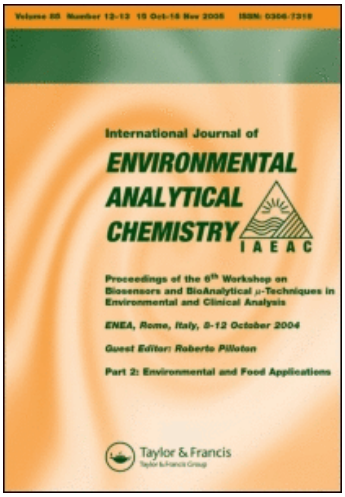

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Development of microformat imaging microplate and membrane immunoenzyme assays of the herbicide atrazine

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Development of microformat imaging microplate and membrane immunoenzyme assays of the herbicide atrazine

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A microformat imaging technique in combination with registration of chemiluminescence by a charge-coupled device, CCD camera, were applied for the detection of the herbicide atrazine by two immunoenzyme assay formats – microplate and membrane assays. For the microplate (ELISA) case the detection limit was 95 pg mL^{-1}, giving a 9.5-fold enhancement in comparison with a traditional colorimetric assay. The membrane dot blot assay allowed detection of up to 40 pg mL^{-1} of atrazine (4.5-fold enhancement). Due to the application of polyelectrolyte carriers for rapid reactants separation the membrane assay may be realized in 15 min. CCD-based registration of signals assures a high reproducibility of measurements; the variation coefficient is in the range 1–7%. The effectiveness of the assays developed for controlling triazine herbicides in mineral and tap water has been demonstrated.

Keywords: Enzyme immunoassay; Atrazine; Herbicides; Chemiluminescence; Charge-coupled device; Microformat imaging

1. Introduction

Immunoenzyme assays are extensively used nowadays in environmental monitoring, medical diagnostics and food quality control [1–4]. Standard detection of an enzyme label requires chromogenic substrates; therefore its sensitivity is limited by the extinction of colored products. The use of emitting light overcomes this disadvantage by dint of amplification and accumulation of the detected signal. Chemiluminescent emission is the preferable variant as it requires neither an excitation source nor (in many cases) a spectral filter [5–7]. Novel enhancers and optical transducers have greatly improved luminescence lifetime and signal read-out for the assays [8–10].

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However, the dynamic nature of the signals causes the necessity in their proper processing. Different imaging systems have been applied for this purpose [11–15]. The quantification of the emitted luminescence by a cooled charge-coupled device (CCD) camera allows the increase of the exposure time without upgrowth of the background. The CCD-based ELISA with chemiluminescent registration was realized previously for the herbicide 2,4-dichlorophenoxyacetic acid [16], but the assay was not confronted with traditional analytical techniques.

The aim of this work was to compare colorimetric and CCD-based chemiluminescent detection for two immunoenzyme assay formats, namely an ELISA in microtitration plates and a novel immunofiltration technique employing polyelectrolyte carriers. Atrazine, a herbicide from the *s*-triazine row, was chosen as the studied analyte due to its widespread agricultural application [17].

2. Experimental

2.1 *Materials*

1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate, *N*-hydroxysuccinimide, 3,3′-diaminobenzidine tetrahydrochloride (DAB), bovine serum albumin (BSA), dimethyl sulphoxide (DMSO), 2,2′-azino-di(3-ethyl-benzthiazolinesulfonic acid) diammonium salt (ABTS), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride and Freund's complete adjuvant were obtained from ICN Biomedicals. Dimethylformamide and Triton X-100 were from Serva. Horseradish peroxidase (HRP), $RZ = A_{403}/A_{275} = 3.2$, was from Biozyme, Great Britain. Protein A from *Staphylococcus aureus* was from Imtek, Moscow, Russia. Atrazine (2-chloro-4-(*N*-ethylamino)-6-(*N*-isopropylamino)-1,3,5-triazine) and its structural analogs, namely propazine, terbuthylazine, atraton, terbutryn, prometryn, desethyldesisopropylatrazine, terbumeton, and 2-amino-4-ethylamino-6-chlor-1,3,5-triazine, were from Riedel–de Haen. Fractionized preparations of polymethacrylic acid (PMA, M_w 260 kDa) and poly-*N*-ethyl-4-vinylpyridinium bromide (PEVP, M_w 2000 kDa) were synthesized as described earlier [18, 19]. These were generously provided by Prof. V. A. Izumrudov (Moscow State University, Russia). Other reactants and buffer components were of analytical grade. Sephadex G-50 (Pharmacia) and Toyopearl HW-55 (Toyosoda) were used for gel-filtration chromatography. The ELISAs were carried out in 96-well polystyrene microplates (Nunc).

2.2 *Apparatus*

Colorimetric registration of ELISA results was realized using a Multiscan EX microplate photometer (Labsystems). The brightness of colored spots formed in the course of filtration assays was detected by a Scan Jet 5P scanner (Hewlett Packard) and processed using Photoshop 7.0 software (Adobe).

For chemiluminescent immunoassays a Photometrix 200 CCD camera (Photometrix) was employed. The camera was thermoelectrically cooled to $-45^{\circ}C$ and fitted with a 50 mm AF Nikkor lens (Nikon). The CCD data obtained in the course of reaction were treated using Photometrix Processing software.

2.3 *Synthesis of atrazine-protein conjugates*

Atrazine-BSA (immunogen) and atrazine-HRP were synthesized according to the following procedure [20]. First, 3.0 mg of atrazine carboxylated derivative [21] (*N*-(6-(*N*-isopropylamino)-2-chlor-1,3,5-triazine-2-yl)-6-aminocaproic acid, 10 μmol) was diluted in 0.2 mL of dimethylformamide. Then 8.5 mg of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (20 μmol) and 2.3 mg of *N*-hydroxysuccinimide (20 μmol) were added, and the mixture was stirred for 1.5 h at room temperature. Then the solution of the activated hapten was cooled to 4°C and added to cooled protein solution (5 mg per 1 mL of 20 mM sodium carbonate buffer, pH 9.5) containing the same percent by volume of dimethylformamide. The hapten : protein molar ratio was 100 : 1 for BSA and 10 : 1 for HRP. The mixture was incubated with orbital shaking for 1 h at room temperature and then for 16 h at 4°C. The resulting conjugates were separated from low molecular weight compounds by gel-filtration on Sephadex G-50 (1 × 20 cm column, in 0.05 M potassium phosphate buffer, pH 7.4, with 0.1 M NaCl, hereafter named PBS) or by dialysis.

2.4 *Conjugation of polymethacrylate with protein A*

The synthesis was carried out as described earlier [22]. A total of 6.0 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (31 μmol) was added to 1.0 mL of PMA sodium salt solution (6 mg mL^{-1}), and the pH was adjusted to 7.8 by addition of 1 M KOH. The mixture was incubated for 5 min under vigorous orbital shaking and room temperature. Then a solution of 6.0 mg of staphylococcal protein A and 6.0 mg of *N*-hydroxysuccinimide (52 μmol) in 1.0 mL of 0.1 M K-phosphate buffer, pH 7.8, was added. The mixture was incubated for 2 h at constant pH and room temperature. The products were separated by gel-filtration on Toyopearl HW-55 (1.6 × 100 cm column) in PBS.

2.5 *Antisera obtaining*

To raise antibodies, Chinchilla rabbits weighing 3–4 kg were immunized according to the following technique [20]. The atrazine-BSA conjugate dissolved in PBS was emulsified with an equal volume of Freund's complete adjuvant to a final concentration of 0.5 mg mL^{-1} (by protein). For the first immunization, 1.0 mL of this mixture was injected four times (days 1, 15, 29 and 43) intradermally on multiple sites on the back from scapula to sacrum. After 2 months (day 103), the first boost was carried out: the rabbits were injected intravenously and intradermally with 0.3 and 0.2 mL of the immunogen, respectively, dissolved in PBS to concentration of 1.0 mg mL^{-1}. Seven days later (days 110), the rabbits were bled. This procedure was repeated on a monthly basis (boosting: days 133, 163 and 193, bleeding: days 140, 170 and 200). Antisera were separated by keeping the blood samples for 16 h at 4°C. Upper layers were collected, divided into aliquots and stored at −20°C.

2.6 *Microplate immunoenzyme assay*

Solution of staphylococcal protein A (100 μL, 3.0 μg mL^{-1} in PBS) was added into wells of microtiter plates and incubated for 2 h at 37°C (or for 16 h at 4°C).

Subsequently, the wells were washed four times with PBST (PBS, containing 0.05% Triton X-100). The specific antiserum was added into the wells (100 μL per well, dilution 1 : 500), incubated for 1 h at 37°C, and the microplates were washed repeatedly. After that 50 μL of antigen (row of dilutions in PBST) and 50 μL of atrazine-HRP conjugate (4.0 μg mL^{-1} for colorimetric detection and 0.5 μg mL^{-1} for chemiluminescent detection) were added into the wells. The microplates were incubated for 1 h at 37°C and then washed. Finally, peroxidase activity of the immobilized immune complexes was measured.

For the photometric detection 100 μL of the substrate contained 0.7 mM ABTS and 2.8 mM hydrogen peroxide in 30 mM sodium acetate buffer, pH 4.5, was added to each microplate well and incubated for 15–30 min at room temperature. Optical densities of oxidation products were measured at 405 nm.

The substrate for the chemiluminescent detection was prepared by mixing equal volumes of commercial luminol/enhancer and peroxide solutions (SuperSignal ELISA Pico Chemiluminescent Substrate, Pierce). A total of 100 μL of the substrate was added into each microplate well and chemiluminescent reaction was monitored for 5 min.

The competitive curves obtained were fitted using Origin 7.5 (OriginLab Corp.) software. Basing on parameters of four-parameter sigmoid fitting, concentrations of cross-reactants caused 50% inhibition of the (labeled antigen–antibodies) binding were used to characterize cross-reactivities of the analytical systems.

2.7 *Membrane immunoenzyme assay*

The reaction mixture was prepared in a microplate by successive addition of atrazine-containing samples (25 μL, a row of dilutions), atrazine-HRP conjugate (25 μL, 0.12 μg mL^{-1} for colorimetric detection and 0.04 μg mL^{-1} for chemiluminescent detection, in PBST), anti-atrazine antiserum (50 μL, dilution 1 : 1000 for colorimetric detection and 1 : 500 for chemiluminescent detection, in PBST), and protein A – PMA conjugate (50 μL, 2.0 μg mL^{-1}, in PBST). The mixture was incubated for 10 min at room temperature with orbital shaking. To separate the mixture compounds, a special holder was used. It consisted of two plain acrylic panels, the top one having a row of conical holes. Nitro-cellulose membrane Ultrabind (Pal-Gelman) and 30–40 layers of filter paper were placed between the plates that could be screwed together. First, 50 μL of PEVP (40 μg mL^{-1}, in PBS) was added into each hole. After full absorption of this solution, the holes were washed with 100 μL of PBST. Then 100 μL of the above reaction mixture was added. After full filtration, the membrane was washed with 100 μL of PBST.

Two methods were used for membrane painting. In the first, the holder was taken apart, and the membrane was placed into the substrate solution. In the second, the substrate solution was introduced directly into the holes. Substrate prepared by diluting DAB (2.5 mg) in 5.0 mL of PBS and then combining with 100 μL of 40 mM $NiCl_2$ and 50 μL of 1 M H_2O_2 aqueous solutions was used for colorimetric peroxidase detection [22]. The resulting mixture was incubated with the membrane (see above) for 1 min at room temperature. The substrate system for chemiluminescent detection as well as the fitting function used are described above.

3. Results and discussion

3.1 *Development and characterisation of microplate immunoassays*

Due to the low molecular weight of atrazine the immunoassay was carried out in competitive format, in which analyte-enzyme conjugate and analyte in the sample tested were incubated with immobilized antibodies, and finally the bound conjugate was quantified. Horseradish peroxidase was used as the enzyme label because of its wide application as well as a number of substrates giving coloured and chemiluminescent products. For preferable orientation of the immobilized antibodies facilitating antigen recognition, the microplate wells were preliminarily covered with staphylococcal protein A [20].

Proper dilutions of antiserum and concentration of the atrazine-peroxidase conjugate as well as duration of the assay steps were chosen for microplate immunoassays with colorimetric and chemiluminescent detection. The optimization criterion was the maximal sensitivity of atrazine revealing under the stipulation that acceptable accuracy of quantitative detection was maintained. A typical image taken with the CCD camera in the course of the conjugate concentration choice is shown in figure 1. Thus, rather low intensities of chemiluminescence can be processed to obtain their reliable quantitative characteristics.

Microplate assay with photometric detection was realized using the (ABTS + H_2O_2) substrate system. Judged from the competitive curve obtained (figure 2) the minimal reliably detected concentration of the analyte was accorded to $IC_{10} = 0.9\,ng\,mL^{-1}$.

Luminol is a widely used chemiluminescent substrate for peroxidase [23]. It requires neither an organic-containing solvent nor a fluorophore for excitation. The luminol/enhancer system from Pierce provides high intensity of luminescence for more than 2 h and a constant readout over a period of 30 min. The chosen exposure time was 5 min.

The chemiluminescent signals were analysed and the integral intensities were plotted as a function of the analyte concentration (figure 3). Under the chosen conditions the limit of detection reached was $0.095\,ng\,mL^{-1}$. The CCD-based detection allows us to increase immunoassay sensitivity (see table 1) as a result of a corresponding decrease of the atrazine-peroxidase conjugate concentration used. Processing of weak chemiluminescent signals makes possible reliable assays for lower concentrations of the conjugate ($0.5\,\mu g\,mL^{-1}$ for chemiluminescent detection as compared with $4.0\,\mu g\,mL^{-1}$ for colorimetric detection). The average deviation of the repeated measurements in the range of quantitative antigen detection did not exceed 5% (data not shown).

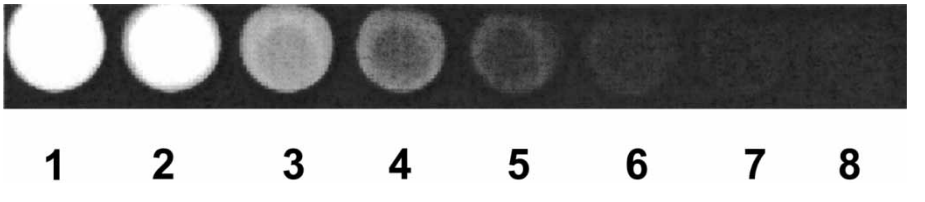


Figure 1. Image taken for atrazine ELISA with chemiluminescent detection. Concentration of atrazine-peroxidase conjugate varied (from left to right) from 8 to $0.06\,\mu g\,mL^{-1}$ with step 2, the chosen concentration ($0.5\,\mu g\,mL^{-1}$) accords to the 5th well.

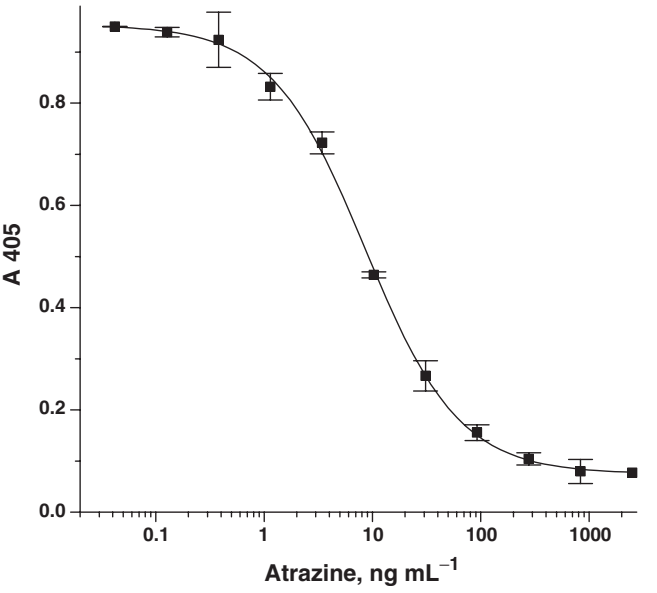


Figure 2. Calibration curve for atrazine ELISA with colorimetric detection. Here and below assay data are presented as Means ($n = 6$) ± SEM (standard error means).

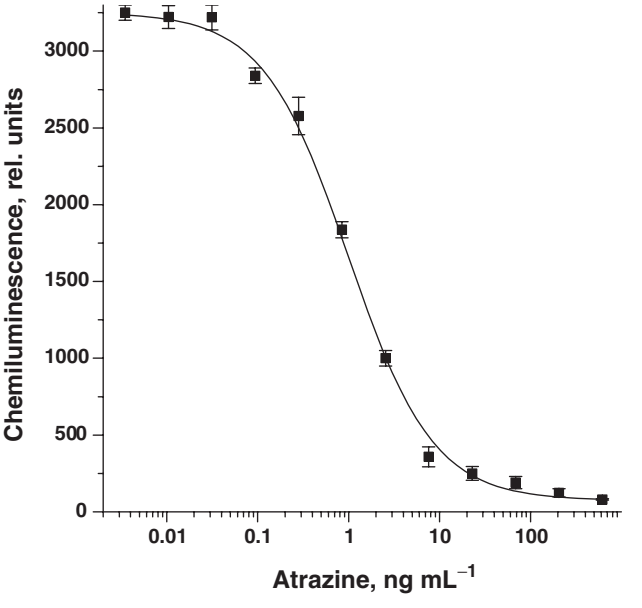


Figure 3. Calibration curve for atrazine ELISA with chemiluminescent detection.

3.2 *Development and characterization of membrane immunoassays*

The second part of the work was directed to developing a membrane immunoassay with possibilities for both qualitative ('yes'–'no') and quantitative registration

Table 1. Analytical parameters for two ELISAs of atrazine.

	Colorimetric detection	Chemiluminescent detection
IC_{50}, ng mL^{-1}	8.24	0.99
Power of sigmoid fitting curve	0.99	1.13
Working range of quantitative antigen determination (IC_{10}–IC_{90}), ng mL^{-1}	0.90–75.6	0.095–10.3

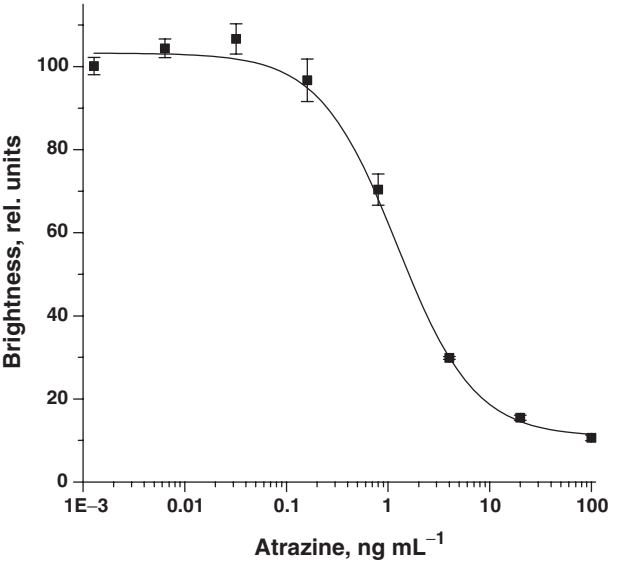


Figure 4. Calibration curve for atrazine membrane immunoassay with colorimetric detection.

of the assay results. The typical format of this assay for low-molecular weight antigens is based on the immobilization of specific antibodies on the membrane and competitive binding of enzyme-labeled and free (to be determined) antigen during the filtration process. The main trouble in realizing this concept is connected with low signals, since the affinity of the antigen–antibody reaction may be insufficient for high yield of the immune complexes.

Polyelectrolyte carriers were proposed earlier as tools to separate the immunoreactants [22]. Due to the high cooperativity of polyvalent interactions between the chains of polymethacrylic acid and poly-*N*-ethyl-4-vinylpiridinium, the interpolymeric complex is formed at extremely high rate and practically irreversible [22]. The interaction between the immobilized polycation and the polyanion conjugated with antibody-binding compound staphylococcal protein A is proposed as a tool for filtration assays. Immune complexes containing polyanion are formed in solution and separated from unreacted compounds in the course of filtration. Such assays with photometric detection were realized earlier for methamphetamine [18], simazine [24] and testosterone [25].

The protocols of the immunofiltration assays with colorimetric and chemiluminescent detection were optimized to reach maximal sensitivity of antigen revealing. In the case of the (DAB + H_2O_2) substrate system up to 0.18 ng mL^{-1} of atrazine can be detected (figure 4), while qualitative visual tests may be realized for nanogram levels

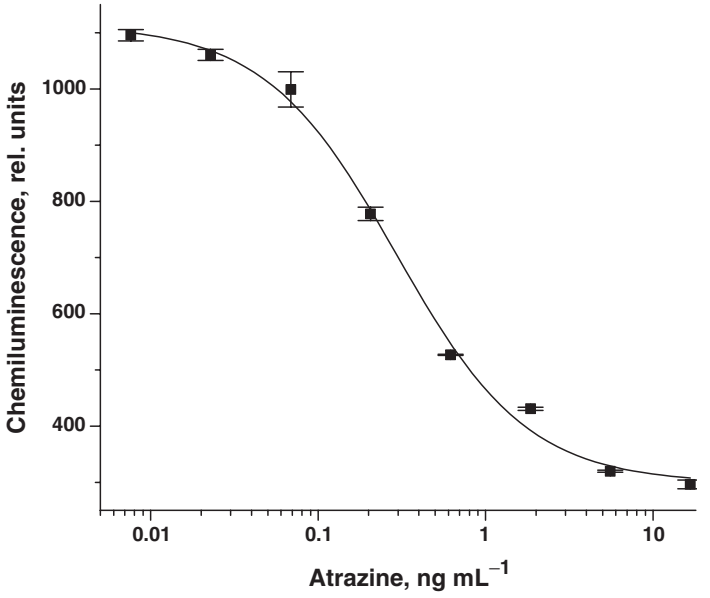


Figure 5. Calibration curve for atrazine membrane immunoassay with chemiluminescent detection.

Table 2. Analytical parameters for two membrane immunoassays of atrazine.

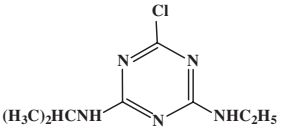
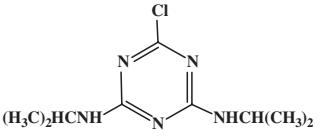
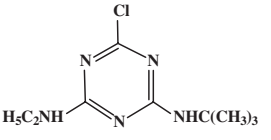
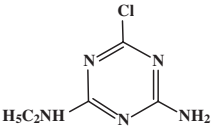
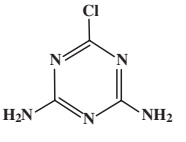
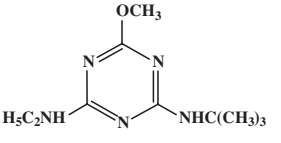
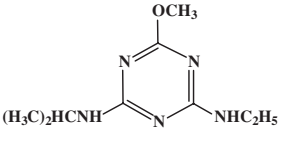
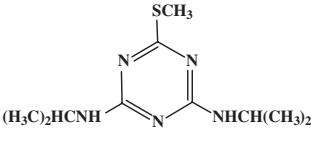
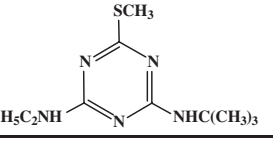
	Colorimetric detection	Chemiluminescent detection
IC_{50}, ng mL^{-1}	1.22	0.29
Power of sigmoid fitting curve	1.14	1.06
Working range of quantitative antigen determination (IC_{10}–IC_{90}), ng mL^{-1}	0.18–8.30	0.040–2.06

Table 3. Coefficients of variation for membrane immunoassay of atrazine with chemiluminescent detection.

Atrazine concentration, ng mL^{-1}	Intra-assay CV, % ($n=6$)	Inter-assay CV, % ($n=6$)
1.9	4.35	3.86
0.6	4.23	4.56
0.2	4.76	5.37
0.07	4.38	7.09

of the analyte. In the case of the (luminol + H_2O_2) substrate system the limit of atrazine detection is equal to 0.04 ng mL^{-1} (figure 5). Analogously to the above described ELISA systems, the sensitivity growth (table 2) corresponds to a decrease of the used concentration of atrazine-HRP conjugate, namely 0.12 μg mL^{-1} for colorimetric detection and 0.04 μg mL^{-1} for chemiluminescent detection. For the proposed CCD-based membrane immunoassay in the range of quantitative antigen detection the average deviation of the repeated measurements did not exceed 7% (table 3).

Table 4. Cross-reactivity of the chemiluminescent immunoassays for the atrazine-related compounds.

s-Triazine derivative	Structure	Cross-reactivity (%) ELISA	Cross-reactivity (%) Immunofiltration
Atrazine		100	100
Propazine		54	77
Terbuthylazine		< 1	8
2-Amino-4-ethylamino-6-chlor-1,3,5-triazine		< 1	< 1
Desethyldesiso-propylatrazine		< 1	< 1
Terbumeton		< 1	10
Atraton		31	66
Prometryn		100	79
Terbutryn		2	17

3.3 *Cross-reactivity of the systems developed*

Nine structurally close atrazine pesticides and their derivatives were tested to evaluate the assay specificity, namely propazine, terbuthylazine, desethyldesisopropylatrazine, 2-amino-4-ethylamino-6-chlor-1,3,5-triazine (representatives of chlorotriazines), terbutrin, prometrin (representatives of methylthiotriazines), atraton and terbumeton (representatives of methoxytriazines). As can be seen from table 4, in the ELISA antibodies interact effectively with prometryn, propazine and atraton. All these compounds have an isopropylamino group at the same position. The immunofiltration assay combines specificity to compounds containing 4-isopropylamino group (prometryn, propazine and atraton) and also to those having 2-ethylamino group (atraton).

3.4 *Matrix influence on the assay performance*

To investigate the matrix effect on the assay performance, atrazine standard curves were obtained in apple juice, pineapple–orange juice, orange juice, milk, mineral and tap water and compared to the one in PBST. The calibration curves obtained in undiluted water matrices showed similar working ranges for atrazine detection, namely 0.12 – 6.02 ng mL^{-1} for mineral water and 0.086–5.13 ng mL^{-1} for tap water (ELISA format). Two-fold dilutions of water samples in the course of the membrane assay were found to be enough for complete atrazine revealing in water matrices. On the other hand, milk and juice samples were required to be properly diluted (to about 500-fold) to avoid the matrix influence on the binding levels (ELISA format). The intra-assay coefficients of variation for the ELISA in different matrices were in the range of 1–3% (table 5) that corresponded to those for measurements in PBST. But for membrane assay milk and juice samples needed to be extremely diluted (about 3000-fold) so as the reaction mixture could be filtered through the membrane. After such dilution the same working ranges for atrazine detection as for tap and mineral water were revealed.

4. Conclusion

Accumulation of signal by CCD camera provides the essential growth of sensitivity in immunoassays. As a result the reached sensitivities of atrazine detection by CCD-based microplate ELISA and immunofiltration assay are 95 and 40 pg mL^{-1}, respectively. The levels given are acceptable for the needs of environmental monitoring.

Table 5. Coefficients of variation for ELISA of atrazine with chemiluminescent detection in matrices.

	Intra-assay CV, % ($n = 6$)			
Atrazine concentration, ng mL^{-1}	Apple juice	Pineapple–orange juice	Orange juice	Milk
8.3	1.97	1.90	1.37	2.76
2.8	1.27	1.97	1.03	2.05
0.9	1.60	2.05	2.34	0.65
0.3	2.34	1.54	1.92	0.49

The investigations carried indicate the effectiveness of the assays proposed for the control of pesticide pollution in water matrices. Adaptation of the assay protocols for other matrices will be the task of further investigations. Besides, the polyelectrolyte separation makes the membrane immunoassay rather rapid (duration – 15 min) and may be used in combination with other immunoreactants for the detection of different compounds.

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