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Development and application of immunoaffinity chromatography for the determination of the triazinic biocides in seawater

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Abstract

The development of an immunoaffinity chromatography (IAC) procedure for the selective extraction of the anti-fouling agent Irgarol 1051 [2-(*tert*.-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine] from seawater is described. The anti-Irgarol 1051 antibodies were covalently bound to agarose-based beads support. IAC column capacities were higher than 400 ng and ethanol–water (70:30) was selected as eluting mixture. After percolation of 250 ml of water sample containing Irgarol 1051 at environmental levels (ng l^{-1}), the breakthrough volume was still not achieved. Other triazine herbicides percolated through the IAC column showed good recoveries. Thus, this IAC procedure may be useful to extract related compounds. The developed IAC column was applied to real seawater samples and compared with RP-C_{18} cartridges. The limit of detection (LOD) reached by using the IAC procedure was twenty times lower than the LOD achieved by the RP-C_{18} cartridges using the same detection system. Irgarol 1051 was detected at ng l^{-1} levels in the Barcelona marina (northwestern Mediterranean Sea). An acceptable correlation between enzyme-linked immunosorbent assay and gas chromatography with nitrogen–phosphorus detection was observed, thus analysis of Irgarol 1051 can be performed by either one of the methods. In this work, further confirmation of the analyte identity for real samples was accomplished by gas chromatography–electron impact mass spectrometry.

Keywords: Immunoaffinity chromatography; Water analysis; Environmental analysis; Triazines; Irgarol

1. Introduction

During the last decade, the use of tri-*n*-butyltin (TBT) as a biocide in anti-fouling paints, has been restricted or even banned in most of developed countries because of its high toxicity to non-target, marine organisms [1,2]. Consequently, a new generation of anti-fouling paints, containing copper and biocides, such as Irgarol 1051, 2-(*tert*.-butylamino)-4-(cyclopropylamino)-6-(methylthio)-1,3,5-triazine has been introduced in the market because of their low toxicity to non target biota.

At present few data has been published regarding the toxicity of Irgarol 1051. However, the Ecotoxicology Structure Activity Relationship (ECOSAR) toxicity model from the United States Environmental Protection Agency (EPA) shows that Irgarol 1051 should be more toxic to algae and aquatic plants than to invertebrates and fish [3]. The minimal inhibition concentration (MIC) reported for algae (*Enteromorpha intestinalis)* and several diatoms (i.e. *Navicula, Nitzschia, Amphora* and *Achnanthes*) are 22 ng l^{-1} and 10 μg l^{-1}, respectively [4,5]. Furthermore, it is toxic to several microalgae [median effective concentration (EC_{50})

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values ranged from 0.45 to 2.12 $\mu g\,l^{-1}$] [6]. Therefore, analytical methodologies are needed to determine triazinic biocides in seawater at the ppt level.

According to the Mackay fugacity model, Irgarol 1051 predominantly occurs in the aqueous phase (95.6%) [3]. Its degradation in sea and freshwater sediments is slow (half-lives of about 100 and 200 days, respectively) [7], and thus it has been included in monitoring programs. In fact, Irgarol 1051 has been detected in waters from several coastal and estuarine areas, harbours and marinas, such as the Côte d'Azur, France [5] the southern coast of the UK [8], the Léman Lake [6], the west coast of Sweden [9], the Western Scheldt estuary in the North Sea [10] and the northwestern Mediterranean, Spain [11]. Since it occurs at trace level in waters, pre-concentration and extraction methods must be used before its detection. Previous extraction methods described in the literature for Irgarol 1051 in aqueous phase are solid-phase extraction (SPE) and liquid–liquid extraction (LLE). Typically, LLE is carried out with dichloromethane (DCM) [8] to extract simultaneously Irgarol 1051 and organotin compounds [12]. Both disks and cartridges of octadecylsilica (C_{18}) [6,11,13–16] or polymeric [10,17] supports are used for SPE. These stationary phases interact with the analytes either by hydrophobic or electrostatic interactions. Therefore, they are not selective and usually several analytes of similar physicochemical properties are co-extracted. Immunoaffinity chromatography (IAC) combines the advantages of the SPE with the specificity of the antibody–antigen (Ab–Ag) interaction (for reviews see [18–21]). In most cases, the solid support employed for the preparation of the immunosorbent is a silica-based material, although agarose-based supports have also been employed [22–24].

The aim of this work was to develop an IAC-based procedure for the analysis of Irgarol 1051 in aqueous matrices followed by enzyme-linked immunosorbent assay (ELISA) or determination by gas chromatography (GC) with nitrogen–phoshorus detection (NPD) and confirmation by GC with electron impact ionization mass spectrometry (EI-MS). Recently, we have reported the preparation of antibodies (Abs) for the detection of Irgarol 1051 by immunoassay [25,26]. In this paper, we report the use of these Abs for the preparation of an immunosorbent and its evaluation and application to the selective extraction of Irgarol 1051 from seawater samples. To our knowledge, this is the first attempt to use IAC for the determination of Irgarol 1051.

2. Experimental

2.1. Chemicals and immunochemicals

Triazines and Irgarol 1051 were obtained as a gift from Ciba-Geigy (Barcelona, Spain). Stock solutions of 100 m*M* Irgarol 1051 and other *S*-triazine derivatives in dimethyl sulfoxide (DMSO) were used to prepare the standard solutions and spiked samples.

Antiserum A17 was obtained by immunization of a New Zealand white rabbit with the (4-*tert*.-butylamino-6-methylthio-[1,3,5]-triazin-2-yl)-4-aminobutanoic acid conjugated to keyhole limpet hemocyanin (KLH) protein and the enzyme tracer used in the ELISA protocol was (4-chloro-6-ethylamino-[1,3,5]-triazin-2-yl)-4-aminobutanoic acid conjugated to horse radish peroxidase (HRP) [25]. Artificial seawater was prepared using a mixture of sea salts purchased from Sigma (Steinheim, Germany) dissolved in Milli-Q water at a concentration of 35 $g\,l^{-1}$.

2.2. Materials

N-Hydroxysuccinimide (NHS)-activated Sepharose columns (Hi-Trap R NHS-activated) were purchased from Pharmacia Biotech (Uppsala, Sweden). Briefly, the columns are made of medical grade polypropylene and their gel consists of highly cross-linked agarose beads activated by NHS. C_{18} columns (LiChrolut RP-18, 500 mg) were purchased from Merck (Darmstadt, Germany). The 15 m×0.25 mm I.D., 0.15 μm DB-225 column used for the GC analysis was purchased from J&W Scientific, (Folsom, CA, USA). Polystyrene microtiter plates used for the ELISA analysis were purchased from Nunc Maxisorb (Roskilde, Denmark).

2.3. Instruments

Samples were pre-concentrated in the C_{18} columns by an automated off-line-SPE system from Aspec, Gilson (Villiers-le-Bel, France). The LC pump used

was from Waters Millipore model 510 (Milford, MA).

For the ELISA, washing steps were carried out using a SLY96 PW microplate washer SLT Labinstruments (Salzburg, Austria). Absorbances were read with a Multiskan Plus MK II microplate reader Labsystems (Helsinki, Finland).

For GC–NPD a Fisons 5300 Mega series gas chromatograph (Milan, Italy) was used, equipped with an A200S auto-sampler and with a NPD 80-FL detector. For GC–EI-MS a MD 800 Fisons gas chromatograph (Manchester, UK) was used.

2.4. Water samples

2.4.1. Spiked samples

The spiked samples were prepared by dilution of the stock solution of Irgarol 1051 or *S*-triazines in artificial seawater with an adjusted pH of 7.6.

2.4.2. Environmental marine samples

Environmental marine samples were obtained from the Barcelona marina at three different depths (1, 5 and 10 m) from the surface. Samples were collected in 2.5 l pre-cleaned amber glass bottles. Samples were filtered through a 0.45 μm membrane by using a vacuum system and the pH was adjusted to 7.6. They were stored in glass bottles at 4°C in the dark.

2.5. Immunosorbent preparation

2.5.1. Ab purification

Polyclonal antiserum [25] and pre-immune serum were purified by 35% $(NH_4)_2SO_4$ precipitation [27]. The precipitate was restored with 10 m*M* phosphate-buffered saline (PBS) solution and dialized against 0.5 m*M* PBS (5 liter×4) and Milli Q water (5 liter×1). The aqueous solution was finally freeze-dried and stored at 4°C until use.

2.5.2. Ab immobilization on the support

Anti-Irgarol 1051 Abs were covalently bound to the NHS-activated Sepharose column following the procedure recommended by the supplier. Briefly, 10 mg ml^{-1} solution (1 ml) of the purified antibodies was prepared in 0.2 *M* $NaHCO_3$ buffer (coupling buffer, pH 8.3, 0.5 *M* NaCl) and injected onto the High-Trap column at a flow-rate of 1 ml min^{-1}. The column was then left to stand for 30 min at room temperature and washed with three column volumes of coupling buffer. Capping of the not coupled active groups was then accomplished by alternate (6 ml each) and repetitive (3 cycles) injections of 0.5 *M* ethanolamine (pH 8.3, 0.5 *M* NaCl) and 0.1 *M* acetate (pH 4, 0.5 *M* NaCl) buffers. Finally, the column was washed with 10 m*M* PBS buffer (2 ml) and stored at 4°C in the presence of 0.02% NaN_3 until used. The coupling efficiency was estimated by comparing the UV absorption at 280 nm of the Ab solution initially injected onto the column and the one eluted with the coupling buffer. A control column with the purified pre-immune serum was also prepared following supplier directions to estimate the unspecific binding.

2.6. SPE procedure

2.6.1. IAC

The protocol used when loading the IAC columns with Irgarol 1051 spiked samples or environmental samples was as follows: First, the column was conditioned by adding 3 ml of the eluting mobile phase (ethanol–water, 70:30, except for the eluent selection experiment where 0.05 *M* glycine–HCl buffer pH 3 was also tested), followed by 5 ml of 10 m*M* PBS pH 7.6. Next, the sample was loaded by using a liquid chromatography (LC) pump at a flow-rate of 1 ml min^{-1}. Immediately after the sample percolation, 10 ml of 10 m*M* PBS pH 7.6 were added in order to elute any analyte unspecifically bound to the immunosorbent. After that, the Irgarol 1051 specifically bound to the immobilized antibodies was eluted with 3–10 ml (10 ml were added in the first experiments and further measurements showed that only 3 ml of eluting mobile phase were required for complete elution of the analyte) of the eluting mobile phase (ethanol–water, 70:30, except for the eluent selection experiment where glycine buffer pH 3, 0.05 *M* was also tested). Finally, 10 ml of 10 m*M* PBS of pH 7.6 were added to condition the column until next use. All the steps were performed at a flow-rate of 1 ml min^{-1} and at room temperature. The IAC columns were preserved in PBS 10 m*M* of pH 7.6 at 4°C when they were used daily. A solution of 0.02% NaN_3 was added to store

them for longer periods of time. Once samples were percolated through the column, one aliquot was separated for ELISA analysis after appropriate dilution. The rest was evaporated under vacuum until dryness for further analysis by GC–NPD.

2.6.2. Reversed-phase C_{18} cartridges

The loading protocol was as following: C_{18} cartridges were conditioned with 9 ml of Milli-Q water followed by 9 ml of methanol at flow-rate of 1 ml min^{-1}. Then, 250 ml of sample was percolated at a flow-rate of 10 ml min^{-1}. Next, cartridges were left to dry for 25 min. Finally, elution was done with 9 ml of methanol at a flow-rate of 1 ml min^{-1}. Samples were evaporated and analyzed by GC–NPD.

2.7. Irgarol 1051 analysis

2.7.1. ELISA

Fractions eluting from the IAC column were appropriately diluted with 10 m*M* PBS buffer and analyzed by ELISA using the procedure already reported [25]. Briefly, samples (50 μl/well) and Irgarol 1051 standards (from 0.012 to 25 n*M*, 50 μl/well) were added to the Ab-coated plates and incubated for 45 min at room temperature. A solution of the enzyme tracer 2a-HRP was then added (50 μl/well) and incubated for 15 min more and plates washed four times with PBST buffer (PBS containing 0.05% of Tween 20). A solution of the substrate (0.01% tetramethylbenzydine and 0.004% H_2O_2) was then added (100 μl/well) and the absorbances measured after 30 min at 450 nm. The standard curve was fitted to a four-parameter equation using the software GraphPad Prism (Graph Pad Software, San Diego, CA, USA). Unless otherwise indicated, the analyses were carried out using three replicates.

2.7.2. GC–NPD

The oven temperature was programmed from 60°C (1 min) to 150°C at 10°C min^{-1} and from 150°C to 220°C (15 min) at 4°C min^{-1}. Injections (1 μl) were performed in splitless mode at an injector and detector temperatures of 270°C. Helium was the carrier gas at 30 cm s^{-1}. The voltage source was 3.5 V. Signal acquisition and handling were carried out with PE-Nelson chromatography software (Model 2600, revision 5.0, Perkin-Elmer Nelson Systems).

Eluted fractions from the IAC or C_{18} columns were evaporated prior to the analysis. Previously, prometryne/terbumeton were added as surrogates, to control any losses of analyte during the evaporation process. After that, samples were reconstituted with an organic solvent (ethyl acetate) and measured by GC–NPD. Cyanazine was used as an internal standard except when cyanazine was analyzed, then terbutryne was used.

2.7.3. GC–EI-MS

The presence of Irgarol 1051 in real samples was confirmed by GC–EI-MS. The source temperature was held at 200°C. The column and gradient conditions were the same as for GC–NPD determination. The chromatograms were acquired in scan and selected ion monitoring(SIM) modes. For the scan mode, the m/z ranged from 100 to 320, and for the SIM mode the ions corresponding to the typical fragments of Irgarol 1051, prometryne (surrogate), cyanazine (internal standard) and 2-(*tert.*-butylamino)-4-amino-6-(methylthio)-1,3,5-triazine (a photodegradation product of Irgarol 1051, described previously in the literature [15]) were recorded.

3. Results and discussion

3.1. Evaluation of the IAC column capacity

The isolated immunoglobulin G (IgG) fraction was immobilized onto the Sepharose gel by means of the amino groups of the Abs through a stable amide bond. Measuring the coupling efficiency of the reaction verified the covalent attachment of a significant fraction of Abs to the solid-phase. During this work, we have prepared three different IAC columns that have shown similar performance properties. Table 1 shows the features of these columns and it can be observed that a high coupling efficiency (80–90%) could be accomplished following the supplier directions for Ab immobilization.

However, it was first necessary to find appropriate eluting conditions to desorb the Irgarol 1051 selectively trapped by the immobilized Abs. The ideal eluent should allow analyte recovery with the lowest

Table 1
Characteristics of the IAC columns prepared for Irgarol 1051 purification[a]

Column	Support (functionalization)	IgG (mg)[b]	Coupling efficiency (%)	IgG immobilized (nmol ml^{-1})[c]	Capacity of the column (ng)[d] (found)
A	Agarose beads (*N*-hydroxysuccinimide)	10	n.d.[e]	n.d.	381±4.6 382±3.2
B	″	″	85	5.7	574±16.3 544±10.3
C	″	″	91	6.1	545±10.2 422±4.8

[a] The data shown correspond to experiments done in duplicates for the three columns prepared and the ELISA analysis were performed in triplicates.
[b] Theoretical mass of IgG introduced into the column per ml of bed.
[c] Considering that only 10% of the total IgG fractions are specific to Irgarol 1051.
[d] Percolating 1 μg of Irgarol 1051 in 1 ml of PBS buffer 0.01 *M*. First and second values correspond to two different experiments.
[e] n.d.=not determined.

volume possible while not damaging the Abs or the support. Common eluents used in IAC are acidic or basic buffers, solutions with high ionic strength, organic solvents, detergents or chaotropic agents [18]. The use of organic solvent–water mixtures such as methanol–water [28–31] or ethanol–water [32,33] has also been reported. This is particularly useful when these SPE procedures are previous to the analysis by chromatographic methods. In this work we have tested two different eluting solutions, one organic–water mixture (ethanol–water, 70:30) and 0.05 *M* glycine–HCl buffer at pH 3, since ELISA is normally carried out under aqueous conditions. Other works on IAC have reported the efficient use of this or other buffers at pH 2 [34–36], however the supplier directions recommended not to use this support at pH values lower than 3. The use of chaotropic agents was initially rejected because of the reported risk of damaging the antibodies [37–40].

First of all, we decided to evaluate the effect that the presence of ethanol in the samples could have over the Irgarol 1051 ELISA. Organic solvents can affect the action of biomolecules, modifying their binding thermodynamics; in the same way, the nature of the solvent has an influence on the Ab binding event [41–43]. Earlier studies showed the effect of the solvent on the optical density, detectability and specificity of the ELISAs when an organic solvent is present in the sample [44–46]. The interest to investigate this influence relies on the possibility of directly measuring by ELISA the eluted fractions from the IAC column. To evaluate the ethanol effect on the Irgarol 1051-ELISA, several calibration curves were prepared in ethanol–water at different percentages of organic solvent (0, 5, 10, 20 and 30%). As shown in Fig. 1, an increase in the organic solvent content produced a gradual increase in the $I-50$ parameter and a decrease of the maximum absorbance, A (in the figure indicated as OD= optical density). Nevertheless, the immunoassay detectability did not vary significantly when the ethanol concentration increased up to 20%.

Considering these previous results, solutions of Irgarol 1051 (10 ml, 10 $\mu g\,l^{-1}$) were introduced into the column and after a washing step eluted with the selected eluting solution, either ethanol–water mixture or a 0.05 *M* glycine–HCl buffer at pH 3. As we can see in Fig. 2a and b, most of the Irgarol 1051 is eluted in the first three fractions, but unfortunately, recoveries were no higher than 30% when the glycine buffer was used. The experiment was repeated three times with little variation on the results obtained. In contrast, the ethanol–water (70:30) mixture afforded better recovery values. Because of the high detectability reached by the present Irgarol 1051 ELISA, it will probably be necessary to dilute the samples prior to the immunochemical analysis, placing thus, the ethanol content on a range where the immunoassay features are not significantly affected. Furthermore, this eluting phase does not damage the immunosorbent since as we have proved in further experiments the IAC column is still effective after 70 loading experiments (results not shown).

3.2. Capacity of the IAC columns

The next step consisted in proving that these IAC

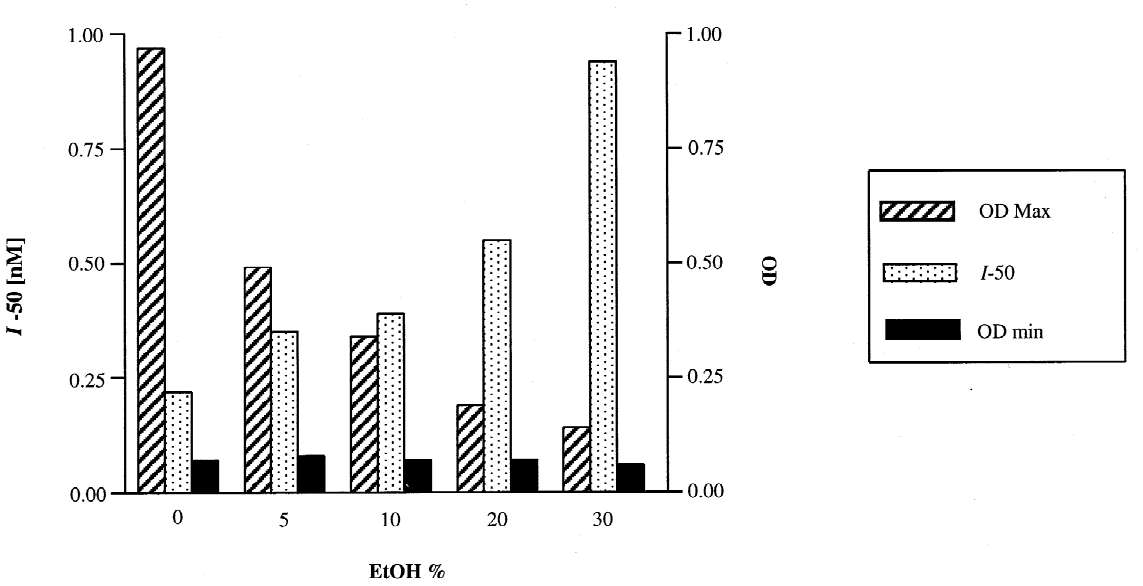


Fig. 1. Ethanol effect on the Irgarol 1051-ELISA features [A_{max} (OD max), $I-50$ and A_{min} (OD min) values]. Five standard curves, prepared with an increasing ethanol content (from 0 to 30%), were compared. *y* axis is dimesionless when OD are represented whereas its units are n*M* when expressed as $I-50$.

columns could readily retain Irgarol 1051. To confirm this fact, we introduced into the column water samples spiked with known amounts of Irgarol 1051. According to the calculated coupling efficiency and considering that only about 10% of specific anti-Irgarol 1051 Abs may be present in the IgG fraction of polyclonal antisera, we could estimate a theoretical column capacity of 3.0 ± 0.1 μg of Irgarol 1051 ml^{-1} bed column. Thus, 1 μg of Irgarol 1051 in 1 ml of Milli-Q water was introduced into the column and the different eluted fractions were analyzed by ELISA. The Irgarol 1051 ELISA used has an $I-50$ value of 100 $ng\,l^{-1}$, with a limit of detection (LOD) of 20 $ng\,l^{-1}$ and a quantification dynamic range from 35 to 300 $ng\,l^{-1}$. As reported [25] the current ELISA is selective for Irgarol 1051. Only terbutryne can be considered as an important interfering substance [cross-reactivity (CR)=148%], followed by terbumeton (18%), propazine (15%) and terbutylazine (10%).

In the determination of the capacity, a ethanol–water (70:30) mixture was used as eluent. These experiments demonstrated that amounts between 380 to 570 ng could be effectively retained by the IAC columns (see Table 1). The data shown corresponds to experiments done in duplicate for the three columns prepared and the ELISA analyses were performed in triplicates. As expected, found capacities were lower than those theoretically estimated as using the reported coupling procedure, the antibodies are randomly oriented which may cause blocking of the paratopes of some IgG molecules. Nevertheless, the results reported in Table 1 indicate that these columns can retain enough Irgarol 1051 for their use as SPE supports for environmental analysis.

3.3. Volume of the sample added into the column

Increasing volumes of artificial seawater samples at pH 8.6 containing 100 $ng\,l^{-1}$ of Irgarol 1051 were percolated through the column (25, 50, 100 and 250 ml) in order to evaluate the effect of the sample volume. The choice of this concentration is due to the fact that Irgarol 1051 occurs in the western Mediterranean sea at ppt levels [17]. Samples were analyzed by ELISA and the results showed that after 250 ml, the breakthrough was still not achieved (Fig. 3). The recovery of Irgarol 1051 when the volume of percolated sample is 250 ml was higher than 80%. In these conditions the eluting fractions had to be

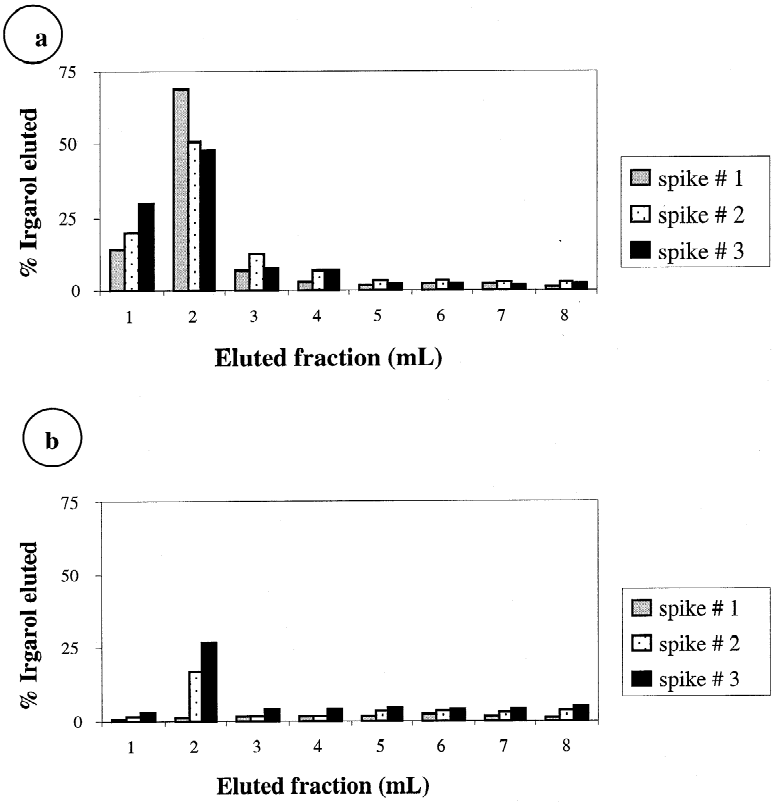


Fig. 2. Recoveries of Irgarol 1051 eluting with (a) ethanol–water (70:30), (b) 0.05 *M* glycine–HCl buffer pH 3. Experiments were performed introducing into the IAC column 10 ml of artificial seawater spiked with 10 $\mu g\,l^{-1}$ of Irgarol 1051. The eluted fractions were analyzed in triplicates by ELISA after an appropiate dilution with PBST 0.01 *M* of pH 7.6 buffer.

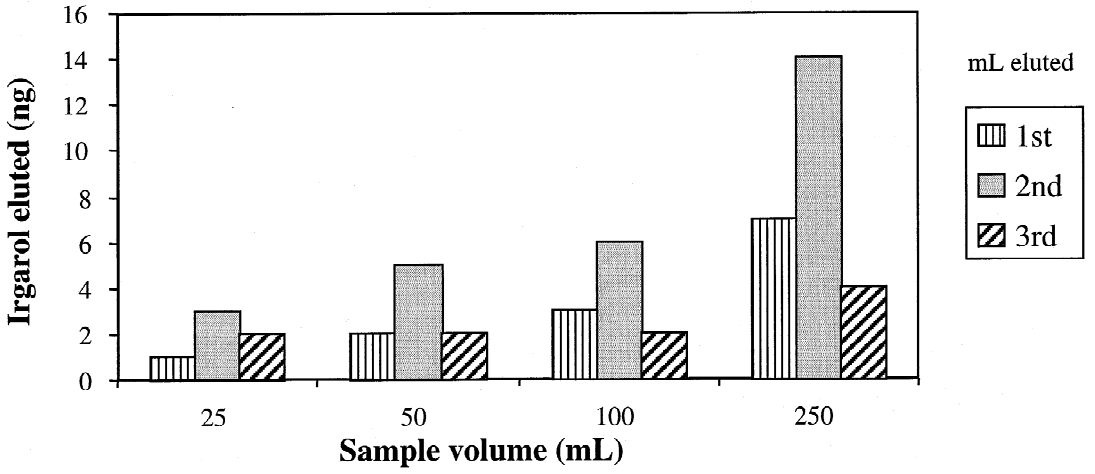


Fig. 3. Absolute amount of Irgarol 1051 recovered in three fractions (1st, 2nd and 3rd) of 1 ml each using ethanol–water (70:30) as eluent from the IAC column previously percolated with different volumes (25–250 ml) of artificial seawater spiked at 100 $ng\,l^{-1}$.

diluted prior to the ELISA analysis, placing the maximum% of ethanol to a value less than 2%, which does not affect the ELISA as demonstrated before.

3.4. Comparison of ELISA and GC–NPD measurements

To quantitate the analyte after the IAC selective extraction, a rapid and sensitive technique with minimal sample manipulation is desirable. ELISA presents these advantages, although overestimation and interferences from the matrix and other analyte related substances may occur, and thus it is often used as a screening technique. In contrast, quantitation by GC–NPD could be quite reliable; however, the eluted fractions have to be manipulated prior to the analysis producing also errors and complicating the measurement process. To evaluate this fact, nine fractions eluted from the IAC column were measured simultaneously with GC–NPD and ELISA. An acceptable correlation was obtained (Fig. 4), and thus, Irgarol 1051 monitoring after IAC clean-up is feasible by ELISA. However, the slope of the correlation obtained in this work (higher than one) shows a slight overestimation of the ELISA. This ELISA was previously evaluated for real and spiked seawater samples [11,26] showing an excellent correlation.

3.5. Specificity of the IAC procedure and evaluation of the non-specific interactions

In order to evaluate the selectivity of the IAC procedure developed, seven triazine herbicides were sequentially percolated through the IAC column at a concentration of 5 $\mu g\,l^{-1}$ in 250 ml of artificial seawater at a pH of 7.6 in order to test the specificity of the IAC procedure towards Irgarol 1051 and related compounds. As shown in Table 2, higher recoveries are obtained when the groups thiomethyl and isopropyl or *tert.*-butyl are present in the chemical structures of the analyzed triazine herbicides. These results are coherent with those obtained for the ELISA, where the same antisera that are binded to the IAC support is used which shows the same pattern of cross-reactivities. To verify that binding was due to specific Ag–Ab interactions three single triazines (Irgarol 1051, prometryne and terbutryne) were passed through the control column prepared with pre-immune serum. All triazines were recovered in the initial fractions of the PBS used to wash the columns. Thus, nonspecific binding was not found for the tested triazines. This fact confirms that

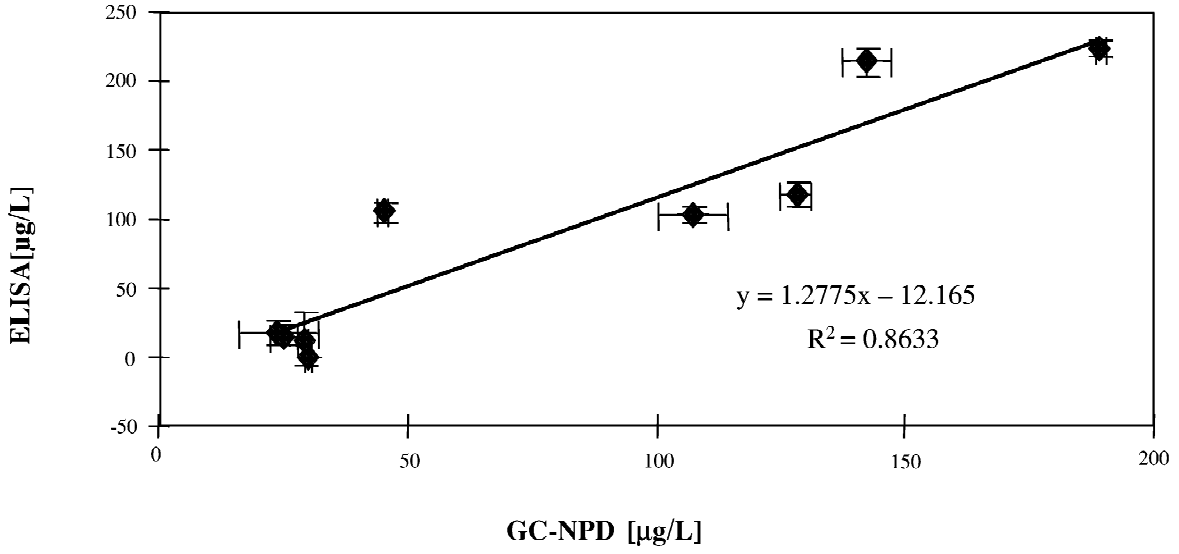
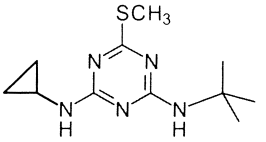
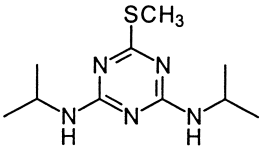
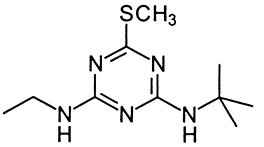
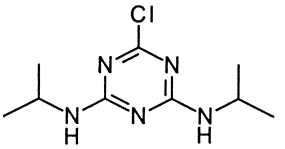
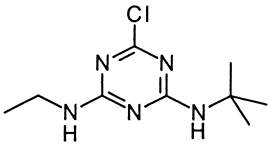
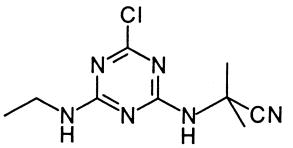
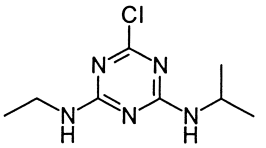
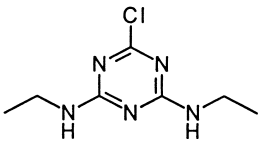


Fig. 4. Correlation between the ELISA and GC–NPD measurements of nine samples (1 ml each) eluted from the IAC column after introducing 1 $\mu g\,l^{-1}$ of Irgarol 1051 in 1 ml of aqueous solution.

Table 2
Recoveries of several *S*-triazine herbicides[a]

Compound	Recoveries (%)	Structure
Irgarol/Target analyte	100	
Prometryne	76	
Terbutryne	72	
Propazine	52	
Terbutylazine	40	
Cyanazine	32	
Atrazine	27	
Simazine	7	

[a] Results were obtained by percolating the triazines into the IAC at 5 $\mu g\,l^{-1}$ in 250 ml of artificial seawater.

Sepharose supports are appropriate to avoid unspecific binding on the IAC, as reported in previous studies [22]. The higher recovery percentages obtained for some of the triazines derivatives, far from being a disadvantage, should permit the simultaneous pre-concentration of some other triazine biocides that may occur in seawater allowing their simultaneous determination [47].

3.6. Application to environmental samples

Seawater samples were pre-concentrated on the anti-Irgarol 1051 IAC column, analyzed by GC–NPD and confirmed by GC–MS. Irgarol 1051 was identified in samples taken at three different depths in the Barcelona marina. Levels found were around 50 ng l^{-1} and are in the same magnitude to those from the Masnou marina previously reported (northwest Mediterranean) [17]. The lower concentration was found at 1 m depth respect to the higher value at 5 m depth which can be attributed to a possible photodegradation of Irgarol 1051 [15] taking into consideration that sampling was carried out in summer. However, the metabolite [2-(*tert*.-butylamino)-4-amino-6-(methylthio)-1,3,5-triazine] was not detected.

To demonstrate the higher selectivity of the IAC comparing with the SPE, the same marine sample (1 m depth) was percolated through the anti-Irgarol 1051 IAC column and the LiChrolut RP-18 cartridge. As expected, the ratio signal-to-noise of the GC–NPD chromatogram was lower for the LiChrolut RP-18-purified sample because not only the analyte of interest was extracted but also other compounds increasing the baseline noises. The LODs reached were 2.5 and 50 ng l^{-1} for the IAC column and LiChrolut RP-18, respectively, and the LOQs, 17 and 174 ng l^{-1} (Fig. 5). Consequently, due to the low concentration of Irgarol 1051 in the environmental samples analyzed, the IAC procedure seems to be more feasible than LiChrolut RP-18 cartridges.

4. Conclusions

An IAC procedure for the selective extraction of the antifouling agent Irgarol 1051 has been developed. The immunopurification system uses Abs

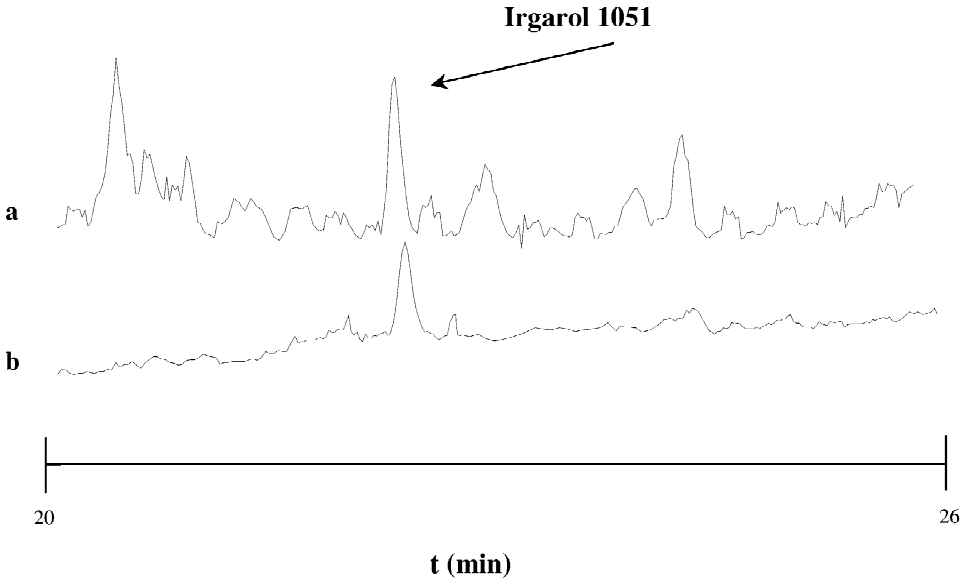


Fig. 5. Comparison of the GC–NPD chromatograms obtained after percolating the same real seawater sample through: (a) LiChrolut RP-18, (b) IAC column. LODs were 3 and 50 ng l^{-1} for the IAC column and LiChrolut RP-18 cartridge, respectively.

previously developed against this analyte [26], covalently attached to highly cross-linked agarose. This support was selected because of its hydrophilic nature and therefore its low nonspecific adsorption properties [20,48]. This character previously described in the literature has also been demonstrated here by preparing an immunoaffinity column with IgGs isolated from preimmune serum. In this case there was no retention of any of the triazine herbicides tested, demonstrating that the results reported have only been due to the presence of specific antibodies in the immunosorbent and not to nonspecific interactions with the support. The capacities of the IAC columns have been determined to be higher than 400 ng per ml of immunosorbent bed. This value is sufficient to analyze environmental samples, since levels of Irgarol are found to be in the low ppb range [11,13]. Furthermore, the breakthrough volume at Irgarol 1051 concentration values of 100 ng l^{-1} is higher than 250 ml. Under these conditions, recoveries are higher than 80% and the Irgarol is eluted in just 3 ml of a ethanol–water (70:30) mixture, which means that a concentration factor of almost 100 times is possible. Furthermore, the developed IAC preconcentration followed by the GC–NPD determination of Irgarol is rugged because the same column can be reloaded over to 70 times without any appreciable lost in sample capacity.

This is the first time that an IAC procedure has been applied to the analysis of real seawater samples. The procedure has been demonstrated to be very efficient in concentrating and selectively isolating Irgarol 1051 from this matrix. It has been reported that there are difficulties in analyzing trace contaminants in samples with high salinity values [49,50], but in this case quantitation of levels around 50 ng l^{-1} has been easily possible by using IAC prior to GC–NPD or GC–MS analysis. Chromatograms were much cleaner when IAC was used in the clean-up step than when a LiChrolut RP-18 solid-phase was employed, increasing thus the reliability of the quantification of trace levels of Irgarol 1051. For screening purposes the efficiency of the monitoring programs can be increased by combining IAC with the immunochemical analysis of the collected fractions. As already reported [11,26] the correlation between the ELISA and the chromatographic methods has proven to be good enough for this purpose.

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