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Enzyme-Linked Immunosorbent Assays Based on Rabbit Polyclonal and Rat Monoclonal Antibodies Against Isoproturon

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This work describes the production and characterization of rabbit polyclonal antisera (pAb) and rat monoclonal antibodies (mAb) against isoproturon. Coating antigen and enzyme-tracer formats were developed. Standard curves for isoproturon were conducted either in 40 mM phosphate buffered saline (PBS) or in Milli-Q water. PAb 352 together with the best enzyme tracer revealed in the optimized ELISA (enzyme tracer format) a test midpoint of 1.06 ± 0.34 μg/L (n = 19, standard set up in Milli-Q water) with a detection limit of about 0.1 μg/L. The comparable ELISA with mAb IOC 7E1 had test midpoints of 0.07 ± 0.04 μg/L (n = 7, standards in Milli-Q water) and 0.11 ± 0.08 μg/L (n = 33; standards in 40 mM PBS). The limits of detection were about 0.003 and 0.01 μg/L in Milli-Q water and PBS, respectively. Noticeable cross reactivities (CRs) were seen with the major metabolites, namely 4-isopropylaniline, 4-isopropylphenylurea, and 1-(4-isopropylphenyl)-3-methylurea. With pAb 352, these CRs were 5%, 7%, and 31%, respectively, and with mAb IOC 7E1, they were 3%, 5%, and ca. 19%, respectively. All arylurea herbicides had only minor CRs, which ranged from no CR (e.g., chlorosulfuron) to a maximum of 3.3% (chlortoluron). Influences of organic solvents (methanol, ethanol, acetonitrile, and acetone) were evaluated. Both pAb- and mAb-based immunoassays showed the highest tolerance for methanol, up to 5%. Ethanol and acetonitrile could not be used above 2% without an influence on the assays. The same was true for acetone, although tested only in the mAb-based assay. Water samples of different origins and matrices were spiked and analyzed with these pAb and mAb ELISAs. The results demonstrated that these immunoassays are useful screening tools.

KEYWORDS: Rabbit polyclonal antiserum; rat monoclonal antibodies; isoproturon; 4-isopropylanilin; 4-isopropylphenylurea; 1-(4-isopropylphenyl)-3-methylurea; urea herbicides; immunoassay; ELISA; water

INTRODUCTION

Arylurea herbicides cause problems in the environment because they are quite often found in surface water or rainwater (*1*). Isoproturon, 3-(4-isopropylphenyl)-1,1-dimethylurea, is the most common representative of the urea herbicides for pre- and post-emergence control of black grass, silky bent grass, wild oats, annual meadow grass, ray grass, many broadleaf weeds in spring and winter wheat, spring and winter barley, and winter rye (*2*). The urea herbicides are slightly water soluble and can easily migrate through the soil to crops and thus enter the food chain. Depending on the particular rainfall conditions and soil properties, the herbicides can also reach the groundwater. Due to the absence of microbial activity, degradation processes are very slow and thus, with accumulation, nontolerable levels may be achieved in drinking water. The European Community has set a MAC (Maximum Admissible Concentration) of 0.1 μg/L for the individual pesticide and 0.5 μg/L for total pesticides [EC 80/778/EEC (*3*) is now repealed and replaced by Council Directive 98/83/EC (*4*)]. To monitor the urea herbicides in water, solid-phase extraction techniques, followed by HPLC or GC/MS, are commonly employed. Although chromatographic methods are advantageous for multiresidue analysis, they need preconcentration and/or derivatization steps before analysis. In addition, they require the use of organic solvents, which are environmentally hazardous. Thus, there is a desire to develop

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rapid, inexpensive, sensitive, and environmentally safe screening methods for analyses, that can be used as an "alarm" and can rapidly detect the presence of pesticides in water. Enzyme-linked immunosorbent assays (ELISAs) are known to be fast screening methods and sensitive, quantitative tools for pesticide analyses (e.g., urea herbicides). After the development of polyclonal antibodies (pAb) for monuron, diuron, and linuron by Schneider et al. (*5*) and monoclonal antibodies (mAb) for diuron by Karu et al. (*6*), the aim of this study was to devise an additional assay for isoproturon, hopefully selective for the target in the presence of the other structurally related ureas. Several antibodies against isoproturon have been developed. Liégeois et al. (*7*) described a quantitative ELISA method (coating antigen format) based on mAbs, which allowed detection of isoproturon of 20−250 μg/L in aqueous solution, and in the range of 1 μg/g in soils. Katmeh et al. (*8*) developed a direct competitive ELISA procedure (based on sheep pAbs) for the determination of isoproturon in water, with a detection limit of 0.03 μg/L. These immunoreagents have also been used in enzyme immunoaffinity chromatography as a rapid semiquantitative technique for the screening of water samples (*9*). Ben Rejeb et al. (*10*) described an indirect enzyme immunoassay (based on rabbit pAbs) for the determination of isoproturon in water matrices (detection limit of 0.02 μg/L) and used a method of antibody purification to reduce the heterogeneity of the medium, when the test is performed within complex surface water matrices. Mallat et al. (*11*) demonstrated an optical immunosensor for isoproturon. Sheep pAbs were used and a detection limit of 0.012 μg/L was described. These antibodies though showed high CRs for diuron, chlortoluron, and linuron (93%, 53%, and 46%, respectively). Another assay for isoproturon was developed by GEC-Marconi Materials Technology, Hirst Division, Herts, UK, which has been evaluated for water and soil samples within a Joint European Union research project (*12*). The Hirst assay uses an antigen coating format that found cross reactivities of 4% and <1%, respectively, for 1-(4-isopropylphenyl)-3-methylurea and 4-isopropylphenylurea, the two demethylated degradation products of isoproturon. The second test within this evaluation was the commercially available test-kit EnviroGard Isoproturon Plate Kit, Strategic Diagnostisc Inc., Newark, DE. The results of this evaluation study showed that ELISA microtiter plates are of interest for the determination of phenylureas in water samples and soil extracts, notably because of the elimination of time-consuming sample preparation. The results of both tests significantly correlated with the HPLC values (*12*). Another plate test-kit on the market is the Isoproturon Plate Kit, Envirologix Inc., Portland, MA.

To obtain our own unlimited source of immunoreagents, we have developed a new set of in-house reagents. All immunoreagents mentioned above are either not commercially available or, if they are, are formatted into ready-to-use test-kits. These immunochemicals developed in-house will be used for a new automated biosensor system for on-line and/or off-line on-site field screening measurements.

To our knowledge, this new mAb is the first rat mAb used successfully in the field of environmental analysis.

In addition, this is the only paper that includes all three major metabolites of isoproturon, namely 4-isopropylaniline, 4-isopropylphenylurea, and 1-(4-isopropylphenyl)-3-methylurea, which might also occur in environmental samples.

MATERIALS AND METHODS

Chemicals, Reagents, and Instruments. Standards of isoproturon, diuron, monuron, chlorbromuron, linuron, neburon, fluometuron, fenuron, metabromuron, metoxuron, 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.

Freund's adjuvants (complete and incomplete), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), protein A, hydrogen peroxide (H_2O_2; 30%), 3,3′,5,5′-tetramethylbenzidine (TMB), *N*,*N*-dimethylformamide (anhydrous DMF), *N*,*N*′-dicyclohexylcarbodiimide (DCC; 99%), *N*-hydroxysuccinimide (NHS; 97%), and goat anti-rabbit (Fc specific) conjugated to HRP were from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. Horseradish peroxidase (HRP; EC 1.11.1.7, lyophilized, ca. 1000 U/mg) was purchased from Serva Electrophoresis GmbH, Heidelberg, Germany.

Slide-A-Lyzer dialysis cassettes, needles and syringes, and goat anti-mouse, Fc specific were purchased from Pierce, Rockford, IL. Mouse anti-rat mAb TIB-172 (κ-specific) can be obtained from American Type Culture Collection [(ATCC); Manassas, VA, USA]. Goat anti-rat conjugated with HRP was purchased from Dianova, Hamburg, Germany. Reagents for synthesis were obtained from either Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO).

Buffer salts (sodium hydrogen carbonate, sodium acetate, sodium chloride, sodium phosphate, disodium hydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate) and solvents [methanol, ethanol, and dimethyl sulfoxide (dried DMSO, GR, max. 0.05% H_2O)] were purchased from Merck (Darmstadt, Germany).

Ultrapure water was prepared by purifying demineralized water in a Milli-Q (MQ) filtration system (Millipore, Eschborn, Germany) and 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) were used in all assays.

Washing steps were carried out with a NUNC Immunowash (Roskilde, Denmark), which was 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 with the four-parameter logistic equation (Softmax Pro, Molecular Devices, Palo Alto, CA).

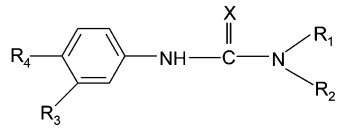
During the preparation of conjugates centrifugations were carried out with a Heraeus Sepatech Biofuge 15 (Heraeus, Hanau, Germany).

Hapten Syntheses. Using the criteria previously developed by Goodrow and Hammock (*13*), idealized immunogen haptens (**I** and **II**, **Table 1**) and a potential coating/tracer hapten (**III**, **Table 1**) were prepared from the appropriate aryl isocyanate (or isothiocyanate) and amino acids. The two heterologous haptens, **I** and **II**, were used for immunization and screening purposes. These isoproturon derivatives mimic the structure of the target compound to which is covalently attached an innocuous methylene handle that is terminated by a carboxylic acid group to facilitate conjugation to proteins. Hapten **III**, a thiourea mimic of the immunizing hapten except for a shorter methylene protein-attachment handle, was prepared as an ideal coating/enzyme tracer hapten. Very sensitive assays for isoproturon were thus produced. Cross-reactivities of the numerous other arylurea herbicides were negligible.

The ease of synthesis encouraged the evaluation of additional structural modifications (**IV**−**X**) that would be most (or least) important in target molecule recognition by the antibody (or antibodies). The purity of each hapten was assessed by thin-layer chromatography (TLC) employing 0.2 mm precoated silica gel 60 F254 on plastic sheets from E. Merck (Darmstadt, Germany). Sample-spotted plates were eluted with an ethyl acetate−hexane (1:1, v/v) plus 2% acetic acid solution, viewed first under UV light (254 nm) and after iodine staining for confirmation of purity. Identity and purity were further confirmed by ^{1}H and ^{13}C NMR [General Electric QE 300 spectrometer (Bruker NMR, Billerica, MA) operating at 300.1 and 75.5 MHz, respectively] and infrared (IR) spectra [Mattson Galaxy Series FTIR 3000 spectrometer (Madison, WI)]. Mobile protons were checked with D_2O.

1-(5-Carboxypentyl)-3-(4-isopropylphenyl)-1-methylurea (I). A mixture of 0.355 g (2.00 mmol) of 4-isopropylphenylisocyanate, 0.362 g (2.00 mmol) of 6-methylaminohexanoic acid dihydrate, and 4.2 mL

Table 1. Cross Reactivities of Haptens



						% CR			
compd	X	R_1	R_2	R_3	R_4	pAb (352)[a]	pAb (352)[b]	mAb (IOC 7E1)[a]	mAb (IOC 7E1)[b]
isoproturon	O	CH_3	CH_3	H	$CH(CH_3)_2$	100	100	100	100
I (immunogen hapten)	O	CH_3	$(CH_2)_5COOH$	H	$CH(CH_3)_2$	345	396	89	69
II	O	CH_3	$(CH_2)_3COOH$	H	$CH(CH_3)_2$	199	184	41	25
III (enzyme-tracer hapten)	S	CH_3	$(CH_2)_3COOH$	H	$CH(CH_3)_2$	9	13	0.05	0.08
IV	O	CH_3	CH_2COOH	H	$CH(CH_3)_2$	22	50	1	0.7

[a] Coating antigen format; coating Ag: **I**-BSA. [b] Coating antigen format; coating Ag: **II**-BSA.

(4.2 mmol) of 1.0 M NaOH was agitated for 2 h and filtered through Celite, then the filtrate was acidified to pH 2. Crystallization of the resultant solid from ethanol−water (1:2, v/v) provided 0.520 g (85%) of **I** as white crystals: mp 95.5−96.5 °C; TLC R_f 0.32; IR (KBr) 3376 (s, NH), 1717 (vs, COOH), 1623 (vs, amide C═O), 1525 (vs, amide II), 1244 (m, C−O), 814 (w, 1,4-disub) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 8.09 (s, 1 H, NH), 7.35 (d, J = 8.1 Hz, 2 H, ArH-2,6), 7.08 (d, J = 8.1 Hz, 2H, ArH-3,5), 3.27 (t, J = 7.2 Hz, 2 H, CH_2-6), 2.90 (s, 3 H, NCH_3), 2.80 (heptet, 1 H, CH), 2.20 (t, J = 7.2 Hz, CH_2-2), 1.5 (m, 4 H, CH_2-3,5), 1.2 (m, 2 H, CH_2-4), 1.17 (d, J = 6.8 Hz, 6 H, 2 CH_3); ^{13}C NMR (DMSO-d_6) δ 174.5 (COOH), 155.6 (urea C═O), 141.8 (ArC-4), 138.5 (ArC-1), 125.9 (ArC-3,5), 120.3 (ArC-2,6), 48.0 (C-6), 34.4 (NCH_3), 33.8 (C-2), 32.9 (CH), 27.3 (C-5), 26.0 (C-4), 24.5 (C-3), 24.1 (2 CH_3). COSY, HETCOR, and APT experiments confirmed ^1H and ^{13}C NMR assignments.

1-(3-Carboxypropyl)-3-(4-isopropylphenyl)-1-methylurea (II). This urea was prepared on a 5.00-mmol scale by using 0.805 g of 4-isopropylphenylisocyanate and 0.806 g (5.25 mmol) of 4-methylaminobutanoic acid hydrochloride in 10.5 mL of 1.0 M NaOH according to the procedure for **I** and provided 1.29 g (93%) of **II** as a white powder: mp 134.5−135.5 °C dec with gas evolution; TLC R_f 0.23; IR (KBr) 3343 (m, NH), 1715 (vs, COOH), 1657 (s, amide C═O), 1536 (vs, amide II), 1231 (s, C−O), 833 (m, 1,4-disub) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.2 (br, 1 H, OH), 8.13 (s, 1 H, NH), 7.35 (d, J = 8.3 Hz, 2 H, ArH-2,6), 7.08 (d, J = 8.3 Hz, 2 H, ArH-3,5), 3.29 (t, J = 7.2 Hz, 2 H, CH_2-4), 2.91 (s, 3 H, NCH_3), 2.81 (heptet, J = 6.9 Hz, 1 H, CH), 2.22 (t, J = 7.2 Hz, 2 H, CH_2-2), 1.72 (quin, J = 7.2 Hz, 2 H, CH_2-3), 1.17 (d, J = 6.9 Hz, 6 H, 2 CH_3); ^{13}C NMR (DMSO-d_6) δ 174.4 (COOH), 155.6 (urea C═O), 141.9 (ArC-4), 138.4 (ArC-1), 125.9 (ArC-3,5), 120.2 (ArC-2,6), 47.5 (C-4), 34.4 (NCH_3), 32.9 (C-2), 30.9 (CH), 24.1 (2 CH_3), 23.0. C-3). COSY, HETCOR, and APT experiments confirmed ^1H and ^{13}C NMR assignments.

1-(3-Carboxypropyl)-3-(4-isopropylphenyl)-1-methylthiourea (III). Employing the procedure for **I**, 4-isopropylphenylisothiocyanate, 0.355 g (2.00 mmol), 0.323 g (2.10 mmol) of 4-methylaminobutanoic acid hydrochloride, and 4.2 mL of 1.0 M NaOH provided 0.570 g (97%) of **III** as white flakes: mp 88.0−89.0 °C; TLC R_f 0.36; IR (KBr) 3408 (m, NH), 3207 (m, NH), 1711 (vs, COOH), 1532 (vs, amide II), 1326 (vs, C═S), 1214 (m, C−O), 830 (w, 1,4-disub) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.16 (s, 1 H, OH), 8.90 (s, 1 H, NH), 7.2 (m, 4 H, ArH-2,3,5,6), 3.77 (t, J = 7.1 Hz, 2 H, CH_2-4), 3.21 (s, 3 H, NCH_3), 2.86 (heptet, J = 6.8 Hz, 1 H, CH), 2.26 (t, J = 7.1 Hz, 2 H, CH_2-2), 1.83 (quin, J = 7.1 Hz, 2 H, CH_2-3), 1.20 (d, J = 6.8 Hz, 6 H, 2 CH_3); ^{13}C NMR (DMSO-d_6) δ 181.1 (C═S), 174.3 (COOH), 144.8 (ArC-4), 138.8 (ArC-1), 126.2 (ArC-2,6 or −3,5), 125.7 (ArC-2,6 or −3,5), 52.1 (C-4), 38.7 (NCH_3), 33.1 (CH), 30.9 (C-2), 24.1 (2 CH_3), 22.4 (C-3). HETCOR and APT experiments confirmed ^1H and ^{13}C NMR assignments.

1-(Carboxymethyl)-3-(4-isopropylphenyl)-1-methylurea (IV). Emulating **I**, a mixture of 0.178 g (2.00 mmol) of sarcosine, 2.2 mL of 1.0 M NaOH, and 0.322 g (2.00 mmol) of 4-isopropylphenylisocyanate provided 0.416 g (83%) of **IV** as a white powder: mp 153.0−154.0 °C dec with gas evolution; TLC R_f 0.10; IR (KBr) 3401 (s, NH), 1721 (s, COOH), 1634 (s, amide C═O), 1536 (vs, amide II), 1214 (vs, C−O), 836 (m, 1,4-disub) cm^{-1}; ^{13}C NMR (DMSO-d_6) δ 171.9 (COOH), 156.2 (urea C═O), 142.3 (ArC-4), 138.3 (ArC-1), 126.3 (ArC-3,5), 120.4 (ArC-2,6), 50.4 (C-2), 35.9 (NCH_3), 33.1 (CH), 24.4 (2 CH_3).

1-(3-Carboxypropyl)-1-methyl-3-phenylthiourea (V). Compound **V** was prepared and worked up in the same manner as **I** but on a 10.0-mmol scale from phenylisothiocyanate and 4-methylaminobutanoic acid hydrochloride, providing 2.15 g (85%) of **V** as a white solid: mp 85.0−86.0 °C dec with gas evolution; TLC R_f 0.36; IR (KBr) 3387 (m, NH or OH), 3243 (m, NH or OH), 1716 (vs, COOH), 1527 (s, amide II), 1324 (s, C═S), 1242 (m, C−O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.20 (s, 1 H, OH), 8.98 (s, 1 H, NH), 7.3 (m, 4 H, ArH-2,3,5,6), 7.1 (m, 1 H, ArH-4), 3.78 (t, J = 7.3 Hz, 2 H, CH_2-4), 3.22 (s, 3 H, NCH_3), 2.26 (t, J = 7.3 Hz, 2 H, CH_2-2), 1.84 (quin, J = 7.3 Hz, 2 H, CH_2-3) (with added D_2O the 12.20 and 8.98 ppm peaks were undetectable); ^{13}C NMR (DMSO-d_6) δ 181.3 (C═S), 174.9 (COOH), 141.4 (ArC-1), 128.4 (ArC-3,5), 126.6 (ArC-2,6), 125.1 (ArC-4), 52.5 (C-4), 39.1 (NCH_3), 31.2 (C-2), 22.7 (C-3). HETCOR and APT experiments confirmed ^1H and ^{13}C− assignments.

1-(5-Carboxypentyl)-3-(4-chlorophenyl)-1-methylthiourea (VI) and 1-(3-carboxypropyl)-3-(4-chlorophenyl)-1-methylurea (VII) were synthesized as described by Schneider et al. (*5*).

1-(3-Carboxypropyl)-1-methyl-3-phenylurea (VIII). The reaction of 4-(methylamino)butanoic acid dihydrate (1.15 g, 7.50 mmol), 16 mL of 1.0 M NaOH, and 0.85 mL (0.93 g, 7.8 mmol) of phenylisocyanate under conditions and workup similar to **I** was followed by the crystallization of the crude product from acetonitrile providing 1.47 g (83%) of **VIII** as white crystals: mp 112.5−113.5 °C dec with gas evolution; IR (KBr) 3371 (vs, N−H), 1694 (vs, COOH), 1624 (vs, urea C═O), 1530 (s, amide II), 1242 (s, C−O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.1 (br, 1 H, OH), 8.20 (s, 1 H, NH), 7.46 (d, J = 8.2, Hz, 2 H, ArH-2,6), 7.22 (dd, J = 8.2 Hz, 7.2 Hz, 2 H, ArH-3,5), 6.92 (t, J = 7.2 Hz, 1 H, ArH-4), 3.30 (t, J = 7.2 Hz, 2 H, CH_2-4), 2.93 (s, 3 H, NCH_3), 2.23 (t, J = 7.3 Hz, 2 H, CH_2-2), 1.73 (t, J = 7.2 Hz, 2 H, CH_2-3); ^{13}C NMR (DMSO-d_6) δ 174.7 (COOH), 155.6 (urea C═O), 140.9 (ArC-1), 128.4 (ArC-3,5), 121.9 (ArC-4), 120.1 (ArC-2,6), 47.6 (C-4), 34.5 (NCH_3), 31.0 (C-2), 23.1 (C-3). COSY, HETCOR, and APT experiments confirmed ^1H and ^{13}C assignments.

1-(3-Carboxypropyl)-3-(3,4-dichlorophenyl)urea (IX). Employing the procedure for **I**, a mixture of 1.36 g (6.5 mmol) of 90% 3,4-dichlorophenylisocyanate, 0.67 g (6.5 mmol) of 4-aminobutyric acid, and 6.6 mL of 1.0 M NaOH gave crude **IX**. Recrystallization from acetonitrile yielded 1.70 g (6.05 mmol, 93%) of **IX** as white needles: mp 151.0−152.0 °C dec; IR (KBr) 3340 (s, NH), 3190 (s, NH), 1693 (s, COOH), 1642 (vs, urea C═O), 1577 (vs, amide II), 1222 (s, C−O) cm^{-1}; ^1H NMR (DMSO-d_6) δ 12.1 (s, 1 H, OH), 8.77 (s, 1 H, ArNH), 7.84 (d, J = 2.4 Hz, 1 H, ArH-2), 7.44 (d, J = 8.8 Hz, 1 H, ArH-5), 7.24 (dd, J = 8.4 Hz, 2.4 Hz, 1 H, ArH-6), 6.35 (t, J = 5.6 Hz, 1 H, NH), 3.10 (dt, J = 6.2 Hz, 6.6 Hz, 2 H, CH_2-4), 2.24 (t, J = 7.4 Hz, 2 H, CH_2-2), 1.66 (quin, J = 7.1 Hz, 2 H, CH_2-3). A COSY experiment confirmed ^1H assignments.

1-(5-Carboxypentyl)-3-(3,4-dichlorophenyl)-1-methylthiourea (X). A mixture of 1.0 g (5.0 mmol) of 3,4-dichlorophenylisothiocyanate, 1.0 g (5.8 mmol) of 6-methylaminohexanoic acid dihydrate, and 1.0

M NaOH to pH 10 was treated as per compound **I**. Crystallization of the crude product from 1:1 (v/v) ethanol/water yielded 1.60 g (4.58 mmol, 92%) of **X**: mp 97.5−98.5 °C; TLC R_f 0.43; ^{1}H NMR (DMSO-d_6) δ 12.0 (br, 1 H, OH), 9.10 (s, 1 H, NH), 7.63 (d, J = 2.4 Hz, 1 H, ArH-2), 7.54 (d, J = 8.7 Hz, 1 H, ArH-5), 7.35 (dd, J = 2.4, 8.7 Hz, 1 H, ArH-6), 3.77 (t, J = 7.4 Hz, 2 H, CH_2N), 3.22 (s, 3H, CH_3N), 2.22 (t, J = 7.3 Hz, 2 H, CH_2-2), 1.6 (m, 4 H, CH_2-3,5), 1.3 (m, 2 H, CH_2-4); ^{13}C NMR (DMSO-d_6) δ 180.5 (C═S), 174.6 (C═O), 141.5 (ArC-1), 130.1 (ArC-3), 129.7 (ArC-5), 127.1 (ArC-2), 126.2 (ArC-4), 125.8 (ArC-6), 53.1 (C-6), 38.8 (N−CH_3), 33.8 (C-2), 26.5 (C-5), 25.9 (C-4), 24.5 (C-3). A DEPT experiment revealed the 38.8 ppm peak among the DMSO peaks.

Preparation of Hapten−Protein Conjugates for Immunogens and Coating Antigens. Haptens **I** and **II** were conjugated to KLH and BSA, respectively, according to Krämer et al. (*14*). Briefly, the following procedure was employed: 0.200 mmol of hapten **I** (MW 306.4) or hapten **II** (MW 278.4), respectively, 0.202 mmol of NHS, and 0.223 mmol of DCC were dissolved in 1 mL of anhydrous DMF. The mixture was stirred for 4 h at room temperature, followed by 12 h at 4 °C (refrigerator). This mixture was centrifuged (6 min, 1400 rpm) to remove the precipitate (urea). The clear supernatants were slowly (20 μL/10 min) pipetted to the stirred KLH and BSA solutions (50 mg/5 mL 0.05 M borate buffer, pH 7.8), respectively. Stirring was continued for additional 16 h at 4 °C (refrigerator). The solutions were centrifuged, and the clear supernatant was transferred to Slide-A-Lyzer cassettes (Pierce, Rockford, IL) for dialysis against aqueous 0.2 M PBS (0.9% (w/v) NaCl) for 4 days. Solutions were then freeze-dried and stored at −25 °C. KLH conjugates were used for immunizations, and BSA conjugates as coating antigens (pAb). For mAb development, BSA conjugates were used for immunizations and KLH and BSA conjugates utilized for screening.

Preparation of Hapten−Enzyme Conjugates for Enzyme Tracers. Enzyme tracers were prepared according to the method described by Schneider et al. (*5*). Briefly, 3 μmol of hapten **I** (MW 306.4), hapten **II** (MW 278.4), or hapten **III** (MW 294.4) separately, 15 μmol of NHS, and 30 μmol of DCC were dissolved in 130 μL of anhydrous DMF. The solution was stirred for 4 h at room temperature followed by 12 h of stirring at 4 °C (refrigerator). The solution was centrifuged (10 min, 1400 rpm), and the clear supernatant was added slowly to the enzyme solution (2 mg of HRP in 3 mL of 130 mM sodium hydrogen carbonate, pH 8.4). This solution was stirred overnight at 4 °C (refrigerator) and centrifuged (10 min at 1400 rpm), and the clear solution was transferred to Slide-A-Lyzer cassettes for dialysis against 130 mM sodium hydrogen carbonate, pH 8.4, for 4 days. The solutions were then stored in dark glass vials at 4 °C (refrigerator) and appeared to be stable for at least 4 years.

Production of Polyclonal Antisera (pAb) for Isoproturon. *Immunization of Rabbits and Screening of Sera.* Six female Chinchilla bastard rabbits (2.5 kg) were used for immunizations: three were immunized with **I**-KLH conjugate and three with **II**-KLH conjugate. Immunogens were dissolved in a sterile, 1.7% NaCl aqueous solution at a concentration of 1 mg/mL. Conjugates (300 μL) and NaCl solution (1200 μL) were mixed with 1500 μL of Freund's complete (first immunization) or Freund's incomplete (all boost injections), respectively, so that 100 μg/rabbit was injected. Boost injections were performed at monthly intervals. All antisera were screened for anti-isoproturon antibodies with the same haptens **I** and **II** conjugated to BSA as coating antigens. For the last boost (14th), 300 μg/rabbit was used. Ten days after the last boost, rabbits were bled and the clear sera were stored either at −25 or −80 °C.

Production of Monoclonal Antibodies (mAbs) for Isoproturon. *Immunization of Rats, Primary Screening, Fusion, Secondary Screening.* Approximately 50 μg of **I**-BSA or **II**-BSA conjugates was injected intraperitoneally (ip) and subcutaneously (sc) into LOU/C rats, using 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. (*15*). Hybridoma supernatants were tested in a solid-phase immunoassay with **I**-KLH or **II**-KLH adsorbed on polystyrene microtiter plates. Following incubation with culture supernatants for 1 h, bound monoclonal antibodies were detected by 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 (5 μg/mL) and mouse anti-rat lambda (5 μg/mL). Tissue supernatants were added for 1 h. After 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 a 2-h incubation, HRP-labeled tracers (50 μL, 1:100) were added without washing. After 30 min, the plates were washed with PBS, and the substrate added. Eleven mAbs reacted positively with the enzyme tracer and were inhibited by isoproturon in binding the tracer.

The immunoglobulin type of monoclonal antibodies was determined by using biotinylated anti-rat IgG subclass-specific monoclonal antibodies (ATCC, Manassas, VA). The best clone, IOC 7E1 (rat IgG2a,κ), was finally used for further studies because of its high affinity to isoproturon.

ELISA-Coating Antigen Format with Polyclonal Antisera. Microtiter plates were coated with **I**-BSA or **II**-BSA, respectively (250 μL/well, 0.1 μg/mL in 0.05 M sodium carbonate buffer, pH 9.6, overnight at 4 °C). The next day, the plates were washed with 4 mM PBST (pH 7.4.-7.6), and 150 μL of isoproturon standard (different concentrations in Milli-Q water) was added to each well. Then 50 μL of pAb 352 (1:40 000 in 40 mM PBST (PBS buffer containing 0.05% (v/v) Tween 20), pH 7.4−7.6) was added per well and the solutions were incubated for 2 h at room temperature. The plates were washed with 4 mM PBST, and 200 μL of goat anti-rabbit conjugated to HRP (dilution 1:17 000 in 40 mM PBST, pH 7.4−7.6; Sigma-Aldrich, Schnelldorf, FRG) was added to each well and incubated for 1 h at room temperature. After another washing step (4 mM PBST), substrate and chromogen for HRP were added (200 μL/well, H_2O_2/TMB in 0.1 M sodium acetate buffer, pH 5.5). The enzyme reaction was stopped after 20−25 min with 50 μL of 2 M H_2SO_4/well. Microtiter plates were read at 450 nm (reference 650 nm). Standard curves were calculated with the 4-parameter equation.

ELISA-Coating Antigen Format with Monoclonal Antibody. Microtiter plates were coated with **I**-BSA or **II**-BSA, respectively (250 μL/well, 0.1 μg/mL in 0.05 M sodium carbonate buffer, pH 9.6, overnight, 4 °C). The next day, the plates were washed with 4 mM PBST, pH 7.6, and 150 μL of isoproturon standard (different concentrations in Milli-Q water)/well and 50 μL of mAb IOC 7E1/well (1:2000 in 40 mM PBST, pH 7.6) was added successively and incubated for 2 h at room temperature. After the washing step with 4 mM PBST, 200 μL of goat anti-rat-mAb-HRP/well (1:25 000 or 16 ng/mL in 40 mM PBST, pH 7.6, Dianova, Hamburg, FRG) was added and incubated for 1 h at room temperature. Another washing step was used to remove unbound material, and 200 μL of substrate/chromogen (H_2O_2/TMB) solution per well was added. The reaction was stopped after 10 min with 50 μL of 2 M H_2SO_4/well and the microtiter plates were read at 450 nm (reference 650 nm).

Enzyme Tracer Format with Polyclonal Antisera. Microtiter plates were coated with 250 μL/well of protein A (2 μg/mL in 0.05 M sodium carbonate buffer, pH 9.6) and maintained overnight at 4 °C. The next day, the plates were washed with 4 mM of PBST and 200 μL of anti-isoproturon-pAb 352 (1:10 000 in 40 mM PBST, pH 7.6) was added to each well and incubated for 2 h at room temperature. For the inhibition step, plates were washed again, and 150 μL of standard (prepared in Milli-Q water) or sample was added to the wells. After a preincubation of 60 min, enzyme tracer **III**-HRP (50 μL, 1:1000 in 40 mM PBS, pH 7.6) was added to each well. Then in 30 min, the plates were washed and 200 μL of HRP substrate/chromogen solution (H_2O_2/TMB in 0.1 M sodium acetate buffer, pH 5.5) was added. The enzyme reaction was stopped after 20−25 min with 50 μL of 2 M H_2SO_4 per well and the absorbance was measured at 450 nm (reference 650 nm). Standard curves were evaluated with the 4-parameter equation.

Enzyme-Tracer Format with Monoclonal Antibody. Microtiter plates were coated with mouse anti-rat-antibodies (200 μL/well, mouse anti-rat kappa TIB-172, 2 μg/mL in 0.05 M carbonate buffer, pH 9.6, overnight, 4 °C, or 2 h at room temperature). The plates were washed

with 4 mM PBST and rat mAb IOC 7E1 (150 μL/well; 1:2000 in 40 mM PBS, pH 7.6) was added. After an incubation for 2 h at room temperature, plates were washed again (4 mM PBST), and then 100 μL/well of isoproturon standards was added (either set up in 40 mM PBS or in Milli-Q water) and incubated for 1 h at room temperature. Then 50 μL/well of enzyme tracer (**III**-HRP 1:500 or 1:1000 in 40 mM PBS, pH 7.6) was added and the mixtures were incubated with shaking for another 30 min at room temperature. After the plates were washed 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 and incubated for 10 min at room temperature. Finally, the 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 4-parameter equation.

Enzyme Tracer Format in Different Organic Solvents. Tests for solvent tolerances were performed with both the pAb and the mAb. ELISA procedures were performed as described above, using the protocols for the pAb and the mAb, respectively. The only difference was that isoproturon standards were set up in different percentages (v/v) of methanol, ethanol, acetonitrile, or acetone. Standard curves were evaluated with the 4-parameter equation.

Analysis of Spiked Water Samples. Water samples from different origins (tap water, creek water, river water, and pond water) were drawn. The pH of the water samples ranged from 7.5 to 9.3. Water samples were spiked in the following way: 30 μL of the corresponding standard concentration was added to each water sample to a final volume of 3 mL; this means the spiked water samples contained 1% of the standard solution. For the spike with 0.5 μg/L, the water sample contained only 0.5% of the standard solution (15 μL in 3 mL). Samples were run in parallel with a standard curve for isoproturon on each microtiter plate. Standards and samples were determined in quadruplicate. The assays were performed as described previously in the paragraphs Enzyme Tracer Format with Polyclonal Antisera and Enzyme Tracer Format with Monoclonal Antibody. Samples measured with polyclonal antiserum were not identical with those measured with monoclonal antibody. The concentrations of the spikes were chosen to fit into the linear dose−response region of the standard curves, so that it was possible to calculate the obtained results.

RESULTS AND DISCUSSION

Synthesis of Haptens. Haptens **I** and **II** were designed specifically for immunizing purposes as they possess the ideal features expressed earlier (*13*). They are exact representations of the target structures with a chemically inert methylene handle sufficiently long to allow flexibility of the target structure that extends from the carrier protein. Note that the longer five-methylene handle provided better test results (**Table 1**) than the three-methylene handle, suggesting that handle length and/or flexibility were important factors in assay utility. Hapten **III**, a thiourea, was designed as a surrogate tracer hapten based on the observation that the best coating/tracer hapten has previously contained a shorter methylene handle, and thus less flexibility, than does the immunizing hapten. The use of the thiourea moiety was based on earlier success with the urea herbicides monuron immunoassay (*5*), and diuron flow injection immunoaffinity analysis (FIIAA) developments (*16*). The rationale for diminished recognition at the "active" site is the loss of H-bonding by the thiourea with the active site as well as a steric rejection due to the larger size of the sulfur moiety compared to oxygen.

Compounds with structure **IV**−**X** were available or synthesized for the purpose of evaluating appendage recognition and potential use for the assay. Thus, there was negligible recognition of all thiourea structures (**III**, **V**, **VI**, and **X**). A 4-isopropylphenyl group was a required appendage for antibody recognition (**I**−**IV**), whereas a phenyl or 4-chlorophenyl group in this position of a urea or thiourea displayed negligible recognition. It is not surprising then that the 13 other arylurea herbicides except isoproturon (**Table 4**) have negligible cross-reactivities in these assays.

Table 2. Assay Optimization with Polyclonal Antisera Enzyme Tracer Format

antiserum dilution	enzyme tracer dilution	IC50% [μg/L]	% CR	
			monuron	diuron
347 1:10 000	**I**-HRP 1:1 000	0.70	0.5	0.9
352 1:20 000	**I**-HRP 1:2 000	0.95	0.5	0.7
352 1:10 000	**II**-HRP 1:8 000	0.96	0.3	0.3
352 1:10 000	**III**-HRP 1:1 000	0.57	0.5	0.3

Although different combinations of immunizing and enzyme tracer hapten could be used, the best combination in optimized assays were **I**-KLH for immunization and **III**-HRP as the enzyme tracer. This was true for the pAb- and mAb-based assays.

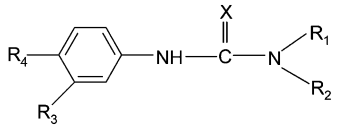
Coating Antigen Format. *Screening of Antisera.* For the determination of the antisera titers, **I**-BSA and **II**-BSA were used as coating antigens for screening the blood sera. Generally, both haptens were recognized by all six antisera, but not all sera showed inhibition with isoproturon. Nevertheless, several sensitive assays for isoproturon were obtained with different sera. With serum 347 (immunizing hapten **II**-KLH) the test midpoint (IC50% value or the concentration of isoproturon that inhibits 50% of the antigen-binding sites of the antibodies) of the optimized standard curves for isoproturon was about 45 μg/L when **I**-BSA was used as the coating antigen, and about 99 μg/L when **II**-BSA was used (data not shown). With the sera 351 and 352 (immunizing hapten: **I**-KLH) the test midpoint of the optimized assay was about 55 μg/L when serum 351 was used and about 102 μg/L when serum 352 (using coating antigen **I**-BSA) was used; the averages of assay midpoints for these sera were about 78 μg/L (pAb 351) and 309 μg/L (pAb 352), when coating antigen **II**-BSA was used. Although all of these antisera were processed further, the most detailed characterizations focused on serum 352.

Table 1 shows the results of the cross reactivities of haptens **I**, **II**, **III**, and **IV** in the coating antigen format with serum 352. These haptens were tested as cross reactants to aid in selecting the best hapten for use as an enzyme tracer (**Table 2**). When coating antigen **I**-BSA was used **(Table 1**, footnote a), the CRs were 345% (**I**), 199% (**II**), 9% (**III**), and 22% (**IV**). When coating antigen **II**-BSA was used (**Table 1**, footnote b), the CRs were 396% (**I**), 184% (**II**), 13% (**III**), and 50% (**IV**).

Enzyme Tracer Format with Polyclonal Antisera. With use of the information from the coating antigen format, enzyme tracers were made with several haptens. Basically, haptens **I**, **II**, and **III** were useful to different extents in the enzyme tracer format. Although the CRs for the different haptens varied from over 300% down to about 10%, the development of sensitive assays was possible with antiserum 352, when all of these derivatives were investigated as potential enzyme tracer haptens. When all derivatives were compared and also the %CR for monuron and diuron were determined, the assay with hapten **III** as enzyme tracer hapten demonstrated a slightly better inhibition curve for isoproturon (IC50% = 0.6 μg/L; **Table 2**). Hapten **IV**, which showed 22% CR (coating antigen **I**-BSA) and 50% (coating antigen **II**-BSA) with antiserum 352 failed to meet our criteria for an enzyme tracer hapten. A selection of four assays is shown in **Table 2**, which could be used for the determination of isoproturon at the lower ppb levels. In addition to serum 352, serum 347 also displayed good sensitivity.

In summary, serum 352 (immunogen **I**-KLH) and enzyme tracer **III**-HRP were selected for extensive evaluation, because

Table 3. Cross Reactivities of Haptens, Using Enzyme Tracer Format with **III**-HRP



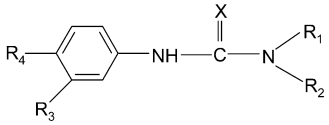
compd	X	R_1	R_2	R_3	R_4	% CR pAb (352)[a]	% CR mAb (IOC 7E1)[b]
isoproturon	O	CH_3	CH_3	H	$CH(CH_3)_2$	100	100
I (immunogen hapten)	O	CH_3	$(CH_2)_5COOH$	H	$CH(CH_3)_2$	128	35
II	O	CH_3	$(CH_2)_3COOH$	H	$CH(CH_3)_2$	78	20
III (enzym tracer hapten)	S	CH_3	$(CH_2)_3COOH$	H	$CH(CH_3)_2$	6	0.4
IV	O	CH_3	CH_2COOH	H	$CH(CH_3)_2$	24	2
V	S	CH_3	$(CH_2)_5COOH$	H	H	<0.01	<0.01
VI	S	CH_3	$(CH_2)_5COOH$	H	Cl	0.01	0.02
VII	O	CH_3	$(CH_2)_3COOH$	H	Cl	0.3	0.2
VIII	O	CH_3	$(CH_2)_3COOH$	H	H	<0.01	<0.01
IX	O	H	$(CH_2)_3COOH$	Cl	Cl	0.6	0.6
X	S	CH_3	$(CH_2)_5COOH$	Cl	Cl	0.01	0.08

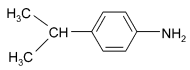
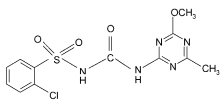
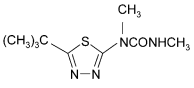
[a] Analyte in Milli-Q water for pAb. [b] Analyte in 40 mM PBS for mAb.

this pair demonstrated the best combination of high absorbances, IC50% value, and the lowest CR for monuron and diuron.

Screening of Monoclonal Antibodies. For screening of positive clones of mAbs, **I**-BSA and **II**-BSA were evaluated as coating antigens. Of the positive clones which recognized **I**- and/or **II**-BSA, only one clone was characterized further, because it showed very good inhibition with isoproturon [IOC 7E1 (sub class IgG2a,κ)]. The coating antigen format was optimized with both coating antigens, **I**- and **II**-BSA. Both coating antigens were recognized by the mAb IOC 7E1 and could be used in assays for isoproturon. The IC50% for isoproturon were very similar with both coating antigens, which was ca. 1.2 μg/L. The CR pattern was similar to that of the polyclonal sera, but here the amount of CR was generally lower (**Table 1**, footnotes a and b). When using coating antigen **I**-BSA, mAb IOC 7E1 had a CR of 89% for hapten **I**, 41% for **II**, 0.05% for **III**, and 1% for **IV**. CR were even lower when coating antigen **II**-BSA was used with the CR being 69% for **I**, 25%

Table 4. Cross Reactivities of Metabolites and Pesticides, Using Enzyme Tracer Format with **III**-HRP



Compound	X	R_1	R_2	R_3	R_4	%CR[a] pAb (352)	%CR[a] mAb (IOC 7E1)
1-(4-Isopropylphenyl)-3-methylurea[b]	O	H	CH_3	H	$CH(CH_3)_2$	31	19
4-Isopropylphenylurea[b]	O	H	H	H	$CH(CH_3)_2$	7	5
4-Isopropylaniline[b]						5	3
Diuron	O	CH_3	CH_3	Cl	Cl	0.4	1.8
Monuron	O	CH_3	CH_3	H	Cl	0.1	0.9
Linuron	O	CH_3	OCH_3	Cl	Cl	0.1	0.2
Chlorbromuron	O	CH_3	OCH_3	Cl	Br	0.2	0.6
Chlortoluron	O	CH_3	CH_3	Cl	CH_3	0.3	3.3
Neburon[b]	O	CH_3	$(CH_2)_3CH_3$	Cl	Cl	1.0	0.4
Fluometuron[b]	O	CH_3	CH_3	CF_3	H	0.1	0.2
Metoxuron	O	CH_3	CH_3	Cl	OCH_3	<0.01	nd
Metabromuron	O	CH_3	OCH_3	H	Br	<0.01	nd
Fenuron	O	CH_3	CH_3	H	H	<0.01	nd
Monolinuron	O	CH3	OCH3	H	Cl	nd	0.09
Chlorsulfuron						<0.01	nd
Tebuthiuron						<0.01	nd

[a] Isoproturon set as 100%; [b] in 1% Methanol (Milli-Q water for pAb; 40 mM PBS for mAb); nd not determined

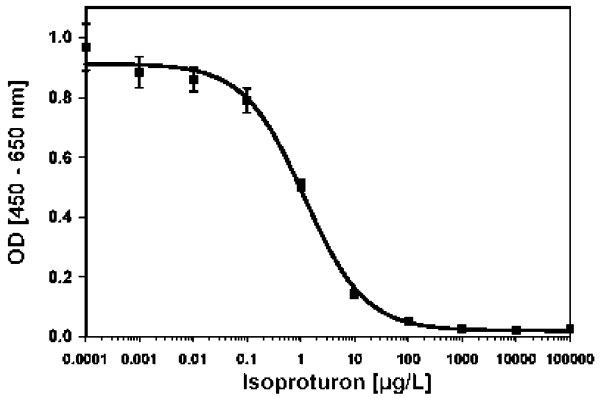


Figure 1. Representative standard curve with rabbit pAb 352 (immunogen **I**-KLH), using **III**-HRP as the enzyme tracer. Microtiter plates were coated with Protein A (2 μg/mL in 50 mM sodium carbonate buffer, pH 9.7); pAb 352 1:10 000 in 40 mM PBST, enzyme tracer **III**-HRP 1:1000 in 40 mM PBS. Standards were set up in Milli-Q water. The standard curve was calculated according to the 4-parameter equation $y = (A - D)/1 + (x/C)\wedge B)) + D$ with the following values: $A = 0.913$; $B = 0.775$; $C = 1.16$ μg/L; $D = 0.021$; $n = 2$. The first concentration, which is presented in the graph as 0.0001 μg/L, refers to 0 μg/L (Milli-Q water).

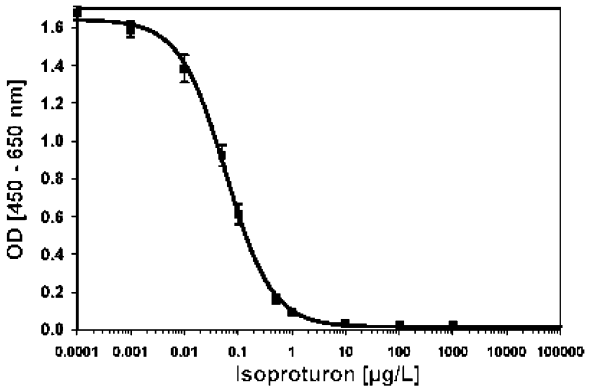


Figure 2. Representative standard curves with rat mAb IOC 7E1 (immunizgen **I**-BSA) and **III**-HRP as the enzyme tracer. Microtiter plates were coated with TIB-172 (2 μg/mL in 50 mM sodium carbonate buffer, pH 9.7); mAb IOC 7E1 (supernatant 1:2000 in 40 mM PBS, enzyme tracer **III**-HRP 1:500 in 40 mM PBS). Standards were set up in Milli-Q water. The standard curve was calculated according to the 4-parameter equation $y = (A - D)/1 + (x/C)\wedge B)) + D$ with the following values: $A = 1.643$; $B = 1.013$; $C = 0.059$ μg/L; $D = 0.017$; $n = 4$. The first concentration, which is presented in the graph as 0.0001 μg/L, refers to 0 μg/L (Milli-Q water).

for **II**, 0.08 for **III**, and 0.7% for **IV**. It is very interesting to point out that derivative **III**, which was later used successfully as an enzyme tracer hapten, has a CR less than 1%. Again, hapten **IV**, which has a slightly higher CR, was not useful as a tracer hapten in the enzyme tracer format.

Standard Curve and Cross Reactivities. *Polyclonal Antiserum.* **Figure 1** shows a representative standard curve for isoproturon, using the serum 352 (dilution 1:10 000; produced from immunizing conjugate **I**-KLH) and enzyme tracer **III**-HRP (dilution 1:1000). The IC50% value was 1.06 $\pm$ 0.34 μg/L ($n = 19$, standard set up in Milli-Q water). This is a slightly less sensitive assay than a former development with sheep pAb (Katmeh et al. (*8*)), and comparable to the assay in the indirect format shown by Ben Rejeb et al. (*10*).

Other candidates with related structures were assayed for recognition, but only derivatives **I**−**IV** showed noticeable CRs (**Table 3**).

Cross reactivities for the major metabolites 4-isopropylaniline, 4-isopropylphenylurea, and 1-(4-isopropylphenyl)-3-methylurea were relatively high, namely 5%, 7%, and 31%, respectively (**Table 4**). All urea herbicides tested demonstrated only minor cross reactivities. Most CRs observed were <1%, with average values for monuron and diuron of 0.1% and 0.4%, respectively. Only neburon showed a CR of ca. 1% (**Table 4**).

Standard Curve and Cross Reactivities. *Monoclonal Antibody.* **Figure 2** shows a typical standard curve for isoproturon, using the rat mAb IOC 7E1 (dilution of culture supernatant: 1:2000; here immunizing conjugate was **I**-BSA) and **III**-HRP as enzyme tracer (dilution: 1:500). When the standards were performed in Milli-Q water, the IC50% value was 0.07 $\pm$ 0.04 μg/L; $n = 7$. When standards were set up in 40 mM PBS, the IC50% value was 0.11 $\pm$ 0.08 μg/L ($n = 33$). Both IC50% values are about 10 times lower than the one obtained with polyclonal serum.

Similarly as shown for pAb immunoassay, standard curves for isoproturon could also be obtained with **I**- or **II**-HRP as enzyme tracer, respectively (data not shown). These standard curves were slightly less sensitive than with **III**-HRP, and also higher amounts of antibody and enzyme tracer had to be used. Therefore, **III**-HRP was utilized in all further optimizations.

In comparison to the polyclonal serum, the cross reactivities for the metabolites 4-isopropylaniline, 4-isopropylphenylurea, and 1-(4-isopropylphenyl)-3-methylurea were lower, being 3%, 5%, and ca. 19%, respectively (**Table 4**).

Among all other urea herbicides tested, only chlortoluron showed a slightly higher CR of about 3% (**Table 4**). Monuron and diuron showed only minor cross reactivities (0.9% and 1.8%, respectively). Other relevant cross reactivities were not seen (**Table 4**).

Although these mAbs were developed in rats, they can be used in the same way as the usually used mouse mAbs. Only the catching antibody or protein have to be adapted to these rat antibodies. Here, we used either mouse anti-rat mAb TIB-172 (κ-specific) or protein G. For conventional ELISA on microtiter plate, the use of culture supernatants was sufficient. For examination of immunosensor formats, it is recommended that protein G purified antibodies will be utilized.

Besides mAb IOC 7E1, a second clone, namely IOC 10G7 (sub class IgG2b,κ), showed a relatively good inhibition curve with an IC50% of about 8 μg/L. Although this mAb was not thoroughly characterized, it is both promising and selective for use in immunoaffinity columns, as it showed only a 0.3% CR for both monuron and diuron.

Effect of Organic Solvents on Isoproturon Immunoassay. **Figure 3** summarizes the effects of organic solvents, namely methanol, ethanol, and acetonitrile, on the isoproturon immunoassay, using both pAb 352 and mAb IOC 7E1, respectively (**Figure 3A**−**C**). Acetone was also tested with mAb IOC 7E1 (**Figure 3D**). Effects were always evaluated in comparison to 0% solvent on the same microtiter plates. The same enzyme tracer **III**-HRP was used throughout all tests.

The isoproturon immunoassay with pAb 352 shows less tolerance to organic solvents, especially when the changes in IC50% values are considered. Methanol can be tolerated up to 5% without a significant change in IC50%, whereas as little as 2% ethanol or acetonitrile display an increase in the IC50%. Organic solvents also show an effect on the maximum absorbance of the assays. Generally, the absorbance decreases with the increase of organic solvent. It should be noted though, that usually higher amounts of organic solvents can be used, when the standards and the sample are set up in the identical amount

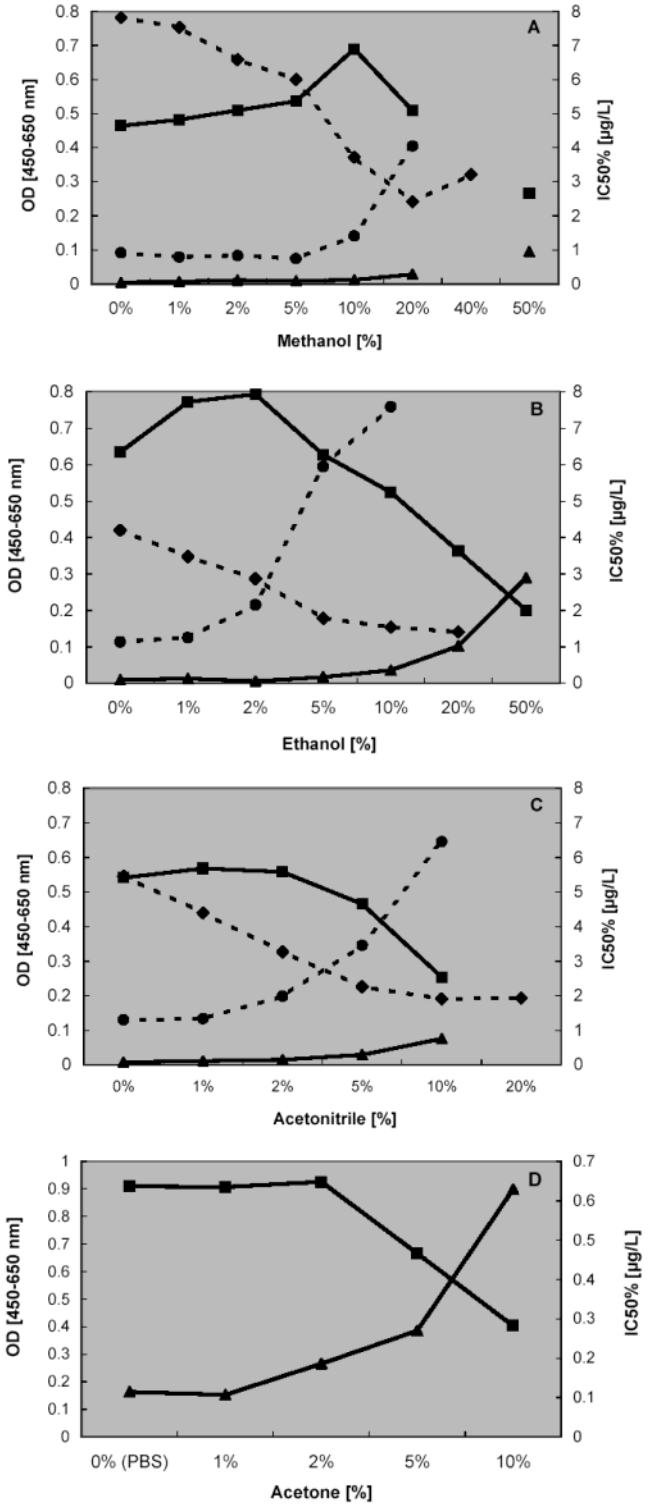


Figure 3. Effect of organic solvents on isoproturon immunoassay, using pAb 352 and mAb IOC 7E1. Solvents: methanol (A), ethanol (B), acetonitrile (C), and acetone (only mAb IOC 7E1 (D)). Dotted lines: pAb 352. Solid lines: mAb IOC 7E1. Key: ●, IC50% pAb 352; ▲, IC50% mAb IOC 7E1; ◆, absorbance pAb 352; ■, absorbance mAb IOC 7E1. Zero percent corresponds to Milli-Q-water when pAb 352 was tested, and to 40 mM PBS when mAb IOC 7E1 was tested.

of solvent. This is especially the case when only the absorbance is affected, but not the IC50%.

Analysis of Spiked Water Samples. Different sets of water samples from different origins were spiked and analyzed either with the isoproturon immunoassay using the pAb 352 or with the isoproturon immunoassay using mAb IOC 7E1. Without spike, all water samples were analyzed as negative (below the limit of detection). When isoproturon was analyzed with the pAb immunoassay, the spiked concentrations in different water matrices were generally found (recovery 93 ± 41%, see **Table 5**). Drinking water showed the best recovery data.

Table 5. Immunoassay Results of Water Samples from Several Sources, Using Isoproturon PAb 352 (immunogen **I**-KLH) and Enzyme Tracer **III**-HRP

water source	pH	amount spiked [µg/L]	amount determined [µg/L][a]
drinking water (tap water)	7.7	without spike	<0.08[b]
		0.1	0.09 ± 0.09
		0.5	0.51 ± 0.17
		1.0	1.2 ± 0.5
		10.0	10.2 ± 4.2
pond water	8.2	without spike	<0.03[b]
		0.1	0.19 ± 0.04
		0.5	0.59 ± 0.05
		1.0	1.3 ± 0.3
		10.0	5.5 ± 1.1
creek water, English Garden, Munich	8.3	without spike	<0.08[b]
		0.1	0.02 ± 0.02[b]
		0.5	0.3 ± 0.22
		1.0	0.63 ± 0.36
		10.0	6.3 ± 0.29
River Isar, Munich	8.5	without spike	<0.08[b]
		0.1	0.14 ± 0.04
		0.5	0.46 ± 0.03
		1.0	0.86 ± 0.08
		10.0	5.8 ± 2.0

[a] $n = 4$. [b] ≤ limit of detection (of the corresponding standard curve on the same plate as the samples).

By using mAb IOC 7E1 the analyses of the water samples could be performed at lower concentrations, which are especially interesting for complying with the EU drinking water directive. Water samples drawn from different locations and matrices were spiked and analyzed. The analysis of tap water showed very good correlations between the spiked and analyzed concentrations (**Table 6**). With some other matrices, the recovery was not as good. Thus it is recommended that the standard curves be performed in the corresponding matrix. In the future, the latter should be checked before screening by conventional analysis for potential contaminations. Nevertheless, this immunoassay would be a very powerful tool for routine sensitive and selective screening of certain water matrices.

General Remarks on the Availability of Immunoreagents. For the success and the further development of the immunoanalytical field, the availability of antibodies and haptens (either for the usage in the coating antigen or in the enzyme tracer) is still a critical factor. Although immunoassay test-kits are commercially available for nearly all relevant pesticides and other environmental contaminants, the immunoreagents alone are not. The latter are the ones needed for new and innovative formats and assay developments. Even if some reagents are available, agreements have to be signed that limit their usage to research only. Up to a certain stage, this might be sufficient, but to bring new immunochemical technologies into a wider range of applications, for example, engineering companies have to be involved. They typically do not have the biochemical expertise in-house and need the access to immunoreagents to guarantee the future customers the needed support. This is up to now not the case. To get out of this "*catch-22*" situation, it would be necessary to find a public storage place, through which both antibodies and haptens would be available to everybody. This might be accompanied by payments, so that the original

Table 6. Immunoassay Results of Water Samples from Several Sources, Using Isoproturon MAb IOC 7E1 (immunogen **I**-BSA) and Enzyme Tracer **III**-HRP

water source	pH	amount spiked [μg/L]	amount determined [μg/L][a]
drinking water (tap water)	7.5	without spike	<0.005[b]
		0.01	0.01 ± 0.003
		0.05	0.05 ± 0.01
		0.1	0.1 ± 0.02
		0.5	0.45 ± 0.06
		1.0	0.8 ± 0.1
		5.0	2.4 ± 0.3[c]
creek water 1 English Garden, Munich	8.3	without spike	≤0.005[b]
		0.01	0.009 ± 0.007
		0.05	0.03 ± 0.004
		0.1	0.08 ± 0.02
		0.5	0.62 ± 0.1
		1.0	1.1 ± 0.6
creek water 2 Schwabinger Bach English Garden, Munich	8.4	without spike	<0.002[b]
		0.01	0.003 ± 0.001
		0.05	0.04 ± 0.02
		0.1	0.06 ± 0.02
		0.5	0.61 ± 0.49
		1.0	1.1 ± 1.0
		5.0	3.2 ± 1.4[c]
creek water 3 Nymphenburg Castle, Munich	8.4	without spike	≤0.003[b]
		0.01	0.008 ± 0.005
		0.05	0.08 ± 0.04
		0.1	0.13 ± 0.03
		0.5	0.98 ± 0.27
		1.0	2.1 ± 0.5
lake water Nymphenburger Castle, Munich	8.3	without spike	≤0.004[b]
		0.01	0.004 ± 0.001[b]
		0.05	0.03 ± 0.01
		0.1	0.07 ± 0.008
		0.5	0.7 ± 0.2
		1.0	1.8 ± 0.5
fountain water 1 Nymphenburg Castle, Munich	8.6	without spike	<0.002[b]
		0.01	<0.002[b]
		0.05	0.02 ± 0.006
		0.1	0.05 ± 0.008
		0.5	0.77 ± 0.24
		1.0	1.2 ± 0.3
fountain water 2 Nymphenburger Castle, Munich	9.3	without spike	≤0.004[b]
		0.01	0.002 ± 0.001[b]
		0.05	0.02 ± 0.02
		0.1	0.05 ± 0.03
		0.5	0.86 ± 0.36
		1.0	3.6 ± 2.2[c]
		5.0	9.8 ± 6.2[c]

[a] $n = 4$. [b] ≤ limit of detection (of the corresponding standard curve on the same plate as the samples). [c] ≥ limit of detection (of the corresponding standard curve on the same plate as the samples).

developer of the reagents can get some of the invested money back, or in the case of a public funded development, the government could use the money for reinvestment in new research.

Future Developments. Conventional immunoassays, such as the ones described herein, are often applied to different formats, both for continuous automated analysis and for discontinuous on-site tests. For isoproturon, the latter has been already realized by using polyclonal sheep antisera (*9*, *17*, *18*). Advantages of these formats are automation, speed, and/or ease of usage.

Our next goals are to apply these newly developed immunoreagents to different formats and applications will be developed with partners in novel projects. One format will be the FIIAA (flow injection immunoaffinity analysis), which was already successfully carried out for atrazine and diuron (*16*, *19*, *20*).

Other formats will be the so-called express immunotechniques based on polyelectrolytes, which were in part already shown by Dzantiev et al. (*21*). In the latter, polyelectrolytes are used for homogeneous or semihomogeneous immunoassay formats, which can be carried out within 30 min (instead of several hours for conventional ELISAs).

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LITERATURE CITED

(1) Bossi, R.; Vejrup, K. V.; Mogensen, B. B.; Asman, W. A. H. Analysis of polar pesticides in rainwater in Denmark by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2002**, *957*, 27−36.

(2) Tomlin, C., Ed. *The Pesticide Manual*, 10th ed.; Royal Society of Chemistry and the British Crop Protection Council: Farnham, Surrey, UK, 1994.

(3) EC Directive water directive relating quality of water intended for human consumption (80/778/EEC) *Off. J. Eur. Commun.* 1980, L229/11.

(4) Council Directive of 3.11.1998 relating to the quality of water intended for human consumption (98/83/EC) *Off. J. Eur. Commun.* 1998, L330, p 32 ff.

(5) Schneider, P.; Goodrow, M. H.; Gee, S. J.; Hammock, B. D. A highly sensitive and rapid ELISA for the arylurea herbicides diuron, monuron, and linuron. *J. Agric. Food Chem.* **1994**, *42*, 413−422.

(6) Karu, A. E.; Goodrow, M. H.; Schmidt, D. J.; Hammock, B. D.; Bigelow, M. W. Synthesis of haptens and derivation of monoclonal antibodies for immunoassay of the phenylurea herbicide diuron. *J. Agric. Food Chem.* **1994**, *42*, 301−309.

(7) Liégeois, E.; Dehon, Y.; de Brabant, B.; Perry, P.; Portetelle, D.; Copin, A. ELISA test, a new method to detect and quantify isoproturon in soil. *Sci. Total Environ.* **1992**, *123/124*, 17−28.

(8) Katmeh, M. F.; Frost, G.; Aherne, W.; Stevenson, D. Development of an enzyme-linked immunosorbent assay for isoproturon in water. *Analyst* **1994**, *119*, 431−435.

(9) Katmeh, M. F.; Godfrey, A. J. M.; Stevenson, D.; Aherne, G. W. Enzyme immunoaffinity chromatography−a rapid semiquantitative immunoassay technique for screening the presence of isoproturon in water samples. *Analyst* **1997**, *122*, 481−486.

(10) Ben Rejeb, S.; Fischer-Durand, N.; Martel, A.; Le Goffic, F.; Lawrence, J. F.; Yeung, J.; Abbott, M. A. Development and validation of an enzyme immunoassay for the detection of the herbicide isoproturon in water matrices. *Intern. J. Environ. Anal. Chem.* **1998**, *69*, 13−30.

(11) Mallat, E.; Barzen, C.; Abuknesha, R.; Gauglitz, G.; Barceló, D. Part per trillion level determination of Isoproturon in certified and estuarine water samples with a direct optical immunosensor. *Anal. Chim. Acta* **2001**, *426*, 209−216.

(12) Mouvet, C.; Broussard, S.; Riolland, H.; Baran, N.; Abuknesha, R.; Ismail, G. Evaluation of ELISA microtiterplate-based assays for the direct determination of isoproturon in water samples and soil extracts. *Chemosphere* **1997**, *35*, 1099−1116.

(13) 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.

(14) Krämer, P. M.; Marco, M.-P.; Hammock, B. D. Development of a selective enzyme-linked immunosorbent assay for 1-naphthol—the major metabolite of carbaryl (1-naphthyl *N*-methylcarbamate). *J. Agric. Food Chem.* **1994**, *42*, 934−943.

(15) 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.

(16) Krämer, P. M.; Franke, A.; Standfu*β*-Gabisch, C. Flow injection immunoaffinity analysis (FIIAA)—a screening technology for atrazine and diuron in water samples. *Anal. Chim. Acta* **1999**, *399*, 89−97.

(17) Pulido-Tofiño, P.; Barrero-Moreno, J. M.; Pérez-Conde, M. C. Flow-through fluoroimmunosensor for isoproturon determination in agricultural foodstuff. Evaluation of antibody immobilization on solid support. *Anal. Chim. Acta* **2000**, *417*, 85−94.

(18) Pulido-Tofiño, P.; Barrero-Moreno, J. M.; Pérez-Conde, M. C. Sol−gel glass with isoproturon antibody as selective support for the development of a flow-through fluoroimmunosensor. *Anal. Chim. Acta* **2001**, *429*, 337−345.

(19) Krämer, P. M.; Baumann, B. A.; Stoks, P. G. Prototype of a newly developed immunochemical detection system for the determination of pesticide residues in water. *Anal. Chim. Acta* **1997**, *347* (1/2), 187−198.

(20) Krämer, P. M. Automated immunochemical analysis of s-triazine and phenylurea herbicides in drinking water supplies. *FTB* **1998**, *36*, 111−118.

(21) Dzantiev, B. B.; Zherdev, A. V.; Yazynina, E. V. Application of water-soluble polymers and their complexes for immunoanalytical purposes. In *Smart Polymers for Bioseparation and Bioprocessing*; Mattiasson, B., Galaev, I., Eds.; Taylor & Francis: New York, 2002; pp 207−229.

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