Performance of two immunoassays for the determination of atrazine in sea water samples as compared with on-line solid phase extraction–liquid chromatography-diode array detection

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Abstract

Two immunoassay formats, magnetic particles-based assay (Atrazine RaPID assay and Atrazine High-Sensitivity RaPID assay) and microtiter plate based assay (Department of Entomology and Environmental Toxicology, University of California in Davis) were evaluated for the determination of atrazine in sea water samples. The results obtained were compared and validated with those obtained by using on-line solid phase extraction followed by liquid chromatography-diode array detection (on-line SPE-LC-DAD). The correlation between both techniques was good when analyzing levels of atrazine ranging from 0.01 to 5 µg/l in samples showing salt concentration values varying from 0 to 35 g/l and pH values from 2 to 10. One of these immunoassays (Atrazine High-Sensitivity RaPID assay) was employed to directly analyze atrazine in real estuarine and coastal water samples. The same samples were analyzed after filtration and C\textsubscript{18} Empore disks extraction.

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1. Introduction

The s-triazines herbicides are among the most commonly detected pesticides in water. Their widespread use combined with overapplications, accidents, runoff from mixing–loading areas, spills and faulty waste disposal creates environmental concerns [1]. The stability of these chemicals (atrazine half life in soil is about 50 days) together with their solubility (for atrazine, 33 mg/l) and their mobility in surface and ground water prompts s-triazine herbicides to reach estuarine areas carrying contamination to the sea. The transport of atrazine through pore and ground waters to surface and coastal sea waters is favored in the dissolved phase when compared to particulate matter [2–3]. All these reasons justify its detection in sea water samples from the Mediterran-
nean, through different rivers such as Ebre (Spain), Po (Italy), Rhone (France), and Axios (Greece) and from other European regions [2,4–7].

Implementation with new analytical techniques for monitoring trace quantities of contaminants in the sea and river water samples is now-a-days an important issue in environmental research. Because of their specificity, high sensitivity, adaptability for field use and ability to recognize a wide range of substances, immunochemical techniques can be particularly suited to this type of measurements. Numerous immunoassays and related techniques have been developed during the preceding years, covering a broad range of pesticides and contaminants of industrial origin (for review see [8,9]). Although today some of them are commercially available, its acceptance as regular screening method depends upon validation compared to chromatographic methods. Recently the good correlation existing between ELISA (enzyme-linked immunosorbent assay) techniques and GC-MS to determine triazines in water was shown [10]. Similar agreement was obtained on a validation study comparing ELISA and liquid chromatography followed by postcolumn derivatization fluorescence detection to analyze carbaryl in ground waters [11]. Interferences on immunoassay methods can be categorized into two major classes: (i) those that affect binding of antigen by competing for the specific binding site on the antibody (often referred as cross-reactants) and (ii) those that affect the binding event between the antibody and an antigen in a general way. Sea water presents concrete features that may interfere on the immunological reaction and to our knowledge no previous work was reported on the evaluation of immunochemical methods for these samples. In recent review articles [9,12] the necessity to study the performance of ELISA techniques in real environmental samples and the difficulties met with some complex matrices was emphasized. In general, for immunoassay, matrix effects are manifested as a reduction of the color development. These effects may result of a non-specific binding (NSB) of the analyte to the matrix, non-specific binding of the matrix to the antibody or enzyme, or to denaturation of the antibody and/or enzyme. In a recent book Sherry [13] described some key challenges for immunoassays to become widely used analytical methods, e.g., the establishment of clear performance criteria such as detection limit, selectivity, working range, precision as well as data on the recoveries.

In this context, the first objective of this work was to evaluate the influence of selected physico-chemical parameters of sea water matrix, such as ionic strength and pH on the performance of the immunoassays by comparing the obtained data to an on-line solid phase extraction-liquid chromatography procedure using a diode array detector (on-line SPE-LC-DAD). Among the numerous immunoassays available to analyze triazines we have chosen for this study two RaPID magnetic particle-based ELISAs (Atrazine and High-Sensitivity Atrazine) from Ohmicron [14] and the microtiter plate ELISA developed at the University of California in Davis [15]. A second objective was to monitor atrazine in real estuarine and coastal water samples together with the evaluation of the effect of extreme pH conditions on the atrazine bound to humic acids.

2. Materials and methods

2.1. Chemicals and immunochemicals

Pesticide-grade solvent ethyl acetate, ethyl ether, methanol and dichloromethane, were obtained from SDS (Peypin, France). Atrazine and simazine were purchased from Polyscience (Niles, IL, USA). Deethylatrazine and deisopropylatrazine were gifts from Ciba–Geigy (Basel, Switzerland). The RaPID®-magnetic particle-based ELISA from Ohmicron (Newtown, PA, USA) was purchased through J.T. Baker (Deventer, NL, USA). Immunoreagents employed for the microtiter plate ELISAs were generously provided by Professor Dr. Bruce D. Hammock (Department of Entomology and Environmental Toxicology, University of California, Davis, USA). Sea salts and other immunochemical reagents were obtained from Sigma (St. Louis, MO).

2.2. Buffers and solutions

All buffers and solutions were prepared using Milli-Q water: (1) Coating buffer was 0.1 M carbonate buffer pH 9.6. (2) PBS (phosphate-buffered saline) was 0.2 M phosphate buffer with
0.8\% (w/v) NaCl, pH 7.5. (3) PBST was PBS buffer containing 0.05\% (v/v) Tween 20. (4) 10\% PBST is PBST 2M, 8\% (w/v) NaCl and 0.5\% (v/v) Tween 20. (5) Substrate buffer was 0.1 M citrate pH 5.5. (6) Substrate solution for peroxidase enzyme was prepared mixing 400\mu l of TMB (3,3',5,5'-tetramethylbenzidine, 0.6\% (w/v) in DMSO) with 100\mu l of 1\% (v/v) H\textsubscript{2}O\textsubscript{2} in 25 ml of substrate buffer. (7) Enzymatic reaction was stopped with aqueous 4 N H\textsubscript{2}SO\textsubscript{4}.

2.3. Sample preparation

Water samples used throughout this study were Milli-Q water, estuarine and Ebre river water (Amposta, Tarragona, Spain). Samples with different salinity values were prepared by dissolving the corresponding amounts of sea salts (Sigma, St. Louis, MO) in Milli-Q water. Measurements of the sea salt content of the different real environmental samples were performed by using a portable conductimeter from Crison (Alella, Barcelona, Spain). Previously a calibration graph was constructed measuring the conductivity of solutions containing known amounts of sea salts.

_Ebre river water samples from Amposta_ (pH=7.9 and salinity 6.4 g/l). About 2.51 of water was collected, filtered through 0.45 \mu m filters (Millipore, Bedford, MA) to remove suspended particles and stored at 4\textdegree C until analyzed. _Estuarine–river water samples_ were collected at several points of the estuarine area showing salinity values of 25, 15, 8, 4 and 0.1 g/l. Estuarine–river water samples were first filtered through fiber-glass filters (Millipore, Bedford, MA) of 0.70 \mu m and subsequently through 0.45 \mu m filters to eliminate particulate matters as described [16]. The SPE off-line method used a Millipore 47 mm filtration apparatus. The membrane extraction disks were manufactured by 3M (St. Paul, MN) under the trademark Empore and are distributed by J.T. Baker and Analyticchem International. The disks used in these experiments were 47 mm in diameter and 0.5 mm thick. Each disk contains about 500 mg of C\textsubscript{18} bonded silica material. The disk, placed in a conventional Millipore apparatus was washed with 2×10 ml of methanol under vacuum avoiding the solid phase to become dry and one liter of water was extracted with the vacuum adjusted to yield a 1 h extraction time. The disks containing the pesticides were used for transportation and storage. Immediately before the analysis, the pesticides trapped in the disk were collected with 20 ml of methanol. After careful evaporation of the methanol to dryness, the samples were re-dissolved in 480 ml of Milli-Q water for atrazine determination by on-line-SPE-LC-DAD. Water samples for immunoassay determination were taken through the different steps of this clean-up procedure.

2.4. _On-line SPE-LC-DAD system_

A PROSPEKT (Spark, Emmen, The Netherlands) was used in combination with an LC-DAD system. The PROSPEKT consisted on a solvent delivery unit, a six-port valve connected to the gradient pumps, and an automated cartridge exchange module. For SPE 10 mm×2 mm i.d. precolumns packed with 40 \mu m C\textsubscript{18} (Baker, Deventer, The Netherlands) were used. The precolumns were conditioned by way of a solvent delivery unit from Spark. The LC analyses were performed with a Waters 600-MS solvent delivery unit provided with a 20 \mu l injection loop and combined with a Waters 996 photodiode array detector (Waters, Millipore, MA). A 25 cm×4.6 mm i.d. analytical column packed with 5 \mu m Hypersil BDS C\textsubscript{8} (Shandon) was used. Before LC analyses, Milli-Q water and sea water samples were filtered through 0.45 \mu m filters (Millipore, Bedford, MA) to remove suspended particles.

Approximately 50–140 ml of sample were percolated through a preconditioned SPE column (20 ml of acetonitrile followed by 10 ml of Milli-Q water at 1 ml/min) where the sample was preconcentrated at a flow rate of 4 ml/min. After a washing step with Milli-Q water (3 ml for Milli-Q water samples and 9 ml when samples of high-salinity value were analyzed), the analytes are desorbed by the mobile phase into the LC-system in the backflush mode by using a switching valve. Separation was achieved in the reversed phase C\textsubscript{8} chromatographic column. A gradient elution was employed starting from a mobile phase containing 20\% A (acetonitrile) and 80\% B (water) to one containing 60\% A and 40\% B in 20 min; to 100\% acetonitrile in 20 min and then back to the initial conditions in 5 min at a flow rate of 1 ml/min. Quantitation was done using the external
standard calibration method with the detector set at 220 nm. Calibration graphs were constructed for atrazine by analyzing spiked aqueous samples prepared with Milli-Q water and 35 g/l sea salts in Milli-Q water.

2.5. ELISA methods

RaPID assay measurements were determined using the RPA-I RaPID Photometric Analyzer™ (Ohmicron, Newton, PA, USA) at 450 nm. A two-piece magnetic separation rack consisting of a test holder which fits over a magnetic base containing permanently positioned rare earth magnets is required. This two-piece design allows for a 60-tube immunoassay batch to be set up, incubated and magnetically separated without removing the tubes from the holders. Microtiter plate ELISAs were performed using polystyrene microtiter plates bought from Nunc (Maxisorb, Roskilde, Denmark). The absorbances were read at 450 nm in a microtiter plate ELISA reader Multiskan Plus (Labsystems, Helsinki, Finland). Data acquisition and calculations were performed using the commercial software package Genesis (Labsystems, Helsinki, Finland). A four-parameter logistic equation was used for the calibration of the standard curves.

2.5.1. Atrazine RaPID assay

All samples were assayed according to the RaPID Assay package insert. The RaPID magnetic particle-based ELISA (RaPID-ELISA, RaPID®, assays, Ohmicron, Newtown, PA, USA) has polyclonal antibodies coated on paramagnetic beads. A total amount of 200 μl of the sample water to be analyzed is added to a disposable test tube, along with 250 μl of pesticide (atrazine) hapten-horseradish peroxidase (HRP) enzyme conjugate, and 500 μl of rabbit anti-pesticide magnetic particles (anti-atrazine) attached covalently. Tubes were vortexed and incubated for 15 min at room temperature. Atrazine of the sample and the enzyme labeled pesticide compete for the antibody binding sites on the magnetic particles. The reaction mixture was magnetically separated using a specially designed magnetic rack. After separation, the magnetic particles were washed twice with 1.0 ml of distilled water to remove unbound conjugate and eliminate any potential interfering substances. Pesticide and enzyme labeled pesticide remained bound to the magnetic particles in concentrations proportional to their original concentration. The presence of labeled pesticide was detected by adding a total of 500 μl of a 1:1 mixture of a solution containing substrate and chromogen (hydrogen peroxide and 3,3’,5,5’-tetramethylbenzidine (TMB)). The tubes were vortexed to resuspend particles and incubated for another 20 min at room temperature to allow color development. The color reaction was stopped by the addition of 500 μl of 2 M H₂SO₄ solution. The final concentrations of pesticide for each sample were determined by comparing their absorbance at 450 nm to a standard curve where the logarithm of the pesticide concentration was plotted versus %B/B₀ (B=absorbance of each concentration of the standards, B₀=absorbance at zero concentration). The standard curve was prepared from calibrators containing known levels of atrazine at 0, 0.1, 1.0, and 5.0 μg/1. All standards were analyzed in duplicate. Test kits were stored at 4°C but temperature and time are important parameters that must be controlled for this ELISA to work properly. In all cases, solution of immunoassay and samples were allowed to equilibrate to room temperature before use, and reaction times were consistent throughout the experiment. A negative control had to be tested concurrently with each set of water samples. The negative control was used to standardize the water sample’s absorbance measurements.

2.5.2. High sensitivity atrazine RaPID assay

Samples were processed as described above, except the 250 μl of sample water are necessary for atrazine determination and the competition step takes 30 min. Calibrators used to build the standard curves were 0, 35, 250 and 1000 ng/l.

2.5.3. Atrazine microtiter plate ELISA

Plates were double coated first with goat antimouse (0.5 μg/ml in coating buffer, 100 μl/well) and incubated in the microtiter plates overnight at 4°C. The day after plates were washed five times with PBST and the second coating was performed with monoclonal anti-atrazine antibodies (AM7B2.1 diluted 1/3000 in coating buffer, 100 μl/well) incubating for 12 h at 4°C. For running the assays, 50 μl of standards (stock solution 0.1 mM in DMSO, 5000,
500, 50, 25, 12.5, 6.25, 3.1, 1.55, 0.77, 0.77, 0.38 and 0 nM in PBST) or buffered samples (225 μl of water sample + 25 μl of 10x PBST) and 50 μl of enzyme tracer (1/4000 in PBST) were incubated on the coated plate for about 15 min at room temperature. Plates were rinsed five times with PBST and developed by adding 100 μl of the substrate solution. Plates were stopped by adding 50 μl of 4N H₂SO₄ after approximately one hour. Finally the absorbances were read at 450 nm. All samples were run on triplicates unless otherwise indicated.

2.6. Matrix effect studies

2.6.1. Immunoassay standard curves parallelism studies

To study the effect of the salinity on the immunoassay determinations, solutions containing different sea salt concentrations (0 2.5, 5, 10, 15, 20, 25, 30 and 35 g/l) were prepared dissolving sea salts (Sigma, St. Louis) in Milli-Q water and used for preparing standard curves. Another batch of solutions was prepared with PBS varying the pH value (3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) by adding small amounts of 1N HCl or 1N NaOH. Each of these solutions were used to prepare standard curves and to analyze their parallelism by ELISA. For the RAPID High-Sensitivity atrazine immunoassay we only checked 0 and 35 g/l sea salt concentrations while pH effect was studied on solutions at 2.5, 5.7, 7.0 and 9.1. Water from the Ebre estuarine–river was also tested to confirm the absence of matrix effects.

2.6.2. Effect of the salinity on the on-line-SPE-LC-DAD system

Calibration graphs were prepared with Milli-Q water containing 0, 2.5, 15 and 35 g/l sea salt concentrations.

2.6.3. Effect of the pH on the on-line-SPE-LC-DAD system

Ground water at pH 2.5, 7.4 and 10 was spiked with 0.5 μg/l of atrazine and used to determine breakthrough volumes.

2.6.4. Recovery studies

Milli-Q water (sea salt content 0 g/l), Milli-Q water (sea salt content 35 g/l) and Ebre river water were spiked with atrazine at concentration values ranging from 0.01 to 5 μg/l and used for HPLC and ELISA determinations as described above.

2.6.5. Correlation studies

Spiked samples were split for ELISA or HPLC analyses. Regression studies comparing both techniques were performed using statistical software with a confidence level of p<0.05.

2.6.6. Real samples

Real samples from the Ebre river were used for checking the effect of humic acids on atrazine containing waters. Atrazine-bound to humic acids could be released to the liquid phase with extreme pH conditions. Water samples with a pH of 7.9 were acidified until pH 2.0 with 1N HCl. Both samples were tested directly to determine atrazine concentrations in the liquid phase using the High-Sensitivity atrazine RAPID assay. In addition, five water samples from estuarine–river were chosen to carry out an evaluation of the performance of the High-Sensitivity atrazine immunoassay on water with high salt content at different steps of the clean-up procedure. Two of these samples were analyzed directly without any kind of filtration, while all of them were analyzed before (only with a filtration through 0.7 and 0.45 μm filters) and after purification by C₁₈ extraction disks. Results obtained were compared to those obtained by a robust analytical technique such as on-line SPE-LC-DAD.

3. Results and discussion

3.1. General remarks

Factors such as anions, cations, pH and organic content are responsible for what is known as matrix effect and may interfere non-specifically with the immunochemical reaction. In fact, a tendency to overestimate the amount of pollutants in environmental samples was often observed [10,11,17,18] by ELISA when compared to chromatographic methods. Regardless, antibodies are made to work under physiological conditions (which means on a liquid well-defined media in terms of its physico-chemical
parameters) immunochemical reaction has proven to occur satisfactorily in many other aqueous environments, or even gas-phase media (i.e., some piezoelectric immunosensors have shown to be effective measuring parathion on contaminated air samples [19]). Under these conditions, however, the affinity of the antibody versus the analyte or the enzyme tracer may vary changing thus the kinetics of the immune-reaction. Therefore, before start performing immunoassays in a new matrix it is advisable to study if the kinetic of the antibody reaction in such media remains identical to that of the standards on the assay buffer. A way to do that is just to study the analogy of the standard curves performed on the matrix. This was the first aim of the work presented here.

3.2. Effect of the salinity and the pH

Main features of sea water samples are the ionic strength, usually about 0.7, and the pH, generally varying from 7.5 to 8.5. As it is shown in Fig. 1, the evaluation of the effect of these parameters demonstrated that there was no effect on the immunoassay performance when salinity content in water raised to 35 g/l. Standard curves run at different salinity values (0–35 g/l) were parallel in all immunoassays employed in this study (see Fig. 1(A)), thus, suggesting that reliable analyses could be made through the river–estuarine waters without changing the immunoassay protocol or applying clean-up procedures. Similar results regarding salinity and pH effect were found for the RaPID atrazine immunoassay and for microtiter ELISA. Analogously, these immunoassays were not affected by extreme pH conditions since the standard curves did not show variations on a pH range between 3 and 9 (see Fig. 1(B)). Only a slight decrease on the absorbance was observed when analyzing atrazine in pH 2.0 water samples with the High-Sensitivity RaPID atrazine kit (see Fig. 1(C)), although the slope of the assay did not show any significant difference when compared to the standard curve run at pH 7.4.

Similarly effect of salinity on the on-line SPE-LC-DAD system was evaluated by running water samples having a salinity content of 0, 2.5, 15 and 35 g/l. These samples were spiked with different amounts of atrazine to construct calibration graphs. Linearity obtained was good even at the highest sea salt concentration, although the slope of the linear regression equation decreased (see Fig. 2). Additionally chromatograms showed broadening of the peaks causing problems while quantifying small concentrations of atrazine. Main reasons of this behavior were attributed to the precolumn, to which high-salt containing solutions shortened lifetime considerably. The slight bias observed on the slope could be corrected by changing the precolumn in every five injections. In contrast analyses by on-line SPE-LC-DAD of atrazine were easily carried out independent of the pH. No breakthrough for this analyte occurred at any of the pHs used when the water volume varied between 50 and 140 ml. However, it is predictable that a lifetime reduction of the precolumn when large number of water samples, showing a pH value around 2, were introduced into the system.

3.3. Recovery studies

In order to prove that the matrix effect was negligible, Milli-Q water (sea salt content 0 g/l), Milli-Q water (sea salt content 35 g/l) and Ebre estuarine–river water were spiked with different amount of atrazine and used for HPLC and ELISA determinations. Table 1 shows the recoveries obtained with every analytical methods employed in this study. The chromatographic method was always more accurate since the recovery values obtained were always close to 100%; however, for high salt content samples this was only true when frequent renewal of the precolumn was performed. In this context, we should note that whereas immunoassays could perform several replicates of the spiked samples in any of the conditions tested in this study, some analyses carried out by the chromatographic method had to be rejected since quantification of atrazine was not possible at low concentration levels (see samples marked with a star in Table 1). Quantification with both RaPID and microtiter plate ELISAs in contrast did not cause any problem even under extreme pH or salinity conditions, although precision of the chromatographic method was always higher (see the average of the coefficients of variation in Table 1). The RaPID atrazine ELISA kit tends to overestimate while the microtiter plate ELISA shows more accuracy.
3.4. Correlation studies

Spiked samples were used to establish the correlation existing between the immunoassay methods and the chromatographic method. As shown in Fig. 3 agreement between on-line SPE-LC-DAD and ELISA was always good with a coefficient of correlation higher than 0.96 and slopes were in every case close to one. The best correlation was obtained when analyzing Milli-Q water (Fig. 3(A)), although 35 g/l sea salt containing Milli-Q water also showed satisfactory values (Fig. 3(B)). On the other hand the overestimation of the RaPID atrazine assay is clear when Ebre estuarine–river water was analyzed (Fig. 3(C)).

3.5. Analyses of real estuarine and coastal water samples

Effect of the pH on the amount of soluble atrazine in estuarine water. The pH tolerance presented by the immunoassays used in this study prompted us to
High-Sensitivity atrazine immunoassay when decreasing down to pH 2.0, although other parameters of the curve such as slope and $I_{50}$ remained constant. To correct this effect we quantified the atrazine-IR of these acidic samples with corresponding standard curve run at the same pH. Results obtained demonstrated an increment on the atrazine-IR from a value of 0.26 to 0.36 µg/l suggesting a release of the atrazine from particulate matter or humic substances. Studies on the effect of the pH on these kind of samples will continue on following studies.

River-estuarine and coastal water samples. Since no strong effect of the salinity on the immunoassay performance was observed and as a part of this validation study, we faced the analysis of real samples by immunoassay comparing the results obtained with those of the chromatographic method. Water samples showing salinity levels ranging from 0.1 to 24, as it appears in Table 2, were taken at different points of estuarine area. Samples were processed by on-line-SPE-LC-DAD after the clean-up procedure described on the experimental section, whereas analyses at three different steps of the purification process were performed with the High-Sensitivity atrazine RaPID assay: (i) two of the samples were analyzed on-site without any processing and all samples were analyzed (ii) after passing the samples through 0.7 and 0.45 µm filters and (iii) after extracting atrazine with C$_{18}$ extraction disks. Samples before any processing showed dark brown color with abundantly suspended material and gave the highest values of atrazine-IR when analyzed directly by ELISA. Analysis performed either after

![Graph showing calibration graphs obtained when injecting standards dissolved in water with different salt content. Linearity is good in all cases, although the slope decreases with increasing sea salt concentration.](image-url)

perform a preliminary experiment aimed to detect changes on the levels of soluble atrazine as a function of the pH. We thought that atrazine-bound to humic acids could be released to the liquid phase by decreasing the pH. Therefore, a fraction from the Ebre estuary, showing a pH of 7.9, was acidified to pH 2.0 and both samples were tested for their atrazine-like immunoreactive content (atrazine-IR). The true analogy existing on the standard curves run either in buffer or Ebre water was shown (Fig. 1(C)). A slight effect is observed on the response of the

### Table 1
Recoveries of Microtiter Plate, RaPID atrazine ELISA and on-line SPE/LC-DAD at different spiked levels of atrazine with Milli-Q water at 0 g/l and 35 g/l of salinity and Ebre river water

<table>
<thead>
<tr>
<th>Spiked (µg/l)</th>
<th>0 g/l Salinity</th>
<th>35 g/l Salinity</th>
<th>Spiked (µg/l)</th>
<th>Ebre river</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microtiter</td>
<td>RaPID</td>
<td>LC-DAD</td>
<td>Microtiter</td>
</tr>
<tr>
<td>0.108</td>
<td>126.39</td>
<td>125.00</td>
<td>92.59</td>
<td>90.28</td>
</tr>
<tr>
<td>1.078</td>
<td>93.60</td>
<td>135.43</td>
<td>107.61</td>
<td>98.24</td>
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<tr>
<td>2.157</td>
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<td>122.86</td>
<td>104.78</td>
<td>92.86</td>
</tr>
<tr>
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<td>105.19</td>
<td>118.08</td>
<td>101.08</td>
<td>81.98</td>
</tr>
<tr>
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<td>107.14</td>
<td>106.86</td>
<td>105.47</td>
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</tr>
<tr>
<td>Mean</td>
<td>103.49</td>
<td>121.65</td>
<td>102.31</td>
<td>93.43</td>
</tr>
<tr>
<td>(% CV)</td>
<td>(15.09)</td>
<td>(8.56)</td>
<td>(5.79)</td>
<td>(8.81)</td>
</tr>
</tbody>
</table>

*Quantification of these samples was difficult due to matrix effects.
Fig. 3. Graphics showing the correlation encountered when comparing the chromatographic method to the immunoassay techniques. (A) Milli-Q water; (B) 35 g/l sea salts (C) Ebre estuarine water.

Table 2: Variability of atrazine-IR measurements in real samples with different salinity levels by HS-Atrazine ELISA and on-line SPE/LC-DAD techniques

<table>
<thead>
<tr>
<th>Sample</th>
<th>Salinity (g/l)</th>
<th>HS-Atrazine RaPID ELISA</th>
<th>On-line SPE/LC-DAD (ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before extraction,</td>
<td>Before extraction,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>without filtration (ng/l)</td>
<td>with filtration (ng/l)</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>n.d.</td>
<td>49.93 (8.8)</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>n.d.</td>
<td>85.23 (7.8)</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>n.d.</td>
<td>173.97 (5.1)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>311.41 (6.2)</td>
<td>231.44 (6.7)</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>492.81 (2.4)</td>
<td>222.21 (7.3)</td>
</tr>
</tbody>
</table>

*Quantification was difficult due to matrix effects.

n.d.: non determined; n=6.
filtration or filtration/extraction procedures showed a good correlation with the data obtained by liquid chromatography, except for samples 4 and 5 that gave values significantly higher before extraction by C_{18} disks. We attributed these differences to the presence of deethylatrazine, the main metabolite of atrazine, since these samples were collected from areas showing lower salinity values and higher contamination levels, which could justify the presence of metabolite. This compound contributes to the measured immuno-reactivity before C_{18} extraction on a 25% according to the cross-reactivity data provided by the supplier. However, we could not quantify deethylatrazine in the samples due to higher interferences in the LC-DAD signal. It is also worth to note that the atrazine-IR values obtained are in general lower than the atrazine concentration found by on-line SPE-LC-DAD method, when usually immunoassay methods tend to overestimate reducing thus the possibilities of false negative results. We attributed this difference to the presence of large quantities of humic acids that interfered with the quantification of the eluting peaks. Actually, despite the purification process applied to all samples, two of them, sample 3 and 5, were difficult to quantify by on-line SPE-LC-DAD.

4. Conclusions

Standard curves run with the atrazine immunoassays evaluated through this study, were completely analogous under a broad range of salinities or pH conditions, suggesting that analyses of water samples can be directly performed through river–estuarine areas, as shown with samples of this study. At pH 2.0 a slight bias was observed with the High-Sensitivity atrazine RaPID assay. However, by using a buffer mimicking the matrix, these samples could be quantified. This advantage will allow us to perform future studies aimed to better understand the exiting equilibrium between unbound and bound-atrazine-IR under different pH conditions. On a preliminary study we have already shown in this paper how the amount of soluble atrazine-IR in the Ebre estuarine water samples increases at low pH.

This paper displays the potential of immunochemical techniques to analyze environmental samples directly, and in particular, matrices showing high salinity values. The concentration of atrazine on coastal areas decreased when salinity increased [5]. Therefore, higher volumes of these water samples should be preconcentrated before the analysis can be performed. In this context, we have shown in this paper how the presence of high concentrations of salts interferes with the standard on-line SPE-LC-DAD procedure due to a decrease of the lifetime of the precolumns. Because of the high-sensitivity of the immunochemical methods, measurements of high salinity samples can be carried out directly with only 200–250 µl of sample (see Table 3 for comparison of the three methods employed throughout this study). This is without any doubt an important benefit that immunoassay technology brings to the environmental area. As it is shown in Table 2 direct measurement of environmental estuarine waters gave already atrazine-IR values on the same range that those obtained by on-line SPE-LC-DAD. Note that we were working on the ng/l level with water samples contained a high amount of particulate matter and other interfering substances. Nevertheless, just a filtration procedure was sufficient to obtain results that closely match those obtained by the chromatographic method.

Table 3
Comparison between the three analytical techniques used with 0 g/l–35 g/l of salinity for the analysis of atrazine

<table>
<thead>
<tr>
<th></th>
<th>SPE/LC-DAD</th>
<th>ELISA microtiter</th>
<th>RaPID-ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy (%)</td>
<td>102/106</td>
<td>103/93</td>
<td>121/110</td>
</tr>
<tr>
<td>Precision (% CV)</td>
<td>6/8</td>
<td>15/9</td>
<td>8/18</td>
</tr>
<tr>
<td>Detection Limit (µg/l)</td>
<td>0.02/0.1</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>Volume of sample (ml)</td>
<td>60</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Speed (samples/h)</td>
<td>1</td>
<td>&gt;100</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Filtration necessary</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Difficulty</td>
<td>No / Yes*</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

* Difficult at low concentrations.
Benefits of the immunoassay technology were often recognized, specially due to the low cost, high sensitivity and simplicity of these techniques that make them suitable for screening procedures when large amount of samples are going to be analyzed (see speed rate on Table 3). However, everyone working on immunoassay is aware that measuring absorbances on a colored solution is not an identifiable method. For this reason, results from immunoassay tests are often reported as analyte-equivalents or analyte-like immunoreactive material, unless other confirmatory analytical techniques were used. This fact is in contrast with other more specific methods such as GC- or HPLC–mass spectrometric systems, where a fragment of the analyte is directly detected by the mass selective detector. By accepting that such kind of equipments are expensive and not accessible to every laboratory, we could place half way these methods based on UV detection (HPLC) or NP detection (GC). But, in fact, we would like to point out the fact that on the same way that a decrease in the absorbance does not necessarily has to be attributed to the antibody-analyte specific interaction, the appearance of a peak at a certain retention time may also be caused by the presence in the matrix of other compounds with similar chromatographic behaviors, making thus also necessary to use a confirmatory technique. In this context, two samples from the estuary presented problems to be quantified by on-line SPE-LC-DAD due to interferences that we have attributed to the high amount of humic acids present. On the other hand we have also to consider the increase of the cost of the analysis when performing frequent renewal of the precolumns in order to make accurate analysis.

Finally, this paper shows that immunoassay technology may be an excellent complement to other more robust analytical techniques since allow rapid processing of environmental samples while providing sufficient accuracy. Because of the interferences encountered while quantifying atrazine on these samples containing a high salinity value by on-line SPE-LC-DAD, we envisage that on certain kind matrices results obtained by the use of immunochemical technologies may truly support those of obtained by chromatographic methods.

Acknowledgements

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