JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Extending the Working Range of Immunoanalysis by Exploitation of Two Monoclonal Antibodies

PETRA M. KRÄMER,*,†,‡ ELISABETH KREMMER,§ STEPHAN FORSTER,‡ AND MARVIN H. GOODROW#

Technische Universität München, Wissenschaftszentrum Weihenstephan, Lehrstuhl für Ökologische Chemie und Umweltanalytik, Weihenstephaner Steig 23, D-85350 Freising, Germany; Institute of Ecological Chemistry, GSF-National Research Center for Environment and Health, Ingolstädter Landstrasse 1, 85764 Neuherberg (Oberschleissheim), Germany; Institute of Molecular Immunology, GSF-National Research Center for Environment and Health, Marchioninistrasse 25, 81377 Munich, Germany; and 109 Almond Drive, Winters, California 95694

A newly established rat monoclonal antibody (mAb) for isoproturon, namely, IOC 10G7, is described. This mAb shows a standard curve for isoproturon in phosphate-buffered saline with a test midpoint of 5.5 ± 1.8 μg/L ($n = 15$). In combination with the formerly developed mAb IOC 7E1, IOC 10G7 can be exploited to extend the working range for the analysis of isoproturon. Both antibodies were formatted into a competitive enzyme-linked immunosorbent assay (ELISA), using the same enzyme−tracer. MAb IOC 7E1 and mAb IOC 10G7 have different affinities for the target compound, but the signal−response curves of the single antibodies overlap. Cross-reactivity (CR) patterns of both antibodies are comparable, showing the highest CR for the metabolite 1-(4-isopropylphenyl)-3-methylurea (IOC 10G7, 9%; IOC 7E1, 19%). The system described here includes the combined, but individual, usage of both assays on one microtiter plate, as well as the strategy for mixing the two antibodies for the utilization in one assay. When standards are performed in Milli-Q water, the working range for isoproturon with the individual ELISAs using mAb IOC 7E1 is from 0.01 to 1 μg/L (test midpoint = 0.11 ± 0.03 μg/L; $n = 17$) and with IOC 10G7, it is 1−100 μg/L (test midpoint = 10.3 ± 1.6 μg/L; $n = 32$). The working range with mixed antibodies is usually on the order of 0.03−30 μg/L (test midpoint = 0.5 ± 0.2 μg/L; $n = 17$). These strategies (mAbs individually and mixed) cover a range of 4 and 3 orders of magnitude, respectively. As a demonstration, water samples of different origins and an extract of mixed sediment were analyzed. The advantages of these strategies are discussed.

KEYWORDS: Rat monoclonal antibodies; mixed monoclonal antibodies; isoproturon; ELISA; extended working range; water; sediment

INTRODUCTION

Immunochemical analysis is an analytical tool that enables the analyst to screen samples, especially water samples, in a very effective, inexpensive, environmentally friendly, and fast manner (*1*, *2*). Immunochemical assays are currently available for a wide range of pesticides (*3*). They can be conducted in different matrices and usually do not require sample cleanup or preconcentration (see, e.g., refs *4*−*6*). A disadvantage, though, especially when using monoclonal antibodies (mAb), within this method is that the working range is usually on the order of only two magnitudes, and the concentration of the analyte in the unknown sample has to fall within the dose−response region of the standard curve. Solutions to this problem are (1) to use several dilutions/preconcentrations of the samples from the beginning or (2) to prescreen and then subsequent measure selected samples that did not fall within the dose−response region during the first measurement. These strategies need either more measurements or more time. For a more time- and cost-effective strategy, it would be advantageous to have a broader working range. This objective was realized in this paper by exploiting two mAbs with different binding affinities for the target isoproturon. In addition to the earlier described rat mAb IOC 7E1 (*7*), which had a working range for isoproturon down to 10 ng/L, we also established a second rat mAb, IOC 10G7, which can detect isoproturon up to 100 μg/L. Both antibodies were raised against the same hapten, both belong to the same

* Address correspondence to this author at TU München, c/o GSF-National Research Center for Environment and Health, Institute of Ecological Chemistry, Ingolstädter Landstr. 1, 85764 Neuherberg (Oberschleissheim), Germany (telephone 49-89-3187-2772; fax 49-89-3187-3371; e-mail kramer@gsf.de).

† Technische Universität München.

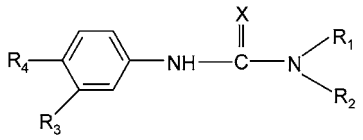
‡ Institute of Ecological Chemistry, GSF-National Research Center for Environment and Health.

§ Institute of Molecular Immunology, GSF-National Research Center for Environment and Health.

Winters, CA.

10.1021/jf0488918 CCC: $27.50
Published on Web 09/25/2004

Table 1. Cross-Reactivities of Haptens: Enzyme–Tracer Format Using **III**–HRP



compound	X	R_1	R_2	R_3	R_4	%CR[a] mAb (IOC 10G7)	%CR[a,b] mAb (IOC 7E1)
isoproturon	O	CH_3	CH_3	H	$CH(CH_3)_2$	100	100
I (immunogen hapten)	O	CH_3	$(CH_2)_5COOH$	H	$CH(CH_3)_2$	452	35
II	O	CH_3	$(CH_2)_3COOH$	H	$CH(CH_3)_2$	42	20
III (enzyme–tracer hapten)	S	CH_3	$(CH_2)_3COOH$	H	$CH(CH_3)_2$	14	0.4
IV	O	CH_3	CH_2COOH	H	$CH(CH_3)_2$	3.2	2
V	S	CH_3	$(CH_2)_5COOH$	H	H	<0.01	<0.01
VI	S	CH_3	$(CH_2)_5COOH$	H	Cl	0.05	0.02
VII	O	CH_3	$(CH_2)_3COOH$	H	Cl	0.1	0.2
VIII	O	CH_3	$(CH_2)_3COOH$	H	H	<0.01	<0.01
IX	O	H	$(CH_2)_3COOH$	Cl	Cl	0.08	0.6
X	S	CH_3	$(CH_2)_5COOH$	Cl	Cl	0.05	0.08

[a] Standards were set up in 40 mM PBS. [b] Data are taken from ref *7*.

antibody subclass, use the identical enzyme−tracer and dilution within the competitive enzyme-linked immunosorbent assay (ELISA) format, and show comparable cross-reactivity patterns. Both assays are optimized in such a way that the procedure is identical with the only variation being the antibodies and their concentrations. In addition to the usage of the antibodies separately, a mixture of both antibodies was used. A similar approach was described earlier by Ohmura et al. (*8*) utilizing two mAbs with different affinities for estriol and demonstrating this technique in a flow immunoassay system.

We will demonstrate how our strategies were devised and exploited for the analysis of isoproturon in different water samples and sediments.

MATERIALS AND METHODS

Chemicals, Reagents, and Instruments. Standards of isoproturon, diuron, monuron, chlorbromuron, chlorsulfuron, chlortoluron, monolinuron, linuron, neburon, fluometuron, and tebuthiruron were purchased from Riedel de Haën (Seelze, Germany; now available through Sigma-Aldrich, Taufkirchen, Germany). Stock solutions were prepared in ethanol with a concentration of 1 mg/mL and stored at 4 °C. 1-(4-Isopropylphenyl)-3-methylurea, 4-(isopropylphenyl)urea, and 4-isopropylaniline were purchased from Dr. Ehrenstorfer, Augsburg, Germany.

Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), hydrogen peroxide (H_2O_2; 30%), and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. Horseradish peroxidase (HRP; EC 1.11.1.7, lyophilized, ∼1000 units/mg) was purchased from Serva Electrophoresis GmbH, Heidelberg, Germany.

Mouse anti-rat mAb TIB-172 (κ-specific) is an in-house clone; it is in general available through the American Type Culture Collection (ATCC; Manassas, VA). Goat anti-rat, HRP-conjugated, was purchased from Dianova, Hamburg, Germany. Buffer salts (sodium hydrogen carbonate, sodium acetate, sodium chloride, sodium phosphate, disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate) and ethanol (p.a.) were purchased from Merck (Darmstadt, Germany).

Demineralized water was prepared by a Milli-Q (MQ) filtration system (Millipore, Eschborn, Germany) and was used for preparation of the standards and solutions and in some assays for the determination of the zero concentration (100% control value).

Microtiter plates (96 well, MaxiSorp, Nunc, Wiesbaden, Germany) covered with lids were used in all assays.

Washing steps were carried out using a Nunc Immunowash (Roskilde, Denmark) connected to a vacuum pump (KNF Neuberger, Laboport, K&K Laborservice, München, Germany). Absorbances were read with a ThermoMax microtiter plate reader (Molecular Devices, Palo Alto, CA) at 450 nm (reference 650 nm). The inhibition curves were analyzed using the four-parameter logistic equation (Softmax Pro, Molecular Devices, Palo Alto, CA).

During the preparation of conjugates centrifugations were carried out using a Heraeus Sepatech Biofuge 15 (Heraeus, Hanau, Germany).

Hapten Syntheses and Conjugation to Proteins. Hapten syntheses of immunogen hapten 1-(5-carboxypentyl)-3-(4-isopropylphenyl)-1-methylurea (**I**) and the enzyme−tracer hapten 1-(3-carboxypropyl)-3-(4-isopropylphenyl)-1-methylthiourea (**III**) were described earlier (*7*) using criteria developed by Goodrow and Hammock (*9*). Haptens **I** and **III** (**Table 1**) were prepared from the appropriate aryl isocyanate (or isothiocyanate) and amino acids. All haptens were conjugated via the active ester method to KLH, BSA, and HRP as described by Krämer et al. (*7*).

Immunization of Rats, Primary Screening, Fusion, and Secondary Screening. The preparation of mAb IOC 10G7 was parallel to that of mAb IOC 7E1 using ∼50 μg of **I**−BSA conjugate, which also was injected intraperitoneally (ip) and subcutaneously (sc) into LOU/C rats with CPG2006 (TIB MOLBIOL, Berlin, Germany) as an adjuvant. After a 2 month interval, a final boost was given ip and sc 3 days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to a standard procedure described by Kremmer et al. (*10*). Hybridoma supernatants were tested in a solid-phase immunoassay using **I**−KLH adsorbed on polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound mAbs were detected using peroxidase-labeled goat anti-rat IgG + IgM antibodies (Dianova, Hamburg, Germany) and *o*-phenylenediamine as chromogen in the peroxidase reaction. BSA was used as a negative control. Positive-reacting hybridomas were further tested for inhibition of HRP-labeled **I**, **II**, and **III** derivatives, respectively. For this, microtiter plates were coated with mouse anti-rat kappa TIP-172 (5 μg/mL) and mouse anti-rat lambda (5 μg/mL). Tissue supernatants were added for 1 h. After a washing with PBS, either 50 μL of an isoproturon solution (10 μg/L in PBS) or 50 μL of PBS as control was added. After 2 h of incubation at room temperature, HRP-labeled tracers (50 μL, 1:100) were added without washing. After 30 min, the plates were washed with PBS, and the substrate was added.

Immunoglobulin type of the mAbs was determined using biotinylated anti-rat IgG subclass-specific mAbs (ATCC). Besides clone IOC 7E1 (rat IgG2a,κ), this screening revealed clone IOC 10G7 (rat IgG2b,κ), which was characterized further and used for these studies.

Purification of Monoclonal Antibodies. Purification of mAbs was performed via protein G. Culture supernatants were added to protein G−Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden). Elution of mAbs from the column was carried out with 0.1 M citrate buffer, pH 2.7. Dialysis was done against 40 mM PBS, pH

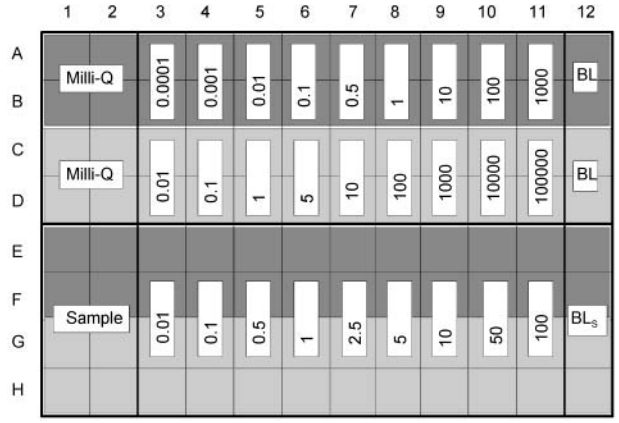


Figure 1. Setup of the plate with mAbs IOC 7E1 and IOC 10G7 individually. Two standard curves were positioned on the same plate, and standards were determined in duplicate with each antibody. Note that the concentration ranges of the standards (μg/L) were different with the two antibodies. For mAb IOC 7E1 (dark gray; 37.5 ng/mL), standard concentrations ranged from 0.0001 to 1000 μg/L; for mAb IOC 10G7 (light gray; 20 ng/mL) isoproturon standards from 0.01 to 100000 μg/L were used and were applied in duplicate. On the same plate, samples were analyzed in duplicate on two different locations, on which the different antibodies were positioned.

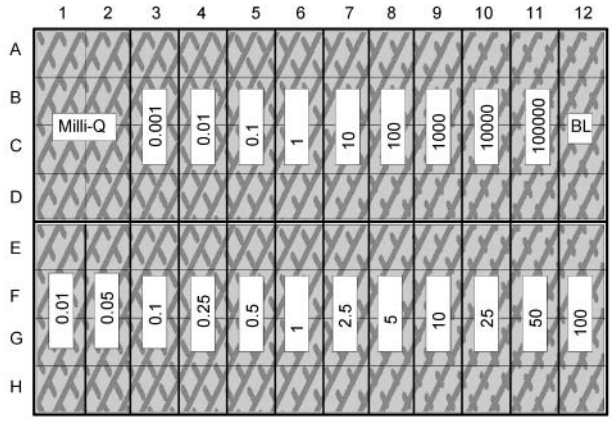


Figure 2. Setup of the plate with mAbs IOC 7E1 and IOC 10G7 mixed. Together with the samples (e.g., water), a single standard curve in Milli-Q water, which was determined with the mixed antibodies (final concentrations of mAbs: IOC 7E1, 37.5 ng/mL; IOC 10G7, 20 ng/mL) was applied on the same microtiter plate. The standards were determined in quadruplicate (rows 1–12, **A**–**D**), and the range of concentration was from 0.001 to 100000 μg/L. BL was determined without mAb and enzyme–tracer. Serial dilutions of the water sample were placed on the lower part of the microtiter plate (rows 1–12, **E**–**H**). They were also determined in quadruplicate. Concentrations were set up from 0.01 to 100 μg/L.

7.6, with 3 times of 100 times of the antibody volume. Antibody solutions were filtered sterile through a 0.2 μm filter and stored at 4 °C until used.

Determination of Protein Concentration. The protein concentrations of the antibodies were determined spectrophotometrically, based on the absorbance measurements at 235 and 280 nm according to the method of Whitaker and Granum (*11*).

Enzyme−Tracer Format with Both Monoclonal Antibodies. *(a) Individual Usage of Both Antibodies.* Microtiter plates were coated with mouse anti-rat κ mAb TIP-172 (200 μL/well, 2 μg/mL in 0.05 M carbonate buffer, pH 9.6, overnight, 4 °C, or 2 h at room temperature). Plates were washed with 4 mM PBST, pH 7.6, and either 150 μL/well rat mAb IOC 7E1 (protein G purified, 1.5 mg/mL; 1:20000 or 1:40000, referring to 75 or 37.5 ng/mL, respectively, in 40 mM PBS, pH 7.6) or IOC mAb 10G7 (protein G purified, 1.4 mg/mL, 1:70000 or 20 ng/mL) was added. Plates were then incubated for 2 h at room temperature and washed again (4 mM PBST). In the next step, 100 μL/well of isoproturon standards or samples was added and incubated for 1 h at room temperature. Standards of isoproturon were set up either in 40 mM PBS (cross-reactivity studies) or in Milli-Q water (when water and sediment samples were analyzed). Samples were put two times on the same plate at two different locations, depending upon the location of the corresponding antibodies (**Figure 1**). After 1 h of incubation, 50 μL/well of enzyme−tracer (**III**−HRP 1:2000 in 40 mM PBS, pH 7.6) was added, and the mixture of analyte/sample and enzyme−tracer was incubated with shaking for another 30 min at room temperature. After the final washing step (three times with 4 mM PBST), 150 μL/well of substrate/chromogen solution for the HRP reaction (TMB/H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5) was added to the plates and incubated for 10 min at room temperature. The enzyme reaction was stopped with 50 μL/well of 2 M H_2SO_4, and the absorbance was read at 450 nm (reference 650 nm). Standard curves were evaluated with the four-parameter equation (Softmax Pro).

(b) Mixture of Both Antibodies. Microtiter plates were treated as described under Individual Usage of Both Antibodies. The only difference was that rat mAb IOC 7E1 and mAb IOC 10G7 were set up double concentrated and then mixed 1 + 1 for use in the assay. The final antibody concentrations were 37.5 ng/mL for mAb IOC 7E1 and 20 ng/mL for mAb IOC 10G7. All steps in the ELISA were performed as described before. Using this strategy either more samples could be analyzed on one plate or a quadruplicate determination of each sample was possible, because they were put only once on the plate (**Figure 2**).

Analysis of Water Samples. Tap water was obtained from the laboratory water supply of the GSF-National Research Centre. Water samples were taken from different creek waters (Landshut and Munich, Germany), from the rivers Isar (Munich, Germany) and Danube (Landshut, Germany), and from pond water (Weiher Baumgarten, LK Freising, Germany). Samples were collected and stored at 4 °C. Water samples were analyzed without further treatment in the assays. The pH of the samples ranged from 7.4 to 8.3. Isoproturon was added to a final concentration of 100 μg/L and then serially diluted with the corresponding water matrix. The software program Softmax Pro was used to determine the concentration of the water samples via unknowns on the same microtiter plate as the corresponding standard curves.

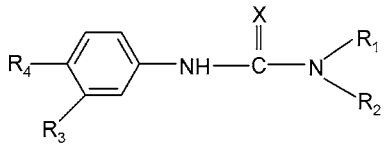
Analysis of Sediment Samples. Three grams (final concentration = 0.1 g/mL) or 0.3 g (final concentration = 0.01 g/mL) of a mixture of sediments [0.25% organic carbon; drawn from Liao-He, China, in June 1999 (*12*)] was weighed into a 75 mL centrifuge tube, mixed with 30 mL of Milli-Q water, and extracted in an ultrasonic bath for 30 min. Samples were then centrifuged (2000 rpm; 18 °C; Heraeus Cryofuge 8500i, Heraeus, Hanau, Germany), and the water extract of the sediment was decanted and stored in the refrigerator (4 °C) until used. Isoproturon was added to the water extract to a final concentration of 100 μg/L, followed by a serial dilution in the same matrix. Standards of the isoproturon standard curve were set up in Milli-Q water. Concentrations in the sediments were determined by the software program Softmax Pro.

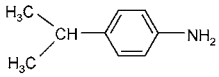
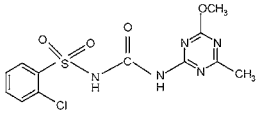
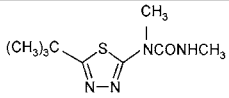
RESULTS AND DISCUSSION

Characterization of MAb IOC 10G7 in Enzyme−Tracer Format. Rat mAb IOC 10G7 was established as the second clone for isoproturon during the earlier established mAb IOC 7E1 (*7*). As described for IOC 7E1, IOC 10G7 was also raised against hapten **I**−BSA. The subclass of mAb IOC 10G7 is IgG2b,κ. This allowed for coating of the microtiter plates the usage of the same catching antibody [mouse anti-rat (κ-specific) TIB-172] as for rat mAb IOC 7E1 (IgG2a,κ). For the use of both anti-isoproturon mAbs on the same microtiter plate, either separated or in a mixture, this was a considerable advantage.

MAb IOC 10G7 was characterized in detail with regard to its performance in PBS buffer. With the optimized ELISA, a standard curve for isoproturon was obtained with an IC50%

Table 2. Cross-Reactivities of Metabolites and Pesticides: Enzyme–Tracer Format Using **III**–HRP



Compound	X	R_1	R_2	R_3	R_4	%CR[a] mAb (IOC 10G7)	%CR[a,b] mAb (IOC 7E1)
Isoproturon	O	CH_3	CH_3	H	$CH(CH_3)_2$	100	100
1-(4-Isopropylphenyl)-3-methylurea	O	H	CH_3	H	$CH(CH_3)_2$	10 / 9[c]	19[c]
4-Isopropylphenylurea	O	H	H	H	$CH(CH_3)_2$	0.6 / 0.8[c]	5[c]
4-Isopropylaniline						0.06 / 0.04[c]	3[c]
Diuron	O	CH_3	CH_3	Cl	Cl	0.2	1.8
Monuron	O	CH_3	CH_3	H	Cl	0.2	0.9
Linuron	O	CH_3	OCH_3	Cl	Cl	0.1	0.2
Chlorbromuron	O	CH_3	OCH_3	Cl	Br	0.1	0.6
Chlortoluron	O	CH_3	CH_3	Cl	CH_3	0.09 / 0.12[c]	3.3
Neburon	O	CH_3	$(CH_2)_3CH_3$	Cl	Cl	1.8	0.4
Fluometuron	O	CH_3	CH_3	CF_3	H	0.2	0.2
Monolinuron	O	CH_3	OCH_3	H	Cl	0.05	0.09
Chlorsulfuron						≤0.01	nd
Tebuthiuron						0.03	nd

[a] Standards were set up in 40 mM PBS. [b] Data are taken from ref *7*. [c] Analyte in 1% methanol/40 mM PBS; nd, not determined.

(test midpoint) of 5.5 ± 1.8 μg/L (n = 15), using the protein G purified antibody (20 ng/mL) and the enzyme−tracer **III**−HRP (1:2000). In Milli-Q water, the IC50% shifted to the right and was 10.3 ± 1.6 μg/L (n = 32).

Cross-reactivities (CR) for synthesized haptens **I**−**X** (*7*) were determined in comparison to a standard curve carried out on the same plate (**Table 1**). In comparison to mAb IOC 7E1, an important result of the determination of CR was that mAb IOC 10G7 showed much higher cross-reactivities (%CR) to the corresponding haptens and—most important—a considerably better recognition of the immunogen hapten **I** (452%CR versus 35%CR). As a consequence also structurally related hapten **II**, which has only a shorter methylene chain spacer, was recognized better (42%CR versus 20%CR). The higher affinity was also true for hapten **III**, which was used in the enzyme−tracer (14%CR versus 0.4%CR). This higher recognition of these structures is apparently the cause for the lower IC50% for isoproturon, which is a factor of ∼100 higher than the one with IOC 7E1. The latter immunoassay with purified IOC 7E1 (37.5 ng/mL) and **III**−HRP (1:2000) showed a test midpoint in Milli-Q water of 0.11 ± 0.03 μg/L (n = 17). This result corresponds well with the earlier reported test midpoint, which was determined with the culture supernatants (*7*).

As a next step, the cross-reactivities with major metabolites of isoproturon, namely, 1-(4-isopropylphenyl)-3-methylurea, 4-isopropylphenylurea, and 4-isopropylaniline, were determined with IOC 10G7. MAb IOC 10G7 recognized these metabolites, but also other urea pesticides, slightly less than IOC 7E1. For example, 1-(4-isopropylphenyl)-3-methylurea had only about 10%CR versus 19%CR with IOC 7E1. The patterns of recognition, though, were for both antibodies very similar (**Table 2**).

As a result of this characterization, it was concluded that mAb IOC 10G7 is not useful for monitoring isoproturon according to the Drinking Water Directive 98/83/EEC (*13*) but that it is

Table 3. ELISA Results Using MAb IOC 7E1 and MAb IOC 10G7, Separately and Mixed: Analysis of Water Sample in Different Concentration Ranges

		amount determined (μg/L)		
water source, pH	spike amount added (μg/L)	mAb 7E1[a]	mAb 10G7[a]	mAb IOC 7E1/ mAb IOC 10G7[b]
tap water (lab), 7.5		<0.01[d]	<1.0[d]	nd
	0.01	0.02 ± 0.001	<1.0[d]	0.004 ± 0.003[d]
	0.05	nd	nd	0.05 ± 0.02
	0.1	0.13 ± 0.02	<1.0[d]	0.11 ± 0.03
	0.25	nd	nd	0.39 ± 0.11
	0.5	0.80 ± 0.17	<1.0[d]	0.70 ± 0.19
	1.0	1.50 ± 0.41	0.5 ± 0.1	1.1 ± 0.4
	2.5	2.86 ± 1.42[d]	1.7 ± 0.3	2.5 ± 0.2
	5.0	>1.0[d]	5.3 ± 0.9	3.4 ± 0.6
	10	>1.0[d]	11.4 ± 1.1	6.1 ± 1.4
	25	nd	nd	25.0 ± 4.8
	50	>1.0[d]	37.4 ± 6.2	74.9 ± 14.6
	100	>1.0[d]	73.2 ± 16.9	>100[d]
creek water, 7.6		<0.01[d]	<1.0[d]	nd
Mühlbach, Landshut	0.01	0.02 ± 0.01	<1.0[d]	0.01 ± 0.005[d]
	0.05	nd	nd	0.06 ± 0.004
	0.1	0.12 ± 0.005	<1.0[d]	0.14 ± 0.02
	0.25	nd	nd	0.41 ± 0.10
	0.5	0.66 ± 0.04	<1.0[d]	0.70 ± 0.13
	1.0	1.19 ± 0.20	1.8[c]	1.1 ± 0.1
	2.5	3.81 ± 0.15[d]	2.7[c]	2.2 ± 0.4
	5.0	>1.0[d]	3.7[c]	3.5 ± 0.6
	10	>1.0[d]	6.9 ± 2.2	6.6 ± 1.0
	25	nd	nd	17.2 ± 2.7
	50	>1.0[d]	38.3 ± 4.9	41.7 ± 7.6
	100	>1.0[d]	65.7 ± 6.9	>100[d]
creek water, 8.3		0.01 ± 0.00	<1.0[d]	nd
Gretelmühle, Landshut	0.01	0.03 ± 0.01	<1.0[d]	<0.02[d]
	0.05	nd	nd	0.06 ±0.01[d]
	0.1	0.14 ± 0.00	<1.0[d]	0.15 ± 0.05
	0.25	nd	nd	0.45 ± 0.02
	0.5	0.56 ± 0.01	<1.0[d]	0.73 ± 0.03
	1.0	1.02 ± 0.04	1.9[c]	1.3 ± 0.4
	2.5	2.49 ± 0.00[d]	4.8 ± 0.9	2.5 ± 0.6
	5.0	>1.0[d]	4.7 ± 0.9	3.6 ± 0.3
	10	>1.0[d]	9.5 ± 2.4	7.3 ± 1.5
	25	nd	nd	22.3 ± 2.1
	50	>1.0[d]	40.4 ± 6.8	51.4 ± 4.7
	100	>1.0[d]	78.7 ± 6.4	139.5 ± 18.0[d]
creek water, 7.9		<0.01[d]	<1.0[d]	nd
Munich	0.01	0.01[c]	<1.0[d]	<0.02[d]
	0.05	nd	nd	0.04 ± 0.01
	0.1	0.11 ± 0.01	<1.0[d]	0.12 ± 0.07
	0.25	nd	nd	0.4 ± 0.1
	0.5	0.51 ± 0.00	0.9[c,d]	0.6 ± 0.1
	1.0	0.91 ± 0.01	1.2[c]	1.3 ± 0.6
	2.5	1.93 ± 0.02[d]	2.7 ± 1.7	2.0 ± 0.1
	5.0	>1.0[d]	4.7 ± 0.9	3.3 ± 0.4
	10	>1.0[d]	11.8 ± 3.5	9.0 ± 2.2
	25	nd	nd	29.0 ± 5.8
	50	>1.0[d]	58.6 ± 7.0	76.9 ± 9.0[d]
	100	>1.0[d]	110.4 ± 7.1	>100[d]
river water, 7.4		0.01 ± 0.01[d]	<1.0[d]	nd
Isar, Munich	0.01	0.02 ± 0.01	<1.0[d]	<0.02[d]
	0.05	nd	nd	0.06 ± 0.01
	0.1	0.13 ± 0.00	<1.0[d]	0.13 ± 0.06
	0.25	nd	nd	0.34 ± 0.13
	0.5	0.51 ± 0.04	<1.0[d]	0.8 ± 0.3
	1.0	0.99 ± 0.05	0.7[c,d]	1.3 ± 0.2
	2.5	2.02 ± 0.07	3.4[c]	2.6 ± 0.6
	5.0	>1.0[d]	6.2[c]	4.1 ± 0.4
	10	>1.0[d]	8.5 ± 3.6	7.2 ± 2.5
	25	nd	nd	24.6 ± 3.8
	50	>1.0[d]	41.2 ± 7.1	57.9 ± 6.0[d]
	100	>1.0[d]	79.7 ± 15.1	122.1 ± 15.0[d]

Table 3 (Continued)

water source, pH	spike amount added (μg/L)	amount determined (μg/L) mAb 7E1[a]	mAb 10G7[a]	mAb IOC 7E1/ mAb IOC 10G7[b]
river water, 8.2		0.02 ± 0.00	<1.0[d]	nd
Danube, Deggendorf	0.01	0.02 ± 0.01	< 1.0[d]	0.01 ± 0.00[d]
	0.05	nd	nd	0.07 ± 0.01
	0.1	0.13 ± 0.01	<1.0[d]	0.16 ± 0.04
	0.25	nd	nd	0.45 ± 0.05
	0.5	0.69 ± 0.32	<1.0[d]	1.0 ± 0.07
	1.0	0.70 ± 0.23	1.6[c]	1.6 ± 0.2
	2.5	2.0 ± 0.3[d]	3.0[c]	3.2 ± 0.04
	5.0	>1.0[d]	3.4 ± 0.1	4.7 ± 0.3
	10	>1.0[d]	nd	9.2 ± 1.1
	25	nd	nd	25.7 ± 2.6
	50	>1.0[d]	50.5 ± 10.5	63.3 ± 1.7[d]
	100	>1.0[d]	87.3 ± 9.0	130.3 ± 21.4[d]
pond water, 8.2		<0.01[d]	<1.0[d]	nd
Weiher Baumgarten, LK Freising	0.01	0.02 ± 0.004	<1.0[d]	<0.02[d]
	0.05	nd	nd	0.03 ± 0.01
	0.1	0.12 ± 0.01	<1.0[d]	0.09 ± 0.02
	0.25	nd	nd	0.25 ± 0.06
	0.5	0.72 ± 0.14	<1.0[d]	0.45 ± 0.07
	1.0	1.30 ± 0.33	0.6 ± 0.2	0.8 ± 0.1
	2.5	2.6 ± 1.3[d]	2.2 ± 0.5	1.7 ± 0.4
	5.0	>1.0[d]	4.8 ± 0.3	2.6 ± 0.6
	10	>1.0[d]	9.9 ± 1.0	5.4 ± 1.3
	25	nd	nd	21.0 ± 3.2
	50	>1.0[d]	36.0 ± 5.9	42.3 ± 8.3
	100	>1.0[d]	69.8 ± 14.2	103.2 ± 56.6

[a] Values are means ± SD of duplicate determinations. [b] Values are means ± SD of quadruplicate determinations. [c] Single determination. [d] Value is outside the working range of the standard curve; nd, not determined

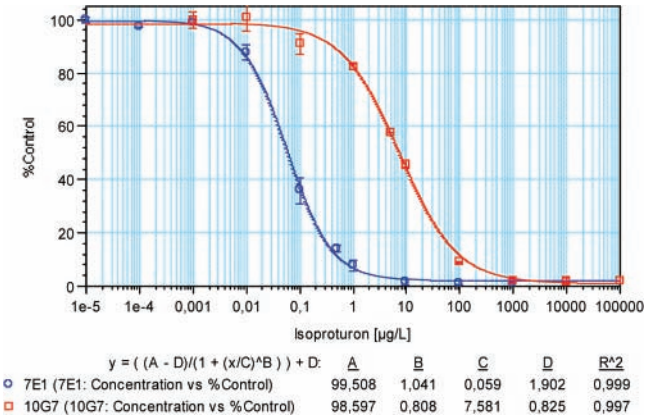


Figure 3. Representative standard curves in Milli-Q water (%Control) for isoproturon with mAbs separated: mAbs IOC 7E1 (37.5 ng/mL) and IOC 10G7 (20 ng/mL), enzyme–tracer **III**–HRP 1:2000. Both assays were performed on one plate as seen in **Figure 1**. Plotted values are means ± SD of duplicate determinations. Original graph and curve-fitting values were from the program Softmax Pro. The lowest concentration (0.00001 μg/L) refers to 0 μg/L (Milli-Q water).

very useful in combination with IOC 7E1, because it extends the working range of the assay.

Extension of the Working Range for Isoproturon Using Two Monoclonal Antibodies Separately. With the first strategy, mAbs IOC 10G7 and IOC 7E1 were used individually to determine standard curves for isoproturon on the same microtiter plate (setup in **Figure 1**). This tactic reveals two different standard curves (**Figure 3**). The IC50% of this representative standard curve in Milli-Q water with mAb IOC 7E1 is 0.06 μg/L. The working range is from 0.01 to 1 μg/L. The standard curve with IOC 10G7 shows an IC50% of ~8 μg/L with a working range of ~1−100 μg/L. When environmental samples were analyzed, they were applied in duplicate at two different locations on the same plate, where the corresponding mAbs were coated. Depending upon the isoproturon concentration, at least one antibody should provide a result.

When the same water samples were analyzed with both assays individually, only a small fraction of the whole concentration range fell into the working range of the corresponding assay (**Table 3**), but it was always indicated correctly into which category of assay the sample would fall. If one does not know the analyte concentration in the samples, this will give more security in the screening process of samples than only one assay does. On the other hand, a duplicate determination is sometimes a problem, when the variation of the absorbance values is high. Some of the variations between the amounts spiked and determined, especially when it was observed with both assays, might be caused either by an influence of the water matrix on the enzyme−tracer or by the fact that for the standard curves Milli-Q water was used, which differs from the matrix of the samples. This result supports again the usage of uncontaminated matrices in the standard curve. Unfortunately, this kind of matrix is not always available. Therefore, Glass et al. (*14*) suggested, for example, as one solution to this problem the use of labeled and unlabeled antibodies, which should enable a self-calibration, thus reducing the sample matrix effect. Although this strategy focused on the effects on the antibody, it was expected to have utility for other classes of matrix effects also.

Extension of the Working Range for Isoproturon Using a Mixture of Two Monoclonal Antibodies. In the second strategy, both mAbs were mixed together (**Figure 2**) and a

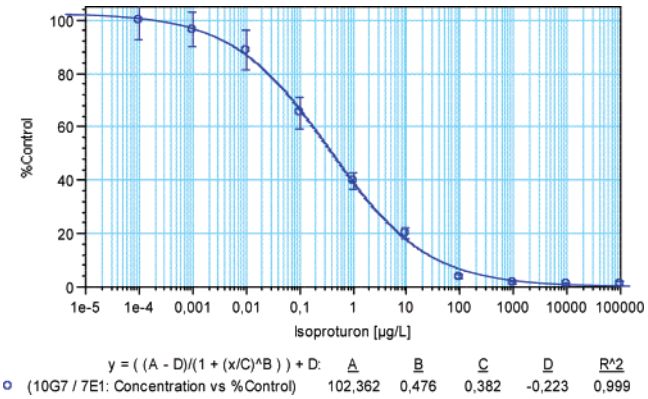


Figure 4. Standard curves in Milli-Q water (%Control) for isoproturon with mAbs mixed: mAb IOC 7E1 (final concentration = 37.5 ng/mL) and IOC 10G7 (final concentration = 20 ng/mL), enzyme–tracer **III**–HRP 1:2000. The setup of the standards was performed as shown in **Figure 2**. Plotted values are means ± SD of quadruplicate determinations. Original graph and curve-fitting values were from the program Softmax Pro. The lowest concentration determined (0.0001 μg/L) refers to 0 μg/L (Milli-Q water).

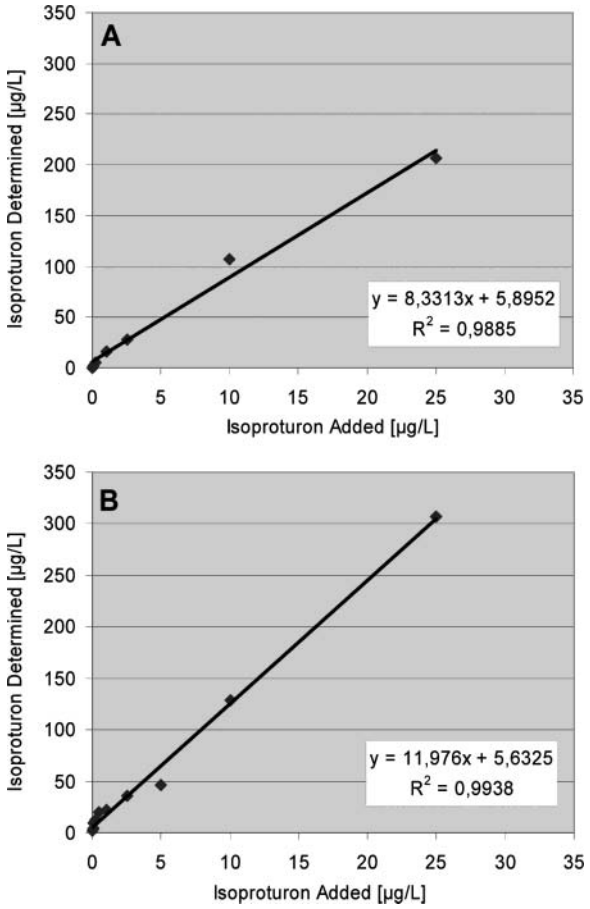


Figure 5. Influence of sediment matrix on the analysis of isoproturon using mAbs IOC 7E1 and IOC 10G7 mixed. Different amounts of sediment matrix were used: (**A**) sediment 0.01 g/mL; (**B**) sediment 0.1 g/mL. With these results a clear influence of the matrix is demonstrated, which is dependent upon the amount of matrix present: mAb IOC 7E1 (37.5 ng/mL), mAb IOC 10G7 (20 ng/mL), **III**–HRP 1:2000; isoproturon standards in Milli-Q water. For better comparison, scales of both graphs were kept the same. The values plotted are means of quadruplicate determinations.

standard curve for isoproturon in Milli-Q water was obtained (**Figure 4**). This approach is recommended only when mAbs such as the ones described here are available. As pointed out by Ohmura et al. (*8*), for an extension of the working range of a standard curve with mixed antibodies, it is necessary to have an overlap of the signal−response curves of the single antibodies used. This was the case with the antibodies used in this study. In addition, the CR of the antibodies should not be extremely different. The two mAbs showed slightly different CRs, but the overall patterns were essentially the same. The standard curves of this setup are less steep (less sensitive) and reveal an IC50% of 0.5 ± 0.2 μg/L (n = 17). The working range was here at least an order of 3 magnitudes, ranging from about 0.03 to 30 μg/L (**Figure 4**). In addition, environmental samples have to be applied only once, which enables the analysis of at least two times more samples (with duplicate determination) or the same number of samples in quadruplicate determination.

When complex matrices, such as sediments, are analyzed, a clear advantage will be the exploitation of the mixed mAb assay, because usually a wide concentration range can be assumed. This will then allow in one step to get a fast screening. Although there is an 8 (with 0.01 g/mL) to nearly 12 times (with 0.1 g/mL) overestimation of the isoproturon concentration, both calibration graphs show good linear correlations, thus indicating that even in complex matrices the mixture of antibodies can be applied (**Figure 5**). The setup of standards in uncontaminated sediment matrix would again be advantageous.

Conclusions. The combination of high- and low-affinity mAbs, used either individually on one plate or in a mixture, presents an enormous potential for greater confidence in the results during the analysis of environmental samples. These strategies enable the analyst to cover a much wider working range and will provide an even more powerful tool in environmental immunochemical analysis.

ACKNOWLEDGMENT

We thank Sabine Rottmüller for excellent technical assistance. We acknowledge Dr. Dieter Martens for a fruitful discussion regarding the antibody mixture and for making the sediment mixture available to us.

LITERATURE CITED

(1) Hammock, B. D.; Gee, S. J.; Cheung, P. Y. K.; Miyamoto, T.; Goodrow, M. H.; van Emon, J.; Seiber, J. N. Utility of immunoassay in pesticide trace analysis. In *Pesticide Science and Biotechnology*; Greenhalgh, R., Roberts, T. R., Eds.; IUPAC, Blackwell Scientific Publications: Oxford, U.K., 1987; pp 309−316.

(2) Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M. H.; Li, Q, X.; Lucas, A. D.; Szekacs, A.; Sundaram, K. M. S. Immunochemical technology in environmental analysis. Addressing critical problems. In *Immunochemical Methods for Environmental Analysis*; van Emon, J. M., Mumma, R. O., Eds.; ACS Symposium Series 442; American Chemical Society:, Washington, DC, 1990; pp 112−139.

(3) Dankwardt, A. Immunochemical Assay in Pesticide Analysis. *Encyclopedia of Analytical Chemistry*; Meyers, R. A., Ed.; Wiley: Chichester, U.K., 2000.

(4) Abad, A.; Moreno, M.-J.; Pelegrí, R.; Martínez, M.-I.; Sáez, A.; Gamón, M.; Montoya, A. Monoclonal enzyme immunoassay for the analysis of carbaryl in fruits and vegetables without sample cleanup. *J. Agric. Food Chem.* **2001**, *49*, 1707−1712.

(5) Moreno, M.-J.; Abad, A.; Pelegrí, R.; Martínez, M.-I.; Sáez, A.; Gamón, M.; Montoya, A. Validation of a monoclonal enzyme immunoassay for the determination of carbofuran in fruits and vegetables. *J. Agric. Food Chem.* **2001**, *49*, 1713−1719.

(6) Casino, P.; Morais, S.; Puchades, R.; Maquieira, Á. Evaluation of enzyme-linked immunoassay for the determination of chloroacetanilides in water and soil. *Environ. Sci. Technol.* **2001**, *35*, 4111−4119.
(7) Krämer, P. M.; Goodrow, M. H.; Kremmer, E. Enzyme-linked immunosorbent assays based on rabbit polyclonal and rat monoclonal antibodies against isoproturon. *J. Agric. Food Chem.* **2004**, *52*, 2462−2471.
(8) Ohmura, N.; Tsukidate, Y.; Shinozaki, H.; Lackie, S. J.; Saiki, H. Combinational use of antibody affinities in an immunoassay for extension of dynamic range and detection of multiple analytes. *Anal. Chem.* **2003**, *75*, 104−110.
(9) Goodrow, M. H.; Hammock, B. D. Hapten design for compound-selective antibodies: ELISAs for environmentally deleterious small molecules. *Anal. Chim. Acta* **1998**, *376*, 83−91.
(10) Kremmer, E.; Kranz, B. R.; Hille, A.; Klein, K.; Eulitz, M.; Hoffmann-Fezer, G.; Feiden, W.; Herrmann, K.; Delecluse, H.-J.; Delsol, G.; Bornkamm, G. W.; Mueller-Lantzsch, N.; Grässer, F. A. Rat monoclonal antibodies differentiating between the Epstein−Barr virus nuclear antigens 2A (EBNA2A) and 2B (ERNA2B). *Virology* **1995**, *208*, 336−342.
(11) Whitaker, J. R.; Granum, P. E. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. *Anal. Biochem.* **1980**, *109*, 156−159.
(12) Final report. *Monitoring of Chlorine and Other Organic Trace Contaminants in Selected Stretches of the Liao and Yangtse Rivers (China)*; Gawlik, G. M., Platzer, B., Muntau, H., Eds.; Joint Research Centre: Ispra, Italy; 2000; Annex 2, 320−323, EC 2000, EUR 19531 EN.
(13) Council Directive of 3.11.1998 relating to the quality of water intended for human consumption (98/83/EC). *Off. J. Eur. Communities* **1998**, *L330*, 32 ff.
(14) Glass, T. R.; Ohmura, N.; Saiki, H.; Lackie, S. J. A combination of labeled and unlabeled antibody enables self-calibration and reduction of sample matrix effects in immunoassay. *Anal. Biochem.* **2004**, *331*, 68−76.

Received for review July 6, 2004. Revised manuscript received August 15, 2004. Accepted August 16, 2004. Part of this work was funded by the EU Project ICA2-CT-2001-10007 (INCO-Copernicus).

JF0488918