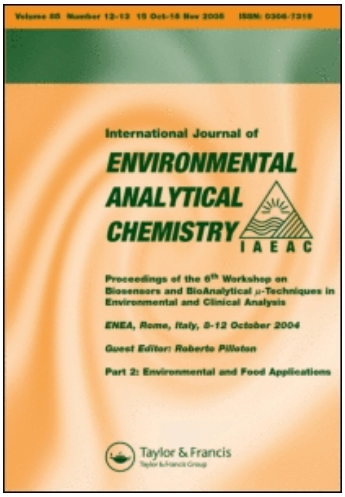

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Development and Comparative Study of Different Immunoenzyme Techniques for Pesticides Detection

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DEVELOPMENT AND COMPARATIVE STUDY OF DIFFERENT IMMUNOENZYME TECHNIQUES FOR PESTICIDES DETECTION

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Different ELISA techniques have been developed for the detemination of four widely used pesticides: 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), simazine and atrazine. Dependences between the assay scheme and the limiting detectable concentration of the pesticide were studied. The cases of preferential applying of the scheme with immobilized antibodies or one with immobilized pesticide-protein conjugate have been revealed. The following approaches resulting in lowering of ELISA sensitivity were proposed: preliminary incubation of the tested sample with antibodies, immobilization of antibodies via staphylococcal protein A, usage of monovalent fragments of antibodies instead of native ones and chemical modification of the pesticide molecules in the sample. Optimal combinations of these approaches permitted to lower the detection limit of the assays in about 5–30 times. The achieved sensitivities were 3 ng/mL for 2,4-D, 5 ng/mL for 2,4,5-T, 0.05 ng/mL for simazine, and 0.1 ng/mL for atrazine, being acceptable for purposes of ecological monitoring.

KEY WORDS: Pesticides, enzyme immunoassay, optimization, sensitivity, phenoxyacetic acids, triazines.

INTRODUCTION

Usage of pesticides has become an indispensable part of agricultural technologies nowadays. However, toxicity of a great number of pesticides causes the necessity to control their content in the environment. Therefore, the development of effective techniques for pesticides detection in soil, water and agricultural products is a problem of increasing significance[1].

Many different approaches are practiced for this purpose now[2–4]: chromatographic assays, mass-spectrometry, catalytic reactions and immunochemical methods. One of the most acceptable methods of pesticides quantitative determination is solid phase enzyme immunoassay (ELISA). Being based on the usage of specific antibodies and high-active enzyme labels, this method corresponds to the current practical requirements. This is a matter of intensive elaboration of the ELISA techniques for different pesticides[5–9].

Several ELISA modifications differing in consequence of stages and used derivatives of antibodies and antigens are known. Therefore, the choice of a technique with the highest sensitivity is an important problem in assay elaboration. This is why in the present work two tasks were set: (1) the development of ELISA step for some widely

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implemented pesticides and (2) the determination of optimal assay techniques and approaches for the detection limit lowering. Four pesticides were studied: two derivatives of phenoxyacetic acid—2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and two derivatives of the symmetric triazine—simazine (2-chloro-4,6-di(N-ethylamino)-1,3,5-triazine) and atrazine (2-chloro-4-(N-ethylamino)-6-(N-isopropylamino)-1,3,5-triazine).

EXPERIMENTAL

Chemicals

2,4-D and 2,4,5-T were from Serva. Phenol, 2,4,5-trichlorophenol, pentachlorophenol and phenoxyacetic acid (all from Sigma) were used to analyze specificity of obtained antibodies against 2,4-D and 2,4,5-T.

Purified preparations of simazine, atrazine, their 2-ethylcarboxy- and 2-propylcarboxy- derivatives were generously provided by Dr. S. A.Eremin, Moscow State University.

Bovine serum albumin (BSA, Sigma), rabbit serum albumin (RSA), haemocyanine of *Paralithodes camtschatica* (HC, generously provided by Dr. I. Yu.Sakharov, Moscow State University), soybean trypsin inhibitor (STI, Reanal), ovalbumin (OA, Serva) and horseradish peroxidase (HRP, RZ = A_{403}/A_{280} = 3.0, Biolar, Latvia) were used for syntheses of protein-pesticide conjugates.

1-Cyclohexyl-3(2-morpholinoethyl) carbodiimide (CalBiochem), dicyclohexylcarbodiimide, o-phenylenediamine (both from Fluka), N-hydroxysuccinimide, hexamethylenediamine, sodium dodecylsulfate (all from Sigma), hexamethylenediamine (Reachim, Russia), protein A of *Staphylococcus aureus* (*Vostok*, Russia), 2,4,6-trinitrobenzensulfonic acid (Chemapol), sodium periodate, sodium borohydride, dimethylformamide, polyethylene glycol (M_w 6 kDa), dithiothreitol, sodium ethylenediaminetetraacetate, Tween-20 (all from Serva), cystein, lysine (both from Reanal) and alpha-amylase of *Bacillus subtilis*[10] were also used in the experiments.

Synthesis of protein conjugates with 2,4-D/2,4,5-T[11]

For the pesticide activation 15 mg 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide and 4 mg N-hydroxysuccinimide were added to 4.0 mg 2,4-D (or 4.6 mg 2,4,5-T) dissolved in 0.5 mL of dimethylformamide. The mixture was incubated with stirring for 2 h at room temperature and then added to protein solution in water (10 mg/mL). In the cases of immunogen syntheses the initial hapten:protein molar ratios were 50:1 (BSA, RSA) or 200:1 (HC); when the conjugates were obtained for usage at antisera testing (STI, OA), it was 20:1; while for the HRP conjugates it varied from 2:1 to 200:1.

The conjugates prepared were separated from low molecular weight compounds of the reaction mixture by dialysis and/or gel-filtration on Sephadex G-25 (Pharmacia) column.

Synthesis of HRP conjugates with 2,4-D/2,4,5-T containing hexamethyl spacer group

20 mg HRP was diluted in 0.5 mL of 1 mM Na-acetic buffer, pH 4.5; 20 mg sodium periodate was diluted in 0.5 mL of the same buffer. The two solutions were combined

and incubated for 20 min at room temperature with stirring. After this activation, HRP was dialyzed against the same buffer. Next, 3 mg hexamethylenediamine were diluted in 1 mL of 0.1 M Na-carbonic buffer, pH 9.2. The peroxidase solution was added, whereupon pH was adjusted to 9.2 by NaOH. The mixture was incubated for 3 h at room temperature under pH controlling. Then 0.2 mL of freshly prepared $NaBH_4$ solution (4 mg/mL) in water was added dropwise with stirring. The obtained mixture was incubated with intensive stirring for 15 min at room temperature.

Modified peroxidase was separated from low molecular weight compounds of the reaction mixture by gel-filtration on Sephadex G-25 column in 0.1 M Na-carbonic buffer, pH 9.2. The solution obtained was diluted to 2 mg/mL HRP concentration.

In the next stage, 2 mg N-hydroxysuccinimide and 10 mg dicyclohexylcarbodiimide were added to 1.2 mL of the pesticide solution in dimethylformamide (2.0 mg/mL for 2,4-D or 2.3 mg/mL 2,4,5-T) and incubated for 1.5 h at room temperature. During this time 10 mg dicyclohexylcarbodiimide was supplementary added in portions of approximately 1 mg. After the incubation, the obtained product was mixed with the activated peroxidase solution. The resultant mixture was incubated with stirring for 1 h at room temperature, and further—for 16 h at +4°C.

Final separation from low molecular weight compounds was carried out by gel-filtration on Sephadex G-25 column.

Synthesis of protein conjugates with simazine/atrazine [12,13]

2.5 mg carboxylated derivative of the pesticide was diluted in 0.1 mL of dimethylformamide. Then, 2 mg N-hydroxysuccinimide and 4 mg dicyclohexylcarbodiimide were added. The mixture was stirred at room temperature for 1.5 h with followed removing of the precipitated dicyclohexylurea by centrifugation. Then, the activated hapten was added to the protein solution (BSA, HC, STI, OA or HRP; concentration for each protein—10 mg/mL) in 0.2 mL of 1 M Na-carbonate buffer, pH 9.2, containing 20% (v/v) dimethylformamide. Initial hapten:protein molar ratio varied from 2:1 to 200:1. The resultant mixture was incubated with stirring for 2 h at room temperature, and further—for 16 h at +4°C.

The conjugates obtained were separated from low molecular weight compounds using the techniques described above.

Determination of the pesticide-protein conjugates composition

To calculate hapten:protein ratios for the obtained conjugates, we compared the numbers of surface amino groups in the original protein and in one conjugated with the hapten. These groups were detected by means of 2,4,6-trinitrobenzensulfonic acid using the traditional technique of amino groups titration[14,15].

Immunization

The following conjugates were used as immunogens: 2,4-D-BSA, 2,4-D-RSA, 2,4,5-T-BSA, 2,4,5-T-HC, simazine-BSA, simazine-HC and atrazine-BSA.

For raising antibodies against the phenoxy-containing pesticides, Chinchilla rabbits weighting 3–4 kg were immunized according to the following procedure. An immunogen

dissolved (1 mg/mL) in 0.05 M K-phosphate buffer, pH 7.4, with 0.1 M NaCl (further named as PBS) was emulsified with an equal volume of Freund's complete adjuvant (Difco). On days 1, 15 and 29, 1.0 mL of the prepared mixture was injected intracutaneously at multiple sites on the back from scapula to sacrum. After two months the first cycle of reimmunization was carried out: the rabbits were boosted intravenously on day 89 with 0.3 mL of the immunogen dissolved in PBS at the same concentration, and were bled 7 days later. This boosting/bleeding procedure was repeated 2-4 times on a monthly basis.

Immunization of Chinchilla rabbits in the case of triazine pesticides was differed in the following. Immunogen mixed with Freund's complete adjuvant was injected fourfold two weeks apart. At each reimmunization cycle the immunogen solution in PBS was injected both intravenously (0.3 mL) and intracutaneously (0.2 mL)

Antiserum separation[16]

Antisera were extracted by settling blood samples for 12 h at +4°C. Upper layers were carefully collected, divided into aliquots and stored at –20°C.

Antiserum testing[17]

Antisera titres were determined by the standard technique of indirect ELISA of antibodies. Conjugate of STI or OA with the pesticide was immobilized in the wells of optically transparent polystyrene microplate (Dynatech), to which the antiserum dilution, goat-anti-rabbit peroxidase conjugate (or conjugate of protein A with peroxidase, see below) and HRP substrate were added at sequential stages. The limiting dilution for which the measured optical density of the peroxidase reaction product had been reliably ($p = 0.95$) higher comparing with non-specific binding was regarded as titre of the antiserum tested.

Isolation of antibodies[16]

The IgG was precipitated from antisera by equal volume of 20% polyethylene glycol. The sedimentation was repeated twice.

Preparation of monovalent antibody fragments[18]

The following technique based on S-S-bords reducing was employed. Solutions of cystein (0.15 mL, 2 mg/mL), sodium ethylenediaminetetraacetate (0.075 mL, 2 mg/mL) and $Na_2S_2O_4$ (0.1 mL, 17.5 mg/mL) in water were added to solution of IgG (1.0 mL, 3 mg/mL) in 0.1 M K-phosphate buffer, pH 7.0. The mixture was incubated for 2 h at 37°C. Next, low molecular weight compounds were removed by dialysis. Preliminary the complete transformation of IgG molecules into the monovalent fragments at these conditions had been shown by application of gel-filtration on Sephadex G-100 (Pharmacia) column.

Syntheses of peroxidase conjugates with IgG or protein A

HRP was conjugated with IgG and with staphylococcal protein A by the traditional technique[19] based on periodate oxidation of the enzyme. The conjugates obtained were separated from unreacted molecules by gel-filtration on Sephadex G-200 (Pharmacia) column.

2,4-D/2,4,5-T ELISAs with immobilized antibodies

The basic technique 0.1 mL aliquots of IgG (from the anti-pesticide serum) in PBS were dispensed into microplate wells and incubated for 1.5 h at 37°C (or for 16 h at +4°C). The wells were then fourfold washed by PBS with 0.05% Tween-20 (further named as PBST). After washing, 0.05 mL of 2,4-D (or 2,4,5-T) sample and 0.05 mL of 2,4-D-HRP (2,4,5-T-HRP) solution were added. The microplate was incubated at 37°C, washed and then peroxidase activity was measured.

Technique with antibody immobilization via protein A 0.1 mL aliquots of protein A in PBS were dispensed into microplate wells and incubated for 1.5 h at 37°C (or for 16 h at +4°C). Next, after PBST washing, 0.1 mL aliquots of IgG were added into the wells and incubated for 45 min at 37°C. The following steps were the same as those described above.

Usage of monovalent antibody fragments Protein A was immobilized as mentioned above. After washing, 0.1 mL aliquots of monovalent antibody fragments were added and incubated at 37°C. The following steps were the same as those described previously.

Usage of preliminary modification of the sample analyzed 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide and N-hydroxysuccinimide were added to the analyzed sample in the final concentrations 3 mg/mL and 0.8 mg/mL, accordingly. The mixture was incubated for 15 min at room temperature. Then, bacillary alpha-amylase or lysine were added (their final concentration should be 1 mg/mL) and the obtained mixture was incubated for 15 min at room temperature. Next, the quantification of 2,4-D (or 2,4,5-T) was carried out as mentioned above.

Optimal reagent concentrations and stage durations chosen in the course of the work for the 2,4-D/2,4,5-T ELISAs with immobilized antibodies are given in Table 1.

2,4-D/2,4,5-T ELISAs with immobilized hapten-protein conjugate

0.1 mL aliquots of STI or HC conjugated with the pesticide, both in PBS, were dispensed into microplate wells and incubated for 1.5 h at 37°C (or for 16 h at +4°C). The conjugate concentration was 5 ug/mL for STI or 20 ug/mL for HC. After washing of the microplate, 0.05 mL of analyzed sample and 0.05 mL of specific antiserum (in dilution 1:5000 for the 2,4-D case or 1:8000 for the 2,4,5-T one, in PBST) were added to the wells and incubated for 1 h at 37°C. The microplate was washed again, 0.1 mL aliquots of HRP-protein A conjugate (at an HRP concentration 0.8 ug/mL) in PBST were added and incubated for 45 min at 37°C. After washing peroxidase activity was measured.

Table 1 Optimal conditions of the developed 2,4-D and 2,4,5-T ELISAs.

Antigen	*2,4-D*	*2,4,5-T*
Concentration of adsorbed protein A, ug/mL	2.5	2.5
Concentration of IgG or its monovalent derivative, ug/mL	6	2
(Antiserum dilution)	1:2000	1:7500
Duration of reaction between protein A and antibodies, min	45	45
Concentration of pesticide-HRP conjugate, ug/mL (by HRP)	0.25	0.1
Duration of competitive immunochemical reaction, min	60	60
Duration of peroxidase reaction, min	15	15

Simazine/atrazine ELISAs with immobilized antibodies

The basic technique 0.1 mL aliquots of IgG (from the anti-pesticide serum) in 0.1 M K-phosphate buffer, pH 7.4 (further named as PB) were dispensed into microplate wells (3 ug/mL) and incubated for 1.5 h at 37°C (or for 16 h at +4°C). The wells were washed and 0.05 mL of simazine (atrazine) sample and 0.05 mL of simazine-HRP (atrazine-HRP) solution (at an HRP concentration 0.05 ug/mL) were added in PB containing 0.05% Tween-20 (further named as PBT). Then the microplate was incubated for 45 min at 37°C, washed and peroxidase activity was measured.

Technique with antibody immobilization via protein A 0.1 mL aliquots of protein A (2.5 ug/mL) in PB were dispensed into microplate wells and incubated for 1.5 h at 37°C (or for 16 h at +4°C). Next, after PBT washing, 0.1 mL of IgG from the anti-pesticide serum (6 ug/mL) or the native antiserum (in dilution 1:2000) were added to the wells and incubated for 45 min at 37°C. The following steps were the same as those described above.

Simazine/atrazine ELISAs with immobilized hapten-protein conjugate

The basic technique 0.1 mL aliquots of pesticide-BSA (or pesticide-OA) conjugate were immobilized for 1.5 h at 37°C (or for 16 h at +4°C). After washing, 0.05 mL of the pesticide sample and 0.05 mL of the specific antiserum diluted were added to the wells and incubated at 37°C. The microplate was washed, 0.1 mL aliquots of HRP-protein A conjugate were added and incubated at 37°C. After washing peroxidase activity was measured.

Usage of preliminary sample incubation with the antiserum 0.05 mL of the pesticide sample and 0.05 mL of the specific antiserum were previously incubated. Then, these preparations were added into washed wells with immobilized pesticide-protein conjugate. The following steps were the same as those described above.

Usage of HRP-IgG conjugate instead of antiserum The pesticide-protein conjugate was immobilized. After washing, mixture of 0.05 mL of the pesticide sample and 0.05 mL of HRP-IgG conjugate (at an HRP concentration 2 ug/mL) being previously incubated were added to the wells and incubated. After washing, the peroxidase activity was measured.

Optimal reagent concentrations and stage durations chosen in the course of the work for the simazine/atrazine ELISAs with immobilized hapten-protein conjugate are given in Table 2.

Measurement of peroxidase activity[16]

A solution of o-phenylenediamine (0.4 mg/mL) in 30 mM Na-acetate buffer, pH 4.5, containing 1.8 mM of H_2O_2, was used as a peroxidase substrate. The ELISA microplate was incubated with the substrate for 15 min at room temperature in darkness and then the reaction was stopped by H_2SO_4. The optical density was measured at 490 nm by vertical photometer MR-580 (Dynatech).

RESULTS AND DISCUSSION

Choice of antisera

At first the antisera obtained had been compared. The highest titres were equal to: for 2,4-D—1:150000 (immunogen—2,4-D-BSA), for 2,4,5-T—1:300000 (immunogen—2,4,5-T-HC), for simazine—1:200000 (immunogen—simazine-HC), and for atrazine—1:150000 (immunogen—atrazine-BSA). All these levels were achieved after 2-3 reimmunizations. It is important to note that titres for different pesticides are close to each other.

Table 2 Optimal conditions of the developed simazine and atrazine ELISAs.

Antigen	*Simazine*	*Atrazine*
Concentration of adsorbed pesticide-STI conjugate, ug/mL	0.2	0.5
Concentration of IgG, ug/mL	2.5	5.5
(Antiserum dilution)	1:5000	1:2000
Duration of preliminary incubation between sample and antibodies, min	15	15
Duration of competitive immunochemical reaction, min	30	45
Concentration of HRP-protein A conjugate, ug/mL (by HRP)	0.5	0.5
Duration of reaction with HRP-protein A conjugate, min	45	45
Duration of peroxidase reaction, min	15	15

Comparison of ELISA techniques with antigen and antibody labelling

The indispensable approach in ELISA of monovalent antigens (haptens) is the competition between the hapten to be determined and the hapten-protein conjugate for binding with antibodies. The main two variants of competitive ELISA are:

— the scheme with antibodies in solution (they are directly or indirectly labelled by an enzyme at this case)

— and the scheme with immobilized antibodies (a hapten-enzyme conjugate is used at this case).

From the above reasoning necessary immunoreagents for the accomplishment of both schemes had been prepared and sensitivities of the ELISA schemes were analyzed for each pesticide studied.

All pesticides tested demonstrated practically equal limits of detection in the scheme with immobilized antibodies. The limits were in the range from 3 to 10 ng/mL, but for the scheme with antibodies in solution essential differences in sensitivities were observed. While for both triazine pesticides, the limits of detection were slightly lower as compared with the first case (1–3 ng/mL), for 2,4-D and 2,4,5-T the ELISA sensitivities increased significantly (up to 100 ng/mL).

Study of the antibody specificity

To determine an optimal ELISA scheme for each pesticide and the strategy of its improvement, specificities of the produced antibodies have been analyzed.

For assays of simazine and atrazine no effects were observed causing with cross-reactions of the antibodies. Even other triazines have elicited low levels of competition (no greater than 5%) with the antigens for antibody binding.

Cross-reactions with chemical pesticide analogs in the ELISAs developed for 2,4-D and 2,4,5-T were absent, if their molecules did not contain oxyacetic group or had other disposition of chlorine atoms (Figure 1, curves 2–4). However, the cross-reactions were examined between 2,4-D and 2,4,5-T: the level of 2,4,5-T revealing in ELISA of 2,4-D was 20%, ELISA of 2,4,5-T revealed 0.2% of 2,4-D. Far more considerable level of the cross-reaction at the former combination correlates with data obtained for other immunoassays of phenoxy-containing pesticides[20–22].

But the most important particularity of the antibodies against 2,4-D and 2,4,5-T resided in their interactions with the so named "bridge"—a conjugate region between hapten and protein. The antibodies (both against 2,4-D and 2,4,5-T), obtained on all immunization cycles, were bound to a conjugated hapten more effectively in comparison with a free one (Figure 1, curves 1,5; Figure 2A, curves 1,2). Sensitivities for the two objects detection differed considerably; the distinction varied from 10 to 200 times. Hence, the greater part of the antibodies in the obtained preparations discerns not only a hapten, but also its surroundings in a conjugate. Therefore, the antibodies studied do not distinguish the "bridge" as a separate determinant. This is proved by the fact that they hardly react with conjugates synthesized by the same technique (i.e. containing the same "bridge") with another hapten (Figure 1, curve 6). On the other hand, the antibodies interacted significantly worse with the 2,4-D-HRP (or 2,4,5-T-HRP) conjugate, in which the "bridge" was prolonged by a hexamethyl chain.

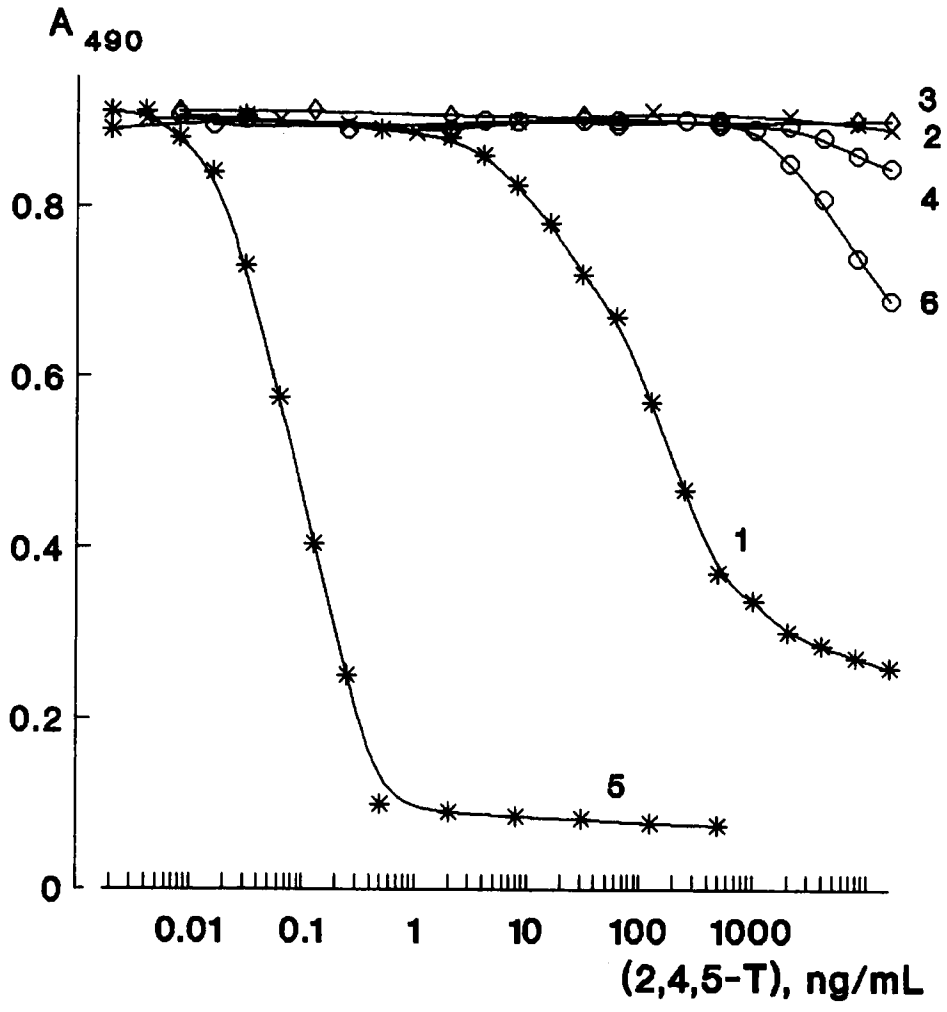

Figure 1 Specificity of the developed 2,4,5-T ELISA with labelled antigen. Competitive curves for 2,4,5-T (1), 2,4,5-trichlorophenol (2), pentachlorophenol (3), phenoxyacetic acid (4), 2,4,5-T-STI conjugate (5) and phenoxyacetic acid-STI conjugate (6).

Approaches for ELISA sensitivity increasing: 2,4-D and 2,4,5-T

In the ELISA systems for 2,4-D and 2,4,5-T the antibodies interact with free pesticide molecule to be detected much more worse comparing with the competitive agent—pesticide-protein conjugate. But this disadvantage may be turned into a possibility of increasing the sensitivity of native pesticides quantification. To achieve this aim we propose to incubate the analyzing sample with the linking reagents of the conjugate synthesis before the sample interaction with the antibodies. Both for 2,4-D and 2,4,5-T there was shown an increase of the ELISA detection limit up to 10 fold caused by

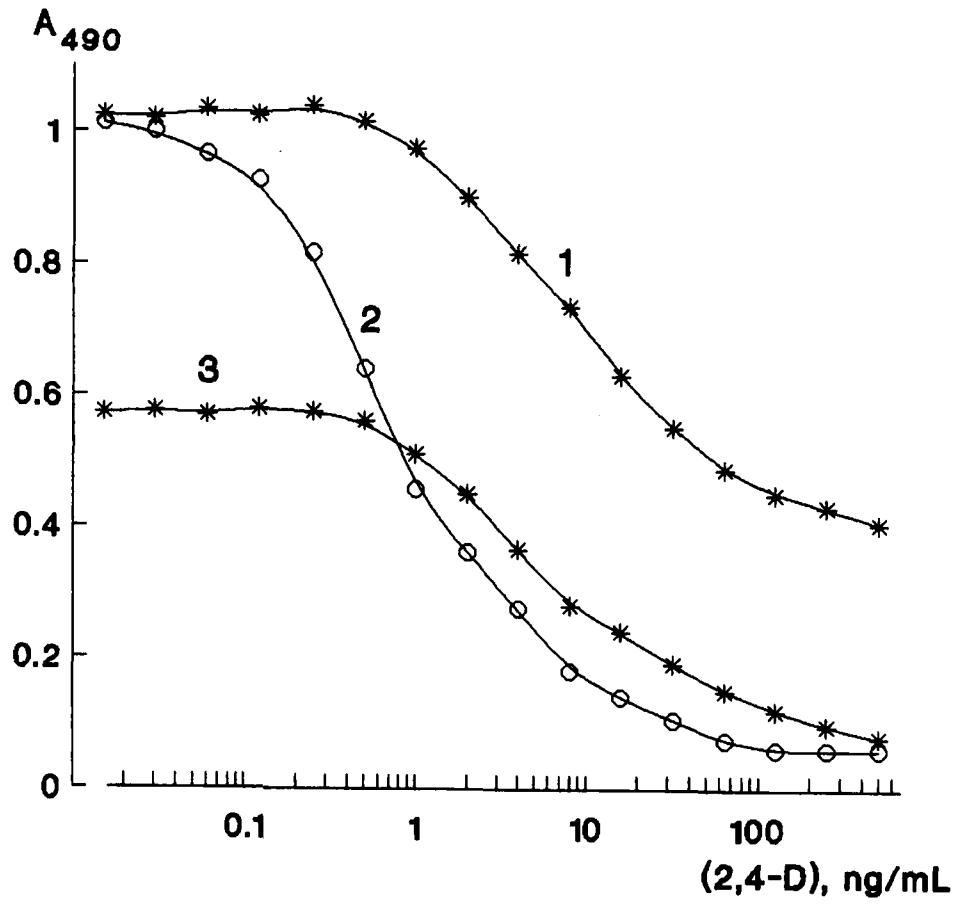

Figure 2 Determination of native and modified 2,4-D by the developed ELISA with labelled antigen. A. Competitive curves for 2,4-D (1), 2,4-D-STI conjugate (2) and 2,4-D in the presence of phenoxyacetic acid-STI conjugate at an STI concentration 5 ug/mL (3).

applying this approach (Figure 2B, curve 2). The sensitivity of the assays became to be nearly to one for specially synthesized conjugates (Figure 2A, curve 2). Carrying out the activation in excess of amines (when protein or amino acid solution had been added) caused further increasing of the assay sensitivity (Figure 2B, curves 3,4). Therefore, the latter regime permits to obtain correct values of the pesticide content, irrespectively of whether the sample analyzed contains other compounds with the reactive groups or not.

As free 2,4-D concentration increased, lowering of detected signal for its ELISA with immobilized antibodies slowed (Figure 2A, curve 1). This phenomenon results in less accuracy of the assay. Therefore, it is reasonable to eliminate the peroxidase conjugate interactions with those antibodies which do not recognize the free hapten. For this purpose we added during the immune reaction also another conjugate, which was distinguished both by the hapten and the protein used (for example, the conjugate of STI with phenoxyacetic acid) but had been synthesized by the same technique as 2,4-D-HRP. The latter approach did not change the sensitivity of the assay, but improved its

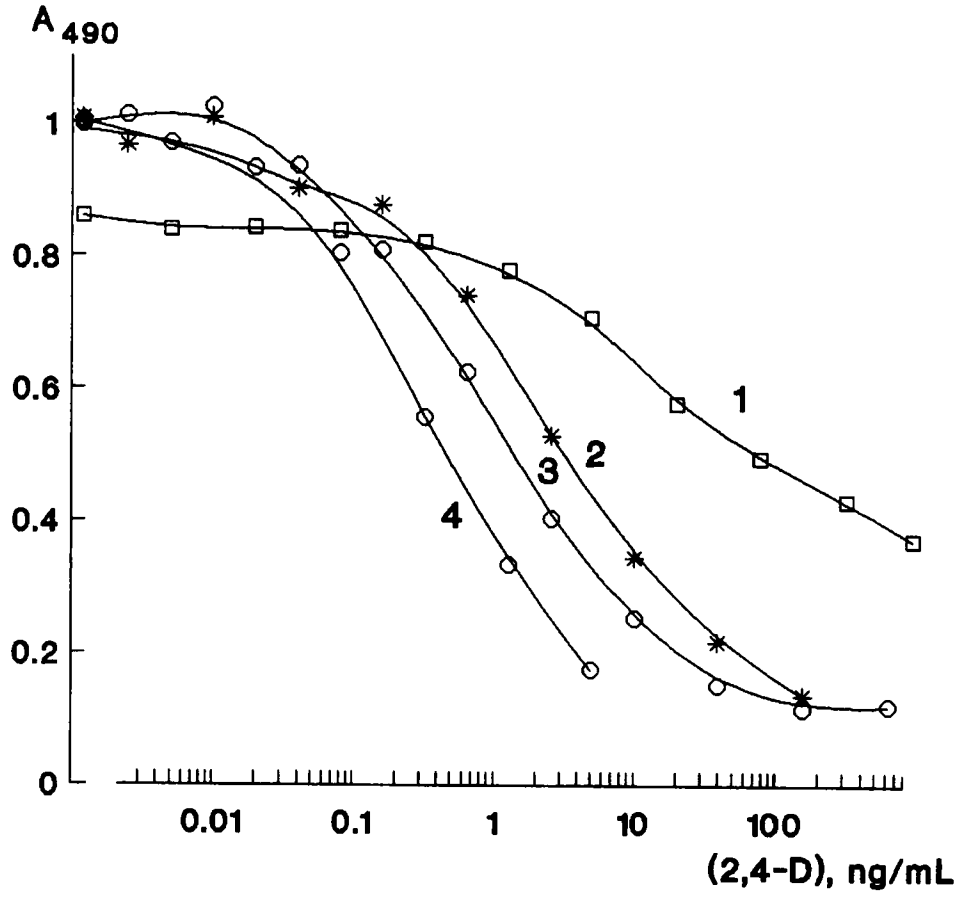

Figure 2 B. Competitive curves for 2,4-D (1) and for 2,4-D activated by N-hydroxysuccinimide: in the absence of amino-containing compounds (2), in the presence of bacillary alpha-amylase (3) and in the presence of lysine (4).

accuracy, because the competition was detected on the background of less non-specific level (Figure 2A, curve 3).

Lesser binding properties of free pesticide comparing with pesticide-protein conjugates may be explained as a result of formation of the bivalent immune complexes (usually being about two orders more affine than monovalent ones[23,24]) only by the conjugate molecules. In this connection the choice of optimal composition of the conjugate used is an important task. Bivalent interactions probability and hindrances for compete binding of the native hapten strongly correlate with the density of antigentic determinants on the conjugate surface. We had established that the highest ELISA sensitivity was reached if molar compositions of 2,4-D-HRP and 2,4,5-T-HRP conjugates were in the range 2:1–5:1. For conjugates with power ratios the detected signal lowered, whereas non-specific HRP binding increased. On the other hand, usage of the conjugates with higher ratios resulted in a tenfold decrease of the sensitivity (Figure 3).

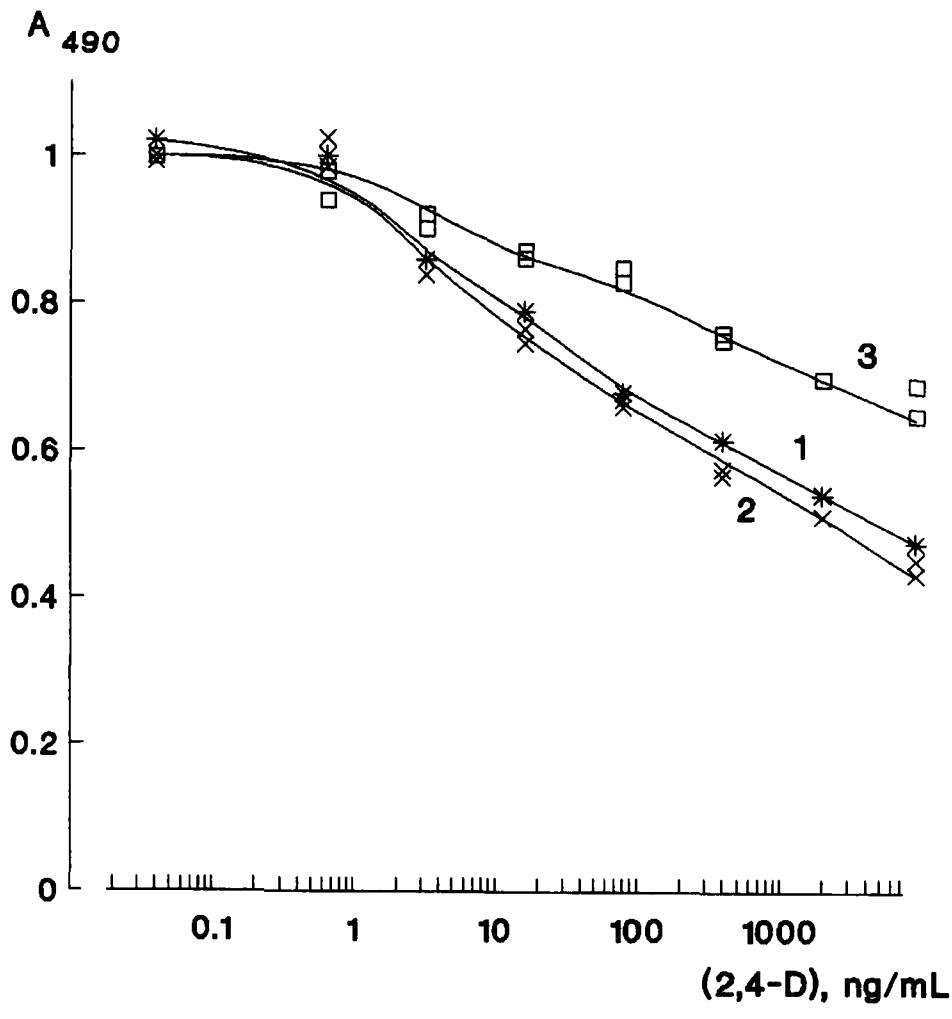

Figure 3 Influence of the peroxidase conjugate composition on calibration curves for the developed 2,4-D ELISA with labelled antigen. Competitive curves for 2,4-D obtained with usage of the 2,4-D-HRP conjugates having hapten:protein molar ratios 2:1 (1), 5:1 (2) and 15:1 (3).

Another approach developed dealt with the modification of immobilized antibodies. Their attachment through adsorbed protein A resulted in a twofold increase of the assay sensitivity (Figure 4, curves 1,2). Previously, we have described the same phenomenon for testosterone ELISA[25]. It may be explained as a consequence of either a favorable orientation of the antigen-binding sites or a selection of IgG subclasses with higher affinity to the antigen during the antibody-protein A reaction.

As has already been intimated, the bivalent interactions of the conjugate molecules with IgG may induce a decrease of the ELISA sensitivity. To eliminate these interactions monovalent derivatives of antibodies may be applied. Usage of F_{ab}-fragments is not reasonable in this case because they cannot be immobilized through protein A. Therefore, necessary derivatives had been obtained by reducing the S-S-bonds in the IgG

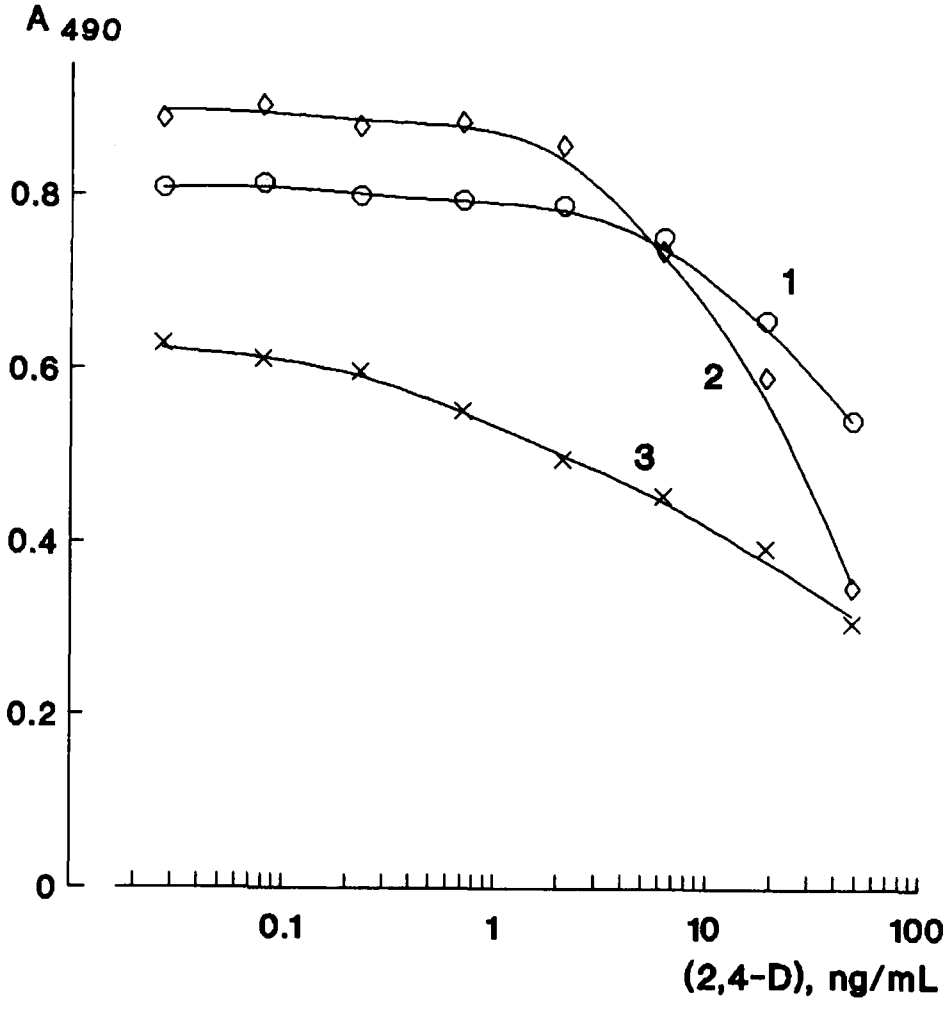

Figure 4 Comparison of antibody immobilization ways in 2,4-D ELISA with labelled antigen. Calibration curves for 2,4-D with directly adsorbed IgG molecules (1), with IgG immobilized through protein A (2) and with their monovalent derivatives also immobilized through protein A (3).

molecule. This approach allowed to increase the sensitivity of the 2,4-D ELISA in about 3 folds (Figure 4, curve 3).

Approaches for ELISA sensitivity increasing: simazime and atrazine

It is known that the sensitivity of competitive ELISAs often has risen after preliminary incubation of analyzed sample with antibodies[17]. Such pre-incubation for the samples containing simazine (prior to adding into microtiter plate coated by simazine-BSA conjugate) resulted in tenfold increase of the ELISA sensitivity (Figure 5, curves 1,2).

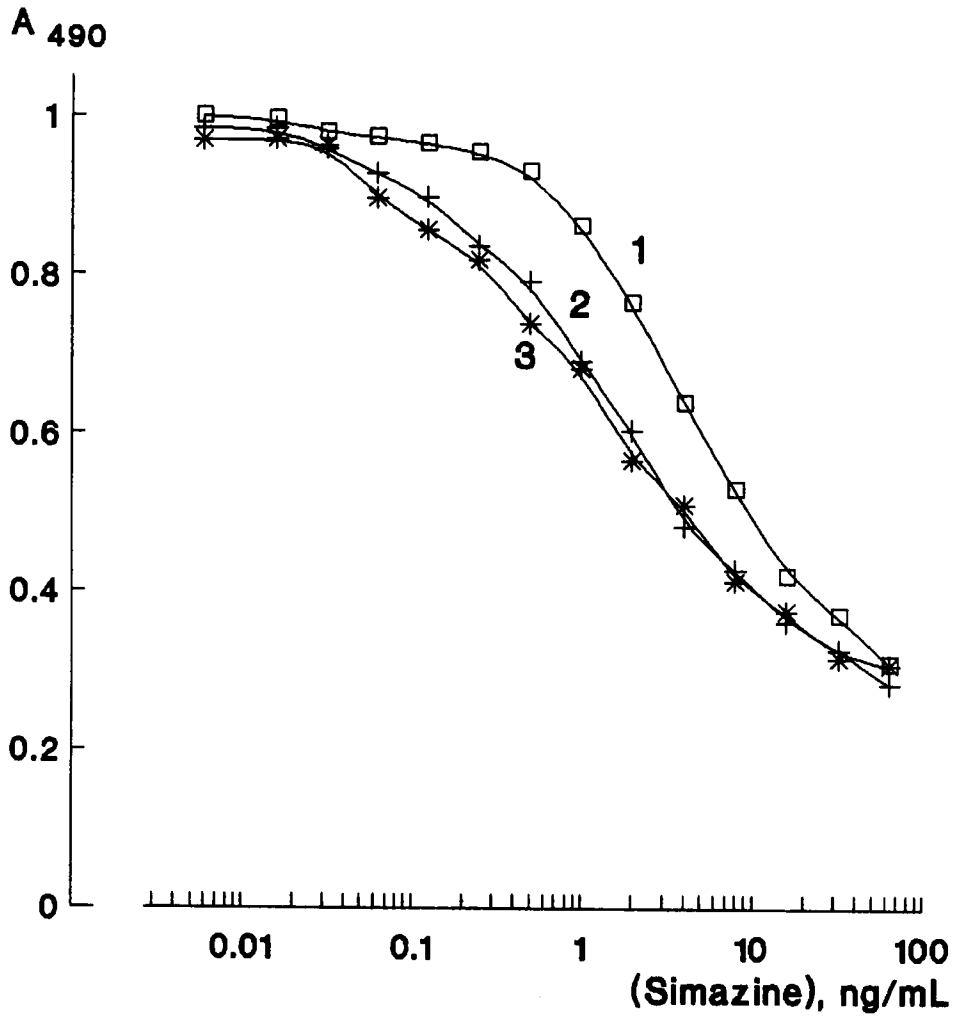

Figure 5 Calibration curves for simazine ELISA with labelled antibodies or protein A: scheme with HRP-protein A conjugate and without preliminary incubation of sample and antibodies (1), the same, but with the incubation for 15 min at 37°C (2) and scheme with HRP-IgG conjugate and the incubation for 15 min at 37°C (3).

Applying of this approach to atrazine permitted to reach practically the same increasing of the sensitivity (Figure 6).

At the same time the techniques with direct labelling of antibodies and of indirect one (by usage of HRP-protein A conjugate) had shown equal sensitivities both for simazine (Figure 5, curves 2,3) and atrazine. So, the choice between the two variants depends on practical purposes. On the one hand, direct labelling reduces the assay duration. On the other, protein A labelled by peroxidase is an universal reagent permitting to exclude syntheses of labelled antibodies for each new ELISA technique and even to work with native antisera.

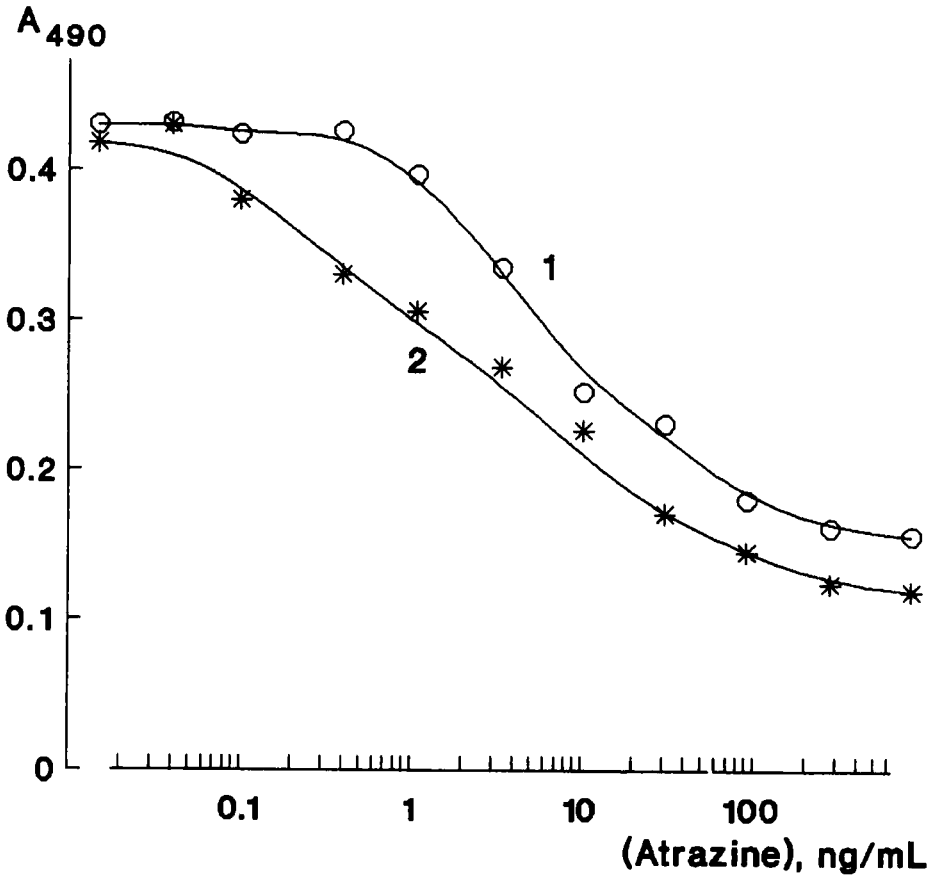

Figure 6 Calibration curves for atrazine ELISA with labelled protein A: scheme without preliminary incubation of sample and antibodies (1) and one with the incubation for 15 min at 37°C (2).

Optimization of the developed techniques

After the determination of the optimal ELISA techniques for different pesticides we have carried out their optimization by the choice of the reagent concentrations and the stage durations. Maximal assay sensitivity and minimal expense of chemicals were the optimization criteria. The chosen parameters of the ELISA techniques are given in Tables 1, 2. The variation coefficients for the assays at these conditions are shown in Table 3.

Table 3 Sensitivity and accuracy of the pesticide ELISAs (for their optimal conditions).

Antigen	*2,4-D*	*2,4,5-T*	*Simazine*	*Atrazine*
Limit of sensitivity, ng/mL	2	3	0.05	0.1
Middle error in seria*, %	12	10	7	10
Middle error between series*, %	20	16	14	12

*—when the sample contains 5 ng/mL of the pesticide

The reached sensitivities are in accordance with other developed ELISAs of phenoxy-containing[20–22,26–29] and triazine[12,13,30–38] pesticides.

CONCLUSION

ELISA techniques for four widely used pesticides have been developed. Their sensitivities are consistent with up-to-date requirements to the systems of pesticide control. The possibility of the reliable quantification of pesticides in water samples by these techniques has been revealed.

The approaches proposed permit to lower the ELISA detection limits in the average of one order of magnitude. Their principles are suitable for employing in determination of other low molecular weight compounds.

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