG2a,λ), 125 ng/mL		mAb DDT 1B2 (Rat IgG2a,κ), 500 ng/mL	
Coating antigen	DDT-hapten-OVA conjugate,[b] 0.5 μg/mL		DDT-hapten-OVA conjugate,[b] 1 μg/mL		DDT-hapten-OVA conjugate,[b] 1 μg/mL	
Analyte	IC_{50}, μg/L	%CR[c]	IC_{50}, μg/L	%CR	IC_{50}, μg/L	%CR
p,p′-DDT	6.1 ± 0.9 (*n* = 3)	75 ± 3.5	1.8 ± 0.7 (*n* = 15)	100	7.5 ± 2.2 (*n* = 12)	100
o,p′-DDT	4.2 ± 0.5 (*n* = 13)	100	2.7 ± 0.9 (*n* = 4)	56 ± 11.3	≥1000	≤0.1
p,p′-DDD	30.2 ± 4.5 (*n* = 3)	12 ± 2.5	1.5 ± 0.4 (*n* = 3)	98.3 ± 2.9	13.0 ± 1.8 (*n* = 3)	65.0 ± 3.5
o,p′-DDD	3.9 ± 0.8 (*n* = 3)	91 ± 10.1	6.4	15	223	4
p,p′-DDE	35.1 ± 4.1 (*n* = 3)	13 ± 1.2	3.4	51	106.4 ± 6.3 (*n* = 3)	8.3 ± 1.2
o,p′-DDE	3.2 ± 0.3 (*n* = 3)	141 ± 16.7	31	6	≥10000	≤0.01
p,p′-DDA	≥10000 (*n* = 3)	≤0.01	≥10000	≤0.01	≥10000	≤0.01
DDT-hapten	1.0 ± 0.1 (*n* = 6)	421 ± 33	0.7 ± 0.1 (*n* = 3)	255 ± 54	2.4 ± 0.4 (*n* = 3)	282 ± 32

[a] Parameters of the assays were obtained from the four-parameter curve fitting. The DDT compounds were set up in 40 mM PBS.

[b] Conjugate was DDT-hapten-OVA1/4, which means that only one-quarter of the usual hapten amount was used for conjugation to OVA; *see* text for details.

[c] %CR refers to the results obtained on the same microtiter plate in comparison to the main analyte; *n* is the same as given for IC_{50} values.

and easier to handle; this also leads to reduced auto quenching. Oyster dyes are available in NHS-ester form and, as such, have a MW of about 1000. Oyster-645 is similar to Cy5™, but is 2–3 times brighter than Cy5 (Denovo Biolabels GmbH, product information). Prior to conjugation, anti-DDT mAbs were dialyzed against 50 mM sodium borate buffer, pH 7.8. The mAb concentrations were 1.5 mg/mL for DDT 7C12, 1.3 mg/mL for DDT 1B2, and 0.6 mg/mL for DDT 1C1. Twenty microliters of DMF were added to the fluorophore (one vial of activated dye contains an amount to label 1.5 mg protein). One aliquot (1/4) of the dissolved dye was added directly to the mAb solutions. The vials were shaken continuously at ambient temperature. After 10 min of incubation, active NHS esters were blocked by addition of 2 μL 10% (w/v) glycine (aminoacetic acid)/100 μL antibody solution. For the purification of the mAb-Oyster-645 conjugates, Slide-A-Lyzer dialysis cassettes were used, and conjugates were dialyzed against 10 mM phosphate buffer, pH 7.6. The final concentrations of the mAb-Oyster-conjugates were for DDT 7C12 Oyster-645 1.5 mg/mL (or 0.7 mg/mL), for DDT 1B2-Oyster-645 1.3 mg/mL, and for DDT 1C1-Oyster-645 0.6 mg/mL.

Optical Immunosensor Device

The optical immunosensor enables measurements of any parameters carried out by means of immunoassay technology. It consists of a bench-top optical readout device (Figure 1A, with open cover; length × width × height: 320 × 260 × 135 mm; weight: 3.3 kg; Siemens AG, Munich, Germany), which holds the sensor chip (here inserted), and which is controlled by a laptop computer. The disposable single-use, low-cost sensor chips have an optical prism [Figure 1B, left side, unmounted; polymethyl methacrylate (PMMA), 11 × 23 mm] and include a fluidic system (Figure 1B; pes diagnose systeme GmbH, Leipzig, Germany). The surface of the prism served as the solid phase for the immobilization of the DDT-hapten-KLH conjugate (coating antigen). The optical prism was connected to the carrier with an adhesive film that had a capillary aperture of 50 μm, which defined the flow channel for the fluidics. The flow channel had an inlet and outlet for the fluid. The mixture of the mAb 7C12-Oyster-645 conjugate and the standard or water sample was pipetted to the charging hole of the sample container. The sensor chip had an integrated piston pump, which controlled the transport of the fluid. The valve and the pump were interfaced with a cock drive and a plunger, the latter being a part of the bench-top device. The technology is based on evanescent field excitation of fluorescent markers and was described elsewhere (46). The combination of both fluorescence measurements and evanescent wave excitation provided extremely sensitive detection and avoided washing or separation steps.

Measurements were commenced in accordance with the software settings made previously on the computer (Figure 1C). All steps were controlled by the settings about defined movements of the cock drive and the plunger. The sample was pumped through the capillary aperture over the surface of the prism that carried the immobilized coating antigen. The small capillary aperture ensured that the binding

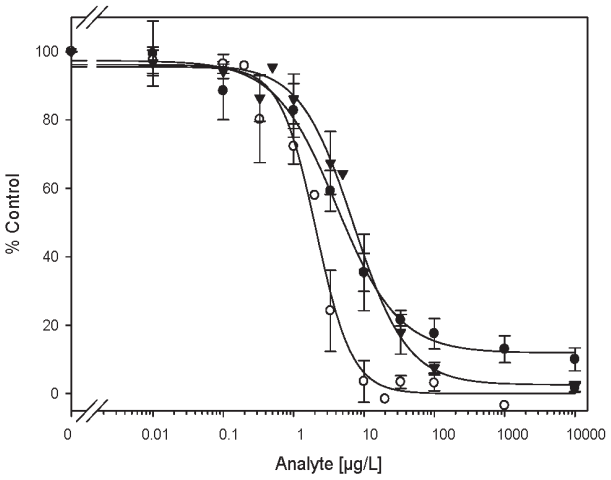

Figure 3. Mean of five optimized standard curves for *p,p′*- or *o,p′*-DDT in ELISA (coating antigen format) expressed as % control. The standard curves were fitted (SigmaPlot software) according to the four-parameter equation with the following coefficients: (○) *p,p′*-DDT with DDT 7C12: A = 95.5, B = 1.7, C (IC_{50}) = 2.1 μg/L, D = 0.0, R^2 = 0.988 (*n* = 5); (▼) *p,p′*-DDT with mAb DDT 1B2: A = 96.1, B = 1.1, C (IC_{50}) = 7.1 μg/L, D = 2.6, R^2 = 0.991 (*n* = 5); and (●) *o,p′*-DDT with mAb DDT 1C1: A = 97.3, B = 1.0, C (IC_{50}) = 4.1 μg/L, D = 11.9, R^2 = 0.994 (*n* = 5). The zero standard is 0 μg/L (40 mM PBS). Absorbance values (OD) for zero concentration ranged from 0.350 to 1.800 and for maximum concentrations from 0.0009–0.076.

of the fluorophore-labeled mAb to the immobilized coating antigen was only insignificantly limited by diffusion processes. The light of a laser beam (λ_{ex} 635 nm) was used for the excitation of the fluorophore. An evanescent field was hereby generated by total internal reflection of the beam at the interface between the high-refractive PMMA prism and the low-refractive liquid sample inside the flow channel. Only the fluorescence dye of the bound antibody to the coating antigen was excited to fluoresce, due to the low penetration depth of the evanescent field of approximately 150 nm. A photomultiplier tube was used for detection (λ_{em} 675 nm). The whole system was temperature-controlled with an operating temperature at 35.5°C. After about 2 min, the measurement was complete, and the instrument indicated the end. Data were then transmitted from the bench-top device to the computer via the serial interface RS 232 for further evaluation. The results were recorded via Aqua-Optosensor software (Figure 1C), Version 1.00 (Siemens AG). For evaluation of the results, the maximum range of a linear slope in mV/s of the peak must be determined. Therefore, the position of the peak must be localized by setting cursor position A and B to the beginning and the end of the peak, respectively. Cursor position (seconds) A and B were set to maximize the period of time in which the slope was linear. The values were displayed in mV/s. The change in time of the fluorescence drawn from the evaluation is directly proportional to the binding concentration of the fluorophore-labeled mAb to the immobilized coating antigen and, therefore, indirectly proportional to the concentration of analyte in the standard or sample.

Analysis of DDT with the Optical Immunosensor

The DDT-hapten-KLH conjugate (3.1 mg/mL, 0.1 μL) was pipetted onto the PMMA prism and was immobilized via physical adsorption to the plastic surface (Figure 2). The optimized concentrations of the anti-DDT mAb-Oyster-645 conjugates were for DDT 7C12-Oyster-645 2.5 μg/mL, for DDT 1B2-Oyster-645 4.3 μg/mL, and for DDT 1C1-Oyster-645 1.2 μg/mL in 40 mM PBS with 2% (w/v) BSA. The mAb-Oyster conjugates were preincubated for 30 min with the analyte [either *p,p′*-DDT or *o,p′*-DDT in 5% (v/v) isopropanol in 40 mM PBS] or with the water sample, into which 5% (v/v) isopropanol also was added (150 μL mAb with 150 μL standard or sample). The mixture was then pipetted into the chip and pumped automatically over its surface (2 min). The fluorescence emission of the fluorophore within the labeled anti-DDT antibody is measured, and the signal is inversely proportional to the DDT concentration. Figure 2 shows as an example mAb DDT 7C12-Oyster conjugate for the detection of *p,p′*-DDT. The slope obtained by the photomultiplier (mV/s) was stored via the software, and the peaks were evaluated.

Water Samples

Three different surface water samples were collected in glass bottles (1 L of each) from different sources: sample 1 (Lake Riem, Munich, Germany), sample 2 (River Isar, Munich, Germany), and sample 3 (creek water, English Garden, Munich, Germany), in August 2005. After collection, they were stored refrigerated (4°C) until use. These samples were spiked with different concentrations ranging from 0.1 to 33.33 μg/L *p,p′*-DDT and analyzed with the optical immunosensor or by ELISA in the coating antigen format, using mAb DDT 7C12-Oyster-645 conjugate or mAb DDT 7C12, respectively. Standard curves for *p,p′*-DDT were set up in 5% (v/v) isopropanol, in 40 mM PBS (sensor), or in 40 mM PBS (ELISA).

Results and Discussion

Conjugation of the DDT-Hapten to Different Proteins

The conjugation reaction between the DDT-hapten and the proteins was confirmed spectrophotometrically by comparing the DDT-hapten-protein conjugates with their corresponding precursors. The DDT-hapten was measured at a concentration of 20 or 50 μg/mL in methanol; the protein concentrations were 1 mg/mL in 20 mM PBS, pH 7.6; and DDT-hapten-protein conjugates were diluted 1:20 or 1:50 (final concentrations between 60 and 370 μg/mL) in 20 mM PBS, pH 7.6.

The DDT-hapten alone showed a main peak at about 260 nm. The main peaks of the proteins alone were at 277.5 nm for KLH, 278.5 nm for OVA, and 277.2 nm for

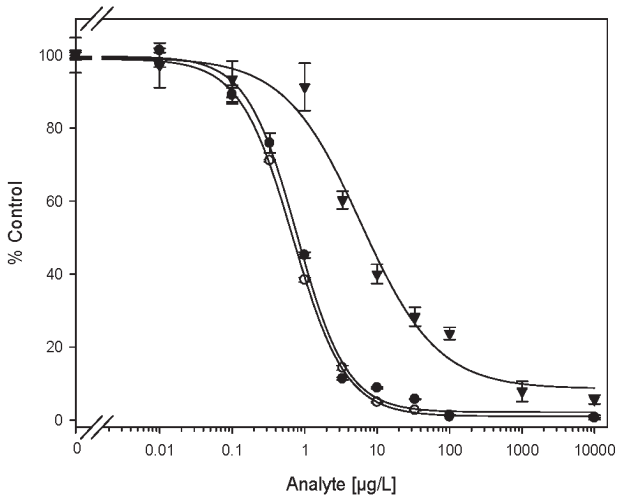

Figure 4. Optimized standard curves as % control in enzyme-tracer format with DDT-hapten-HRP. The standard curves were fitted (SigmaPlot software) with the four-parameter equation with the following coefficients: (○) *p,p′*-DDT with mAb DDT 7C12: A = 99.0, B = 1.2, C (IC_{50}) = 0.7 µg/L, D = 1.0, R^2 = 0.999 (*n* = 2); (▼) *p,p′*-DDT with mAb DDT 1B2: A = 99.4, B = 0.8, C (IC_{50}) = 6.0 µg/L, D = 8.6, R^2 = 0.985 (*n* = 4); and (●) *o,p′*-DDT with mAb DDT 1C1: A = 99.7, B = 1.3, C (IC_{50}) = 0.8 µg/L; D = 2.2, R^2 = 0.996 (*n* = 2). For details, *see* text and Table 2. The first concentration, which is presented in the graph as 0 µg/L, refers to 40 mM PBS. Absorbance values (OD) for zero concentration ranged from 0.460 to 0.760 and for maximum concentrations from 0.003–0.043.

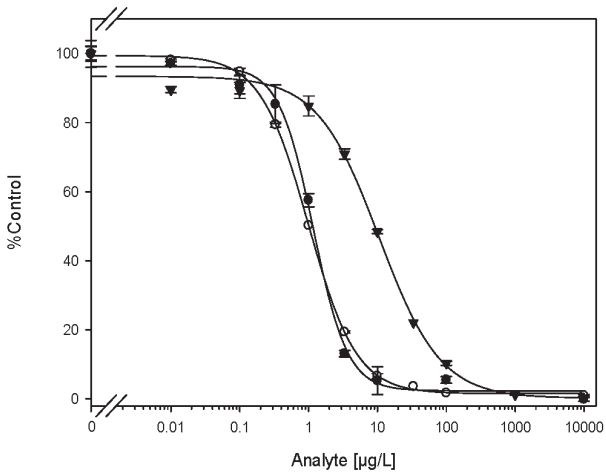

Figure 5. Optimized standard curves as % control in enzyme-tracer format with DDT-hapten-GOD. The standard curves were fitted (SigmaPlot software) according to the four-parameter equation with the following coefficients: (○) *p,p′*-DDT with mAb DDT 7C12: A = 99.4, B = 1.2, C (IC_{50}) = 1.0 µg/L, D = 1.5, R^2 = 0.999 (*n* = 2); (▼) *p,p′*-DDT with mAb DDT 1B2: A = 93.4, B = 1.0, C (IC_{50}) = 10.5 µg/L, D = 0.2, R^2 = 0.995 (*n* = 2); and (●) *o,p′*-DDT with mAb DDT 1C1: A = 96.2, B = 1.7, C (IC_{50}) = 1.2 µg/L; D = 2.4, R^2 = 0.996 (*n* = 3). For details, *see* text and Table 3. The first concentration, which is presented in the graph as 0 µg/L, refers to 40 mM PBS. Absorbance values (OD) for zero concentration ranged from 0.200 to 0.480 and for maximum concentrations from –0.001–0.002.

BSA. This meant that the peaks of the proteins overlapped the peak of the DDT-hapten. Therefore, a calculation of the haptens/protein was not performed. Nevertheless, it was clearly visible that the spectra of the DDT-hapten-protein conjugates were different from those of the proteins alone.

These DDT-hapten-protein conjugates were used for immunization of rats and/or in the coating antigen format during the assay optimization. After first screenings and characterizations of the mAbs, DDT-hapten-OVA1/4 turned out to be the best coating antigen. During the preparation of DDT-hapten-OVA1/4, only one-fourth of the DDT-hapten concentration was used (0.050 mM instead of 0.200 mM), which resulted in a reduction of hapten density. With this reduced hapten density in the coating antigen, the analyte could obtain a better competition at lower concentrations, thus resulting in lower detection limits for the target analytes (*o,p′*-DDT or *p,p′*-DDT).

Characterization of Selected Clones

During the first screening procedure, 44 clones showed binding to the DDT-hapten-protein conjugates (with KLH or BSA), but not to a hapten-protein conjugate with an unrelated hapten. These culture supernatants were then tested again in the coating antigen format using, in addition, DDT (*o,p′*- and *p,p′*-DDT, 20 µg/L) for inhibition. Only 21 of these clones showed inhibition with one or both of the DDT isomers. No inhibition was found for the rest of the clones. Out of these 21 clones, six clones showed inhibition >50% with either *o,p′*- or *p,p′*-DDT at a concentration of 10 µg/L. Two of the latter clones showed an inhibition >50% with 10 µg/L *o,p′*-DDT, three with 10 µg/L *p,p′*-DDT, and one clone equal inhibition with *o,p′*-DDT and *p,p′*-DDT at 10 µg/L. From these six selected clones, only three were stable during further subcloning. They displayed high inhibition and were, therefore, characterized finally for sensitivity and selectivity to DDT isomers and metabolites in two different ELISA formats. These mAbs were DDT 1C1 (IgG2b,κ), DDT 7C12 (IgG2a,λ), and DDT 1B2 (IgG2a,κ).

Characterization of the mAbs in Different Assay Formats

Immunoassay performance, especially the sensitivity, can be affected by the formats used. Usually, the first format developed in immunoassays is the coating antigen format, because it is also needed in the clone screening and selection prior to fusion to hybridoma cells. The next step of

Table 2. Test midpoints (IC_{50}) and cross-reactivities (%CR) of DDT isomers and metabolites in enzyme-tracer format, using DDT-hapten-HRP as enzyme-tracer[a]

	DDT-hapten-HRP					
mAb	mAb DDT 1C1 (Rat IgG2b,κ), 300 ng/mL		mAb DDT 7C12 (Rat IgG2a,λ), 125 ng/mL		mAb DDT 1B2 (Rat IgG2a,κ), 250 ng/mL	
Enzyme-tracer	DDT-hapten-HRP, 1:8000		DDT-hapten-HRP, 1:8000		DDT-hapten-HRP, 1:1000	
Analyte	IC_{50}, μg/L	%CR[b]	IC_{50}, μg/L	%CR	IC_{50}, μg/L	%CR
p,p′-DDT	4.6 ± 0.8 (*n* = 5)	23	0.5 ± 0.2 (*n* = 10)	100	4.2 ± 1.6 (*n* = 9)	100
o,p′-DDT	1.0 ± 0.3 (*n* =12)	100	2.4 ± 1.7 (*n* = 3)	71	≥10000	≤0.01
p,p′-DDD	54.7	2	0.8	90	32.3	19
o,p′-DDD	3.6	23	6	11	415.2	1
p,p′-DDE	26.2	6	3.5	37	186.5	4
o,p′-DDE	3	32	28.9	5	≥10000	≤0.01
p,p′-DDA	≥10000	≤0.01	≥10000	≤0.01	≥10000	≤0.01
DDT-hapten	0.5	102	0.3	88	0.9	395

[a] Parameters of the assays were obtained from the four-parameter curve fitting. The DDT compounds and enzyme-tracers were set up in 40 mM PBS.

[b] %CR values were always calculated with the test midpoint of the main analyte and the cross-reactant on the same microtiter plate.

development is the establishment of the enzyme-tracer format, because this format has one step less than the coating antigen format and is, therefore, faster. Both formats have advantages and disadvantages. The coating antigen format is usually less sensitive and takes longer, but the environmental sample has no influence on the enzyme. The enzyme-tracer format, on the other hand, is typically more sensitive and faster, but an influence of the environmental matrix on the enzyme cannot be excluded. In order to obtain the most suitable ELISAs for the developed antibodies, both formats were investigated. Furthermore, this was significant for potential future applications of these antibodies within different immunosensors. For both formats, optimal immunoreagent dilutions were chosen to obtain high absorbance, high sensitivity, and low background.

Coating Antigen Format and Cross-Reactivities

A study of seven compounds (DDT isomers and metabolites, and the DDT-hapten) as cross-reactants in this ELISA format with all three developed mAbs concluded that these mAbs were suitable for the sensitive and selective detection of DDT isomers and metabolites. The IC_{50} values and cross-reactivities with these ELISAs are summarized in Table 1. For reasons mentioned earlier, these assays were set up with DDT-hapten-OVA¼ as coating antigen. The mAb DDT 1C1 showed preferential recognition for all tested *o,p′*-isomers. The best recognized compound was *o,p′*-DDT with an IC_{50} of 4.2 ± 0.5 μg/L (*n* = 13) and a working range (90–20% control) of 0.4–40 μg/L. This mAb showed in addition 75%CR to *p,p′*-DDT, which will be relevant when both isomers are present in the sample.

The mAb DDT 7C12 was complementary to mAb DDT 1C1 because it revealed the highest CR for *p,p′*-isomers, with *p,p′*-DDT being the major analyte with an IC_{50} of 1.8 ± 0.7 μg/L (*n* = 15) and a working range of 0.4–8 μg/L. With this mAb, one could not distinguish between *p,p′*-DDT and *p,p′*-DDD.

The least sensitive mAb, but the most selective of the three rat mAbs, was DDT 1B2, which recognized *p,p′*-DDT as the main analyte with an IC_{50} value of 7.5 ± 2.2 μg/L (*n* = 12) and a working range of 0.7–60 μg/L. This mAb did not recognize *o,p′*-DDT, and the only metabolite with relevant CR was *p,p′*-DDD (65%). The latter dropped substantially in the enzyme-tracer format (19% with HRP and 33% with GOD as enzyme). Thus, to the best of our knowledge, this is the most selective mAb for DDT published to date.

The coating antigen format was also used to check the recognition of the immunizing DDT-hapten. As shown in Table 1, the DDT-hapten itself was recognized with very high CRs by all mAbs. The highest recognition was observed with mAb DDT 1C1 (about 420%CR), followed by mAb DDT 1B2 (about 280%CR) and mAb DDT 7C12 (about 260%CR). The competition between the DDT-isomers and the DDT-hapten was in favor of the DDT-hapten, which was the same one used in the immunogen, the coating antigen, and the enzyme-tracers. Changing to a structurally different hapten in assay development can lead to higher sensitivity for the target analyte (26). This strategy was not applied here though.

Figure 3 shows representative standard curves for *o,p′*- and *p,p′*-DDT, with the corresponding most suitable mAb.

Table 3. Test midpoints (IC_{50}) and cross-reactivities (%CR) of DDT isomers and metabolites in enzyme-tracer format, using DDT-hapten-GOD as enzyme-tracer[a]

	DDT-hapten-GOD					
mAb	mAb DDT 1C1 (Rat IgG2b,κ), 600 ng/mL		mAb DDT 7C12 (Rat IgG2a,λ), 300 ng/mL		mAb DDT 1B2 (Rat IgG2a,κ), 1000 ng/mL	
Enzyme-tracer	DDT-hapten-GOD, 1:1000		DDT-hapten-GOD, 1:1000		DDT-hapten-GOD, 1:1000	
Analyte	IC_{50}, μg/L	%CR[b]	IC_{50}, μg/L	%CR[b]	IC_{50}, μg/L	%CR[b]
p,p′-DDT	7.8 ± 2.7 (*n*=4)	27	1.4 ± 0.7 (*n* = 4)	100	8.6 ± 2.6 (*n* = 7)	100
o,p′-DDT	1.6 ± 0.4 (*n*=8)	100	3.0 ± 1.9 (*n* = 3)	71	≥10000	≤0.01
p,p′-DDD	144	1	1.1	90	32.5	33
o,p′-DDD	5.6	26	6.2	16	556.7	2
p,p′-DDE	45.7	5	4.2	47	218.9	5
o,p′-DDE	4.7	34	38.4	5	≥10000	≤0.01
p,p′-DDA	≥10000	≤0.01	≥10000	≤0.01	≥10000	≤0.01
DDT-hapten	1.0	115	0.7	86	2.3	238

[a] Parameters of the assays were obtained from the four-parameter curve fitting. The DDT compounds and enzyme-tracers were set up in 40 mM PBS.

[b] %CR values were always calculated with the test midpoint of the main analyte and the cross-reactant on the same microtiter plate.

Enzyme-Tracer Format and Cross-Reactivities

The same mAbs were applied in ELISAs in the enzyme-tracer format, using either HRP or GOD as enzymes. GOD is an enzyme that is widely used in electrochemical transducers of immunosensors (e.g., 52), and, therefore, this enzyme-tracer was interesting for potential applications within these biosensors. The mAbs were tested in competitive ELISAs with both enzyme-tracers. Using DDT-hapten-HRP, representative standard curves for the main compounds (*o,p′*-DDT with mAb DDT 1C1, and *p,p′*-DDT with mAbs DDT 7C12 and DDT 1B2) are shown in Figure 4; DDT-hapten-GOD standard curves are shown in Figure 5.

In comparison to GOD, all optimized assays showed lower IC_{50} values when HRP was used in the enzyme-tracer. The reason for this was most likely the different number of hapten molecules that could be conjugated to these enzymes via the NH_2 group of lysine residues. HRP has six lysines (53), of which only 1–3 can be used for coupling (e.g., 54). GOD, on the other hand, has 30 lysine residues, 15 per subunit (55). In addition, HRP has about 1 to 3 times higher catalytic activity than GOD, which allowed higher dilutions of DDT-hapten-HRP (1:8000 with DDT 1C1 and DDT 7C12); higher dilutions are more favorable for sensitivity and avoidance of unspecific binding. Furthermore, with GOD in the enzyme-tracer, HRP was also used in a coupled enzymatic reaction (H_2O_2 from GOD reaction was used as substrate for HRP). Nevertheless, GOD in the enzyme-tracer revealed very good and useful standard curves for DDT isomers and metabolites with all mAbs and could be used in electrochemical immunosensors.

The mAb DDT 1C1 showed with DDT-hapten-HRP (Figure 4, Table 2) an IC_{50} value for *o,p′*-DDT of 1.0 ± 0.3 μg/L (*n* = 12), with a working range of 0.2 to 6 μg/L. With DDT-hapten-GOD (Figure 5, Table 3), the IC_{50} value for *o,p′*-DDT was 1.6 ± 0.4 μg/L (*n* = 8), with a working range of 0.3 to 5 μg/L. The pattern of cross-reactivities was independent of the enzyme in the enzyme-tracer (Tables 2 and 3). As described for the coating antigen format, mAb DDT 1C1 generally revealed higher CR for *o,p′*-isomers.

With mAb DDT 7C12, the selectivity was again best for *p,p′*-isomers (Tables 2 and 3). The best test midpoint was obtained for *p,p′*-DDT using DDT-hapten-HRP (0.5 ± 0.2 μg/L, *n* = 10). The working range for this assay was 0.1 to 5 μg/L (Figure 4). Using GOD in the enzyme-tracer, the IC_{50} value for *p,p′*-DDT was 1.4 ± 0.7 μg/L (*n* = 4), and the working range was 0.2 to 7 μg/L (Figure 5). Additional relevant CRs were with both enzymes *p,p′*-DDD (90%), *o,p′*-DDT (71%), *p,p′*-DDE (37 and 47%), and *o,p′*-DDD (11 and 16%; Tables 2 and 3). The most selective assay, as already shown with the coating antigen format, was again with mAb DDT 1B2, independent of the enzyme used. The optimized assay with DDT-hapten-HRP showed an IC_{50} value of 4.2 ± 1.6 μg/L (*n* = 9) for *p,p′*-DDT, and a working range (90–20% control) of 0.4 to 60 μg/L (Figure 4). With GOD in the enzyme-tracer, the assay had an IC_{50} value for *p,p′*-DDT of 8.6 ± 2.6 μg/L (*n* = 7), and a working range of 0.4 to 100 μg/L.

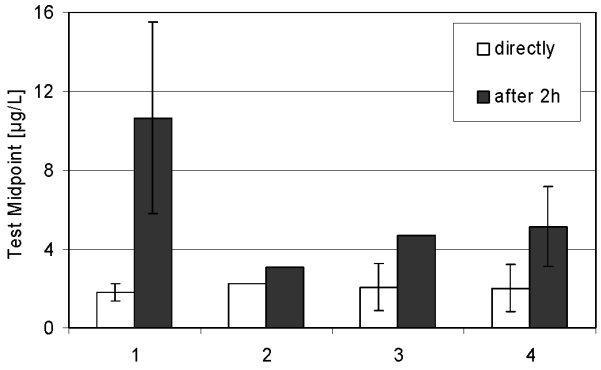

Figure 6. Effect on the test midpoint of the standard curve when *o,p′*-DDT standards were added directly to the microtiter plate after their setup (white), or 2 h after their setup (dark grey). The mAb DDT 1C1 and DDT-hapten-HRP were used. Analyte was set up in 1 = 40 mM PBS (*n* = 5), 2 = 10% isopropanol in 40 mM PBS with 0.01% (v/v) Tween 20 (*n* = 1–2), 3 = 10% (v/v) isopropanol–DMSO mixture (4 + 1) in 40 mM PBS (*n* = 2–3), and 4 = 10% isopropanol in 40 mM PBS (*n* = 3–4). Assay conditions were described under the *Experimental* section.

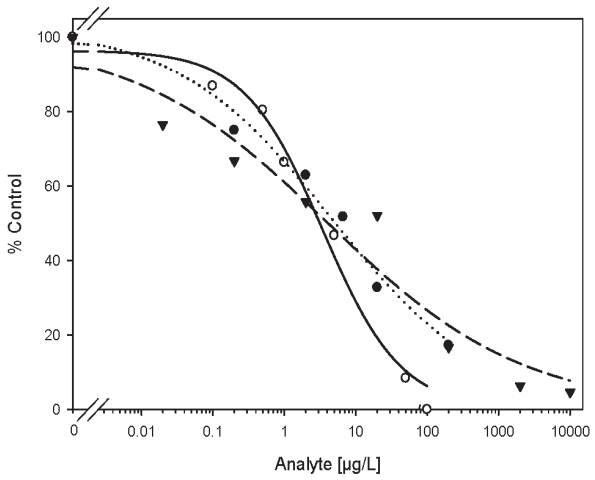

Figure 7. Representative standard curves as % control for *p,p′*-DDT and *o,p′*-DDT with the optical immunosensor. (○, solid line) *p,p′*-DDT with mAb DDT 7C12; (▼, dashed line) *p,p′*-DDT with mAb DDT 1B2; and (●, dotted line) *o,p′*-DDT with mAb DDT 1C1. For details, *see* the *Experimental* section.

Comparing the coating antigen and the enzyme-tracer formats, it could be concluded that both immunoassay formats were suitable for the sensitive (DDT 1C1 and DDT 7C12) and selective (DDT 1B2) detection of DDT isomers. All cross-reactivity patterns were very similar in both format types. In the coating antigen format, the assays obtained usually showed higher optical density (OD) values in comparison to the enzyme-tracer format.

The newly developed mAbs showed comparable to slightly lower IC_{50} values for *p,p′*-DDT to those formerly developed antibodies for *p,p′*-DDT. The mAb 7C12 had an IC_{50} of 0.5 µg/L, mAb LIB5-25 was published with an IC_{50} of 0.7 µg/L (37), polyclonal Ab IV had an IC_{50} of 2 µg/L (38), and purified polyclonal Ab 930/6 had an IC_{50} of 2.5 µg/L (39). In addition, the high selectivity of mAb DDT 1B2 for *p,p′*-DDT was unique and distinctive.

Effect of Time Between Setup of o,p′-DDT Standards and Their Use in the Standard Curve

A usual practice during the setup of standard curves is the preparation of standards on the same day on which the assay will be carried out. Several h can pass, though, before the standards will be pipetted to the microtiter plate. This time will most likely differ and depend upon the number of standard curves that have to be set up during one experiment. As an example, the effect of this storage time was shown for *o,p′*-DDT using mAb DDT 1C1 and DDT hapten-HRP.

Being an organochlorine pesticide, DDT is almost insoluble in water. Adsorption effects on the surface of glass vials were observed. Longer storage resulted in a shift of the standard curve to the right, which increased with an increase of time between the setup of the standards and their addition to the microtiter plate. This was observed especially when the standards were set up in 40 mM PBS (Figure 6, column 1). Test midpoints are shown when standards were set up immediately before pipetting to the plate took place (white bars) and after 2 h (black bars). High SDs in test midpoints were observed, especially when the standards were left for about 2 h in the glass vials. The addition of small amounts of solvents or mixtures of solvents and the addition of detergent, which were chosen as examples, showed clearly a reduction of this effect (Figure 6, columns 2–4). Similar effects were observed with *p,p′*-DDT in the optimized ELISA with mAb DDT 7C12 and DDT-hapten-HRP (data not shown).

Optical Immunosensor for DDT (Isomers)

All optimized standard curves for *p,p′*-DDT and *o,p′*-DDT in the immunosensor used as coating antigen DDT-hapten KLH (3.1 mg/mL). This coating antigen was immobilized via adsorption to the PMMA surface of the sensor prism. Compared with the mAbs in ELISA, the mAb-Oyster-645 conjugates in the optical immunosensor platform showed analogous performances for the corresponding DDT-isomers. The mAb-Oyster-645 conjugates DDT 7C12-Oyster-645, DDT 1B2-Oyster-645, and DDT 1C1-Oyster-645 were optimized and characterized for these measurements. The test midpoints were again lower than 10 µg/L for the analogous DDT-isomers. Representative standard curves for *p,p′*-DDT with DDT 7C12-Oyster-645 [5 µg/mL in 2% (w/v)

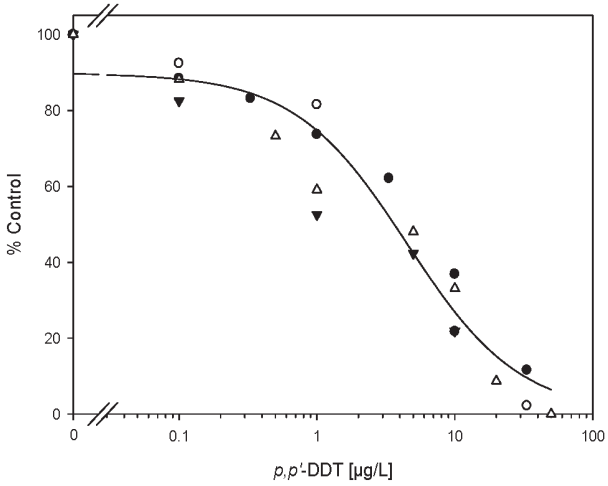

Figure 8. Interday reproducibility of optimized standard curve for *p,p′*-DDT with the optical immunosensor system using coating antigen DDT-hapten KLH (3.1 mg/mL) and mAb DDT 7C12-Oyster-645 conjugate. DDT standards were set up in 5% (v/v) isopropanol in 40 mM PBS. The mAb DDT 7C12-Oyster-645 [0.7 μg/mL in 2% BSA (w/v) in 40 mM PBS; new optimization with new batch of conjugate] and *p,p′*-DDT were preincubated together for 30 min at 22°C prior to the injection into the one-way chip. The standard curve (as % control) was fitted according to the four-parameter equation with the following coefficients: A = 89.7, B = 1.1, C (IC_{50}) = 4.5 μg/L, D = 0.0, R^2 = 0.964 (*n* = 1–4). Different symbols refer to different days.

BSA/40 mM PBS] had an IC_{50} of 3.5 μg/L with a working range (85–15% control) of 0.3–30 μg/L; with DDT 1B2-Oyster-645 [4.3 μg/mL in 2% (w/v) BSA/40 mM PBS], the IC_{50} was 4.1 μg/L with a working range of 0.02–1000 μg/L. For *o,p′*-DDT using mAb DDT 1C1-Oyster-645 [1.2 μg/mL in 2% (w/v) BSA/40 mM PBS], the IC_{50} was 4.6 μg/L and the working range 0.1–300 μg/L (Figure 7).

Immunosensor analysis was carried out as single measurements that were run sequentially. Unlike assays on microtiter plates, on which measurements of standards and controls were determined in parallel and at least in triplicate, the immunosensor system revealed results for standards or samples that could only be compared (as % control) to zero concentrations (buffer) that were run sequentially on the same day.

As an example for interday reproducibility of standard measurements, DDT 7C12-Oyster-645 was selected (Figure 8). All conditions were kept constant, except for the mixtures of mAb DDT 7C12-Oyster-645 conjugate and analyte, which were prepared fresh every day. For these interday tests, a new batch of DDT 7C12-Oyster-645 conjugate was used and optimized again. To confirm that the result of the measurement was independent of the previously measured concentration, the competition tests were carried out by changing the sequence of analyte concentration measurement: chips were measured from lower to higher analyte concentration and vice versa. The results with the optical immunosensor system [slopes (mV/s) and IC_{50}] showed good repeatability of these interday measurements (Figure 8). The average standard curve of the interday measurements showed an IC_{50} of 4.5 μg/L and a working range from 0.3 to 20 μg/L.

Analysis of Surface Water Samples with Optical Immunosensor and ELISA

As an example for the applicability of immunochemical analysis of *p,p′*-DDT, surface waters from different origins were sampled, spiked, and analyzed without any sample pretreatment. Table 4 presents an overview of results both with the optical immunosensor and the ELISA (coating antigen format). This ELISA format was chosen for better comparison with the immunosensor platform and to avoid the influence of matrix on the enzyme of the enzyme-tracer.

The mAb DDT 7C12 (ELISA) and mAb DDT 7C12-Oyster-645 (immunosensor) were used. Standard curves for *p,p′*-DDT were set up in 40 mM PBS for the microtiter plate format and in 5% (v/v) isopropanol in 40 mM PBS for the immunosensor platform. Within the working range of both immunochemical techniques, the majority of results revealed recoveries in the range of 80–120%. In addition, samples with spiked DDT concentrations outside the working ranges of the formats were also determined as such. The working range in ELISA was very narrow, i.e., namely only one order of magnitude. For screening purposes, this would be advantageous because it could be used to distinguish contaminated from noncontaminated samples at a certain threshold concentration (e.g., maximum allowable concentration).

ELISAs and the optical immunosensor for DDT isomers were also applied for the analysis of water and sediment samples from the Nairobi River, Kenya. Results showed that screening of water and sediment samples was possible (56).

For all applications described here, the optical immunosensor was applied only for single-analyte determination. It was noticed, though, that multianalyte analysis would be an enormous advantage. This was already demonstrated with up to three analytes, namely diuron, isoproturon, and phenothrin (57), and was also already performed for up to six analytes (work in progress). Future applications in environmental analysis with multianalyte determinations will allow a range of new perspectives.

Conclusions

These newly developed immunoreagents for the analysis of DDT showed different sensitivities and selectivities for the isomers and metabolites. This aspect can be used for different applications, depending upon the environmental question.

Both immunochemical techniques have advantages and limitations. On the one hand, ELISA is the method of choice when many samples have to be analyzed. At least 12 samples

Table 4. Analysis of different water samples (including spiked amounts of *p,p′*-DDT) with ELISA and optical immunosensor

		ELISA mAb DDT 7C12[a]		Optical immunosensor mAb DDT 7C12-Oyster-645[b]		
Water sample	Spikes *p,p′*-DDT, μg/L	Detected, μg/L	Recovery, %	Slope, mV/s	Detected, μg/L	Recovery, %
Lake Riem, Munich, Germany, pH 8.4	0	—[c]	—	10.4	<1	—
	0.1	ND[d]	ND	9.5	<1	—
	1	1.09 ± 0.14	109	8.8	<1	—
	2	1.75 ± 0.16	88	ND	ND	ND
	3.33	2.44 ± 0.45	73	ND	ND	ND
	10	>8	—	3.1	8.9	89
	33.33	ND	ND	0.7	>30	—
River Isar, Munich, Germany, pH 8.1	0	—	—	9.2	<1	—
	0.1	ND	n.d.	8.5	<1	—
	1	0.46 ± 0.04	46	7.3	1.2	124
	2	1.52 ± 0.18	76	ND	ND	ND
	3.33	3.00 ± 0.67	90	ND	ND	ND
	10	>8	—	3.1	8.9	89
	33.33	ND	ND	0.7	>30	—[c]
Creek water, English Garden, Munich, Germany, pH 8.3	0	—	—	9.6	<1	—
	0.1	ND	ND	8.5	<1	—
	0.33	ND	ND	8.3	0.4	121
	1	1.10 ± 0.07	110	7.1	1.4	140
	2	1.93 ± 0.09	97	ND	ND	ND
	3.33	2.94 ± 0.29	88	6.0	2.6	78
	10	>8	—	4.0	8.5	85
	33.33	ND	ND	0.6	>30	—

[a] *p,p′-DDT standards were set up in 40 mM PBS.*

[b] *p,p′-DDT standards were set up in 5% (v/v) isopropanol in 40 mM PBS.*

[c] Outside working range.

[d] ND = Not determined.

can be quantified (with quadruplicate determination) simultaneously together with a standard curve on the same microtiter plate. The same time (about 4 h) will be needed, though, for only one sample. The ELISA technique consists of a lot of manual pipetting steps. The experience of the person carrying out the assay is, therefore, a crucial factor. On the other hand, with the optical immunosensor, many steps are automated, and a single measurement takes only about 32 min. For many samples, though, serial measurements would take a lot of time. The results obtained with the immunosensor were still burdened by SDs that were too high for reliable measurements with single-use chips. The reason for this uncertainty in the results was mainly the manual spotting of the immunoreagents on the sensor surface. More reproducibility could only be assured with the use of an automated system spotter. First improvements have been obtained already, and experiments will be continued.

The described optical immunosensor shows a lot of potential for future applications, which are not limited to environmental analysis alone. The sensor can be also adapted to food analysis and medical diagnostics. Multianalyte determinations will lead to an even broader usage of this technology.

Acknowledgments

We thank Herbert Walleri, Lothar Löf, Christian Kassel, and Franz Drobner (Siemens AG) for their excellent collaboration and help regarding the optical immunosensor. Norbert Hertkorn (IEC, Helmholtz Center Munich, Germany) is acknowledged for structure confirmation of the DDT-hapten by NMR spectrometry. Some results were presented earlier in posters at the 2nd Asian International Conference on Ecotoxicology and Environmental Safety

(SECOTOX 2004), Songkla, Thailand, September 26–29, 2004; at the Eighth World Congress on Biosensors, Granada, Spain, May 24–26, 2004; and in an oral presentation at Pacifichem, Honolulu, HI, December 15–20, 2005. Major parts of this work were funded by the EU Project AQUA-SCREEN (Contract ICA4-CT-2002-10008).

References

(1) Müller, P.H. (1948) Dichloro-diphenyl-trichloroethane and newer pesticides, Nobel Lecture, December 11, 1948, http://nobelprize.org/medicine/laureates/1948/muller-lecture. pdf

(2) Agency for Toxic Substances and Disease and Registry (2000) U.S. Department of Health and Human Services, *Toxicological profile for DDT, DDE and DDD (update), Draft for public comment,* Agency for Toxic Substances and Disease Registry, Atlanta GA, http://www.atsdr.cdc.gov/toxprofiles/tp35.pdf

(3) EPA Press Release: *DDT ban takes effect* (December 31, 1972) http://www.epa.gov/history/topics/ddt/01.htm

(4) Law about the trade with DDT (DDT law), dated August 7, 1972 (BGBl.I , p. 1385), last changed through Article 8, §2 Health Care Institutions Reform dated June 24, 1994 (BGBl.I, p. 1416)

(5) The Stockholm Convention on Persistent Organic Pollutants, Stockholm, Sweden, May 2001, http://www.pops.int/documents/convtext/convtext_en.pdf

(6) Attaran, A., Roberts, D.R., Curtis, C.F., & Kilama, W.L. (2000) *Nat. Med.* **6**, 729–731

(7) Roberts, D.R., Manguin, S., & Mouchet, J. (2000) *Lancet* **356**, 330–332

(8) Wendo, C. (2004) *Lancet* **363**, 1376

(9) Rinaldi, A. (2004) *EMBO Reports* **5**, 847–851

(10) Lubick, N. (2007) *Environ. Sci. Technol.* **41**, 6323A–6325A

(11) Metcalf, R.L. (1995) in *Kirk-Othmer Encyclopedia of Chemical Technology*, J. Kroschwitz & M. Howe-Grant (Eds), Vol. 14, John Wiley and Sons, Inc., New York, NY, pp 524–602

(12) World Health Organization (1989) *Environmental Health Criteria 83, DDT and Its Derivatives: Environmental Aspects*, Geneva, Switzerland

(13) Howard, R.R. (1996) *Sci. Total Environ.* **185**, 3–26

(14) Martens, D., Schramm, K.-W., & Kettrup, A. (2000) in *Monitoring of Chlorinated and Other Organic Trace Contaminants in Selected Stretches of the Liao and Yangtse River (China)*, B.M. Gawlik, B. Platzer, & H. Muntau (Eds), European Commission, EUR 19531 EN, ECSC-EC-EAEC, Brussels, Luxembourg, p. 95

(15) Kishimba, M.A., Mwevura, H.H., Mmochi, A.J., Mihale, M., & Hellar, H. (2004) *Talanta* **64**, 48–53

(16) Hong, Z., Yonglong, L., Dawson, R.W., Yajuan, S., & Tieyu, W. (2005) *Chemosphere* **60**, 762–769

(17) Scheyer, A., Graeff, C., Morville, S., Mirabel, P., & Millet, M. (2005) *Chemosphere* **58**, 1517–1524

(18) Villa, S., Negrelli, C., Maggi, V., Finizio, A., & Vighi, M. (2006) *Ecotoxicol. Environ. Saf.* **63**, 17–24

(19) Hammock, B.D., Gee, S.J., Cheung, P.Y.K., Miyamoto, T., Goodrow, M.H., Van Emon, J.M., & Seiber, J.N. (1987) in *Pesticide Science and Biotechnology*, R. Greenhalgh & T.R. Roberts (Eds), Symposium Proceedings of the International Union of Pure and Applied Chemistry, Blackwell Scientific Publications, pp 309–316

(20) Rogers, K. (1995) *Biosens. Bioelectron.* **10**, 533–541

(21) Marty, J.-L., Gracia, D., & Rouillon, R. (1995) *Trends Anal. Chem.* **14**, 329–333

(22) Krämer, P. (1996) *J. AOAC Int.* **79**, 1245–1254

(23) Van Emon, J.M., & Gerlach, C.L. (1998) *Anal. Chim. Acta* **376**, 55–59

(24) Van Emon, J.M. (2001) *J. AOAC Int.* **84**, 125–133

(25) Mallat, E., Barcelo, D., Barzen, C., Gauglitz, G., & Abuknesha, R. (2001) *Trends Anal. Chem.* **20**, 124–132

(26) Krämer, P.M., Goodrow, M.H., & Kremmer, E. (2004) *J. Agric. Food Chem.* **52**, 2462–2471

(27) Krämer, P.M., Kremmer, E., Weber, C.M., Ciumasu, I.M., Forster, S., & Kettrup, A.A. (2005) *Anal. Bioanal. Chem.* **382**, 1919–1933

(28) Skark, C., Zullei-Seibert, N., Schöttler, U., & Schlett, C. (1998) *Int. J. Environ. Anal. Chem.* **70**, 93–104

(29) Thurman, E.M., & Cromwell, A.E. (2000) *Environ. Sci. Technol.* **34**, 3079–3085

(30) Van Emon, J.M., & Lopez-Avila, V. (1992) *Anal. Chem.* **64**, 79A–88A

(31) Farre, M., Brix, R., & Barcelo, D. (2005) *Trends Anal. Chem.* **24**, 532–545

(32) Rogers, K.R. (2000) *Mol. Biotechnol.* **14**, 109–129

(33) Thevenot, R.D., Toth, K., Durst, R.A., & Wilson, S.G. (1999) *Pure Appl. Chem.* **71**, 2333–2348

(34) Centeno, E.R., Johnson, W.J., & Sehon, A.H. (1970) *Int. Arch. Allergy* **37**, 1–13

(35) Banerjee, B.D., Pasha, S.T., & Koner, B.C. (1996) *Med. Sci. Res.* **24**, 553–555

(36) Bürgisser, D., Frey, S., Gutte, B., & Klauser, S. (1990) *Biochem. Biophys. Res. Commun.* **166**, 1228–1236

(37) Abad, A., Manclus, J.J., Mojarrad, F., Mercader, J.V., Miranda, M.A., Primo, J., Guardiola, V., & Montoya, A. (1997) *J. Agric. Food Chem.* **45**, 3694–3702

(38) Beasly, H.L., Phongkham, T., Daunt, M.H., Guihot, S.L., & Skerritt, J.H. (1998) *J. Agric. Food Chem.* **46**, 3339–3352

(39) Giraudi, G., Baggiani, C., Cosmaro, A., Santia, E., & Vanni, A. (1998) *Fresenius' J. Anal. Chem.* **360**, 235–240

(40) Botchkareva, A.E., Fini, F., Eremin, S.A., Mercader, J.V., Montoya, A., & Girotti, S. (2002) *Anal. Chim. Acta* **453**, 43–52

(41) Botchkareva, A.E., Eremin, S.A., Montoya, A., Manclus, J.J., Mickova, B., Rauch, P., Fini, F., & Girotti, S. (2003) *J. Immunol. Methods* **283**, 45–57

(42) Alvarez, M., Calle, A., Tamayo, J., Lechuga, L.M., Abad, A., & Montoya, A. (2003) *Biosens. Bioelectron.* **18**, 649–653

(43) Anfossi, L., Giraudi, G., Tozzi, C., Giovandoli, C., Baggiani, C., & Vanni, A. (2004) *Anal. Chim. Acta* **506**, 87–95

(44) U.S. Environmental Protection Agency (1996) *EPA Method 4042, Soil Screening for DDT by Immunoassay*, http://www.epa.gov/epaoswer/hazwaste/test/pdfs/4042.pdf

(45) Meusel, M., Trau, D., Katerkamp, A., Meier, F., Polzius, R., & Cammann, K. (1998) *Sensor. Actuat. B* **51**, 249–255

(46) Schult, K., Katerkamp, A., Trau, D., Grawe, F., Cammann, K., & Meusel, M. (1999) *Anal. Chem.* **71**, 5430–5435

(47) Langone, J.J., & Van Vunakis, H. (1975) *Res. Commun. Chem. Pathol. Pharmacol.* **10**, 163–171

(48) Schneider, P., Goodrow, M.H., Gee, S.J., & Hammock, B.D. (1994) *J. Agric. Food Chem.* **42**, 301–309

(49) Köhler, G., & Milstein, C. (1975) *Nature* **256**, 495–497

(50) Kremmer, E., Kranz, B.R., Hille, A., Klein, K., Eulitz, M., Hoffmann-Fezer, G., Feiden, W., Herrmann, K., Delecluse, H.-J., Delson, G., Bornkamm, G.W., Mueller-Lantzsch, N., & Grässer, F.A. (1995) *Virology* **208**, 336–342

(51) Geiger, C., Nagel, W., Boehm, T., van Kooyk, Y., Figdor, C.G., Kremmer, E., Hogg, N., Zeitlmann, L., Dierks, H., Weber, K.S.C., & Kolanus, W. (2000) *EMBO J.* **19**, 2525–2536

(52) Keay, R.W., & McNeil, C.J. (1998) *Biosens. Bioelectron.* **13**, 963–970

(53) Welinder, K.G. (1979) *Eur. J. Biochem.* **96**, 483–502

(54) Claassen, E., & Van Rooijen, N. (1985) *J. Histochem. Cytochem.* **33**, 840–844

(55) Mossavarali, S., Hosseinkhani, S., Ranjbar, B., & Miroliaei, M. (2006) *Int. J. Biol. Macromol.* **39**, 192–196

(56) Krämer, P.M., Weber, C.M., Kremmer, E., Räuber, C., Martens, D., Forster, S., Stanker, L.H., Rauch, P., Shiundu, P.M., & Mulaa, F.J. (2007) in *Rational Environmental Management of Agrochemicals: Risk Assessment, Monitoring and Remedial Action*, I.R. Kennedy, K. Solomon, S. Gee, A. Crossan, S. Wang, & F. Sanchez-Bayo (Eds), ACS Symposium Series 966, American Chemical Society, Washington, DC, pp 186–202

(57) Räuber, C., & Krämer, P.M. (2006) *Multianalyte Detection of Pesticides Using an Optical Immunosensor*, Poster at the Ninth World Congress on Biosensors, Toronto, Canada, May 10–12 (Abstract P55, Book of Abstracts)