

## Development of immunosensors for the analysis of l-naphthol in organic media

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### Abstract

Immunosensor systems have been developed for the rapid determination of l-naphthol. In this work, the comparison of performance of immunosensors working in aqueous and organic media was done. Direct, indirect and capture formats were studied. Immunoreagents were immobilized on controlled pore glass (CPG), hidroxysuccinimide agarose gel or on azlactone Protein A/G supports. The Protein A/G-based sensor showed the best performance. In aqueous media, a LOD of  $16.2 \mu\text{g l}^{-1}$  and a DR of 33.7–586.6  $\mu\text{g l}^{-1}$  were achieved employing Tween 20 at a concentration ranging from 0.01 to 0.05% v/v. Maximum sensitivity was reached with 0.025% of surfactant. Binary mixtures of methanol or acetonitrile with aqueous buffer and ternary mixtures of methanol/isopropanol or ethyl acetate/methanol with the same buffer were studied as organic media. The mixture 50% MeOH-50% 20 mM sodium phosphate, pH 8, with 0.05% (v/v) Tween 20 resulted to be the best. A detection limit of  $12.0 \mu\text{g l}^{-1}$  and a dynamic range of 53.6–17 756.0  $\mu\text{g l}^{-1}$  were reached. The recycling of Protein A/G-based sensor working in this media was about 300 assays. Preconcentration factors around 250 were achieved using methanol as extracting solvent. It has been demonstrated that the technique can be successful in carrying out the analysis of low solubility in water analytes, such as l-naphthol. The sensors developed can use higher concentrations of organic solvent (up to 50% methanol) compared to ELISA. On the other hand, the advantage of preconcentration can also be taken for the use of the same procedure as recommended for standard sample treatments. © 2000 Elsevier Science S.A. All rights reserved.

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### 1. Introduction

l-Naphthol is the major hydrolysis product of carbaryl, an *N*-methylcarbamate insecticide widely applied due to its effectiveness against many agricultural (D'Amico and Bollinger, 1991). Different methods have been developed for the analysis of carbaryl and l-naphthol, based mainly on chromatographic techniques (Krämer et al., 1994). However, derivatization and/or careful clean-up of samples is often necessary, making the procedures expensive, difficult and time-consuming. Immunochemical assays are an alternative to these methods due to their sensitivity, rapidity and low cost (Sherma, 1993), and generally enzyme-linked immunosorbent assay (ELISA) is employed. Currently, enzyme

immunoassays we performed in aqueous media, the natural environment of the proteins. However, for determining hydrophobic compounds, organic solvents in antibody (Ab)-antigen (Ag) binding steps would be an interesting approach, even though antibodies are normally inactive at high concentrations of organic solvents (Matsura et al., 1993). This cause the deactivation and/or disruption of immunoreactants, and the organic media have to be carefully selected. On the other hand, the sensitivity reached by that technique is poorer in the presence of organic solvent because tolerance of the immunoreagents to these media is limited and recognition of the analyte is worse (Stocklein et al., 1997).

In general, ELISA can tolerate up to 10% (v/v) of organic solvent in aqueous buffer (Williams et al., 1996), although by choosing the appropriate solvent you can modulate sensitivity four orders of magnitude. For example, a study of the effects of organic solvents

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on Ab–Ag interactions was done by Hedenfalk et al., (1997), in a two phase system using a radioimmunoassay, indicating that the main factor influencing on the sensitivity was the partition coefficient of the analyte between the aqueous and the organic phases. In a recent paper an extensive investigation of the operation of optical immunosensors for pesticides in non-aqueous media has been described (Penalva et al., 1999). The authors performed a thorough investigation of numerous organic mixtures, and correlated the response to their polarity. Although, there are other important factors as solvent hydrophobicity (Russell et al. 1989; Weetall, 1991) molecular mass or solvent induced distortion effects (Ngeh-Ngwainbi et al., 1986; Stocklein et al., 1990, 1995). These facts can be partially solved by working in flow conditions where the contact time between the immunoreagents and the organic solvent is shorter. Also, flow techniques facilitate the manipulation of reagents and analysis automation and can reduce solvent volumes.

Immunosensors are analytical system based on immunochemical principles that can carry out determinations in an automatic way. Heterogeneous-phase immunosensors working in flow conditions (flow-through sensors) combine the sensitivity and selectivity of immunoassays with the accuracy and ease of automation of flow techniques (González-Martínez et al., 1999). Moreover, the flow techniques offer the possibility of reusing the immunosorbent for a large number of assays, thus lowering the analysis cost and raising the reproducibility of the assays. The Ab–Ag complex disruption without affecting the immunological activity of the immobilized reagent is a property required in those techniques.

The current regeneration techniques are based on the exposure of the so-called immunocomplexes to a reagent, which weakens the Ab–Ag interaction and allows the physical separation of both species. Only if the immunoreactor keeps its activity after the regeneration process it can be reused.

In the development of flow-based immunosensors, two different alternatives should be studied: antibody or antigen/hapten immobilization, the first one being the most useful format found in the literature (Puchades and Maquieira, 1996). The main advantage of this option is the economy of expensive antibodies and the reduction of assay steps by the reusability of this immunoreagent. But the antibody could be denatured in the regeneration step and obviously, no successful results would be achieved. On the other hand, the immobilization of the antigen or hapten conjugate, although less employed, is advantageous regarding its reusability. Thus, the regeneration process can be performed without loss of activity of the immobilized reagent and it is able to be recognised by the Ab.

In both formats, a solid phase is used to immobilize either the Ab or the Ag, thus allowing the separation of free fractions from bound immunocomplexes. Thus, the choice of immobilisation support is another important issue (Thompson et al., 1987). To enhance sensitivity, packed bed reactors offer good performance since they minimise sample dispersion (Ho, 1988). Additionally, solid phases should allow the possibility of attaching immunoreagents, in an oriented way and should also be as reusable as possible (Morais et al., 1997). Moreover, immunosorbents should tolerate organic solvents and have good hydrodynamic properties.

The aim of this paper is the study of the effects of organic solvent in the Ab–Ag reaction to develop immunosensors using 1-naphthol as a model analyte. This compound is slightly soluble in water and freely in methanol.

## 2. Experimental

### 2.1. Chemicals and biochemicals

Analytical standards of carbaryl, 1-naphthol, carbofuran, methiocarb and propoxur were purchased from Dr Ehrenstorfer (Augsburg, Germany). Stock solutions of these compounds were prepared in *N-N'* dimethylformamide (DMF), except for carbaryl, which was dissolved in methanol. From stock solutions kept at  $-20^{\circ}\text{C}$ , working standard solutions were daily prepared. 3-(*p*-hydroxyphenyl)-propanoic acid (HPPA), Controlled Pore Glass (CPG, PG-240-200 pore diameter 242, mesh size 120–200), Tween 20 (Tw20), 3-aminopropyltriethoxysilane and bovine serum albumin, fraction V (BSA) were purchased from Sigma Chemical (St. Louis, MO). Horseradish peroxidase (HRP) was from Boehringer (Mannheim, Germany). *N*-hydroxy-succinimide derivatized agarose gel (GASc) was from Bio-Rad Laboratories (Richmond, CA) and Ultralink Immobilized Protein A/G support (Protein A/G) was purchased from Pierce Chemical (Rockford, IL).

#### 2.1.1. Buffer solutions

Phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl 2.7 mM KCl, pH 7.4), PBST (PBS containing 0.05% Tw20), binding buffer (BB, 20 mM phosphate, pH 8.0), BBT1 (BB containing 0.05% Tw20), BBT2 (BB containing 0.025% Tw20) and BBT3 (BB containing 0.01% Tw20) were used as aqueous buffers.

#### 2.1.2. Organic mixtures assayed

Fifty percent methanol (MeOH)-50% PBST; 50% MeOH-50% BBT1; 50% MeOH-50% BBT2; 50% acetonitrile (MeCN)-50% BBT1; 25% MeOH-25% isopropanol (IS)-50% BBT1; 10% ethyl acetate (EtAc)-25% MeOH-65% BBT1.

### 2.1.3. Immunoreagents

Polyclonal anti-1-naphthol antibodies (3898, 3905, 3907) and the hapten 2b: [(5-hidroxy-2-naphthylenyl) oxy] acetic acid were used in this study and previously characterized by ELISA (Krämer et al., 1994). HRP-labelled goat anti-rabbit was from Sigma Chemical (St. Louis, MO). All other reagents used were analytical or biochemical grade.

### 2.2. Preparation of hapten conjugates

The 2b-HRP enzyme tracer was prepared by a modification of the anhydride mixed method (Rajskowsky et al., 1977). The hapten 2b was covalently attached to bovine serum albumin (BSA) using the modified active ester method (Langone and Van Vunakis, 1982).

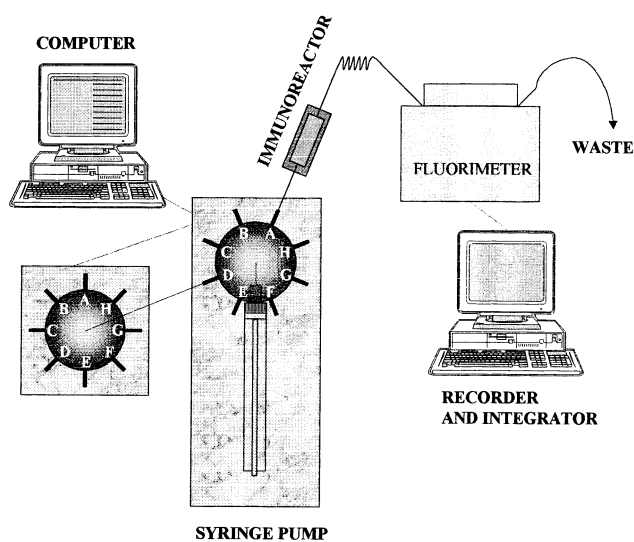


Fig. 1. Scheme of the automatic immunosensor manifold.

Table 1  
Event description corresponding to the direct format

Step	Event <sup>a</sup>
1	Mixing of 0.3 ml tracer and 0.8 ml aqueous buffer or organic mixture <sup>b</sup>
2	Injection of 1 ml of mixture at 0.25 ml min <sup>-1</sup>
3	Washing with 2.5 ml of PBST at 1 ml min <sup>-1</sup>
4	Mixing HPPA (0.3 ml, 0.8 g l <sup>-1</sup> ) with H <sub>2</sub> O <sub>2</sub> (0.2 ml, 0.012%)
5	Injection of 0.2 ml at 0.25 ml min <sup>-1</sup>
6	Incubation for variable time
7	Injection of 1.5 ml of PBST at 2 ml min <sup>-1</sup> . Signal registration
8	Injection of regeneration solution (variable volume) at 0.5 ml min <sup>-1</sup>
9	Washing with 2.5 ml PBST. End of cycle

<sup>a</sup> Previous to each step, the manifold ran a washing cycle of tubes and syringe with the next solution to be used.

<sup>b</sup> The analyte is in the aqueous buffer or in the organic mixture.

### 2.3. Immunosorbent preparation

Three commercial supports: Ultralink immobilized Protein A/G and controlled pore glass (CPG) for direct assays, and *N*-hidroxisuccinimide derivatized (GASc) agarose gel for indirect format, were used to develop the immunosensors. The preparation of these sorbent has been described previously (Morais et al., 1997).

### 2.4. Systems design

The scheme of the flow-through immunosensor is shown in Fig. 1. This system consists of a syringe pump connected to two eight-way distribution valves (Kloehn; Las Vegas, NV). The immunosupports were placed in a polymethylmetacrilate tube of 7 mm length × 4 mm I.D. for the Protein A/G and GASc-based sensors and 5 mm length × 2 mm I.D. for CPG. These packed-bed reactors (immunoreactor) were connected between a port of the first distribution valve and the fluorimeter (Turner, Model 450), equipped with suitable 320- and 405-nm filters and a Hellma flow cell (Type No. 176.052-QS). PTFE tubes (0.5 mm I.D.) were used for all connections. Liquid handling and fluorimetric signals were managed by means of Kloehn Winpump (Kloehn) and Chrom-Card Data Manager software packages (Fisons Instrument, Rodano, Italy), respectively. Sigmaplot software package (Jandel Scientific, Erkrath, Germany) was used for data treatment. For signal normalization the equation used was  $(B - B_{\infty}) / (B_0 - B_{\infty})$ , where  $B$  is the signal (intensity of fluorescence) produced in presence of different analyte concentrations,  $B_{\infty}$  is the background signal and  $B_0$  is the signal in absence of pesticide. The limit of detection (LOD) is calculated from competitive curves as the analyte concentration for which the normalized signal was 90%. The dynamic range (DR) of the method is the analyte concentration which produces a normalized signal between 80 and 20%, and the sensitivity ( $I_{50}$ ) is the analyte concentration which inhibited 50% of the maximum normalized signal. All of these parameters were calculated following the logistic-model of the competitive immunoassays (Rodbard and Fraizer, 1975; Raab, 1983).

### 2.5. Assay protocols

The events for the direct CPG-based sensor are detailed in Table 1. For protein A/G-based immunosensors, the first step involves the injection of a mixture of 300 μl Ab solution, 300 μl of diluted enzymatic tracer and 500 μl of organic media with/without analyte. After washing (step 3), the same events are run. The total assay time averaged about 22 min.

The protocol for indirect assay is given in Table 2 and the total assay time averaged 29 min. In both

Table 2  
Event description corresponding to the indirect format

Step	Event <sup>a</sup>
1	Mixing of 0.4 ml Ab and 0.7 ml of aqueous buffer or organic mixture <sup>b</sup>
2	Injection of 1 ml of mixture at 0.25 ml min <sup>-1</sup>
3	Washing with 2.5 ml of PBST at 1 ml min <sup>-1</sup>
4	Injection of 0.2 ml of secondary Ab labelled with HRP
5	Mixing HPPA (0.3 ml, 0.8 g l <sup>-1</sup> ) with H <sub>2</sub> O <sub>2</sub> (0.2 ml, 0.012%)
6	Injection of 0.2 ml at 0.25 ml min <sup>-1</sup>
7	Incubation for variable time
8	Injection of 1.5 ml of PBST at 2 ml min <sup>-1</sup> . Signal registration
9	Injection of 2.5 ml of regeneration solution at 0.5 ml min <sup>-1</sup>
10	Washing with 2.5 ml PBST. End of cycle

<sup>a</sup> Previous to each step, the manifold ran a washing cycle of tubes and syringe with the next solution to be used.

<sup>b</sup> The analyte is in the aqueous buffer or in the organic mixture.

formats, the enzyme incubation time varied as a consequence of reaching the maximum signal with the lower tracer concentration. Also, the volume of regenerating solution (Glycine/HCl 0.1 M, pH 2) was variable in order to achieve the complete disruption of immunocomplexes. These factors affected the total assay time.

## 2.6. Working media

### 2.6.1. Aqueous media

In the Protein A/G-based immunosensor, a 20 mM sodium phosphate buffer, pH 8, at different concentrations of Tween 20, ranging from 0.01 to 0.05% v/v (see Section 2.1) was used. In the CPG and GASc-based sensors, PBS with 0.05% Tw 20 (PBST) was used.

### 2.6.2. Organic media

For this study, a review of the organic solvents used in pesticide extraction methods was performed (Moye, 1990; Sawyer et al., 1990) and the most frequently used were chosen. These were: (a) Water-nonmiscible: hexane, ethyl acetate, dichloromethane; (b) Water-miscible: methanol, acetonitrile, acetone, tetrahydrofurane, 2-propanol and DMF. Three of the above mentioned solvents (tetrahydrofurane, 2 propanol and *N-N'* dimethylformamide) were used, independently of its practice application, as transference solvents to aqueous buffer, mainly for mixtures of nonmiscible solvents.

## 2.7. Preconcentration by solid-phase extraction

Different volumes of distilled water (50–700 ml) fortified with 1-naphthol at 5 µg l<sup>-1</sup> were extracted using C<sub>18</sub> cartridges (Waters, Milford, MA) previously activated with methanol (MeOH). The analyte retained

on the solid-phase was desorbed with 1.5 ml of MeOH, except for the highest volume of sample (700 ml) that was eluted with 2.5 ml, and the extracts were kept at –20°C until the analysis.

## 2.8. Analysis of 1-naphthol in surface waters

A set of surface waters samples from Llobregat River (Barcelona, Spain), called: Sant Joan Despí, Anoia, Sallent, Pont de Vilamare, Pont de Molins, Prese Cairat, Can Burés and Can Carmé, were collected and analysed using the immunosensors. Two fractions of 50 ml from each sample were used for this purpose. In all cases, one of this was used as control and the other was fortified at 40 µg l<sup>-1</sup> of 1-naphthol. After this, the samples were extracted by SPE as described above and the extracts (1.5 ml) were kept at –20°C until required.

## 3. Results and discussion

### 3.1. Studies of organic solvents

When pure organic solvents are used, precipitation of the immunoreagents, incompatibility with the working material and difficulty of handling occurred. In a previous work (Penalva et al., 1999) different studies about miscibility and compatibility with the working conditions were carried out. Consequently, mixtures with the best performances used in this work (see Section 2.1) were always phosphate buffer at different concentrations with variable levels of Tw 20 and methanol as organic solvent. Other assayed compounds such as acetonitrile, isopropanol and ethyl acetate produced poorer results or difficult management. Methanol concentrations < 50% were also rejected in order to develop maximum solvent content assays. In all cases a percentage of 50% of organic solvent was the highest level tested.

### 3.2. CPG based-sensor: direct format

For the CPG-based sensor, the serum 3907 was chosen for immobilization because it shows the best performance in ELISA batch assays. In this format, the immunosorbent reusability is a critical factor to be studied (González-Martínez et al., 1997a). Therefore, once the tracer concentration is defined for each assay media (ranging from 0.01 to 0.1 mg l<sup>-1</sup>) in order to achieve the maximal signals, the volume and pH of the regeneration solution were optimized. In this case, 2.5 ml of 0.1M glycine/HCl solution, pH 2.0, were needed to disrupt the immunocomplexes, indicating that antibody (3907) and the tracer (2b-HRP) interactions are very strong when compared with other systems devel-

Table 3

Percentage of normalized signal obtained after each regeneration cycle using the CPG-based immunosensor in both aqueous and organic media<sup>a</sup>

No. cycle	1	2	3	4	5	6	10
PBST	100	95.9	81.7	71.5	69.3	63.9	60.2
50%MeOH-50%PBST	100	95.1	88.2	77.3	68.5	62.1	59.4

<sup>a</sup> Antibody PAb-3907.

oped (González-Martínez et al., 1997b; Penalva et al., 1999), where the volume of the regeneration solution ranged between 0.2 and 1.5 ml.

These studies were carried out in aqueous buffer (PBST) and in a 50% MeOH-50% PBST mixture as representative of the organic media.

The percentage of normalized signal in absence of analyte after each regeneration cycle in both aqueous (PBST) and organic (50% MeOH-50% PBST) media is shown in Table 3. As it can be seen, the signal decreased after each regeneration cycle, indicating that in direct format the Ab 3907 is not reusable since the signal decreased continuously. This fact is due to the denaturation of the Ab by the regenerating solution, and prevents sensor reusability.

### 3.3. GASc-based sensor: indirect format

In these assays, 2b-BSA conjugate immobilized on GASc and the serum 3907 were used. In this format, the main background signal corresponds to non-specific binding of the secondary antibody. To minimise this fact, the Ab must be dissolved in PBST. Both the optimum concentration of Ab and the secondary Ab concentration were determined by evaluating the obtained signal. The sensor regeneration is complete using 2.5 ml of 0.1M glycine/HCl solution, pH 2.0. The results showed a narrow range of useful signal, defined as the difference between the signal obtained with and without the primary Ab. The competition assay showed a low sensitivity ( $I_{50} = 1066 \mu\text{g l}^{-1}$ ) in aqueous media but in the selected organic media (50% MeOH-50% PBST), there was not enough signal to carry out the competition assays.

### 3.4. Protein A/G based-sensor

For the development of this sensor and in order to obtain the maximum benefits of the system, aqueous media were firstly used because this is the 'ideal' environment for the proteins. The whole study was carried out with the 3907 Ab so it was selected as optimal in previous ELISA experiences (Krämer et al., 1994). The optimum immunoreagent concentrations were determined by preliminary activity assays and ranged from 15 000 to 120 000 for the antibody and from 0.25 to 2 mg l<sup>-1</sup> for enzymatic tracer.

The best immunosensor working conditions (e.g. solutions, volumes and flow rates) are summarized in Table 1. In this case, the regeneration of the sensor is complete by injecting 2.5 ml of 0.1M glycine/HCl, pH 2.0, solution. This process assures the rupture of all Ab-protein A/G bindings, leaving the immunosorbent able to run new assays.

In aqueous media, the signal increased with analyte concentration probably because 1-naphthol was non-specifically bound into the system (tubes, support). To correct this effect, different concentrations of Tw 20 were added to the working buffer. All these conditions were also applied to the other antibodies (3905 and 3898).

Table 4 shows the  $I_{50}$  values obtained for 1-naphthol in aqueous media. As it can be seen, the percentage of Tw 20 in the buffer influences the sensitivity. A concentration of 0.025% Tw 20 (BBT2 aqueous media) produces similar sensitive assays ( $I_{50} 101.2 \mu\text{g l}^{-1}$ ) to that obtained previously by ELISA ( $I_{50} 72 \mu\text{g l}^{-1}$ ) with the same (3907) serum (Krämer et al., 1994). Also a detection limit (LOD) of  $16.2 \mu\text{g l}^{-1}$  and a dynamic range of 33.7–586.2  $\mu\text{g l}^{-1}$  were obtained.

Table 4

Values of  $I_{50}$  ( $\mu\text{g l}^{-1}$ ) obtained with the Protein A/G based immunosensor using different solvent mixtures<sup>a</sup>

Mixtures	Antibodies		
	3898	3905	3907
BBT1	1055.6 ± 146.0	1171.0 ± 113.3	795.3 ± 80.2
BBT2	1128.0 ± 85.4	592.9 ± 146.0	101.2 ± 14.0
BBT3	n.a.	n.a.	433.8 ± 27.7
50% MeOH-50% BBT1	1388.2 ± 266.8	1185.7 ± 194.6	780.8 ± 88.3
50% MeOH-50% BBT2	n.a.	n.a.	1460.5 ± 145.8
50% MeCN-50% BBT1	n.a.	n.a.	n.c. (>10 <sup>4</sup> )
25% MeOH-25% IS-50% BBT1	n.a.	n.a.	n.c. (>10 <sup>4</sup> )
10% EtAc-25% MeOH-65% BBT1	n.a.	n.a.	n.c. (>10 <sup>4</sup> )

<sup>a</sup> n.a., no activity; n.c., no competition; tracer, 2b-HRP; BBT1, binding buffer containing 0.05% Tw20; BBT2, binding buffer containing 0.025% Tw20; BBT3, binding buffer containing 0.0125% Tw20; MeOH, methanol; MeCN, acetonitrile; IS, isopropanol; EtAc, ethyl acetate, values expressed as media ± deviation standard ( $n = 3$ ).

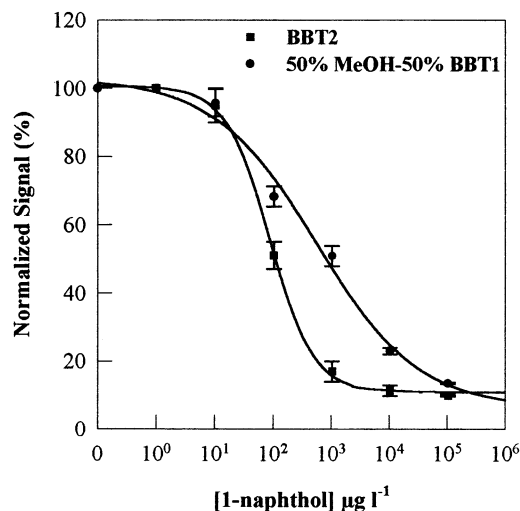


Fig. 2. Comparison of the calibration curves in the best aqueous (BBT2) and organic media (50% MeOH-50% BBT1). Each point represents the mean of three measurements.

Table 5  
Cross-reactivity values (%) using the Protein A/G based sensor in aqueous and organic media<sup>a</sup>

Compound	BBT2		50% MeOH-50% BBT1	
	I <sub>50</sub> (µM)	CR(%)	I <sub>50</sub> (µM)	CR(%)
l-Naphthol	0.7	100	5.4	100
Carbaryl	140.2	0.53	>497.3	<0.15
Propoxur	>478.3	<0.15	>478.3	<0.15
Methiocarb	>444.1	<0.16	>444.1	<0.16
Carbofuran	>452.3	<0.16	>452.3	<0.16

<sup>a</sup> Ab 3907; BBT2, binding buffer containing 0.025% Tw20; BBT1, binding buffer containing 0.05% Tw20.

Table 6  
Analysis of l-naphthol in fortified (40 µg l<sup>-1</sup>) water samples<sup>a</sup>

Sampling stations	Found level (µg l <sup>-1</sup> )	Recovery (%)
S. Joan Despí	37.3 ± 2.7	93.1
Anoia	40.4 ± 6.5	101.1
Sallent	50.3 ± 1.7	125.8
Pont de Molins	43.4 ± 3.6	108.5
Prese Cairat	45.1 ± 7.5	112.8
Can Burés	44.9 ± 0.7	112.3
Can Carné	36.1 ± 3.3	90.1
Pont de Vilamare	41.2 ± 0.7	103.1

<sup>a</sup> Found level expressed as media ± deviation standard (n = 3).

The support resistance, rather than the immunoreagents stability to the working conditions, governs the reusability of this immunosensor. This sensor could be used about 300 assay cycles without loss of performance.

The effect of organic solvent mixtures on sensitivity (I<sub>50</sub>) of the three assayed antibodies is also shown in

Table 4. The methanol mixture (50% MeOH-50% BBT1) is the best regarding sensitivity (I<sub>50</sub> 780.8 µg l<sup>-1</sup>), and only the antibody 3907 was able to work in all organic media. In this case a detection limit of 12.0 µg l<sup>-1</sup> and a dynamic range of 53.6–17 756.6 µg l<sup>-1</sup> were obtained. In all cases the assays are less sensitive than in the aqueous-based assays media. The percentage of Tw 20 in the organic mixtures did not improve the sensitivity dramatically. In these media, higher tracer concentration was needed to obtain an acceptable signal, showing that the diffusion of the Ag towards the recognition sites of the Ab is disregarded, and partial Ab denaturation happened. The competition curves with the best aqueous (BBT2) and organic media (50% MeOH-50% BBT1) are shown in Fig. 2.

### 3.5. Study of cross-reactivity

This study was carried out with the Protein A/G-based sensor using the antibody 3907 in BBT2 and 50% MeOH-50% BBT1 as aqueous and organic media, respectively. To perform the competitive calibration curves, different *N*-methylcarbamate pesticides with structural similarity with l-naphthol: carbaryl, propoxur, methiocarb and carbofuran were used. The cross-reactivity percentage (%CR) is defined as the ratio of I<sub>50</sub> (in molar units) for the interfering compound and I<sub>50</sub> for the specific analyte.

The sensitivity (I<sub>50</sub>) and cross-reactivity (%CR) for each compound are given in Table 5. As it can be seen the %CR values are negligible in both media. Only in aqueous media carbaryl shows some interference. The selectivity of the assay will most likely allow its usage for the monitoring of the degradation of carbaryl or the monitoring of l-naphthol from other sources.

### 3.6. Analysis of water samples: preconcentration studies

The Protein A/G-based sensor that works under the best organic conditions (50% MeOH-50% BBT1) was used for l-naphthol analysis in water samples. The results are shown in Table 6. In this case, two fortified (40 µg l<sup>-1</sup>) fractions of 50 ml each sample were extracted by solid phase extraction (SPE) to study the suitability of the system to the analysis of l-naphthol and a preconcentration factor of 33.4 was obtained, so the level of l-naphthol analysed was in the range of I<sub>50</sub> of the immunosensor. All samples were free of l-naphthol and no matrix effect was detected. With fortified samples, a mean recovery value of 105.8%, ranging from 90.1 to 125.8%, was obtained. These results demonstrate the suitability of the developed system and the extraction procedure used. In all cases, the methanolic extracts were diluted 1/1 with buffer, corresponding to the organic media selected.

To achieve lower detection level a preconcentration study was performed. For this purpose different volumes of distilled water fortified at  $5 \mu\text{g l}^{-1}$ , were extracted by SPE and analyzed using the Protein A/G-based sensor. The preconcentration factors obtained ranged from 33.4 to 280 as a function of the used volume. The recovery curve obtained is shown in Fig. 3. As it can be seen, the best recovery values ( $>90\%$ ) were achieved with higher volumes, from 300 to 700 ml, corresponding to concentrations ranging near the  $I_{50}$ . This is a common property of all heterogeneous immunoassays. According to this study a lower detection level ( $<5 \mu\text{g l}^{-1}$ ) could be achieved if higher volumes in the extraction procedure by SPE are used.

#### 4. Conclusions

Flow immunosensors working in aqueous and organic media have been developed. This fact will allow the analysis of non-polar analytes or those with low water solubility. It has been demonstrated the possibility of carrying out reactions that involve antigens and antibodies in high concentrations of organic solvents. In these cases, although the sensitivity is lower than that obtained in aqueous media, the clean-up SPE procedure allows high preconcentration factors. This fact leads to both selective and sensitive immunosensors.

The regeneration of the antigen–antibody interaction is a critical factor in the immunosensors development. This process should be performed with the least drastic conditions, since it will determine the useful life of the sensor. In this case, the direct CPG-based sensor is not able to work since the antibody is not reusable. The Protein A/G-based sensor is proposed as an alternative to avoid the problems associated with immunosensors regeneration since its useful life is governed by the resistance of the sorbent rather than the immunoreagent stability. It is also remarkable the effect of the Tw-20 in the Ab–Ag binding process, so its concentration in the buffer should be carefully adjusted.

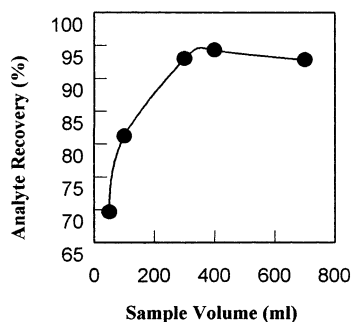


Fig. 3. Recovery curve for l-naphthol obtained in the preconcentration study. Each point represents the mean of three measurements.

In both ELISA and immunosensors described for l-naphthol a low sensitivity was obtained compared to other immunoassays, described for other pesticides. Thus, results obtained in ELISAs may be predictive of the usefulness of these reagents in the sensor format.

We had predicted that the indirect format could be more stable to working conditions since the immobilized conjugate is not the active reagent. Instead, it should only maintain its integrity under disruption conditions in order to be recognised by the antibody. However, this format resulted to be less sensitive than the immobilized antibody format. This indicates that the Ab–Ag recognition is strongly influenced by the immobilized species.

It has also been demonstrated the suitability of the developed sensors for the analysis of l-naphthol in surface waters. In general, the main advantage is the use of the treatment and clean-up procedures recommended by the reference methods. Finally, development of these methodologies will allow to set up analytical devices working on line with high throughput.

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