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Optimization and validation of an enzyme immunoassay for the insect growth regulator fenoxycarb

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Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of the insect growth regulator fenoxycarb. Polyclonal rabbit antisera, raised against protein conjugates of four haptenic derivatives of fenoxycarb, were utilized in immobilized antigen-based, competitive immunoassays. With ELISA systems that were both hapten- and carrier-heterologous, most antiserum titers fell in the range of 1:1000–1:30,000. Assay conditions, including concentrations of antisera and coating antigens, were optimized. The effect of pH, organic solvents, and various blocking agents was also investigated. A hapten-homologous and two hapten-heterologous indirect ELISAs allowed fenoxycarb determination in the range of 0.1–85 ng ml^{-1} with apparent IC_{50} values of 1.2–2.8 ng ml^{-1}. Cross-reactivities with a number of compounds (e.g. pesticides of related structure, hapten synthesis intermediates, fenoxycarb metabolite, photodegradation products) were determined, and the assay proved highly selective for fenoxycarb. In particular, no significant interference was found with selected pyrethroid and juvenile hormone analog insecticides, phenoxyacetic acid herbicides, and photodegradation products of fenoxycarb. Using spiked water samples, assay performance was validated by SPME/GC-MS.
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Keywords: Fenoxycarb; ELISA; Immunoassay optimization; Characterization and validation; GC-MS; SPE; SPME

1. Introduction

Fenoxycarb (Ro 13-5223, Fig. 1, **1**) is an insect growth regulator (IGR) with strong juvenile hormone-mimetic activity [1–6]. This highly selective anti-insect agent, a third generation insecticide, is commonly used in integrated pest management practices [1,5,7–9]. Despite its favorable insect selectivity, it is toxic to certain beneficial insects [4,10–12], aquatic arthropods [13–17] and fish [18]. Because of these features and its environmental persistence, ecotoxicological concerns about some of its applications have been raised. Most traditional methods for the analysis of fenoxycarb are chromatographic techniques such as high performance liquid chromatography (HPLC) [19–24] and gas chromatography (GC) [19,22,24–28]. For rapid analysis of this IGR in environmental samples, an enzyme-linked immunosorbent assay (ELISA) appeared desirable, therefore, development of such assays has been initiated in our laboratories [29,30] and at other research groups [31,32]. Our previous studies focused on the synthesis of haptens **2–5** (Fig. 1) and their protein conjugates

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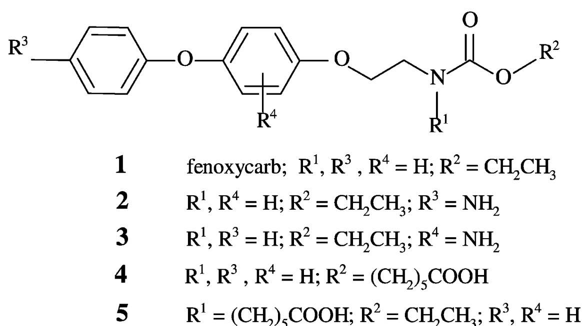

Fig. 1. Chemical structure of fenoxycarb (**1**) and its haptenic derivatives. The haptens contain an amino group on the benzene ring either at the end (**2**) or in the middle (**3**) of the molecule, or a carboxyalkyl moiety either to replace the *O*-ethyl group of the carbamate function (**4**) or on the nitrogen atom of the carbamate group (**5**).

and on the development and characterization of antisera and preliminary ELISAs for fenoxycarb [30]. Here we report on the optimization and validation of hapten-homologous and hapten-heterologous ELISAs.

2. Experimental

2.1. Reagents

Fenoxycarb was isolated from its formulation (Insegar, Syngenta, Basel, Switzerland) purchased from domestic circulation. The commercial solid formulation was dissolved in a mixture of chloroform–ethyl acetate (1:1), the resulting solution was filtered, extracted with water several times, dried over sodium sulfate, and evaporated *in vacuo*. The active ingredient was then purified by recrystallization from a mixture of hexane–ethyl acetate (9:1) to yield fenoxycarb as a white crystalline product (m.p. 52–54 °C, lit. 53–54 °C [6]). Standard solutions of fenoxycarb and related compounds were prepared in methanol and stored at −20 °C. Goat anti-rabbit immunoglobulin (IgG) conjugated to horseradish peroxidase (HRP) was obtained from Institute Human for Serobacteriological and Pharmaceutical Production, Inc. (Gödöllő, Hungary) or from BioRad Laboratories (Hercules, CA, USA). Gelatin from bovine skin and agarose were purchased from Reanal (Budapest, Hungary), various fractions of dextran were from Serva (Heidelberg, Germany) or from Sigma Chemical Co. (St. Louis, MO, USA). Reagent Roti-Block was from Carl Roth (Karlsruhe, Germany), tryptone T (enzymatic casein hydrolyzate) was obtained from Oxoid (Basingstroke, UK). Other chemicals were purchased from Aldrich (Milwaukee, WI, USA), biological reagents and immunochemicals were purchased from Sigma and ICN ImmunoBiological (Lisle, IL, USA), unless otherwise stated. Solvents, obtained from Merck (Darmstadt, Germany) or Aldrich were of analytical grade.

2.2. Instruments

ELISA experiments were carried out in 96-well microplates. Polystyrene microplates were purchased from Nunc (Roskilde, Denmark, No. 442404). Absorbances in the wells of the microplates were read on an iEMS spectrophotometric microplate reader (Labsystems, Helsinki, Finland). The reader was controlled and data were evaluated using the software package Ascent provided by the same manufacturer. GC-MS analyses were carried out on a Saturn 2000 workstation (Varian, Walnut Creek, CA, USA).

2.3. Hapten synthesis and conjugation to carrier proteins

We previously reported the synthesis of haptenic compounds **2**–**5** (Fig. 1) and their protein conjugates [30]. Haptenic compounds containing carboxyl groups (**4** and **5**) were conjugated to carrier proteins using the active ester and mixed anhydride methods, while haptens with aromatic amino groups (**2** and **3**) were linked to proteins by azo coupling as we described earlier [30]. Immunogens were prepared using hemocyanin from keyhole limpet (*Megathura crenulata*) (KLH) and horseshoe crab (*Limulus polyphemus*) (LPH), thyroglobulin (TYG) and conalbumin (ovotransferrin, CONA). Proteins used for sensitizing antigens included bovine serum albumin (BSA), and in certain cases ovalbumin (OVA) or CONA. (The codes of haptenic compounds (**2**–**5**) in our previous publication were identical to those in this paper [30]. Conjugates of haptens **2**, **3**, **4** and **5**, used either as immunogens or sensitizing antigens in the ELISA work, had been designated in the previous article as **14**-protein, **16**-protein, **20″**-protein and **23**-protein,

respectively [30]. Thus, for instance, **14**-BSA conjugate in the previous paper corresponds to **2**-BSA conjugate in this report.)

2.4. Immunization and antiserum collection

An established immunization protocol was followed [33]. Conjugates **2**-KLH, **2**-TYG, **3**-LPH, **3**-TYG, **4**-CONA, **4**-KLH and **5**-KLH were used as immunogens to raise polyclonal antisera in rabbits [30].

2.5. ELISA

Enzyme-linked immunosorbent assays (ELISAs) were carried out according to a modified version of the immobilized antigen-based protocol [30,34]. Thus, ELISA tests were performed on 96-well microplates. Using the appropriate BSA-conjugate diluted in carbonate buffer (0.1 M, pH 9.6, 100 μl per well), conjugates were immobilized by incubating the plates at 4 °C for 12 h. Upon washing the plate three times with 0.01 M phosphate-buffered saline buffer (PBS) (pH 7.4, containing 0.8% NaCl), wells were blocked by incubation with a 1% solution of gelatin (from bovine skin, Reanal) in PBS (150 μl per well) at 4 °C for 1 h. Upon washing the plate three times with PBS containing 0.2% Tween 20 (PBST 0.2), samples or standard solutions diluted in PBS buffer containing 0.05% Tween 20 (PBST 0.05) and 0.5% (v/v) methanol were added to the wells (50 μl per well), followed by the addition of the antiserum diluted in PBST 0.05 (50 μl per well). Plates were incubated at 37 °C for 1 h, washed three times with PBST 0.2, and the enzyme-labeled second antibody (goat anti-rabbit IgG conjugated to HRP) was added at a dilution of 1:12,000 in PBST 0.05 (100 μl per well). The plate was incubated at 37 °C for 1 h, washed three times with PBST 0.2, and enzymatic activity was detected by a chromogenic substrate solution containing 0.32 mg ml^{-1} of 1,2-benzenediamine (*o*-phenylenediamine, OPD) and 0.3 mg ml^{-1} of hydrogen peroxide (100 μl per well). Upon incubation with the substrate at room temperature for 10 min, the enzymatic reaction was stopped with the addition of 4N sulfuric acid (50 μl per well), and absorbancies were read immediately at 492 nm. To prepare standard curves for fenoxycarb and related compounds, a methanolic stock solution (typically 10,000 ng ml^{-1}) of each compound was serially diluted with PBST 0.05 containing 0.5% (v/v) of methanol. Sigmoid standard curves were calculated from absorbance data measures using the Rodbard equation [35].

2.6. Optimization of the ELISA method

2.6.1. pH effect

In order to evaluate the effect of the pH of the medium on assay performance, PBS buffers with pH values between 4.7 and 9.2 with an increment of 0.9 pH unit were prepared. These buffers were supplemented with 0.05% (v/v) Tween 20. Using these buffer-detergent solutions as assay media, fenoxycarb standard inhibition curves were measured in triplicates at each pH.

2.6.2. Solvent effect

Organic solvents are often used for sample preparation. Therefore, several water-miscible common solvents (methanol, ethanol, acetone, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile) were also tested to assess solvent tolerance of the ELISA system. PBS buffers containing 0.05% (v/v) Tween 20 and these organic solvents at various concentrations (0.5, 1, 4, 16 and 32% v/v) were prepared, and fenoxycarb standard curves were measured in triplicates in each buffer as well as in the usual assay buffer, PBST 0.05.

2.6.3. Effect of the blocking reagent

A number of proteins and other biopolymers as well as synthetic polymers were tested for their ability to block the remaining binding capacity of microplates following the immobilization of coating antigens. These compounds included tryptone T, inulin (Sigma No. 3754), agarose, bovine and fish gelatin, milk powder, casein, BSA, OVA, Tween 20, Tween 80, Roti-Block, polyvinylpyrrolidone (PVP), dextran 500, dextran 60, dextran 35, dextran (500) sulfate sodium salt, starch, and agar-agar.

2.7. Application of the ELISA method on aqueous samples

Water samples included distilled water, tap water and various surface water samples (water from the River Danube and surface, lake and river water

samples collected throughout Hungary). In the scope of a national monitoring program during the period of 2000–2002, 281 surface water samples were received from the Soil Conservation and Plant Hygiene Service (SCPHS, Hungary), 41 raw drinking water samples were provided by Wedeco Water and Environmental Technologies (Vác, Hungary), and 10 samples of drinking water were taken as controls.

Tap water and water from the River Danube contained no floating particles, therefore, no filtration step was necessary prior to analysis. Other surface water samples containing floating sediment were filtered, stirred for 1 min, and allowed to stand for 10 min. All water samples were used without any further purification or dilution, their pH was set to 7.4 (surface water samples were slightly alkaline, their pH ranged from 8.1 to 9.1), and 0.5% (v/v) of methanol was added. Standard dilution series of fenoxycarb, starting at 5000 ng ml^{-1} concentration, were prepared in these neutralized water samples, and fenoxycarb content was detected in competitive ELISA.

2.8. Detection of fenoxycarb by GC-MS

Fenoxycarb was detected in water using gas chromatography with a mass spectrometric detector (GC-MS). Distilled and surface water samples were spiked with fenoxycarb at concentrations between 0.25 and 25 ng ml^{-1}, and samples were subjected to solid phase extraction (SPE) prior to GC-MS. Carboprep-90 columns (500 mg, 6 ml, Restek, Bellefonte, PA, USA) were placed on a vacuum suction manifold. The columns were conditioned as follows: a mixture of dichloromethane and methanol (8:2, 5 ml), methanol (2 ml), and then of a 10 g l^{-1} solution of ascorbic acid in distilled water (15 ml) were slowly passed through each column against reduced pressure. Each water sample (1000 ml) was then loaded onto a conditioned Carboprep-90 column, and passed through with a flow rate of 10–15 ml min^{-1}. Each column was rinsed with distilled water (7 ml), remained under suction against air for 10 min to reach air dryness, rinsed with a mixture of methanol and distilled water (1:1, 1 ml), and again remained under air suction for 10 min. Nonacidic contaminants concentrated on the column were eluted with of methanol (1 ml), followed by a mixture of dichloromethane and methanol (8:2, 6 ml) using a low flow rate of 2 ml min^{-1}. Each combined eluate was concentrated under nitrogen to approximately 0.2 ml, isooctane (2 ml) was added, and the solutions were concentrated to 1 ml of final volume.

GC-MS analyses were carried by injecting the above isooctane solutions. GC-MS conditions were as follows: fused-silica column CP-Sil 8 CB, 0.25 μm film thickness, 30 m × 0.25 mm i.d.; injection mode splitless; injection volume 5 μl; injector temperature programmed from 60 °C (held for 0.5 min) to 260 °C a rate of 200 °C min^{-1}, and the final temperature was held for 5 min; column temperature programmed from 70 °C (held for 0.5 min) to 100 °C at a rate of 60 °C min^{-1} and then to 240 °C at a rate of 10 °C min^{-1} and the final temperature was held for 20 min. Helium was used as carrier gas, pressure 0.097 MPa; ionization current, 350 μA; electron energy, 70 eV. The ion trap was scanning in the EI mode from 40 to 650 u. The selected ions for quantitation of fenoxycarb were 116 and 88.

Alternatively, water samples spiked with fenoxycarb were prepared for GC-MS analysis also by solid phase microextraction (SPME). Thus, 4 ml portions of each sample were directly extracted by SPME using a 65 μm thick Carbowax/divinylbenzene (CW/DVB) fiber. SPME fibers and holder assembly were purchased from Supelco (Bellefonte, PA, USA). Extraction time was 20 min at room temperature with stirring by a magnetic stirrer. After extraction, sample desorption from fiber was carried out at 250 °C by direct isothermal injection into the GC system. GC-MS conditions similar as above, except for column temperature that was programmed from 80 °C (held for 1 min) to 300 °C at a rate of 20 °C min^{-1}. The ion trap was scanning in the EI mode from 40 to 650 u.

3. Results and discussion

3.1. Hapten synthesis and conjugation

Haptenic analogues of fenoxycarb and the corresponding protein conjugates (Fig. 1) were synthesized as described before [30]. Because ELISA experiments were carried out in the immobilized antigen (indirect) format, two kinds of hapten-protein conjugates were required. Thus, immunizing antigens (immunogens) for immunization of the experimental animals

and sensitizing (coating) antigens for the ELISA experiments were obtained.

3.2. Antiserum characterization

Antisera and optimized ELISA systems were characterized both by antiserum titers and IC_{50} values detected in the optimized immunoassays. In addition, the lower limit of detection (LOD) was also determined for the studied ELISA systems. Antiserum titer value, by definition, corresponds to the antiserum dilution resulting in uninhibited assay signal three times the background signal under given assay conditions [30]. The IC_{50} value represents the concentration of the analyte resulting in a 50% decrease in the maximal corrected assay signal in the competitive ELISA system. The LOD value is defined as the analyte concentration reducing the mean blank assay signal by three standard deviations of the blank reading.

Immunizations were performed with selected conjugate(s) of each fenoxycarb hapten (**2**, **3**, **4**, or **5**) formed with a carrier protein (KLH, LPH, TYG, or CONA). A number of carrier-heterologous [36] combinations of antisera with conjugates of the protein BSA (in certain cases OVA or CONA) were studied in the immobilized antigen-based ELISA systems. A number of antisera were titrated, and applicable antiserum dilutions were established for hapten-homologous and hapten-heterologous systems [36]. Because antisera based on hapten **5** displayed poor titers (<1:500) in preliminary studies, our subsequent experiments were carried out with antisera raised against only the conjugates of the three other haptens (**2**, **3** and **4**). Fig. 2 displays titration curves for five immunogen/antiserum combinations in three hapten-homologous and two hapten-heterologous ELISA systems. ELISA systems based on the same sensitizing antigen have similar curve shapes (Fig. 2).

Titer values of selected ELISA systems are listed in Table 1. These values ranged mostly between 1:2500 and 1:80,000, although titer values exceeding 1:80,000 have also been obtained in favorable cases. Most antisera displayed relatively high titers with the hapten-homologous sensitizing antigen, but good titer

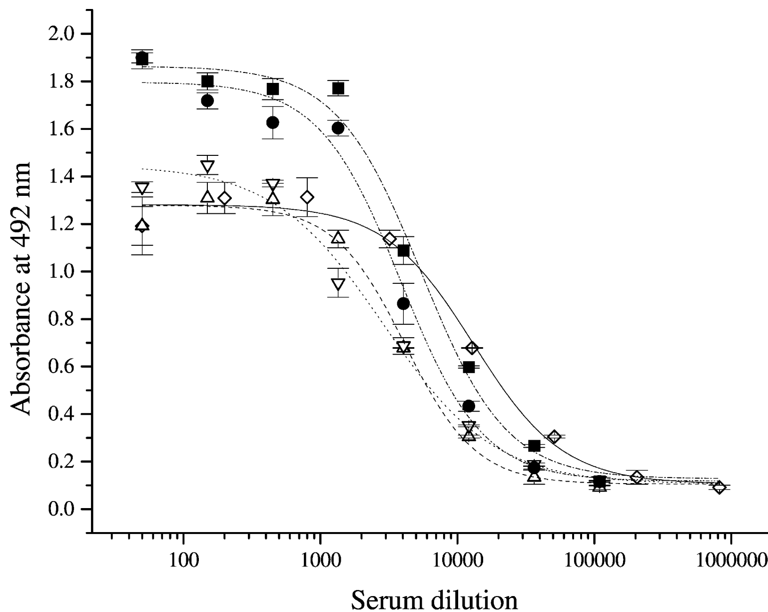

Fig. 2. Titration curves obtained with selected antisera using hapten-homologous and -heterologous coating antigens. Hapten-homologous systems: coating antigen: 1 $\mu g\,ml^{-1}$ **2**-BSA conjugate; antiserum: **2**-KLH(1) (△), **2**-TYG(3) (▽), 5 $\mu g\,ml^{-1}$ **4**-BSA conjugate, antiserum: **4**-KLH (◇); hapten-heterologous systems: 2.5 $\mu g\,ml^{-1}$ **3**-BSA conjugate, **2**-KLH(1) (■); **2**-TYG(3) (◆). Other assay parameters: blocking: 1% gelatin in PBS; anti-IgG-HRP: 1:12,000. Assays were carried out in triplicates in a single microtiter plate using serial dilutions of antisera.

Table 1
Titer values of antisera obtained against various immunogens in immobilized antigen based ELISA systems using various sensitizing antigens at different concentrations

Coating antigen concentration (μg ml^{-1})	Immunizing antigen (antiserum number)	Titer, coating antigen[a]		
		2-BSA	**3**-BSA	**4**-BSA
5	**2**-KLH(1) (4945)	77000	21000	1200
	2-KLH(2) (4946)	30000	21000	160
	2-KLH(3) (4947)	42000	21000	420
	2-TYG(1) (4941)	49000	21000	420
	2-TYG(2) (4943)	37000	8700	240
	2-TYG(3) (4944)	88000	8700	100
	3-HLP(1) (4961)	–	24000	26500
	3-HLP(2) (4962)	–	30000	9500
	3-HLP(3) (4963)	1700	24000	84500
	3-TYG(1) (536)	–	26000	29000
	3-TYG(2) (537)	2500	20000	500
	3-TYG(3) (4960)	14000	20000	5700
2.5	**2**-KLH(1)	85000	18000	–
	2-KLH(2)	28000	13000	–
	2-TYG(1)	39000	13000	–
	2-TYG(3)	70000	12000	–
	3-HLP(3)	16000	38000	–
	3-TYG(3)	32000	45000	–
1	**2**-KLH(1)	55000	4000	–
	2-KLH(2)	30000	1300	–
	2-TYG(1)	53000	1700	–
	2-TYG(3)	80000	2300	–
	3-HLP(3)	70000	11500	–
	3-TYG(3)	15000	9000	–

[a] Titration experiments using conjugate **5**-BSA as coating antigen (5 μg ml^{-1}) with the above antisera gave less satisfactory results.

values were recorded in certain hapten-heterologous systems as well. Using both hapten-homologous and -heterologous systems, the concentration of the sensitizing antigen in the coating buffer has been systematically varied in these experiments. Moreover, titration tests allowed comparisons among antisera obtained against the same immunogen in different experimental animals.

3.3. Competitive inhibition experiments

In the inhibition experiments, calibration curves were established using the standard dilution series of fenoxycarb starting at 5000 ng ml^{-1}. Inhibition curves were measured at fixed antiserum dilutions uniformly set to produce approximately 70% of the maximal signal levels seen in the titration curves. Preliminary observations indicated that the presence of small amounts (0.5–1%, v/v) of methanol as a co-solvent in the assay buffer significantly improved assay sensitivities and standard curve slopes (see Section 3.6). This beneficial effect was seen both in hapten-homologous and -heterologous assays, and resulted in lower IC_{50} values for fenoxycarb, as compared to earlier results [30]. Therefore, 0.5% (v/v) of methanol was used in the sample buffer in all standard curve and sample determinations, and the reported IC_{50} values refer to such conditions unless indicated otherwise.

Sensitizing antigens containing haptens **2**, **3** and **4** were applied to the microplates at three concentrations (5, 2.5, and 1 μg ml^{-1}). Titration experiments indicated different antiserum titers at different sensitizing antigen concentrations; therefore, different antiserum dilutions were applied in the corresponding competitive inhibition tests as well. IC_{50} values detected and antiserum dilutions applied in the various

Table 2
IC_{50} values of antisera obtained against various immunogens in immobilized antigen based ELISA systems using various sensitizing antigens at different concentrations

Coating antigen concentration ($\mu g\,ml^{-1}$)	Immunizing antigen	Coating antigen: **2**-BSA Serum dilution	**2**-BSA IC_{50} ($ng\,ml^{-1}$)	**3**-BSA Serum dilution	**3**-BSA IC_{50} ($ng\,ml^{-1}$)	**4**-BSA Serum dilution	**4**-BSA IC_{50} ($ng\,ml^{-1}$)
5	**2**-KLH(1)		–	1:1000	30	1:650	–
	2-KLH(2)	–	–	1:1000	100	1:150	–
	2-KLH(3)	–	–	1:1000	–	1:200	–
	2-TYG(1)	–	–	1:1000	320	1:180	–
	2-TYG(2)	–	–	1:1000	–	–	–
	2-TYG(3)	–	–	1:1000	10	–	–
	3-HLP(1)	–	–	1:400	10000	1:2200	890
	3-HLP(2)	–	–	1:500	–	1:1600	450
	3-HLP(3)	1:160	250	1:500	~1000	1:6300	110
	3-TYG(1)	–	–	1:2000	10000	1:1000	110
	3-TYG(2)	1:160	1000	1:400	–	1:80	–
	3-TYG(3)	1:500	250	1:500	–	1:500	250
2.5	**2**-KLH(1)	1:160	250	1:2000	2.3	–	–
	2-KLH(2)	1:650	1000	1:1000	80	–	–
	2-TYG(1)	1:650	3160	1:900	2500	–	–
	2-TYG(3)	1:1000	110	1:2000	1.1	–	–
	3-HLP(3)	1:4000	350	1:4600	900	–	–
	3-TYG(3)	1:3200	2510	1:3600	3200	–	–
1	**2**-KLH(1)	1:2000	28	1:460	35	–	–
	2-KLH(2)	1:1000	450	1:380	28	–	–
	2-TYG(1)	1:1200	1410	1:460	–	–	–
	2-TYG(3)	1:4000	2.7	1:600	8	–	–
	3-HLP(3)	1:600	200	1:2500	10000	–	–
	3-TYG(3)	1:1200	1260	1:2500	10000	–	–

ELISA systems are listed in Table 2. The IC_{50} values obtained in the competitive inhibition tests showed a great deal of variation. It is apparent, however, that the IC_{50}s, as expected [37], in most cases decrease with the decrease of the sensitizing antigen concentration.

In our initial studies, two hapten-homologous and a hapten-heterologous assay systems were selected on the basis of assay sensitivity (IC_{50}) [30]. The titration and competition curves, based on a preliminary assay protocol, were presented for the selected systems in our previous paper [30]. For these systems, the coating antigen, antiserum, and IC_{50} ($ng\,ml^{-1}$) were as follows: **2**-BSA (1 $\mu g\,ml^{-1}$), anti-**2**-KLH(1) (1:3000), 102; **4**-BSA (5 $\mu g\,ml^{-1}$), anti-**4**-KLH (1:1000), 95; **3**-BSA (2.5 $\mu g\,ml^{-1}$), anti-**2**-KLH(1) (1:1500), 17 [30].

In this work, several new, highly sensitive ELISA systems were developed beside the selected systems above. Furthermore, the assay protocol was optimized to increase sensitivities. The resulting new standard calibration curves are shown in Fig. 3. With the improved assay conditions, IC_{50}s of the hapten-homologous systems ranged between 2.7 and 95 $ng\,ml^{-1}$ (2.7 $\pm$ 1.6 $ng\,ml^{-1}$ for **2**-BSA/**2**-TYG(3), 28 $\pm$ 7.8 $ng\,ml^{-1}$ for **2**-BSA/**2**-KLH(1) and 95 $\pm$ 23 $ng\,ml^{-1}$ for **4**-BSA/**4**-KLH). IC_{50}s of the hapten-heterologous systems were 1.1 $\pm$ 0.6 $ng\,ml^{-1}$ for **3**-BSA/**2**-TYG(3) and 2.3 $\pm$ 0.3 $ng\,ml^{-1}$ for **3**-BSA/**2**-KLH(1). LODs in the hapten-homologous systems were 0.2–4.6 $ng\,ml^{-1}$, while those in the hapten-heterologous systems were calculated to be 0.11–0.2 $ng\,ml^{-1}$. In certain cases, these values represented a four- to sixfold increase in assay sensitivity relative to our earlier results [30]. It appears that this improvement is due to both the

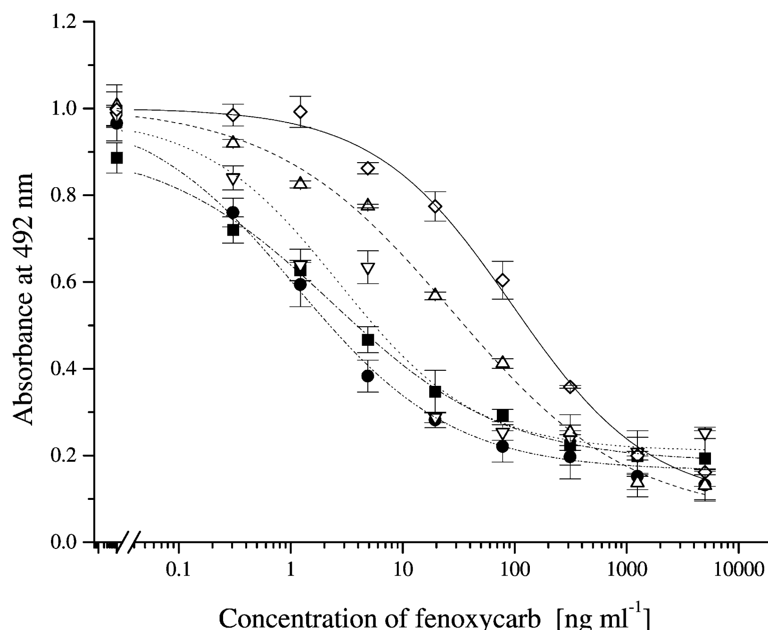

Fig. 3. Standard curves for fenoxycarb in five optimized competitive indirect ELISA systems based on coating antigens **2**-BSA, **3**-BSA and **4**-BSA. Hapten-homologous systems: coating antigen: 1 μg ml^{-1} **2**-BSA conjugate; antiserum: **2**-KLH(1) (1:5000) (△), **2**-TYG(3) (1:4000) (▽), 5 μg ml^{-1} **4**-BSA conjugate, antiserum: **4**-KLH (1:1000) (◇); hapten-heterologous systems: 2.5 μg ml^{-1} **3**-BSA conjugate, **2**-KLH(1) (1:2000) (■); **2**-TYG(3) (1:2000) (◆). Other assay parameters: blocking: 1% gelatin in PBS; assay buffer with 0.5% (v/v) of methanol; anti-IgG-HRP: 1:12,000. Assays were carried out in triplicates in a single microtiter plate using spiked concentrations of fenoxycarb of 5000, 1250, 312.5, 78.1, 19.5, 4.88, 1.22, 0.305 and 0 ng ml^{-1}.

solvent effect of methanol (see below) and other assay parameters. In both our preliminary studies [30] and this work, the hapten-heterologous systems were generally more sensitive than the corresponding hapten-homologous ones, as expected [30,37].

3.4. Cross-reactivity

When developing an ELISA system, it is not sufficient to only prove its efficacy in buffer or given matrices, but several other examinations are also required. One of these is the evaluation of cross-reactivities to reveal which molecules besides fenoxycarb may bind to the anti-fenoxycarb antibodies used in the immunoassay. The assay signal only indicates binding, but does not specify what compound has bound to the antibodies. Thus, cross-reactivities of numerous substances either structurally related to fenoxycarb or potentially appearing together with it as contaminants were determined using the optimized ELISA systems.

Forty-two compounds tested in cross-reactivity (CR) studies included haptens (**2–5**), synthetic intermediates of fenoxycarb (Fig. 4, **8–11**, **14–19**) as well as fenoxycarb photodegradation products (**12**, **21**, **24**) and metabolite (**6**). A fenoxycarb precursor (**7**), pesticides structurally related to fenoxycarb (e.g., W-328 (**13**), pyriproxyfen, phenoxyacetic acid herbicides, pyrethroids), natural and synthetic isoprenoids (e.g. farnesol, methyl farnesoate, methoprene and the ammonium salt of its corresponding carboxylic acid derivative), and some industrial chemicals were also studied. The chemical structures of some of these compounds are seen in Fig. 4. Pesticides tested included chitin synthesis inhibitor (e.g. diflubenzuron (**29**), chlorfluazuron (CGA 112913) and analogue BAY SIR 8514, **30**) and pyrethroid insecticides (permethryn, cypermethrin, deltamethryn) and herbicides (2,4-D, dichlofop, chloroxuron, difenoxuron, acifluorfen, fluorodifen). Several other substances (e.g. clofibrate (**20**), 3-octylthio-1,1,1-trifluoro-2-propanone (**22**), 4-phenyl-acetophenone (**23**), phenoxybenzyl

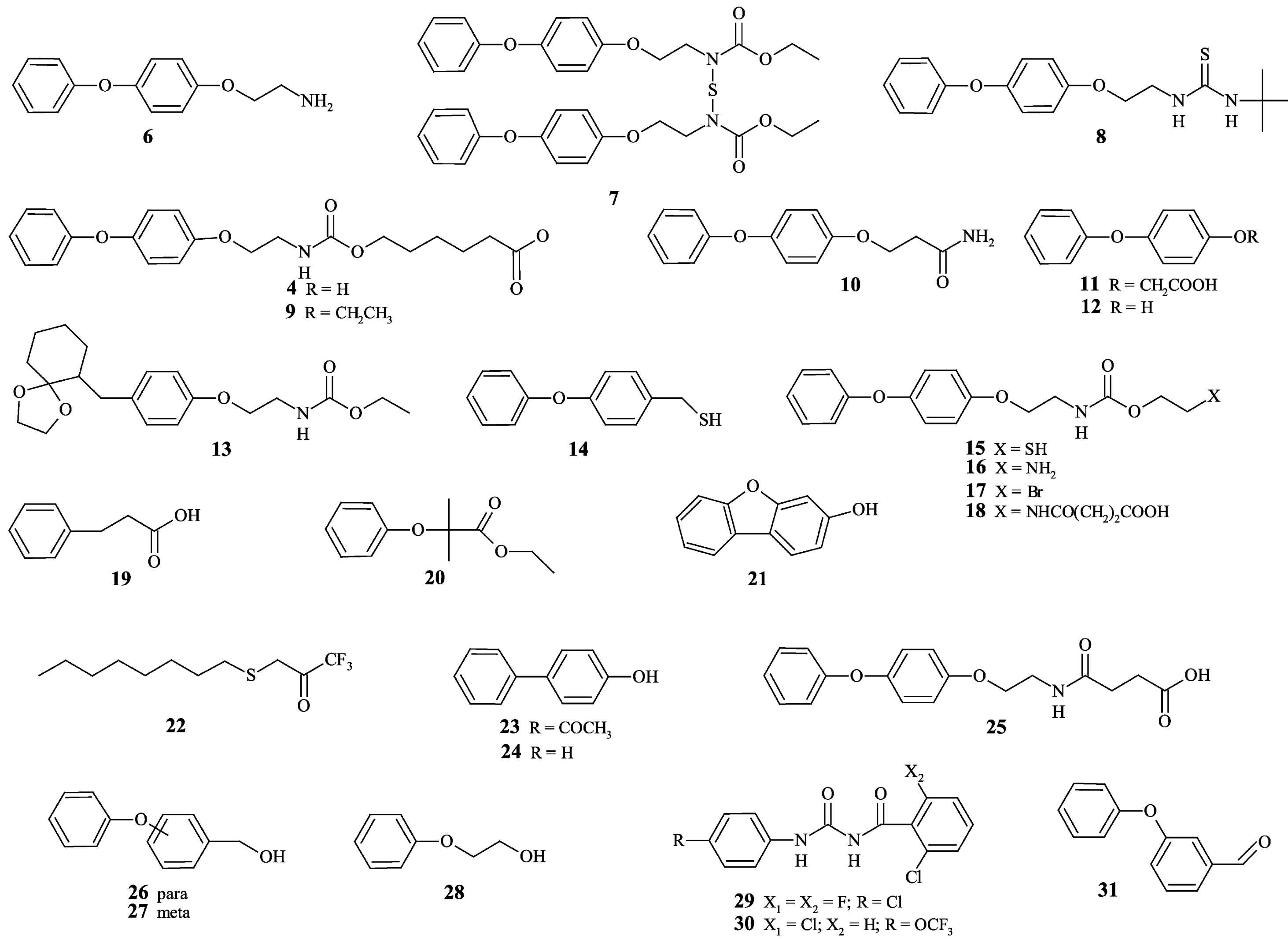

Fig. 4. Chemical structures of various chemicals structurally related to fenoxycarb (**1**), tested in cross-reactivity studies.

Table 3
Cross-reactivities of selected compounds in two optimized fenoxycarb ELISA systems

Cross-reactant[a]	Immunizing antigen							
	2-KLH(1)				**2**-TYG(3)			
	Coating antigen				Coating antigen			
	2-BSA		**3**-BSA		**2**-BSA		**3**-BSA	
	IC_{50} (ng ml^{-1})	CR[b] (%)	IC_{50} (ng ml^{-1})	CR[b] (%)	IC_{50} (ng ml^{-1})	CR[b] (%)	IC_{50} (ng ml^{-1})	CR[b] (%)
1	89.1 ± 20.8	100	7.49 ± 0.44	100	53.6 ± 7.9	100	4.08 ± 1.06	100
4	3577	2.5	300.7	2.4	3647	1.5	269.4	1.5
6	–	–	535.1	1.4	–	–	3.32	122.9
7	576	15.5	35.2	21.3	390	13.7	41.3	9.9
8	761	11.7	98.6	7.6	890	6.03	37.4	10.9
9	912	9.8	96.0	7.8	474	11.3	48.4	8.4
10	1290	6.9	253	2.9	169.4	31.6	168	2.4
11	–	–	–	–	–	–	1208	0.34
12	–	–	3570	0.2	–	–	15800	–
13	5536	1.6	447.2	1.7	2071	2.6	334.5	1.2
14	1274	7.0	1531	0.5	2840	1.9	–	–
15	–	–	–	–	–	–	4170	0.10
19	–	–	(88.4%)[c]	–	–	–	–	–
20	–	–	(70.3%)[c]	–	–	–	–	–
22	–	–	(69.8%)[c]	–	–	–	–	–
23	–	–	(68.1%)[c]	–	–	–	–	–

[a] The table lists only compounds that produced significant inhibition with CR ≥0.1% in most cases. Tested compounds that display CR <0.1% include substances **16**–**18**, **21**, **24**–**28** and **31** shown in Fig. 4, as well as methyl farnesoate, farnesol, methoprene and the ammonium salt of its corresponding carboxylic acid derivative, pyriproxyfen, diflubenzuron (**29**), chlorfluazuron (CGA 112913), BAY SIR 8514 (**30**), permethryn, cypermethrin, deltamethryn, 2,4-D, dichlofop, chloroxuron, difenoxuron, acifluorfen, fluorodifen, 3,4-dimethoxyphenol, 4-nitrobenzyl chloride, 4-nitrophenol, phenylthiourea, as well as reduced and oxidized glutathione.

[b] CR value (%) = (IC_{50} of fenoxycarb/IC_{50} of compound) × 100.

[c] Due to incomplete inhibition curves, IC_{50} values were usually not determined above the 10 μg ml^{-1} analyte level (except for compound **13**, where almost 50% inhibition was reached at 10 μg ml^{-1}). Values in parentheses indicate assay signals at 10 μg ml^{-1} concentration of the given compound as percentage of the maximal (uninhibited) immunoassay signal.

alcohols (**26**, **27**), 2-phenoxyethanol (**28**), phenoxybenzaldehyde (**31**), phenylthiourea, glutathione both in reduced and oxidized forms, 4-nitrobenzyl chloride, 4-nitrophenol, 3,4-dimethoxyphenol) with structures not closely related to that of fenoxycarb were also investigated. Cross-reactivities detected in optimized ELISA systems are listed in Table 3.

Results of the cross-reactivity evaluation indicate that the optimized ELISA systems display cross-reactivity higher than 10% only for a very limited number of compounds tested, and these compounds were all chemical derivatives of fenoxycarb. Other compounds do not show significant cross-reactivity to the antibodies. Cross-reactivities were below 1–2% for the vast majority of compounds tested. Antiserum **2**-TYG(3) showed high affinity towards the backbone amine of fenoxycarb, 2-(4-phenoxyphenoxy)ethylamine (**6**). With three assay systems (Table 3), the highest cross-reactivity was seen for a bis-sulfenyl compound (**7**), a proinsecticide derivative of fenoxycarb [38]. Further compounds with high cross-reactivities included hapten **4** and similar synthetic derivatives (**8**, **9**). Only marginal cross-reactivity was measured for an IGR (W-328, **13**) with structure related to that of fenoxycarb. Low or no cross-reactivity was found for 4-phenoxyphenol (**12**), a fenoxycarb metabolite [30], and for 2-hydroxydibenzofuran (**21**) and 4-phenylphenol (**24**), photodegradation products of fenoxycarb [25]. The lack of considerable interferences with these compounds may be significant in biological and environmental applications of the ELISAs. Furthermore, no cross-reactivity was

observed phenoxyacetic acid herbicides, other IGRs including sesquiterpenoid insect hormone analogues and pyriproxyfen, and benzoyl-phenylurea type chitin synthesis inhibitor insecticides. A low but surprising inhibition was, however, seen by at high concentrations of clofibrate (**20**).

Our cross-reactivity data demonstrate that the antisera are highly fenoxycarb-selective. This leads to two major implications: (a) because no other pesticide active ingredients of related structure displayed cross-reactivity, the immunoassay can be used to detect fenoxycarb in samples possibly containing other pesticides, and (b) because major fenoxycarb metabolites did not cross-react either, the assay can be applied to monitor fenoxycarb as active ingredient. This latter finding served useful when the immunoassay was applied to test fenoxycarb content in tissues of the silkworm, *Bombyx mori* [39,40].

3.5. pH effects

Another factor affecting assay performance was the acidity of the assay medium. Fenoxycarb standard curves were therefore obtained at several pH values, and pH effects evaluated based on the shape and IC_{50} value of each curve (Fig. 5). Both assay signals and curve slopes appeared to decrease with extreme pH values (4.7, 9.2). Maximal signal intensity was seen at neutral pH (7.4). Standard curves indicated that the system better tolerates slightly acidic than alkaline media. Assay performance appears to be only moderately affected by changes in pH between 6.5 and 8, and has an optimum around 7.4.

3.6. Organic solvent effects

Because fenoxycarb is often determined in the presence of organic solvents used for extraction of sample matrices, the effect of various concentrations of some organic solvents in the assay buffer was also evaluated. Organic solvents most commonly used for sample extraction include acetone, methanol, ethanol, acetonitrile, DMF, DMSO and ethyl acetate. These are water-miscible solvents except for the latter one that is slightly soluble in water. Even at low concentrations, DMF, DMSO and acetonitrile dramatically reduced

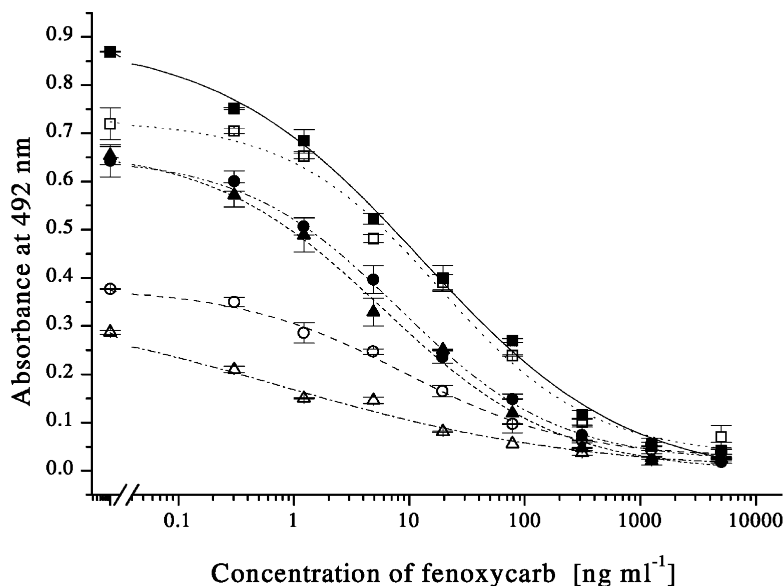

Fig. 5. Standard curves for fenoxycarb in the hapten-heterologous ELISA system at different pH values of the assay medium. (**3**-BSA as coating antigen at 2.5 μg ml^{-1}; anti-**2**-KLH(1) antiserum at dilution 1:2000; other assay parameters: blocking: 1% gelatin in PBS; anti-IgG-HRP: 1:12,000. The assay buffers did not contain methanol in these experiments). Standard curves obtained in assay buffer at pH 4.7 (△), 5.6 (○), 6.5 (□), 7.4 (■), 8.3 (●) and 9.2 (△). Assays were carried out in triplicates in a single microtiter plate. The corresponding IC_{50} values detected were 0.75, 9, 18, 26, 8.2 and 6 ng ml^{-1}, respectively.

the signal intensity. Acetone also did so, yet to a lesser extent (18% decrease in the maximal assay signal at 1% acetone content). When the assay buffer was saturated with ethyl acetate, the resulting standard curves were distorted. Therefore, the use of these solvents for extraction prior to immunoassay should be avoided. Up to 4–5%, ethanol and particularly methanol were, however, well-tolerated in the assay system. Thus, a methanolic sample extract should be diluted with at least 19-fold volume of assay buffer prior to ELISA analysis. The presence of small amounts of methanol (0.5–1% v/v) in the sample buffer improved assay sensitivities and curve slopes without considerable influence on other characteristics of the standard curve (Fig. 6). (The above methanol content refers to the sample buffer; therefore, the corresponding final concentration of methanol in the well (incubation buffer) is 0.25–0.5%.)

A possible interpretation of these results is that a small amount of methanol assisted solvation of the highly lipophilic moiety of the fenoxycarb molecule in the aqueous assay buffer. Due to the favorable effect of low concentrations of methanol, sample buffer containing 0.5% of methanol was further used in the standard assay protocol.

3.7. The effects of blocking

Blocking is an essential step in immunoassays to avoid high background signals due to nonspecific binding of primary or secondary antibodies to the solid surface. In immobilized antigen-based, competitive immunoassays, strong nonspecific binding of the primary antibody may prevent effective competition by the analyte and, thus, result in lower assay sensitivity. It is important to achieve good coverage of the unoccupied sites on the plastic surface after immobilization of the sensitizing antigen. To that end, proteins and other biopolymers as well as synthetic polymers were applied as blocking reagents in this work. Blocking capacities were ranked on the basis of the IC_{50} and slope values of the fenoxycarb standard curves (Table 4). Background and maximum signal intensities of the standard curves were also taken into account.

Blocking agents that are widely used in immunoassays can be classified into three categories: proteins (e.g. BSA, casein, gelatin, OVA, nonfat dry milk), nonprotein substances (e.g. PVP, polyvinyl alcohol, the synthetic blocking agent Roti-Block), and detergents (e.g. Tween 20, Triton X-100) [41]. The latter group mostly comprises nonionic polyoxyethylene

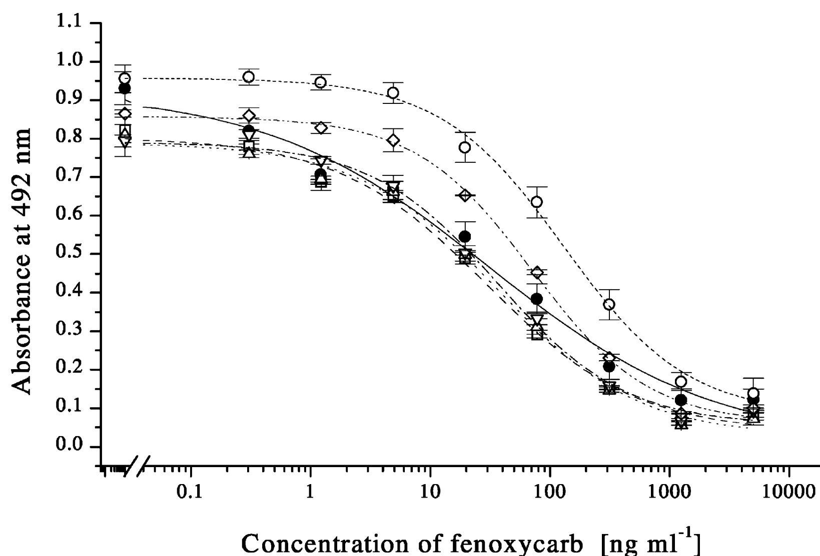

Fig. 6. Standard curves for fenoxycarb in the optimized hapten-homologous ELISA system at different concentrations of methanol in the assay buffer (**2**-BSA as coating antigen at 1 μg ml^{-1}; anti-**2**-TYG(3) antiserum at a dilution of 1:4000; other assay parameters: blocking: 1% gelatin in PBS; anti-IgG-HRP: 1:12,000). Standard curves obtained in assay buffer (●) and in assay buffer containing 0.5% (□), 1% (△), 4% (▽), 16% (◇) and 32% (○) of methanol. Assays were carried out in triplicates in a single microtiter plate.

Table 4
The effect of blocking capacity achieved by various blocking agents in the optimized hapten-heterologous fenoxycarb ELISA system

Blocking agent	IC_{50} [a] (ng ml^{-1})	Slope
Inulin	4.28	0.71
Gelatin from bovine skin	4.52	1.11
Milk powder	5.83	0.67
Agar-agar	6.07	0.72
Agarose	7.34	0.77
Casein	7.58	0.73
Starch	8.02	0.76
Dextran (MW 500000)	8.36	0.95
Dextran sulfate (MW 500000)	8.55	0.72
Gelatin from fish skin	10.5	0.64
Dextran 60 (MW 60000–90000)	10.8	0.62
Tryptone T	11.1	0.82
BSA	11.2	0.53
Tween 80	11.5	1.19
OVA	11.9	1.02
Tween 20	12.5	1.12
Dextran 35 (MW 35000–50000)	12.6	0.72
Polyvinylpyrrolidone (PVP, MW 350000)	15.1	1.10
Roti-Block	16.4	1.02
Without blocking	33.7	1.33

[a] IC_{50} values were detected in the optimized hapten-heterologous ELISA system using antiserum **2**-KLH(1) at a dilution of 1:2000, and 2.5 μg ml^{-1} **3**-BSA as coating antigen. Blocking reagents were applied at a concentration of 1%.

derivatives. Proteins are considered to be the most effective blocking agents [41]. We found that optimal blocking was achieved with gelatin from bovine skin. Somewhat less effective blocking was seen with milk powder, casein or casein hydrolysate (tryptone T), and BSA. Typical nonprotein blocking agents are water soluble, highly polar synthetic polymers. The reported successful use of these polymers and the polyoxyethylene-based detergents for blocking in some immunoassays [41] prompted us to also employ highly polar biopolymers other than proteins in our blocking studies. The use of some of these biopolymers (e.g. inulin, agarose, starch) also resulted in effective blocking in our experiments. When dextran fractions (dextran 35, dextran 60, dextran 500) were used for blocking, the larger the average molecular weight of dextran, the greater the blocking effect was. By the collective adhesive forces of the monomer units, polymers can bind to the surface more tightly than the corresponding lower molecular weight derivatives.

3.8. Validation of the ELISA by GC-MS methods

In order to verify fenoxycarb levels determined by ELISA in spiked water samples, fenoxycarb content was also detected in the same samples using an instrumental technique, GC-MS. For this purpose, SPE and SPME were both used as sample preparation methods. In SPE, significant improvement was achieved when temperature programmed injection was used (allowing a higher injection volume, 5 μl). The retention time of fenoxycarb with temperature programmed injection was 21.2 min. (With isothermal injection at 250 °C, it was 18.2 min.) In spite of the longer retention time, the minimal detectable amount (MDA) decreased to one-fifth (12.5 ng), and therefore the LOD decreased to 3 ng ml^{-1}. Recoveries were tested at 2LOD (6 ng ml^{-1}) and 5LOD (15 ng ml^{-1}) spike levels, and were found to be $102.3 \pm 0.58\%$ and $109.1 \pm 11.2\%$, respectively. SPE/ GC-MS determinations indicated that none of the 118, 119 and 95 water samples collected in Hungary in 2000, 2001 and 2002, respectively, contained fenoxycarb. These water samples included drinking water, tap water, lake water (Lakes Balaton and Velencei, smaller ponds and water reservoirs), river water (Rivers Danube and Tisza, smaller watercourses), as well as surface water samples collected in agricultural, rural and national park areas throughout Hungary. Although fenoxycarb did not occur in the water samples during our pesticide monitoring campaign, it is alarming that this IGR (as the active ingredient of the commercial ant bait Award) has been detected in USA in runoff waters at elevated concentrations (above 600 ng ml^{-1}) [24]. Therefore, along with more intensive applications in Hungary, the compound may also occur as a surface water contaminant.

Because positive field samples could not be used for assay validation, negative surface water samples spiked with fenoxycarb were subjected to both GC-MS and ELISA analyses. Fenoxycarb content was therefore detected in distilled water samples spiked with fenoxycarb and analyzed by SPME/GC-MS and by two optimized ELISA systems (2.5 μg ml^{-1} of **2**-BSA, anti-**2**-KLH(1) antiserum diluted at 1:2000; 1 μg ml^{-1} of **3**-BSA, anti-**2**-TYG(3) antiserum diluted at 1:2000). The retention time of fenoxycarb with SPME was 10.7 min, therefore SPME allowed a more rapid and convenient method of analysis than

Table 5
Detection of fenoxycarb in distilled water by GC-MS and ELISA

Spiked concentration ($ng\,ml^{-1}$)	Concentration by GC-MS ($ng\,ml^{-1}$)	Concentration by ELISA[a] ($ng\,ml^{-1}$)	Concentration by ELISA[b] ($ng\,ml^{-1}$)
0	<3	<0.2	<0.1
0.25	<3	0.23 ± 0.12	0.27 ± 0.12
1.0	<3	1.07 ± 0.42	1.10 ± 0.57
2.5	<3	2.96 ± 0.58	2.66 ± 0.49
5.0	4.29 ± 0.62	4.61 ± 0.71	4.75 ± 0.19
7.6	7.34 ± 0.92	8.46 ± 0.52	7.62 ± 0.30
10	12.3 ± 0.78	11.1 ± 1.25	9.82 ± 0.59
20	17.1 ± 0.49	19.3 ± 1.66	18.6 ± 1.03

[a] Assay parameters: blocking: 1% gelatin in PBS; 2.5 $\mu g\,ml^{-1}$ of **2**-BSA, anti-**2**-KLH(1) antiserum diluted at 1:2000; anti-IgG-HRP: 1:12000.

[b] Assay parameters: blocking: 1% gelatin in PBS; 1 $\mu g\,ml^{-1}$ of **3**-BSA, anti-**2**-TYG(3) antiserum diluted at 1:2000; anti-IgG-HRP: 1:12000.

SPE. Distilled water was spiked at eight concentrations between 0 and 20 $ng\,ml^{-1}$ with fenoxycarb, and the analyte was detected in each sample using SPME/GC-MS and ELISA. Due to its higher LOD, the GC-MS method detected fenoxycarb only at concentrations $\geq 3\,ng\,ml^{-1}$, while the ELISA systems could determine the analyte down to 0.1 $ng\,ml^{-1}$. Although the LOD of the SPME/GC-MS method can be improved using different fibers for SPME, the LOD of the optimized ELISA systems is matching peak sensitivities of SPME/GC-MS seen in the literature [24].

Good correlation between spiked concentrations of 5–20 $ng\,ml^{-1}$ and corresponding peak areas on the GC-MS chromatogram was obtained ($r^2 = 0.973$ and 0.987 for molecule ions *m/z* 116 and 88, respectively), and each sample was cross-validated with the other four. The fenoxycarb content detected by ELISA in these spiked aqueous samples was determined using the standard curves in assay buffer for each optimized assay (Fig. 3). The results obtained by GC-MS and ELISA systems are compared in Table 5. Concentrations detected at each sample within the concentration range, where both GC-MS and ELISA methods are applicable (5–20 $ng\,ml^{-1}$), do not differ from each other significantly for the different methods. Mean concentration values show that both ELISA systems appeared to slightly overestimate the corresponding value detected by GC-MS, except for one point (10 $ng\,ml^{-1}$), where both ELISAs provided lower results than the GC-MS methods. Altogether, the regression between the GC-MS and ELISA methods are good ($r^2 = 0.971$ and 0.954 for the two ELISA systems, respectively), and regression slopes are very close to 1 (1.03 and 0.966 for the two ELISA systems, respectively) indicating correct detection by both ELISA systems. Nonetheless, a small intercept of the regression lines (0.445 and 0.395 for the two ELISA systems, respectively) indicate that the concentrations detected by the ELISA systems are slightly above those detected by GC-MS.

Inhibition curves using drinking, river and lake water samples spiked with fenoxycarb were also measured. The resulting standard curves in these water samples displayed essentially the same IC_{50} values (25.7–31.6 and 2.1–2.9 $ng\,ml^{-1}$ for the ELISA systems, **2**-BSA/anti-**2**-KLH(1) antiserum and **3**-BSA/anti-**2**-TYG(3) antiserum, respectively), LODs and curve shapes as those obtained in distilled water and assay buffer. Moreover, the LODs of the optimized ELISA methods (i.e. 0.2 and 0.1 $ng\,ml^{-1}$ for the hapten-homologous and hapten-heterologous assays, respectively) are one order of magnitude lower than that in the GC-MS method. Thus, the ELISA method appears to be suitable not only for the analysis of environmental water samples, but also for drinking water monitoring requiring more sensitive detection (MRL = 0.1 $ng\,ml^{-1}$).

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