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Atrazine interaction with tropical humic substances by Enzyme Linked Immunosorbent Assay

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Abstract. Enzyme-Linked Immunosorbent Assay (ELISA) has been evaluated by analyzing rich-humic water samples from tropical rivers. The samples were spiked with atrazine at ppb level. Different pHs (4 to 9) and humic concentrations (2.5 to 40 mg L^{-1}) were investigated. The assay performance showed a strong dependence on the pH values and amount of humic matter at low atrazine concentration. From all the conditions studied the low pH (pH 4) and high humic substances concentrations (40 mg L^{-1}) showed the greatest influence. The IC_{50} value to control sample (no humic) diminished from 0.28 nmol L^{-1} to 0.64 nmol L^{-1} to humic acid solution. This effect is specially noted for the humic acid fractions, since fulvic acid fractions showed no significant change on the immunoassay results. Additionally, it has been demonstrated that at basic pH the matrix effect produced by the natural Brazilian water sample containing humic substances even at 40 mg L^{-1} disappears. Therefore, the ELISA method used to determine atrazine, can be employed to determine this pesticide in water samples containing humic substances without prior preparation.

Key words. Atrazine – aquatic humic substances – immunoassays.

Introduction

The main mass of organic carbon distributed in natural aquatic environments and soils is concentrated in humic substances (HS). In general HS are the final products of microbial degradation processes of plants in soils and waters. Aquatic humic substances in freshwater ecosystems are often believed to be of terrestrial origin. The original main source may partially be allocthonous (produced outside the system) or autochthonous (produced within the system). Humic substances generally form the major fraction of the dissolved organic matter (DOM) in natural waters, accounting for up to 80% of the dissolved organic carbon (DOC) [1].

Concentrations of DOC in rivers range from less than 1 mg L^{-1} in alpine streams to more than 40 mg L^{-1} in some tropical or polluted rivers, draining swamps and wetlands [2,3]. Based on their solubility in alkali and acid media, HS are commonly divided into three major fractions: i) humic acids (HA) soluble in dilute alkaline solution but insoluble in acid solution; ii) fulvic acids (FA) soluble in both acid and base; iii) humins, insoluble in both dilute acid and base. Despite their great variety and heterogeneity, HS mostly exhibit comparable functional groups of phenolic and carboxylic types and are characterized by their complexation capabilities towards metal ions and organic pollutants [4,5]. This property in combination with their colloidal properties, makes HA and FA effective agents in transporting both organic and inorganic contaminants in the environment [6].

HS may interact with pesticides by mechanisms involving either physical sorption or chemical reaction. The s-triazine herbicides are among the most commonly detected pesticides in environmental waters and relevant samples, e.g. drinking water, representing therefore a threat to public health [7-10]. It has been shown that atrazine (6-chloro-N-ethyl-N-isopropyl-1,3,5-triazine-2,4-diamine) (AT) adsorption is probably the most important mode of interaction between the pesticide and HS, and is associated principally with the organic matter [11]. For the case of atrazine interacting with humic materials, it has been demonstrated that the atrazine binding of DOC isotherm is of the Langmuir type and not of the partition coefficient type. There is a definite stoichiometric complexing capacity limit [12].

The use of ELISA techniques to study the behavior of atrazine in natural systems has received attention in the literature in recent years. Immunoassay techniques are being used more and more widely for the quantification of herbicides in aquatic environments to complement gas and liquid chromatography [13,14]. A good correlation between ELISA (enzyme-linked immunosorbent assay) and chromatographic techniques to determine triazines in water has been reported [15-17]. Recently we have developed an ELISA for atrazine reaching a detection limit which is far below the limit of the EC Guidelines for Drinking Water [18].

There is a lack of studies on the influence of naturally isolated humic substances on atrazine and the performance of immunoassays. In general, the analysis of pesticides in humic solutions by traditional chromatographic methods is complicated and involves various steps of extraction and sample clean-up [19-21]. 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 for this type of measurement. In fact, most of the published papers applying ELISA in monitoring studies use groundwater water samples or natural river water samples from non-tropical areas with low organic matter content. Therefore, evaluation of alternative or complementary analytical methods to analyze pesticides in these kinds

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of samples is interesting. The presence of agricultural activities in the Environmental Protection Areas in Brazil and the use of pesticides could be an environmental problem. Therefore, this study is of interest for Brazilian environmental protection. In this study we have used the ELISA [18] to investigate the influence of tropical river samples on the assay results. The objectives herein were: i) to study the effect of the HS concentration on the ELISA; ii) to investigate the influence of the rich-humic water samples at various pHs on immunoassay performance and iii) to evaluate the contribution of both humic and fulvic acid fractions on the observed effects.

Materials and methods

Chemicals and immunochemicals

Atrazine was purchased from Ciba-Geigy (Barcelona, Spain). Sea salts employed were acquired from Sigma Chemical Co. (St. Louis, MO). Antiatrazine polyclonal antiserum (As 10) and enzyme tracer 2a-HRP were obtained in our laboratory [18].

Buffers and solutions

PBS is 0.2 mol L^{-1} phosphate buffer, 0.8% saline solution and unless otherwise indicated the pH is 7.5. Coating buffer is 0.5 mol L^{-1} carbonate-bicarbonate buffer pH 9.6. PBST is PBS with 0.05% Tween 20. Citrate buffer is a 0.1 mol L^{-1} solution of sodium citrate pH 5.5. The atrazine standards (0.03 to 200 nmol L^{-1}) were prepared from one stock solution in methanol (33.8 mg/50 mL) by dilution with PBST.

Sample preparation

Rich humic-water samples were collected from two Brazilian tropical rivers: River Negro (pH 4.5), located in the Amazon State and River Iguape (pH 3.8), a river situated in an Environmental Protection Area in Southern Saint Paul State - Brazil. The water samples were collected at surface, acidified at pH 2 (6 mol L^{-1} HCl) directly after sampling, prefiltrated (0.45 µm cellulose membrane), and stored in the dark during the analysis and isolation procedures. Aquatic humic substances were isolated from 150 L of acidified water-samples using Amberlite XAD 8 resin. After elution by 0.1 mol L^{-1} NaOH (pH 13), the XAD concentrated organic dissolved matter (HS) was fractionated at pH 1.5 with 6 mol L^{-1} HCl and allowed to stand overnight. The supernatant was decanted and the resulting humic acid (HA) slurry was washed with 0.5 mol L^{-1} HCl and centrifuged (3 500 rpm, 15 minutes) to remove the remaining fulvic acid (FA). All fractions were treated with the strong cation-exchange resin and dialyzed using a dialysis tube of 1 000 MW cut-off to remove remaining HCl and salts. The external deionized water was replaced until the pH (pH 5) was constant and the Cl was undetectable. The dialyzed fractions were then freeze-dried and stored in a dark-brown container. The humic materials were designated HARI (humic acid river Iguape), FARI (fulvic acid river Iguape), HARN (humic acid river Negro). All humic materials were characterized with respect to elemental analysis and UV-absorbance.

ELISA method

General

Polystyrene microtiter plates were purchased from Nunc (Maxisorb, Roskilde, DK). Washing steps were carried out using a SLT 96PW microplate washer (SLT Labinstruments Ges mbH, Salzburg, Austria). Absorbances were read with a Multikskan Plus MK II microplate reader (Labsystems, Helsinki, Finland) at single wavelength mode at 450 nm. The inhibition curves were analyzed using a four parameter logistic equation (Genesys, Labsystems). All analysis was carried out using three-well triplicates, and experiments were repeated 3 or 4 times on different days.

Immunoassay performance

Microtiter plates were coated with As10 [7] in coating buffer (1/10 000, 100 µl/well) overnight at 4 °C covered with adhesive plate sealers. The following day the plates were washed with PBST (5 times, 300 µL/well). Atrazine standards (0.03 to 2000 nmol L^{-1}) or samples were added to the coated plates (50 µL/well). After 30 min. of incubation at room temperature, a solution of the enzyme tracer 2a-HRP (1/32000 in PBST, 50 µL/well) was added and the mixture incubated for six minutes. The plates were washed as described before and a solution of the substrate tetramethybenzidine (TMB 0.01%, H_20_2 0.004% in citrate buffer) was added (100 µL/well). The enzyme reaction was stopped after 30 min at room temperature with 4 mol L^{-1} H_2SO_4 (50 µL/well) and the absorbances were measured at 450 nm. All standard curves or samples were performed using three well replicates.

Matrix effect studies

Effect of contact time between atrazine and humic substances

HARI sample solutions (40 mg L^{-1} HARI in PBST, and pH 7.5) were spiked with the atrazine stock solution at 2.0 nmol L^{-1}. This concentration value was chosen inside the linear range of the atrazine standard curves. Atrazine-humic complexes were maintained at room temperature and in the dark for defined periods of time of 24, 48, 120 and 192 hours, before analysis was carried out by ELISA.

Effect of dissolved humic substances content

Pesticide-free humic and fulvic acids solutions (PBST, pH 7-8) at concentrations 2.5, 10, and 40 mg L^{-1} were mixed with stock solutions of atrazine (0.03 to 200 nmol L^{-1}) during 24 hours in the dark, and at room temperature.

pH effect

HARI or FARI (40 mg L^{-1}) and HARI:FARI (30:70, similar ratio than natural waters) was dissolved in PBST solutions at pHs 4 and 9, and spiked with atrazine to concentration values ranging from 0.03 to 200 nmol L^{-1}. A River Iguape water sample was also used at pHs 3.8 (natural) and 9 without prior clean-up procedures. The enzyme tracer (2a-HRP) was also prepared with PBST at such pHs values. The effect of pH was evaluated against calibration curve obtained for atrazine in PBST pH 7-8.

Results and discussion

Factors such as anions, cations, reaction time, pH and organic content are responsible for what is known as matrix effect and may interfere non-specifically with the immunochemical reaction. In general, these effects are manifested as a reduction of the color development [17]. Some pesticides can be adsorbed or covalently bound to the humic polymer leading to erroneous results. On the other hand, humic substances if present at high concentrations may exhibit non-specific binding properties in the ELISA [22]. In this study we have used the ELISA developed in our laboratory that has shown a good reproducibility, and the coefficient of variation the IC_{50} (concentration reducing the signal to 50% of the control) was less than 10%. Similarly we have used humic substances from Brazilian rivers. Table I shows the features of the isolated humic and fulvic acids. The composition is in agreement with other reported humic substances [23].

Effect of contact time between atrazine and humic substances

Initially we investigated how long the atrazine had to be in contact with the HS to arrive to an equilibrium. The results obtained showed that the atrazine concentration measurable by ELISA remained constant during the whole period studied. Figure 1 shows a decrease of the atrazine recovery values in humic substances containing solution acids if compared to the control sample. Nevertheless, after 24 h no significant variations were observed in the atrazine concentration values measured by ELISA. Therefore, all solutions were allowed to equilibrate a minimum of 24 h before use.

Effect of organic matter

Figure 2 shows the standard curves run under different concentrations of humic substances (HARN and FARI) likely to be found in natural waters. A slight overestimation of atrazine concentration takes place instead of the expected underestimation due to adsorption of the atrazine by the HA. This behavior is the result of the matrix effect caused by the high organic matter content (40 mg L^{-1}) and the immunoreaction process. This effect is particularly observed at low analyte concentration. However, this will be an extreme case when very low concentration of atrazine < 0.01 ppb and high content of humic substances of 40 mg L^{-1} are present. Simultaneously, up to 10 mg L^{-1} of humic substances, the underestimation is within the expected variability of ELISA when applied to real environmental samples.

Another aspect to be considered is the differences between HARN and FARI. Figure 2 shows that relatively high HARN concentrations (40 mg L^{-1}) produce a decrease of the absorbance values at low atrazine concentration. This effect is less evident for FARI, which shows weak influence on the immunoassay performance. The difference observed could be related to the size of the molecules of each humic material [23]. In this respect, the quotient E_2/E_3 (absorbance at 250 and 365 nm) is widely used, in limnology, as an indicator for the humification (decomposition of organic matter) and correlates with the molecular size and aromaticity of aquatic humic solutes [24]. The values from table I shows the E_2/E_3 values of humic materials, where HARI and HARN quotient values are lower than FARI. These findings

Table 1. Elemental analysis and absorbance values at 250 and 365 nm (*E2*/*E3*).

Humic Material	*% C*	*% H*	*% N*	*% O*	*E2/E3*
HARI	36.06	3.94	1.05	42.45	2.70
FARI	39.5	3.54	0.85	40.55	3.30
HARN	48.08	4.12	2.93	42.25	2.94

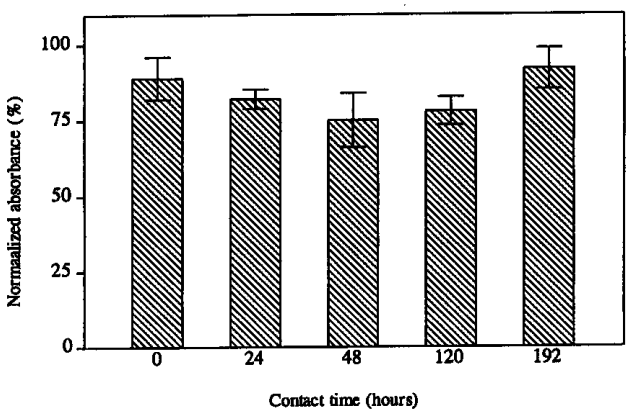

Fig. 1. Recovery of atrazine (2.0 nmol L^{-1}) in HARI sample solutions (40 mg L^{-1} HARI in PBST, and pH 7.5) during 190 hours of contact time. All analyses were carried out using three-well replicates.

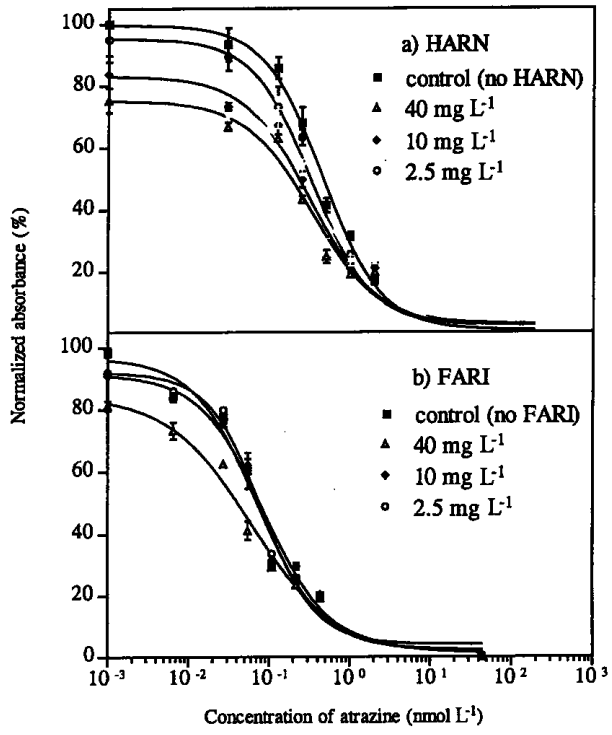

Fig. 2. Standard curves run with different amounts of humic substances (HARN and FARI) with the atrazine ELISA assay. Experiments were carried out at 40 mg L for HARN and FARI substances. Control (no FARI or no HARN) means standard curve in the absence of humic substances. A four-parameter logistic equation was used to fit the standard curves obtained.

suggest that the molecular size of fulvic acids are lower than those of humic acids [24,25].

Effect of pH

The effect of the pH is shown in figure 3. At low pH values a decrease of the signal was observed in the ELISA determination of atrazine. IC_{50} values of 0.24 nmol L^{-1} were observed for the standard atrazine curve and 0.68 nmol L^{-1} at pH 4 and 0.31 nmol L^{-1} at pH 7.5 and 9 for the FARI sample.

Figure 3 shows also that low concentrations of atrazine (0 from 0.074 nmol L^{-1}) and 40 mg L^{-1} of HARI produced a decrease in the absorbance values at pH 4. This effect decreases with increasing pesticide concentration. The values of IC_{50} are the same at pH 4 and 9 (0.45 nmol L^{-1}) and are close at pH 7.5 (0.34 nmol L^{-1}). In the absence of HS, the IC_{50} was 0.27 nmol L^{-1}.

In order to check the behavior of natural water samples, we prepared a standard curve using a solution containing HARI:FARI (30:70) and real water from River Iguape. Figure 4 shows the same behavior for both FARI:HARI solutions and real water sample at pH 4. The IC_{50} values of humic solutions and real water at pH 4 were 0.50 nmol L^{-1} indicating a strong effect in the atrazine measurement. For the control samples (no humic) the IC_{50} was 0.25 nmol L^{-1}.

In previous studies [18] we reported that below pH 4 no signal was obtained by immunoassay, and between pH 5 and pH 9 the immunoassay was more sensitive. By changing the pH the distribution of charged species in the antibody binding sites and the analyte may vary, thus changing the contribution of the electrostatic interactions in the stabilization of the immunocomplex. We should also consider that when pH approaches pKa of the analyte, more problems are expected in the ELISA determinations due to the partial ionization of the analyte. In this respect when lowering pH we are approaching the pKa of atrazine and so more problems may occur. This situation is complex in the presence of antibody, the affinity constant, K_{a1} (antibody-analyte) can be higher than K_{a2} (HA-analyte) and then the antibody can take up the analyte. Moreover, ELISA proteins (HRP; Ab) could also be participating in these interactions.

Additionally, it has been demonstrated that there is a close link between protonation of the carboxylic acid sites of humic acid and the binding of atrazine. At pH > 4, humic acids become anionic and could change conformation, where "open" conformation produces less interaction among the humic acids and the pesticide molecules. These results can be explained because at lower pH the charge on the polymers of the dissolved humic substances was reduced creating a more non-polar environment. Therefore, it is reasonable to think that a more hydrophobic form of the polymer would bind hydrophobic compounds more effectively through association with uncharged portions of humic polymer. The hydrophobic sites, valuable to the binding of nonionic pesticides such as atrazine, exist at pH around 4 and lower in humic materials but are destroyed by conformational changes associated with carboxyl deprotonation at higher pH values [27]. So, as a conclusion, lowering the pH of the ELISA determination may lead to erroneous results. In any case, most of the European natural waters exhibit a

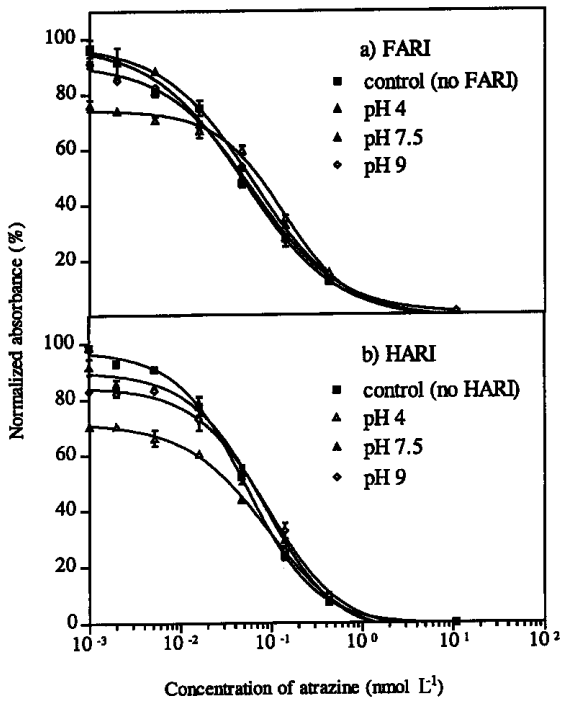

Fig. 3. Effect of pH in the performance of atrazine determination. Experiments were carried out at 40 mg L^{-1} for HARI and FARI. Control (no FARI or no HARI) means standard curve in the absence of humic substances. A four-parameter logistic equation was used to fit the standard curves obtained.

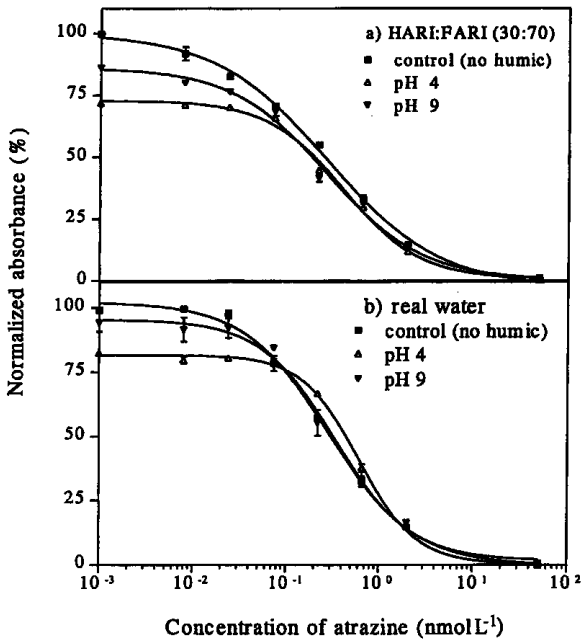

Fig. 4. Comparison of the behavior between a solution of HARI:FARI (40 mg L^{-1}) and a real water sample from River Iguape, at pH 4 and 9. The ratio FARI:HARI was chosen to be similar to natural waters 70:30. A four-parameter logistic equation was used to fit the standard curves obtained.

neutral to basic pH. Although tropical rivers do show often pHs under 6, this is not a problem since buffering the sample prior to the analysis can correct the matrix effect (see figures).

Conclusions

Humic substances constitute a problem, interfering with the analysis of pesticides in most of the analytical procedures. This is especially important with tropical water samples. Immunochemical techniques may provide a good alternative, because of the selectivity showed in front a variety of common interference. In the present paper, we have shown that the effects of humic substances in the atrazine assay is strongly connected to the pH and HS concentration of the solution. The humic substances exhibit non-specific binding if present in high amounts leading to an overestimation of the atrazine concentration. However, this effect is only evident at low atrazine concentration and low pH values. In fact, analyses could be carried out directly in samples containing HARI or FARI which had been adjusted with the pH in the range between 7.5 to 9. Nevertheless when HARI or FARI were present together in the sample we have observed a more pronounced effect on the ELISA, that is only corrected at base pH. Thus, behavior was again confirmed by using a real Brazilian river water sample.

In conclusion, the ELISA for atrazine used in this work could be a useful tool for monitoring this pesticide in tropical countries. The main advantage of this technique is that is easy to perform and could be used directly in the field. Additionally, only a little sample preparation, such as basifying the sample is enough to carry out the analyses. Also its low cost makes it easily available for most in laboratories involved environmental programs.

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