Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 69 (2006) 359–364
Talanta
www.elsevier.com/locate/talanta

Determination of environmental organic pollutants with a portable optical immunosensor

E. Mauriz [a,*], A. Calle [a], A. Montoya [b], L.M. Lechuga [a]

[a] *Microelectronics National Centre (CNM), CSIC, Isaac Newton 8, 28760 Tres Cantos, Madrid, Spain*
[b] *Centro de Investigación e Innovación en Bioingeniería, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain*

Received 31 January 2005; received in revised form 6 May 2005; accepted 29 September 2005
Available online 16 November 2005

Abstract

A portable surface plasmon resonance (SPR) optical biosensor device is described as a direct immunosensing system to determine organic pollutants in natural water samples. Monitoring of organochlorine (DDT), organophosphorus (chlorpyrifos) and carbamate (carbaryl) compounds within the concentration levels stipulated by the European legislation, can be accomplished using this immunosensor. The lowest limit of detection (LOD) was obtained for DDT, at 20 ng L^{-1}, whilst 50 ng L^{-1} and 0.9 μg L^{-1}, were achieved for chlorpyrifos and carbaryl, respectively. Matrix effects were evaluated for the carbaryl immunoassay in different water types with detection limits within the range of carbaryl standard curves in distilled water (0.9–1.4 μg L^{-1}). The covalent immobilization of the analyte derivative through an alkanethiol self-assembled monolayer (SAM) allowed the reusability of the sensor surface during more than 250 regeneration cycles. The quality of the regeneration was proved over a 1-month period of continuous working. The analysis time for a complete assay cycle, including regeneration, comprises 24 min. Our portable SPR-sensor system is already a market product, commercialized by the company SENSIA, SL. The size and electronic configuration of the device allow its portability and utilization on real contaminated locations.

Keywords: DDT; Chlorpyrifos; Carbaryl; SPR; Immunosensor; Environmental analysis

1. Introduction

The pollution caused by certain dangerous substances released into the environment poses a significant risk to aquatic ecosystems and human health. Monitoring of organic pollutants, through real-time on-site methods, is essential for the control of human and animal exposure to the adverse effects derived from several groups of pesticides, pharmaceuticals and endocrine disrupters. Specific measures have been adopted by drinking water legislations to reduce and control the emission of hazardous compounds into environmental waters. The European Union has limited the maximum allowable concentration for a single pesticide to 0.1 μg L^{-1} (Drinking Water Directive: 98/83/EC, 1998) compelling governments to an extensive monitoring of water resources used for human consumption (WFD 2000/60/EC, 2000) [1,2].

Although chromatographic and spectroscopic methods are routinely used in pesticide identification and quantification [3], they can hardly be adapted for field use. The need of fast on-site methods as complementary techniques to highly sensitive HPLC or GC, has led to the application of immunological technology to pesticide analysis [4]. Immunoassay techniques have overcome the requirements of the continuous monitoring of pollutants allowing the screening of a large number of samples [5]. However, these systems (e.g. ELISA formats) use labels to detect the immunological reaction and require several incubation and washing steps that take several hours until the analysis is completed.

The coupling of immunoassay methods with sensor systems allows the determination of analytes in natural water samples rapidly, directly and in a reversible manner [6,7]. This work describes the application of a commercial optical sensor system (β-SPR, SENSIA, SL) based on surface plasmon resonance (SPR) physical principle [8] to the analysis of environmental organic pollutants. The sensing mechanism is based on variations of the refractive index of the medium adjacent to the metal

* Corresponding author. Tel.: +34 91 8060700; fax: +34 91 8060701.
E-mail address: elba@imm.cnm.csic.es (E. Mauriz).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.09.049

sensor surface during the interaction of the analyte to its corresponding recognition element, previously immobilized at the sensor surface [9,10]. Once the biomolecular interaction takes place, the regeneration of the surface allows the reusability of the same sensor surface during a large number of assay cycles.

The aim of this work was to prove the feasibility of this SPR sensor system to measure priority organic substances such as organochlorine (DDT), organophosphate (chlorpyrifos) and carbamate (carbaryl) compounds within the range proposed by the European legislation. To our knowledge, no previous monitoring of the above-mentioned pesticides with an SPR immunosensor has yet been published. The development and optimization of the immunoassay format, the immobilization procedure and the reusability of the biosensing surface was also evaluated in order to show the enormous potential of this immunosensor for on-line analysis of ground, surface and tap water samples. In addition, this sensor has also the capability of a wireless transmission of data from real contaminated locations to a central laboratory.

2. Experimental

2.1. Immunoreagents and chemicals

Immunoassays were developed with the immunoreagents provided by the Centro de Investigación e Innovación en Bioingeniería (Universidad Politécnica de Valencia, Spain), and had been previously characterized by ELISA. The pesticide standards DDT and carbaryl were from Riedel-de Haën (Seelze, Germany) and the standard of chlorpyrifos was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of 1 mM were prepared in dry dimethylformamide (DMF) and stored at −20 °C. From these stock solutions, working standards in the 0.02–200 μg L^{-1} range were prepared daily by serial dilutions in distilled water or PBST (PBS: 10 mM phosphate-buffered saline solution, pH 7.35, containing 0.05% of the surfactant Tween-20 (T20)), depending on the pesticide studied.

The synthesis of DDT, chlorpyrifos and carbaryl haptens [DDT5, 4-{4-[1-(4-chlorophenyl)-2,2,2-trichloroethyhl] phenyl}butanoic acid], [CN4C, O-ethyl O-(3,5,6-trichloro-2-pyridyl) *N*-(3-carboxypropyl) phosphoramido thioate] and [CNH, 6-[[(1-naphtyloxy)carbonyl]-amino] hexanoic acid]; the preparation of the BSA–DDT5, BSA–CN4C and BSA–CNH conjugates; and the production of anti-DDT monoclonal antibody (MAb) LIB–DDT5.25, anti-carbaryl MAb LIB–CNH45 and anti-chlorpyrifos MAb LIB–PO have been described in other papers [11–13].

Common chemicals used in sensor surface immobilization were purchased from Sigma–Aldrich (Steinheim, Germany): mercatoundecanoic acid; *N*-hydroxysuccinimide (NHS) and 1-ethyl-3(-3 dimethyl-amino-propyl) carbodiimide hydrochloride (EDC). Ethanolamine hydrochloride blocking agent was purchased from Acros Organics (Geel, Belgium). Tween-20 was obtained from Quantum Appligene (Heidelberg, Germany) and the organic solvents utilized for the gold-coated film cleaning, ethanol and acetone, were supplied by Merck (Darmstadt, Germany). The additional compounds potassium chloride, sodium chloride, di-sodium hydrogen phosphate and potassium dihydrogen phosphate, used for the preparation of 1 X PBS 10 mM, were provided by Panreac (Barcelona, Spain).

2.2. Instrumentation

A scheme of the commercial SPR used in this work is shown in Fig. 1. It is based on the Kretschmann configuration to achieve the resonant condition by total internal reflection. A polarized 3 mW laser diode operating at 670 nm incides, once divided into two equal beams, on the gold-coated sensing surface where two flow cells with the same volume (300 nL) are placed. The use of two flow cells allows the measurement of two independent samples or the referring of one single analysis. Changes in the intensity of the reflected light indicating shifts in mass at the surface were detected by a multi-element photodiode, amplified and finally converted to a digital signal. During SPR experiments, measurements were carried out at a fixed angle of incidence in order to monitor changes in the refractive index in real-time as the intensity of the reflected light varied.

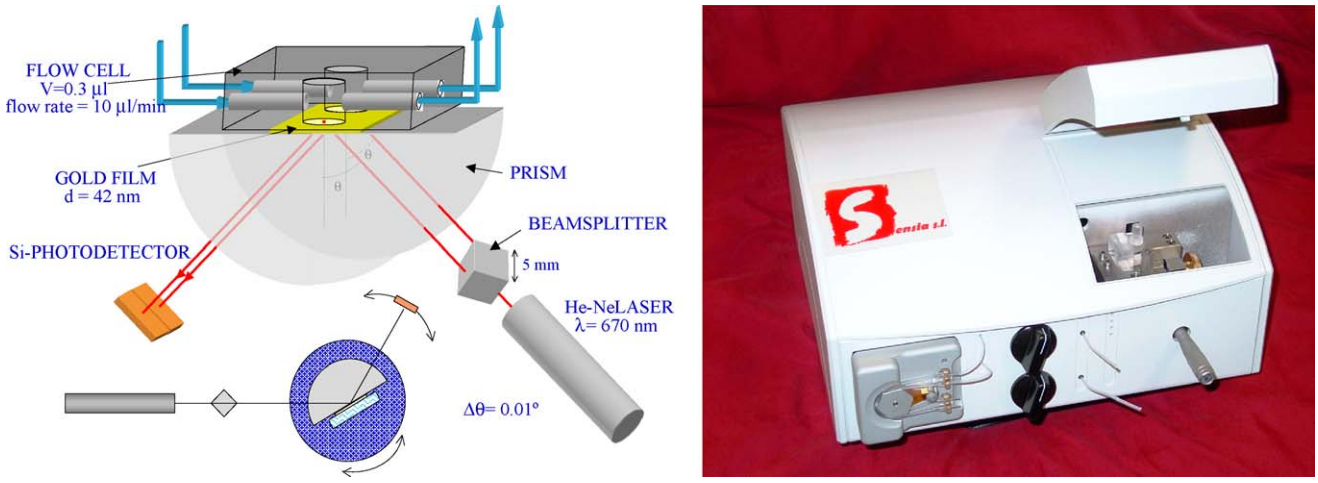

Fig. 1. Portable SPR sensor prototype system including sensor, optics, electronics and flow delivery system: (a) scheme of the SPR device and (b) commercial β-SPR platform.

Samples were transferred to the SPR immunosensor using a flow delivery system incorporated in the β-SPR platform at a constant speed of 30 μL min^{-1}. This system assures the injection of precise volumes of samples of 240 μL, while maintaining a continuous flow of liquid onto the sensor surface. A complete measurement cycle, including injection of sample and regeneration, takes 24 min.

2.3. Preparation of the sensor surface

A gold metallized glass slide was used as sensor surface. To obtain resonance curves with minimum reflectance values, the metal thickness was optimized to 50 nm of gold and 2 nm of chromium. Prior to metallization, glass slides (10 mm × 10 mm × 0.15 mm) were first cleaned with organic solvents: trichloroethylene, acetone and ethanol, immersed in a freshly prepared piranha solution (H_2SO_4/H_2O_2, 3:1), rinsed with water, ultrasonicated for 5 min and dried with N_2. Cleaned glass surfaces were metallized by thermal evaporation. Afterwards, metallized glass slides were cleaned using the same treatment with organic solvents and piranha, previously described. Finally, the gold slide was placed on the sensor system in contact with the flow cells and adhered to the prism through matching oil with the same refractive index ($n = 1.52$).

2.4. Immunoassay format

Biosensor immunoassays, for the determination of the target compounds, were binding inhibition tests [6,7] in which the analyte derivative, conjugated to a carrier protein (BSA), is covalently immobilized onto the sensor surface. For inhibition assays, a fixed amount of antibody is incubated for 10 min with the sample to be analyzed, until the equilibrium of the reaction is reached. The analyte molecules present in the sample bind to the antibody binding specific sites and the mixture is pumped over the hapten-conjugate coated sensor surface. Only antibodies with free binding sites can bind to the surface since the analyte inhibits antibody binding to the immobilized hapten-carrier conjugate. Thus, higher concentrations of analyte will increase the concentration of occupied antibodies reducing the SPR signal and the binding rate to the sensor surface.

After the antibody–hapten coupling, the disruption of the immunocomplexes was accomplished using appropriate regeneration agents for each analyte (HCl for DDT and carbaryl, and NaOH for chlorpyrifos). Reusability of the sensor surface is essential for the development and reproducibility of the assay as well as for the utilization of the biosensor as a portable on-field device. The use of high affinity and specific immobilized recognition elements is not sufficient and hapten conjugates should withstand a large number of regeneration cycles. During the performance of the SPR immunoassays, the same biospecific surface could be reused throughout hundreds of measurements.

2.5. Immobilization

The method of immobilization should provide a stable link between the immobilized component and the sensor surface under working conditions. In order to assure the regeneration and reusability of the sensor surface without denaturation of the immobilized molecule, the formation of self-assembled monolayers (SAM) is required. SAM can provide a covalent attachment of the ligand to the functionalized surface in a controlled way [14]. In this work, a carbodiimide coupling was used to achieve the mentioned covalent linkage between the recognition element and an alkanethiol functionalized surface under non-denaturing conditions [15,16].

The first step of the SAM formation is the adsorption onto the gold surface of a carboxylic terminated thiol (mercaptoundecanoic acid, 0.05 mM in ethanol), to achieve the Au–S attachment. Alkanethiol excess was eliminated by rinsing the surface with ethanol, and afterwards, water was delivered into the flow cells as a continuous flow during the remaining steps of the immobilization procedure. Once the alkanethiol monolayer was formed, the activation of the carboxylic groups took place by using two extremely reactive compounds: *N*-ethyl-*N*-dimethyl-amino-propyl carbodiimide (EDC, 0.2 M) and *N*-hydroxysuccinimide (NHS, 0.05 M). The formation of a stable reactive intermediate (*N*-hydroxisuccinimide ester) allowed the analyte derivative coupling via amine groups. After immobilization of 10 μg mL^{-1} of the corresponding hapten-conjugate, non-covalently bound biomolecules and unreacted NHS-esters remaining at the sensor surface were eliminated using ethanolamine 1 M, pH 8.5, as blocking agent. This procedure ensures that only covalently bound analyte derivatives remain on the sensor surface.

2.6. Water sample preparation

Distilled water was spiked with different concentrations of each pesticide in the range of 0.02–200 μg L^{-1}. Real water samples, not expected to contain carbaryl, were fortified with the target analyte to evaluate potential matrix effects on SPR immunoassays. These samples were spiked with carbaryl at 0.2 μg L^{-1}, 2 μg L^{-1} and 20 μg L^{-1} from a 1 mM stock solution of the correspondent analyte. Water samples tested were drinking water from the tap water of the town of Tres Cantos (Madrid), river water collected from the Samburiel river (Madrid, Spain) and groundwater from León (Spain). Turbid water samples were filtered with a 0.45 μm filter to eliminate particulate matters.

2.7. Data analysis

To optimize the reproducibility of the assay, three spiked analyte-antibody samples were measured for each concentration of pesticide standard. Normalized standard curves were obtained by plotting reflectance (V) against the logarithm of the target analyte concentration. The experimental points were fitted to the four-parameter logistic equation:

$$y = \left\{ \frac{D + (A - D)}{[1 + (x/C)B]} \right\}$$

where A is the asymptotic maximum (maximum SPR signal in absence of analyte, A_{max}), B the curve slope at the inflection point (related to the analyte concentration giving 50% inhibition

of A_{max}: C, I_{50}) and D is the asymptotic minimum (background signal).

3. Results and discussion

3.1. Analytical characterization of DDT, chlorpyrifos and carbaryl immunoassays

As it has been mentioned, the portable β-SPR biosensor was applied to determine three groups of pesticides: the persistent organochlorine pesticide DDT, and the acetylcholinesterase inhibitors chlorpyrifos (organophosphorus insecticide) and carbaryl (carbamate broad-spectrum insecticide). Final assay conditions for these three compounds are summarized in Table 1. The assay sensitivity values were comparable to those obtained by traditional immunological methods (e.g. ELISA) which use the same immunoreagents [5,11,17]. Limits of detection (LODs) were experimentally determined (i) as three times the S.D. of the blank signal and (ii) as the analyte concentration giving a 10% inhibition of the maximal SPR signal. The linear working ranges were also considered as the analyte concentration causing 20–80% inhibition of the maximal SPR signal.

For the DDT assay, the SPR immunosensor working in a dynamic range of 1.76–0.06 μg L^{-1}, proved to be more sensitive than colorimetric ELISAs [11,17]. The sensitivity reached by the SPR immunoassay was comparable to that obtained by chemiluminescent ELISAs [19] with values for the I_{50} of 0.26 μg L^{-1} and of (i) 0.07 μg L^{-1} and (ii) 0.02 μg L^{-1} for the LOD. The improvement in sensitivity could be a consequence of the method of preparation of standards from the stock solution due to the high hydrophobicity of the DDT compound. The immunoassay performance can also be affected by the ionic strength of the PBST used for the standards preparation. Similar effects have been described for other hydrophobic pesticides [5,18,19].

The sensitivity for chlorpyrifos, estimated as the I_{50} value, was 1.95 μg L^{-1}, with a dynamic range between 49.1 μg L^{-1} and 0.23 μg L^{-1} and a limit of detection of (i) 0.06 μg L^{-1} and (ii) 0.05 μg L^{-1} depending on the calculation method. Results obtained for chlorpyrifos in the SPR immunoassays showed also a slight improvement compared with the ELISA assay [20]. With respect to the carbaryl detection, the assay sensitivity was evaluated in the linear working range of 14.7–1.64 μg L^{-1}, with an I_{50} value and a limit of detection of 4.36 μg L^{-1} and (i) 0.1 μg L^{-1} and (ii) 0.9 μg L^{-1}, respectively (Fig. 2). In this case, further optimization of the analytical characteristics of the assay will be needed in order to achieve the sensitivity values reached by the ELISA method [21].

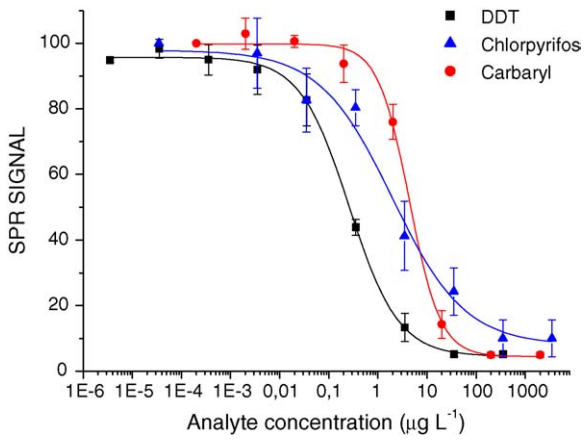

Fig. 2. Standard calibration curves of DDT, chlorpyrifos and carbaryl immunoassays. Standards in the range of 0.02–200 μg L^{-1} were prepared by serial dilution in PBST (DDT) or water (chlorpyrifos and carbaryl) from the stock solution in DMF. Each point represents the mean ± S.D. of three measurements and are fitted to a four-parameter logistic equation.

3.2. Selectivity of the sensor surface and sensor reusability

The affinity of the monoclonal antibodies employed in the assays to their correspondent analytes has been reported previously [11–13]. Similarly, the specificity of the MAbs has

Table 1
Analytical characteristics of the immunoassays for DDT, chlorpyrifos and carbaryl

Characteristics	DDT SPR assay (BSA–DDT5/LIB–DDT5-25)	Chlorpyrifos SPR assay (BSA–CN4C/LIB–PO)	Carbaryl SPR assay (BSA–CNH/LIB–CNH45)
Immunoreagent concentrations			
Hapten conjugate (μg mL^{-1})		10	
MAb (μg mL^{-1})	2.5	1	2
Buffer conditions			
Salt concentration		10 mM phosphate	
%Tween-20		0.05	
pH		7.35	
Standards preparation	PBST	Distilled water	Distilled water
Regeneration conditions			
Desorption agent	HCl 0.1 M	NaOH 0.2 M	HCl 0.1 M
Analytical calibration values			
I_{50} (μg L^{-1})	0.26	1.95	4.36
LODs (10% inhibition concentration, μg L^{-1})	0.02	0.05	0.9
RSD (%)	3.52	6.88	2.95
Working range (μg L^{-1})	1.76–0.06	49.1–0.23	14.7–1.64

been also evaluated by performing competitive assays with compounds structurally similar to DDT, chlorpyrifos and carbaryl, and cross-reactivities (CRs) have been calculated with the obtained I_{50} values [11,19–21]. According to these studies, anti-DDT MAb could be considered as a *class-specific* antibody due to the high cross-reactivity values exhibited for the *p,p′*-isomers of the DDT family. Therefore, LIB–DDT5-25 is adequate for determination of DDT isomers and metabolites [10]. The CR exhibited by chlorpyrifos metabolites and related compounds was negligible for anti-chlorpyrifos MAb LIB–PO and only showed higher affinity for chlorpyrifos-methyl than to chlorpyrifos [20]. Finally, carbaryl immunoassays proved to be very specific as showed by the negligible CRs exhibited by carbaryl metabolites and derivatives [21].

In the assays herein described, hapten–carrier conjugates were covalently attached to the sensor surface in order to confer stability and reproducibility to the analysis. Nevertheless, the selectivity of the sensor surface to avoid non-specific binding of molecules had to be evaluated. With this aim the sensor surface was exposed to the flow of non-related immunoreagents of different molecular weight. Although operating in a single analyte state, a sensing surface coated with the hapten-conjugate of DDT (BSA–DDT5) was exposed to the other MAbs and analytes involved in this study.

The assay proved that the sensor signal was not affected by the presence of the low molecular weight compounds, either carbaryl or chlorpyrifos. In addition, the exposition of the sensing surface to the flow of non-specific antibodies (anti-carbaryl LIB–CNH45 and anti-chlorpyrifos LIB–PO) demonstrated the capability of the SAM to recognize only the complementary reagents. Finally, a mixture of specific antibody (LIB–DDT5-25 2.5 $\mu g\,mL^{-1}$) and non-specific analyte (chlorpyrifos 351 $\mu g\,L^{-1}$) was pumped over the sensor surface. Since only the response corresponding to the anti-DDT MAb LIB–DDT5-25 was obtained, it was assured that the presence of non-related compounds did not interfere the registered sensor signal. Fig. 3 shows the absence of specific sensor signals when measuring the mentioned non-specific analytes.

Once demonstrated, the selectivity of the sensor surface to non-specific binding of biomolecules, the activity of the immunosurface was also evaluated through a large number of regeneration cycles (Fig. 4). As described above, disruption of hapten-antibodies complexes was achieved causing a strong

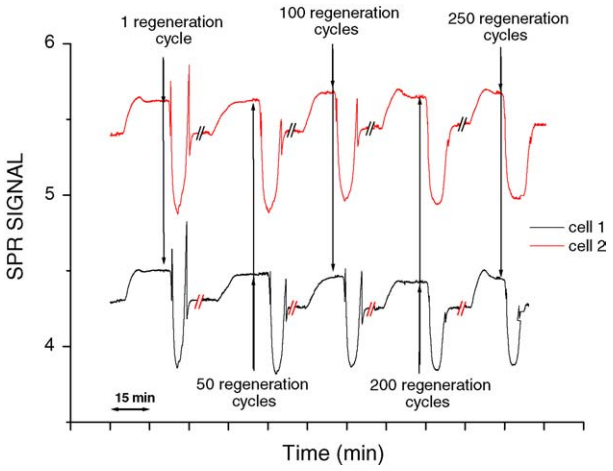

Fig. 4. Representation of the SPR response curves for immunoreaction after 1, 50, 100, 200 and 250 regeneration cycles in the two flow cells of the β-SPR biosensor.

change in pH with HCl 0.1 M for DDT and carbaryl immunoassays, and with NaOH 0.2 M for chlorpyrifos analysis. The SPR signal did not diminish noticeably from the initial analysis value, and neither a significant loss of activity of the hapten–carrier conjugate nor a damage of the physical properties of the SAM was detected on the sensor signal response. The stability of the surface provided by this immobilization method was proved during 250 assay cycles, avoiding the potential denaturation and loss of recognition of interactants given by the antibody-coated formats. As a conclusion, the biospecific surface remained constant after regeneration and the same normalized curve could be used for more than 1 month of continuous operation.

3.3. Analysis of spiked groundwater, river and tap water samples

The influence of different water types (groundwater, tap and river water) on the immunosensor response was evaluated. Standard calibration curves for carbaryl were prepared in water samples and in distilled water as control. The ionic strength of water samples did not need to be adjusted and spiked water samples were prepared like the standard samples. As shown in Fig. 5, the usual variability of carbaryl standard curves was obtained regardless of the nature of the water samples. The

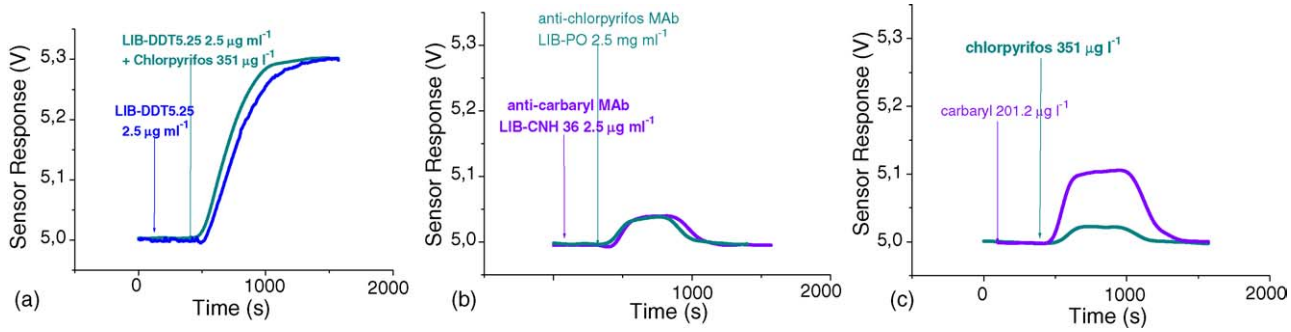

Fig. 3. Sensor response curves for: (a) anti-DDT LIB–DDT5-25 MAb and a mixture of LIB–DDT5-25 and chlorpyrifos standard, (b) anti-carbaryl MAb and anti-chlorpyrifos MAbs and (c) chlorpyrifos and carbaryl standards.

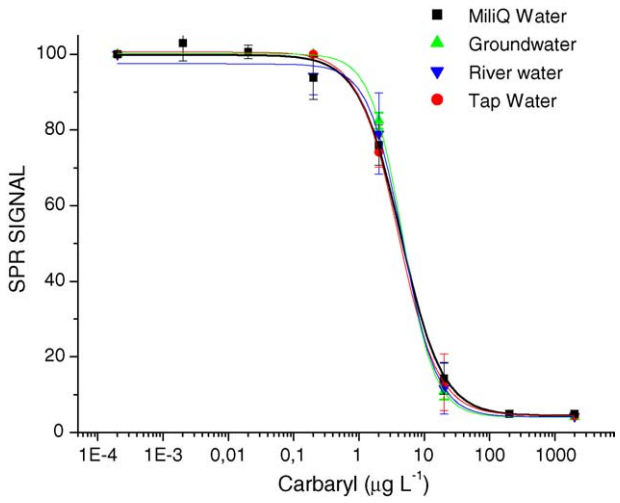

Fig. 5. Standard calibration curves of carbaryl in different water types: groundwater, river and tap water.

Table 2
Influence of matrix effect on the standard curves for carbaryl obtained with spiked water samples (LODs were calculated as the 90% reduction of the SPR maximum signal)

	Carbaryl	
	I_{50} ($\mu g\,L^{-1}$)	LOD ($\mu g\,L^{-1}$)
MiliQ water	4.36	0.9
Groundwater	4.65	1.35
River water	4.66	1.25
Tap water	3.97	0.86

calculated values of I_{50} and LODs for the standard calibrations of carbaryl in different water types are shown in Table 2. LOD values of 1.35, 1.25 and 0.86 were obtained for groundwater, river and tap water, respectively. In the case of the concentration giving the 50% of the SPR signal, the values were in 3.97–4.66 $\mu g\,L^{-1}$ range. Consequently, carbaryl determination seemed to be directly applicable to environmental waters without any sample treatment as matrix effects were not observed.

4. Conclusions

This work has shown the utilization of the β-SPR commercial device as a highly sensitive field-portable analytical method for the environmental monitoring of pollutants. Sensitivity values reached with this immunosensor are comparable to other analytical techniques, and 20 ng L^{-1} was the lowest detection limit obtained for DDT, even better than for ELISA assays. The low time of response, only 12 min for the simultaneous measurement of two samples, allows the application of this biosensor to the determination of a variety of compounds in control and alarm stations. The sensitivity values (I_{50}) of the calibration curves obtained for carbaryl in natural water samples were in the same range, 3.97–4.66 $\mu g\,L^{-1}$, than in distilled water. In addition, the stability of the biospecific sensor surface makes possible the reusability of the sensor for 250 assay-cycles, using the same surface for 1 month of continuous analyses. The small size and the capability to transmit the measuring data by a communication module afford the use of this immunosensor as a valuable screening tool for the continuous monitoring of pollutants from remote locations. Validation of measurements with standard analytical methods is underway.

Acknowledgement

This work has been supported by MCyT (Ministerio de Ciencia y Tecnología), project BIO-2000-P4-05.

References

[1] 98/83/EC, Council Directive (98/83/EC) of 3 November 1998 relating to the water quality of water intended for human consumption, Off. J. Eur. Commun. L330 (1998) 32–54.
[2] 2000/60/EC, Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy, Off. J. Eur. Commun. L327 (2000) 1–72.
[3] H.-R. Buser, M.D. Muller, Anal. Chem. 67 (1995) 2691–2698.
[4] M.C. Hennion, D. Barceló, Anal. Chim. Acta 362 (1998) 3–34.
[5] A. Abad, M.-J. Moreno, R. Pelegrí, M.-I. Martínez, A. Sáez, M. Gamón, A. Montoya, J. Agric. Food Chem. 49 (2001) 1707–1712.
[6] E. Mallat, D. Barceló, TrAC, Trends Anal. Chem. 20 (2001) 124–132.
[7] J. Tschmelak, M. Kumpf, G. Proll, G. Gauglitz, Anal. Lett. 37 (2004) 1701–1708.
[8] J. Homola, Anal. Bioanal. Chem. 377 (2003) 528–539.
[9] E. Mauriz, A. Calle, A. Montoya, J.J. Manclús, L.M. Lechuga, Proceedings of EUROPT(R)ODE VII, Seventh European Conference on Optical Chemical Sensors and Biosensors, 2004, p. 197.
[10] L.M. Lechuga, M. Calleja, J. Tamayo, A. Calle, C. Domínguez, in: D. Morrison (Ed.), Defence Against Bioterror: Detection Technologies, Implementation, Strategies and Commercial Opportunities, NATO Series Books, Kluwer Academic Publishers B.V., Dordrecht, The Netherlands, 2005.
[11] A. Abad, J.J. Manclús, F. Mojarrad, J.V. Mercader, M.A. Miranda, J. Primo, V. Guardiola, A. Montoya, J. Agric. Food Chem. 45 (1997) 3694–3702.
[12] J.J. Manclús, J. Primo, A. Montoya, J. Agric. Food Chem. 44 (1996) 4052–4062.
[13] A. Abad, J. Primo, A. Montoya, J. Agric. Food Chem. 45 (1997) 1486–1494.
[14] S. Ferretti, S. Paynter, D.A. Russell, K.E. Sapsford, D.J. Richardson, TrAC, Trends Anal. Chem. 19 (2000) 530–540.
[15] J.V. Staros, R.W. Wright, D.M. Swingle, Anal. Biochem. 156 (1986) 220–222.
[16] Z. Grabarek, J. Gergely, Anal. Biochem. 185 (1990) 131–135.
[17] I. Hochel, M. Musil, Food Agric. Immunol. 14 (2002) 285–300.
[18] A.E. Botchkareva, F. Fini, S. Eremin, J.V. Mercader, A. Montoya, S. Girotti, Anal. Chim. Acta 453 (2002) 43–52.
[19] A.E. Botchkareva, S.A. Eremin, A. Montoya, J.J. Manclús, B. Mickova, P. Rauch, F. Fini, S. Girotti, J. Immunol. Methods 283 (2003) 45–57.
[20] J.J. Manclús, A. Montoya, J. Agric. Food Chem. 44 (1996) 4063–4070.
[21] A. Abad, A. Montoya, J. Agric. Food Chem. 45 (1997) 1495–1501.