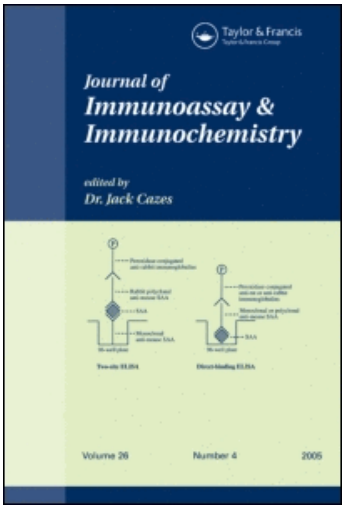

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Rapid Polyelectrolyte-Based Membrane Immunoassay for the Herbicide Butachlor

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Abstract: Oppositely charged water-soluble polyelectrolytes were used in the developed membrane immunoenzyme assay for the herbicide butachlor. High-affinity and rapid binding between polyanion polymethacrylate and polycation poly(N-ethyl-4-vinylpyridinium) was applied to separate reacted and free immunoreactants. Competitive immunoassay format with peroxidase-labeled antigen was realized. The insoluble colored product of the peroxidase reaction was formed by bound labeled immune complexes and was reflectometrically detected. The assay combines short duration (15 min), high sensitivity (0.03 g/mL) and availability for out-of-laboratory testing. Different image processing algorithms were used to determine the herbicide content. Low variation coefficients of the measurements in the proposed quantitative assay, namely 4.8–9.0% for the range of antigen concentrations from 0.1 to 3.0 ng/mL, are evidence of the assay effectiveness. Possibility to control the butachlor content in mineral, artesian, and drinking water was demonstrated.

Keywords: Polyelectrolyte, Membrane immunoassay, Butachlor

INTRODUCTION

The extensive use of pesticides has led to increased health risks due to long-term low-level exposure of humans to these pollutants, thus stipulating the necessity of controlling their levels in soil, water, agricultural products, and other media.[1,2] A major difficulty to evaluate the risk posed by pesticides

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is a lack of analytical techniques that can handle large sample loads with rapid turnover time. Gas chromatography, high-performance liquid chromatography, and capillary electrophoresis are commonly used methods for pesticides detection.[3–5] Although these methods can test different matrixes, they require specific pre-treatment of samples and expensive sophisticated equipment, which in turn requires highly skilled analysts to operate them. The net result is that extraction and analysis are performed at specialized laboratories and information is obtained at considerable cost, days after the samples are taken. Thus, traditional techniques are not suitable for rapid on-site monitoring of pesticides that can allow remedial action to be taken for any arising problem.

In recent years, enzyme immunoassays have been demonstrated to be extremely effective tools for the detection of various substances in ecological monitoring, medicine and biotechnology.[6–9] Due to the use of specific antibodies and enzyme labels, these assays ensure specificity and sensitivity corresponding to the modern practical requirements. A large number of microplate immunoenzyme assays (ELISA) is proposed for pesticides detection, the results of these investigations are summarized in refs.[9–13] The ELISA provides the necessary sensitivity, but needs prolonged incubations (a hour or more) to reach equilibrium of diffusion-dependent immune reactions. Besides, the ELISA requires stationary equipment to manipulate microplates and to register the assay results.

Application of porous membranes with immobilized specific reactants allows significant acceleration of immunoanalyses: interactions in a porous layer ensure faster chemical equilibrium and effective separation of the formed immune complexes.[14–17] However in many cases affinity of the antigen-antibody reaction is insufficient for further reduction the assay time.

This problem may be solved by separation of the reactants through some additional high-affine interaction. Earlier, we proposed a pair of linear water-soluble polyelectrolytes, namely polymethacrylate (PMA) polyanion and poly(N-ethyl-4-vinylpyridinium) (PEVP) polycation, as carriers for such a separation.[18–22] The reaction between them has an extremely high rate and affinity due to co-operative interactions of the polymers' links.[22] Therefore, the polyelectrolyte-based assay may be carried out as a combination of two rapid steps: homogeneous antigen–antibody reaction and separation of the reactants by heterogeneous interpolyelectrolyte reaction. The described approach was applied previously in ecological monitoring for qualitative immunoassay of herbicide simazine, namely, control of exceeding its defined level in samples.[24] However, rapid quantitative determination of pesticides is extremely important to adequately prevent negative consequences of poisoning. In this connection the current investigation has been directed to the development of filtration polyelectrolyte immunoenzyme assay with quantitative determination of antigen content that is based on image processing algorithms.

Butachlor (2-chloro-N-(2,6-diethylphenyl)-N-(butoxymethyl)acetamide) was chosen as the studied antigen. It is a recently registered pre-emergence herbicide from chloroacetanilide family that is widely used against grasses in rice crops. Butachlor may cause different toxic effects[23–25] and, due to long time persistence in water sources, it is an important compound for environmental monitoring. The traditional butachlor control techniques, namely gas or liquid chromatography with mass spectrometric detection,[26–28] have a number of disadvantages mentioned above. In recent years, Guo et al.[29] and our group[30] have developed butachlor ELISAs, while express immunochemical tests for this compound are not known ad interim.

EXPERIMENTAL

Chemicals

The analytical standards of butachlor, acetochlor, alachlor and metolachlor (Fig. 1) were purchased from Riedel-de Haen (Seelze, Germany). Bovine serum albumin (BSA), N,N′-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were from Sigma Chemical Co. (St. Louis, USA), 3,3′-diaminobenzidine tetrahydrochloride (DAB), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride, Freund's complete and incomplete adjuvants—from ICN Biomedicals Inc. (Aurora, USA), Triton X-100—from Serva (Heidelberg, Germany), protein A of Staphylococcus aureus—from Imtek (Moscow, Russia), horseradish peroxidase (HRP,

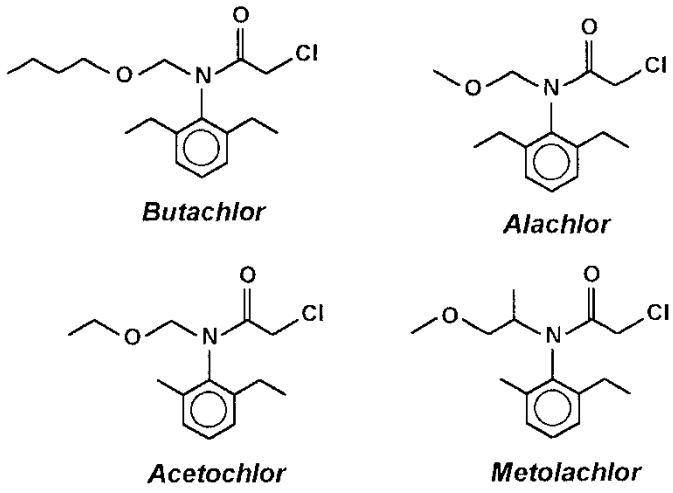

Figure 1. Chemical structures of butachlor and its studied analogs.

RZ = A_{403}/A_{280} = 3.0)—from Biozyme (Pontypool, UK). All other chemicals were of analytical grade.

Obtaining and Characterization of Polyelectrolytes

Polymethacrylic acid (PMA) was synthesized by polymerization of methacrylic acid, and poly(N-ethyl-4-vinylpyridinium) bromide (PEVP)—by polymerization of vinylpyridine with the following exhaustive quaternization by ethyl bromide.[21] Weight-average molecular masses (M_w) of fractionized preparations were 260 kDa for PMA and 2000 kDa for PEVP (light-scattering data). Heterogeneity of molecules in the preparations was characterized by the value of M_w/M_n, where M_n was a number average molecular mass. The ratio was equal to 1.2 and 1.3 for PMA and PEVP, respectively.

Immunogen Synthesis

Butachlor-3-mercaptopropionic acid (BMPA) was used as the hapten for the synthesis. The named carboxylated derivative of butachlor was generously provided by Dr. S.A. Eremin (Moscow State University, Russia). Its obtaining was described in ref.[30] The BMPA was covalently attached to BSA using the following technique of succinimide/carbodiimide synthesis. A mixture of 30 mg (75 μmol) of the BMPA, 47 mg (450 μmol) of NHS and 94 mg (450 μmol) of DCC in 1.0 mL of dimethylsulfoxide was allowed to react overnight under stirring and room temperature. Next the mixture was centrifuged and the supernatant added dropwise to a solution of 66 mg of BSA (1 μmol) in 10 mL of 0.15 M Na-carbonate buffer, pH 9.5. The reaction mixture was incubated overnight at 4°C, and low molecular weight compounds were removed by dialysis against PBS (50 mM K-phosphate buffer, pH 7.4, containing 0.1 M NaCl) during two days at 4°C and then against water. According to the UV spectral data, BPMA:BSA molar ratio in the obtained product was 14:1.

Immunization

Chinchilla rabbits weighting 3–4 kg were immunized by the BMPA-BSA conjugate according to the following procedure. Priming injection was carried out intradermally and subcutaneously to seven points on the back site of the animal's body with 1.0 mg of the immunogen in 0.5 mL of PBS emulsified with 0.5 mL of Freund's complete adjuvant. The rabbits received buster injections by one half dose of the immunogen subcutaneously (43rd day, in PBS with Freund's incomplete adjuvant, v/v = 1:1) and intravenously

(46th day, in PBS). After one week (53rd day) they were blooded. The reimmunization cycles were repeated with 5-week interval (subcutaneous immunizations – 60th, 95th and 130th days, intravenous immunizations – 81st, 116th and 151st days, bleeding – 88th, 123rd and 158th days). The blood was allowed to coagulate for 1 h at 37 °C and then for 2 h at 4°C; the antiserum was decanted and centrifuged.

In the presented experiments the last antisera preparation from rabbit #3 was used as well as it allowed reaching maximal sensitivity of butachlor detection in ELISA.[30]

Synthesis of Antigen-Peroxidase Conjugate

BMPA was covalently attached to HRP by succinimide/carbodiimide technique as described in ref. [30] 1.0 mg of the BMPA (2.5 μmol), 1.7 mg of NHS (16 μmol) and 6.2 mg of DCC (30 μmol) were dissolved in 130 μL of dimethylformamide and allowed to react overnight under stirring and room temperature. The obtained activated ester solution was added dropwise to 1.0 mg of HRP dissolved in 0.5 mL of 130 mM Na-carbonate buffer, pH 9.5. The interaction was performed for 3 h at room temperature, after which low molecular weight compounds were removed by dialysis against PBS and then against water.

Conjugation of Polymethacrylate with Protein A

The synthesis was carried out as described by Yazynina et al.[21] 6.0 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride was added to 1.0 mL of PMA water solution (6.0 mg/mL), and the pH was adjusted from 5.0 to 7.7–7.8 by addition of 1 M KOH. The mixture was incubated for 5 min with rigorous orbital shaking at room temperature. Then a solution of 6.0 mg of NHS and 6.0 mg of protein A in 1.0 mL of 0.1 M K-phosphate buffer, pH 7.8, was added. The obtained mixture was incubated for 2 h at room temperature keeping constant pH. Products of the synthesis were separated by gel-filtration on Toyopearl HW-55 (Toyo Soda, Tokyo, Japan, 1.6 × 100 cm column) in PBS.

Polyelectrolyte-Based Immunofiltration Assay of Butachlor

The following compounds were successively added to microplate wells: butachlor-containing sample (25 μL, a series of dilutions from 0.2 mg/mL to 0.03 ng/mL in PBST (PBS with 0.05% Triton X-100)),

butachlor-peroxidase conjugate (25 μL, 0.15 μg/mL in PBST), anti-butachlor antiserum (50 μL, dilution 1:500 in PBST), and protein A–PMA conjugate solutions (50 μL, 10 μg/mL in PBST). The reaction mixture was incubated for 10 min at room temperature with orbital shaking. A special holder was used for filtration; it consisted of two plain acrylic panels (each of them 1.0 cm thick) that can be assembled together or taken apart using thumb-screws. The top panel had 32 conical holes (each with a volume of 125 mL), and the bottom one was a solid acrylic sheet. Sixteen layers of filter paper were placed on the bottom panel, and the UltraBind US450 (Gelman Sciences—Pall, New York, USA) or Hybond-N^+ (Amersham Biosciences, Piscataway, USA) membrane was placed above. The panels were assembled tightly and 50 mL of PEVP (40 mg/mL, in PBS) was added into each hole. After full imbibing of the polycation solution, the holes were washed by 100 mL of PBST. Then 100 mL of the reaction mixture was added. (In the case of Hybond-N^+ the reaction mixture was deposited on a non-treated membrane.) After filtration the membrane was twice washed with 100 mL of PBST. To prepare the substrate solution for membrane staining 2.5 mg of DAB was dissolved in 5.0 mL of PBS and then combined with 100 mL of 40 mM $NiCl_2$ and 50 mL of 1 M H_2O_2 aqueous solutions. Two methods were used for membrane staining: the holder was taken apart and the membrane was placed into the substrate solution, or the substrate solution was introduced directly into the holder holes. After 2–3 min incubation, brightness of the colored spots was measured by scanner ScanJet 5p (Hewlett-Packard, Palo Alto, USA) or by portable digital camera WAT201A (Watec America Corp., Las Vegas, USA).

Data Processing

The obtained competitive curves (brightness of the colored spots versus antigen concentrations) were fitted by four-parameter sigmoid equation:

$$y = (A - D)/[1 + (x/C)^B] + D$$

The C value accords to antigen concentration causing 50% inhibition of the antibody-conjugate binding (IC_{50}). Cross-reactivity (CR) was calculated using the equation:

$$CR = 100\% \times IC_{50\ \text{butachlor}}/IC_{50\ \text{cross-reactant}}$$

If IC_{50} for cross-reactants cannot be measured, the ratio of IC_{20} values was applied for the CR calculation.

RESULTS

Determination of Optimal Protocol for Membrane Immunoassay of Butachlor

Both formats of the proposed polyelectrolyte-based immunoassay (with immobilized polycation and with positively charged membrane) have been preliminary tested for non-specific interactions. As can be seen from Fig. 2, in the absence of specific antibodies the butachlor-peroxidase conjugate does not interact with membrane carriers under the assay conditions, although electrostatic binding of proteins may be regarded as a potential non-specific obstacle for the formation of the detected labeled complexes. The obtained result corresponds to the prior data about the polyelectrolytes[31] and can be interpreted as a consequence of supplanting initially bound molecules from complexes with polyanion by positively charged carrier that has a significantly higher density of reactionary groups.

Optimization of the assay protocol included choice of the durations of its stages. The incubation of reactants in solution for 5–10 min was found to be sufficient to reach the equilibrium of both antigen–antibody and immunoglobulin–protein A reactions. The 2–3 min filtration permits to separate the formed complexes from non-reacted molecules without a substantial shift of the chemical equilibrium. As well as the polyelectrolytes

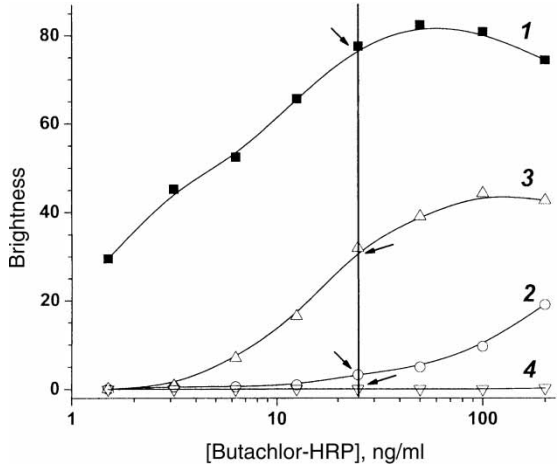

Figure 2. Binding of butachlor-peroxidase conjugate with anti-butachlor antibodies in membrane system (curves 1, 3) and non-specific adsorption of the conjugate (curves 2, 4). Curves 1 and 2 were obtained for Hybond-N^+ membrane, curves 3 and 4—for Ultrabind one. Vertical line with pointers accords to the concentration of the conjugate that is chosen for competitive immunoassay. On X-axis—concentration of the butachlor-peroxidase conjugate, on Y-axis—brightness of the formed spots.

interact with each other with extremely high rate, the indicated above duration of the filtration stage allows to reach sufficient intensity of the detected optical signals. Thus, the total assay time (including handling procedures) is about 15 min.

The second part of the protocol optimization was the choice of reactants concentrations. The studied ranges were 1:200–1:5,000 for antisera dilutions, and 10–100 ng/mL for concentrations of the butachlor–peroxidase conjugate. The competitive curves obtained under different reactants ratios were presented as dependences of spots' brightness versus butachlor concentration. The optimization criterion was maximal sensitivity of butachlor detection under the maintenance of acceptable accuracy. The chosen assay protocol envisages the content of antisera in the final reaction mixture being 1:1,500 and the content of butachlor-peroxidase conjugate being 25 ng/mL. The indicated conjugate concentration corresponds to the absence of non-specific binding on the membrane and is close to the saturation level of the specific binding (see Fig. 2).

Analytical Characteristics of the Developed Immunoassay

Under the chosen conditions, the calibration curves for butachlor detection have been obtained (Fig. 3). Two characterized assay formats differ in approaches for the binding of polyanion-containing complexes. The first format is based on the polycation adsorption on UltraBind membranes, and

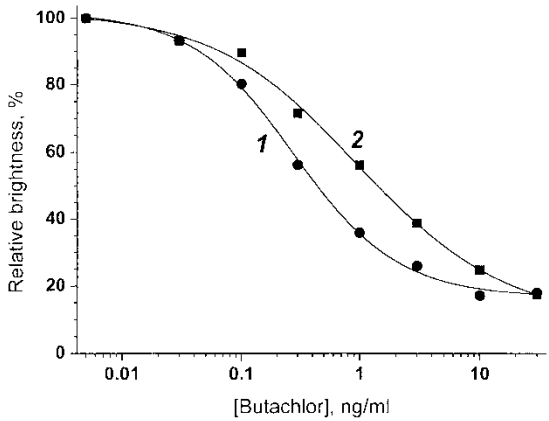

Figure 3. Calibration curves for butachlor detection under optimized regime of the filtration immunoassay: 1—with Ultrabind membrane and adsorbed polycation, 2 with charged Hybond-N^+ membrane. On X-axis—butachlor concentration, on Y-axis—relative brightness of the formed spots (100% is its maximal level in the absence of competitor).

the second one—on the own positive charges of Hybond-N^+ membranes. The both competitive curves are adequately approximated by four-parametric sigmoid equation; R^2 is 0.9986 and 0.9974, correspondingly. The first format has the detection limit of 0.03 ng/mL and the range of quantitative butachlor determination (IC_{20}–IC_{80}) – 0.07–1.1 ng/mL. For the second format the corresponding parameters are 0.05 ng/mL and 0.14–7.0 ng/mL. As well as the first format was found to be more sensitive, it was applied in the further studies.

The results of reproducibility studies are given in Table 1. The relative standard deviation of the spots' brightness is no more that 9% for the range of butachlor concentrations from 0.1 to 3.0 ng/mL. The given value is comparable with characteristics of traditional microplate ELISA. This narrow variation of repeated densitometric measurements ensures correct determination of the antigen content as well as reliable qualitative control of the exceeding of a threshold level of competitor. The "cut-off" level for visual qualitative test corresponds approximately to IC_{50}, being equal to 0.3 ng/mL for UltraBind-based assay and 1.0 ng/mL for Hybond-N^+-based one.

Specificity of the Butachlor Filtration Immunoassay

The earlier developed microplate ELISAs of butachlor[30] were described in terms of their specificity to structurally like pesticides. As well as we used the same immunoreactants, an analogous characterization of membrane immunoassay has been carried out.

We have tested acetochlor, alachlor and metolachlor as cross-reactants because of these pesticides are structurally close to butachlor and are widely used in modern agricultural practice. The determined CR values given in Table 2 confirm similarity of selectivities for the immunofiltration and ELISA in spite of the differences in reactants' ratios and regimes of interactions. The maximal found CR was 0.3% for alachlor (the same as in ELISA), thus providing applicability of the developed assay for selective determination of the target pesticide. Based on the cross-reactivity data one can conclude that

Table 1. Reproducibility of the immunofiltration assay of butachlor

Butachlor concentrations in tested samples, ng/mL	Variation coefficients of repeated measurements (n = 6), %
0.1	7.0
0.3	8.7
1.0	9.0
3.0	4.8

Table 2. CR values of structurally like pesticides in the developed filtration immunoassay and in ELISA of butachlor

Pesticide	CR in filtration immunoassay	CR in ELISA with labeled antigen (Ref. [30])
Butachlor	100%	100%
Acetochlor	0.15%	0.2%
Alachlor	0.3%	0.3%
Metolachlor	0.07%	0.1%

the anti-butachlor antibodies interact both with the alkylation pattern of the benzene ring and acetamide moiety. The specific recognition of butachlor molecule is grounded on its bulky butoxy-group.

Butachlor Revealing in Water Samples

The final task of the presented investigation was to verify availability of the developed analytical system for environmental monitoring, namely for water quality control. Influence of samples matrix on the competitive curves of butachlor detection was tested at first. Mineral, artesian and drinking water samples were taken for these studies. No inhibition of the interaction between butachlor-peroxidase conjugate and specific antibodies was found in their presence. Thus the samples do not contain own butachlor. The samples were spiked with butachlor and after that the competitive filtration immunoassay was realized. The final matrix content (v/v) in the reaction mixture was 17%.

As can be seen from Fig. 4, the matrixes did not cause essential changes of the competitive curves. Statistical analysis shows (Table 3) that IC_{50} of the curves differs no more than at 1.5-fold. Stable affinity of the immune interaction allows correct testing of environmental water samples. Constancy of A_{min} and A_{max} values confirms also the fact that the matrix does not influence neither label activity nor non-specific sorption. Hence, pollution of environmental water sources may be controlled by the developed analytical technique.

DISCUSSION

We used brightness of colored spots as a parameter reflecting the label binding. Digital processing of the membrane images was carried out to obtain quantitative characteristics of the brightness. The difference between middle brightness in and out the filtration zone was calculated by means of standard procedure of Adobe Photoshop program and chosen as the controlled

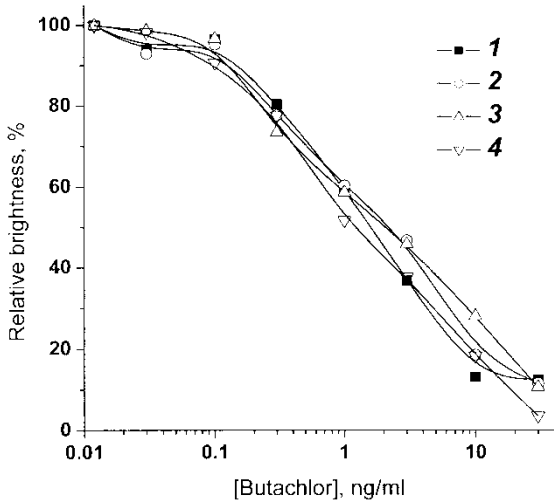

Figure 4. Filtration immunoassay of butachlor in distilled (1), mineral (2), artesian (3) and drinking (4) water. On X-axis—butachlor concentration, on Y-axis—relative brightness of the formed spots (100% is its maximal level in the absence of competitor).

parameter. It is reasonable because colored zones formed in the course of the assay have preset size and location. To verify reliability of this simple algorithm we have studied reproducibility of repeated measurements. The simplest variant of the colored spots formation was used, namely direct sorption of peroxidase label. This variant with minimal manipulation divergences had variation coefficient 3.5% ($n = 8$) that was in accordance with deviations of pipetting procedure. Under appropriate brightness/contrast parameters of measuring technique the graduating dependence is reliable for 100-fold variation of peroxidase dilutions. Programs TotalLab (vers. 2.0) and ImageQuant (vers. 3.3) were tested as alternate tools for image processing. They are also acceptable for quantitative assay, but in spite of additional tools of recognition and processing they do not give preferences in reproducibility of measurements, extend of working range or other analytical parameters. Thus the simple processing algorithm based on brightness averaging in the

Table 3. Four-parametric sigmoid fittings of the competitive curves obtained for the filtration immunoassay of butachlor in water matrixes

	IC_{50}, ng/mL	A_{min}	A_{max}	Power
Distilled water	1.34	16.75	108.82	1.04
Mineral water	2.13	7.22	110.11	0.73
Artesian water	2.06	5.55	114.10	0.62
Drinking water	1.51	3.98	112.68	0.71

controlled zone is sufficient for application in portable photometric detectors for the membrane assays.

In the presented investigation, the polyelectrolyte separation of immunoreactants has been applied for quantitative determination of new target antigen. This assay format combines homogeneous immune interactions with rapid separation of the reaction mixture. In order to realize the assay based on a standard set of immunoreactants for ELISA, we have used the polyanion conjugate with protein A of Staphylococcus aureus. This conjugate allows applying the polyelectrolyte separation technique for different compounds without conjugation or immobilization of specific antibody preparations. The binding of F_c-region of antibodies by the protein A in the course of the assay does not impede effective interaction of the antibodies with the antigen molecules because of $F_{(ab)2}$-region of the antibodies remains to be accessible.

The assay has been realized using the reactants that were earlier used for microplate ELISA. In spite of different formats the reached sensitivity is close to the ELISA with labeled antigen (0.04 ng/mL).[30] High selectivity of the target antigen detection was also preserved as was indicated above. While the conversion from ELISA to non-equilibrium immunofiltration commonly causes coarsening of the assay, the polyelectrolytes ensure preservation of the analytical characteristics.

CONCLUSION

The proposed polyelectrolyte-based filtration immunoassay ensures considerable rapidity of testing (duration of the assay – 15 min). Herbicide butachlor may be determined in concentrations down to 0.03 ng/mL, which is below the threshold level regulated by EU for a single pesticide in drinking water (0.1 ng/mL). Matrixes of water samples do not impede the butachlor detection. The assay can be realized without stationary equipment, making it acceptable for on-site ecological monitoring.

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