Development of an Enzyme-Linked Immunosorbent Assay for 3,5,6-Trichloro-2-pyridinol. 2. Assay Optimization and Application to Environmental Water Samples

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A monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for 3,5,6-trichloro-2-pyridinol (TCP), the major degradation product of chlorpyrifos, chlorpyrifos-methyl, and triclopyr pesticides, was optimized for the analysis of this hydrophilic compound in water. A direct ELISA format was chosen, and the concentration of immunoreagents was first selected to provide the highest sensitivity. Next, the influence of several physical (temperature, time) and chemical (pH, salt, detergent) conditions was studied. Under optimized conditions, the TCP concentration giving 50% reduction of the maximum ELISA signal (I_{50}) in the competitive standard curve was 0.62 nM (0.12 μg/L), and the assay was very specific for TCP. Preliminary evaluation of assay performance in water samples showed the absence of significant matrix effects for the waters tested, whenever the ionic strength of samples was approximately adjusted to that of standards. The assay provides a limit of detection of 0.04 μg/L and a working range of 0.09−0.91 μg/L TCP and allows for a precise and accurate determination of this pesticide degradation product in water at levels as low as 0.1 μg/L without sample cleanup.

Keywords: *3,5,6-Trichloro-2-pyridinol; TCP; chlorpyrifos; ELISA; optimization; environmental waters*

INTRODUCTION

There is a strict legislation on pesticide residues in waters (EC Council, 1980; WHO, 1993). As a consequence, pesticide residue monitoring programs are currently carried out by regulatory and/or control agencies. Less attention has been devoted to pesticide degradation products. Most of them are more hydrophilic than the parent pesticides, so they are not covered by conventional multiresidue methods used in routine monitoring, and their determination requires specific analytical techniques (Somasundaran and Coats, 1991; Barceló, 1993).

TCP (3,5,6-trichloro-2-pyridinol) is the major degradation product of both the widely used insecticides chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and chlorpyrifos-methyl [*O*,*O*-dimethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and of the herbicide triclopyr [[(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid] (Woodburn *et al.*, 1993; Racke, 1993). It is a weak acid, with a pK_a of 4.5, and therefore exists as an anionic compound in solution at pH higher than 5.5. Under neutral to basic conditions, TCP displays different physical and chemical properties as those of the chlorpyrifos insecticides, implying a different environmental fate (Racke, 1993). Photodegradation has been reported as an important dissipation process of TCP in aquatic environments (Dilling *et al.*, 1984). TCP has been demonstrated to be toxic to some aquatic organisms (*i.e.*, salmonids; Wan *et al.*, 1987) and bioactive against several fungal pathogens (Felsot and Pedersen, 1991).

Colorimetric analysis used for phenolic compounds (Rand *et al.*, 1976) and high-performance liquid chromatography determination as used in hydrolysis kinetic studies (Szeto, 1993; Wan *et al.*, 1993) lack the necessary sensitivity for analyzing TCP in environmental samples. The methodology developed for chlorinated acidic pesticide residues (AOAC, 1995) has been applied to determine TCP in water with high sensitivity (Cohen, 1990; Woodburn *et al.*, 1993). This methodology involves extraction, derivatization, and cleanup procedures followed by GC-ECD determination (Edgell *et al.*, 1993). In spite of the widespread use of the parent pesticides, very few data on TCP findings in water samples have been reported (Cohen, 1990). A practical reason for the scarce reports on its environmental monitoring may be the lack of appropriate analytical technology to carry out rapid, simple, and sensitive determinations. Immunochemical analysis has been proved to fulfill such analytical requirements, provided that appropriate antibodies are available. In this respect, highly specific and sensitive monoclonal antibodies (MAbs) have been obtained and used to develop several immunoassay systems to TCP, as reported in the preceding paper (Manclús and Montoya, 1996a). As a continuation of the immunochemical approach to analyze TCP, the objective of the present report was the optimization of the enzyme-linked immunosorbent assay (ELISA) system and the study of its applicability for the determination of TCP in water.

Immunochemical analysis of small molecules such as agrochemicals are based on analyte− and hapten−antibody interactions. These interactions can be affected by the concentrations and properties of the specific components of the assay system, as well as by nonspecific interferences caused by the physicochemical characteristics of the environment where the immunochemical interaction takes place (Hammock *et al.*, 1990; Sherry, 1992; Meulenberg *et al.*, 1995). All these factors can therefore influence the assay performance

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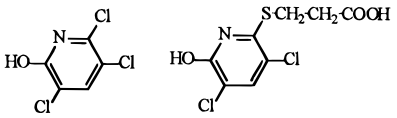

TCP Hapten 1

Figure 1. Structures of TCP and the hapten used in the assay.

in a given matrix, so they should be thoroughly examined to optimize the assay sensitivity and to overcome eventual interferences found in real samples. Herein, the influence of immunoreagent concentrations and of several physical (temperature, time) and chemical (pH, salt concentration, detergent) factors on the performance of the TCP ELISA was investigated. Next, the optimized assay was applied to several spiked water samples of different origins to study matrix effects and assay reliability.

MATERIALS AND METHODS

Reagents. TCP analytical standard was generously donated by DowElanco. The synthesis of hapten (**1**) (Figure 1), preparation of the HRP (horseradish peroxidase)-**1** enzyme tracer, and production of LIB-MC2 MAb are described in the preceding article (Manclús and Montoya, 1996a).

Preparation of Standard Solutions. A working stock solution of 400 μM TCP in *N*,*N*-dimethylformamide (anhydrous, 99.8%; Aldrich Química S.A., Madrid, Spain) was prepared, and from it an intermediate solution of 800 nM TCP in PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.5) was prepared. Standards from 800 nM to 10 pM TCP were then prepared by serial dilutions (factor of 5) in PBS. As the assay procedure involved the addition of the same volume of the appropriate HRP-**1** concentrations, standards were diluted by a factor of 2. Therefore TCP standards in the final assay ranged from 5 pM to 400 nM (1 ng/L to 79 μg/L).

TCP Immunoassay. The competitive direct ELISA with the antibody-coated (enzyme tracer) format used is as follows.

Procedure. Flat bottom polystyrene ELISA plates (Costar High Binding No. 3590, Cambridge, MA) were coated overnight at room temperature with 100 μL/well of the appropriate concentration of LIB-MC2 MAb (0.5 μg/mL in the optimized competitive immunoassay) in coating buffer (50 mM carbonate−bicarbonate buffer, pH 9.6). Plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20) using an ELISA plate washer (Dynatech model Ultrawash II, Sussex, U.K.). Then, 50 μL/well of standards or samples was added to antibody-coated plates followed by 50 μL/well of the appropriate HRP-**1** concentration (10 ng/mL in the final optimized ELISA) in assay buffer (PBST, PBS containing 0.05% Tween 20, as the optimized buffer). Plates were incubated for 1 h at room temperature. After washing, the HRP activity bound to the wells was measured by adding 100 μL/well of the substrate solution (2 mg/mL *o*-phenylenediamine and 0.012% H_2O_2 in 25 mM citrate, 62 mM sodium phosphate buffer, pH 5.4). After 10 min, the reaction was stopped by adding 100 μL/well of 2.5 M sulfuric acid, and absorbance was read in dual-wavelength mode (490 nm as test wavelength and 630 nm as reference wavelength) using an ELISA plate reader (Dynatech model MR-700, Sussex, U.K.).

Data Analysis. Standards and samples were run in triplicate wells, and the mean absorbance values were processed. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration. Using Sigmaplot software package (Jandel Scientific, Germany), sigmoidal competitive curves were fitted to a four-parameter logistic equation

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

where A is the asymptotic maximum (maximum absorbance in absence of analyte, A_{max}), B is the curve slope at the inflexion point, C is the x value at the inflexion point (corresponding to analyte concentration giving 50% inhibition of A_{max}, I_{50}), and D is the asymptotic minimum (background signal). Determination of spiked samples was performed by interpolating their mean absorbance values in the standard curve run in the same plate.

Table 1. Conductivity (μS/cm) of the Water Samples Used in This Study

water source	original sample	final assay solutions: nonconditioned[a]	final assay solutions: conditioned[b]
bottled	310	7930	14310
river	680	7980	14340
well	790	8050	14360
tap	1390	8260	14490
PBS (standards)	14240	14240	14240

[a] In the final ELISA wells, water was diluted 1:1 with PBS. [b] Water was conditioned by adding 1 vol of 10× concentrated PBS to 9 vol of water, before the final 1:1 dilution with PBS.

Sample Spiking and Conditioning. Water from different sources was used to evaluate potential matrix effects and to be spiked with TCP. Waters tested were Valencia tap water, a commercial bottled water, and samples obtained from a well located in the Tavernes de la Valldigna Valley (Valencia, Spain) and from stagnant water of a small river crossing the valley. All water samples showed a pH around neutrality. Only the river water was turbid and filtered prior to use. These water samples were spiked with several TCP concentrations covering the assay working range. Spiked water samples were conditioned, *i.e.*, buffered and roughly normalized for salt concentration, by adding 1 vol of 10-fold concentrated PBS to 9 vol of water. The conductivities of both the original water samples and the final assay solutions are shown in Table 1.

RESULTS AND DISCUSSION

To perform the immunochemical detection of TCP in water, both indirect and direct ELISA formats were considered. As described in the preceding paper, both configurations provided highly sensitive immunoassays for TCP, but the direct one requiring fewer steps was preferred. For this format, the combination of LIB-MC2 MAb and the homologous hapten (**1**) (Figure 1) conjugated to HRP as the enzyme tracer afforded the most sensitive ELISA to TCP, and hence these immunoreagents were selected as specific components of the assay system.

Optimization of the Immunoreagent Concentrations. Concentrations of LIB-MC2 MAb and HRP-**1** were optimized to provide the inhibition curve with the highest affinity (lowest I_{50}), giving adequate maximum absorbance in the 1.0−1.5 range in absence of analyte. First, saturating conditions were determined by a noncompetitive two-dimensional titration covering a wide range of concentrations. Next, under subsaturating conditions, concentrations in a narrow range were combined and examined by competitive assays. In all cases, the minimum absorbance (background signal) obtained with excess of analyte was approximately zero. The parameters of the competitive inhibition curves for each combination tested are shown in Table 2. Even under subsaturating conditions, significant variations of the curve parameters were found. Thus, an increase of coating MAb concentration from 0.5 to 1.0 μg/mL, in combination with the tracer concentration giving appropriate absorbance, produced a variation of I_{50} from 0.58 to 1.40 nM TCP. Coating with MAb in the 0.35−0.50 μg/mL range, and using the corresponding enzyme tracer concentration in the range 10−22 ng/mL, provided the best combinations of immunoreagent concentrations to perform the competitive assay, as indicated

Table 2. Characteristics of TCP Competitive Curves Obtained at Different Subsaturating Concentrations of the Specific Immunoreagents[a]

[LIB-MC2] (μg/mL)	[HRP-**1**] (ng/mL)	parameters[b]		
		A_{max}	slope	I_{50} (nM)
0.25	100	0.99	0.69	0.85
0.35	22	1.22	0.94	0.60
0.40	20	1.48	1.02	0.61
0.50	10	1.28	1.08	0.58
0.65	7	1.28	1.10	0.76
0.80	5	1.40	1.16	1.04
1.00	4	1.38	1.15	1.40

[a] For the competition step, standards in PBS and enzyme tracer in PBST were incubated for 1 h at room temperature. [b] Data obtained from the four-parameter sigmoidal fitting, average of four replicates.

by the lowest I_{50} for TCP (around 0.6 nM), adequate slope (0.9−1.1), and maximum signal (1.0−1.5). For subsequent experiments, ELISA plates were coated with LIB-MC2 MAb at 0.5 μg/mL, and the HRP-**1** enzyme tracer was used at 10 ng/mL. However, if required, sensitive assays with a wider working range, *i.e.*, flatter slope, could be attained simply by using lower MAb (0.25 μg/mL) and higher tracer (100 ng/mL) concentrations.

Influence of Physical and Chemical Factors on the Assay Characteristics. Once the optimum concentrations of the specific components of the assay system were selected, the influence of several nonspecific parameters on assay characteristics was examined.

Temperature. As a physical parameter that accelerates biological interactions, temperature is often used to reduce the incubation time of immunoassays. Nevertheless, it is also recognized as an important source of well-to-well variability on quantitative ELISA, especially by the well-known edge effects caused by a not uniform warming of ELISA plates (Tijssen, 1985). As expected, by performing the competition step of the TCP immunoassay at 37 °C, the incubation time could be shortened while maintaining similar assay sensitivity. However, in these conditions the intra-assay coefficients of variation (CV) increased significantly in comparison with those obtained at room temperature. Moreover, this has frequently been found in routine ELISAs performed at different temperatures in the authors' laboratory, while Jung *et al.* (1989) reported that enhanced reproducibility was obtained by maintaining constant temperature throughout the assay procedure. Therefore, to avoid undesirable effects of temperature changes on assay precision, all incubations were carried out and all assay buffers and solutions were kept at a constant room temperature (20−22 °C).

Time Effects. Time is a variable that can affect several aspects of ELISA. First, the influence of the incubation time of the competition step was investigated. To this purpose, standard curves at different incubation times, from 15 min to 2 h, were performed maintaining a constant antibody coating concentration and using the appropriate enzyme tracer concentration to give maximum absorbance in the range 1.0−1.5. As shown in Table 3, very similar curve parameters were estimated for the conditions tested. Therefore, the assay time can be shortened provided that signals are maintained at a similar level by increasing the tracer concentration. For the purpose of this study, 1 h appeared to be an appropriate incubation time for the competition step using the enzyme tracer at 10 ng/mL.

Another important time effect examined was the dispensation timing. In the direct format, the usual practice is the pipetting of standards and samples into antibody-coated wells followed by the addition of the enzyme tracer. Since the interaction starts at the moment of adding analyte solutions to ELISA wells, it has been suggested that a time-dependent drift in the results may occur as a consequence of different incubation times of analyte solutions (Jung *et al.*, 1989). To study the relevance of this phenomenon in the TCP immunoassay, competitive curves were prepared by dispensing standards into the wells and incubating them for 0, 5, 10, 20, and 30 min prior to the addition of the enzyme tracer. The results showed no significant differences among the characteristics of the competitive curves, indicating the absence of assay drift caused by dispensation timing and suggesting that previous interaction between antibody and analyte has negligible influence on the really important competition step.

Table 3. Influence of the Incubation Time of the Competition Step on the Characteristics of TCP Competitive Curves[a]

time (min)	[HRP-**1**] (ng/mL)	parameter[b]		
		A_{max}	slope	I_{50} (nM)
120	7	1.30	1.2	0.65
60	10	1.24	1.14	0.62
30	13	1.24	1.14	0.64
15	20	1.09	1.17	0.53

[a] Plates were coated with LIB-MC2 MAb (0.5 μg/mL) followed by the competition step between standards in PBS and enzyme tracer in PBST, at room temperature. [b] Data obtained from the four-parameter sigmoidal fitting, average of four replicates.

pH. To examine the influence of pH in the assay system, competitive curves for TCP were obtained using PBS (to prepare standards) and PBST (to dilute the tracer conjugate) at different pHs from 4.5 to 10.5. The conductivity of the different solutions did not vary appreciably as a consequence of pH adjustment. The maximum absorbance, representing the MAb recognition of the enzyme tracer in absence of analyte, and the I_{50} values for TCP, representing the MAb affinity to the analyte, were estimated from the curves obtained at each pH. The variation of these parameters as a function of pH is depicted in Figure 2. Consistent with the ionizable nature of TCP, the influence of pH on the LIB-MC2 affinity to TCP and enzyme tracer was very notorious. MAb recognition of the tracer conjugate (A_{max}) was maximum at neutrality and diminished progressively as pH decreased or increased. Concerning the influence of pH on MAb affinity to TCP, little variations were found at acidic pH, while at basic pH the assay clearly lost sensitivity (increased I_{50}) as pH increased. As the chemical properties of TCP remain invariable in the pH range 5.5−10.5, the effects observed must be related to conformational changes of the macromolecules (MAb and enzyme tracer) participating in the interaction. Similar important pH effects on immunoassays for polar compounds, such as nitrophenol derivatives, have been reported (Li *et al.*, 1991). The highest affinity assay for TCP was found in the pH range 4.5−7.5, where similar I_{50} values were obtained. Within this range, pH 7.5, showing the highest A_{max}/I_{50} ratio, was selected as the optimum pH to carry out the immunochemical determination.

Salt Concentration. The effect of the ionic strength of the assay buffer on the standard curve characteristics was also examined. A simple approach followed to carry out this study was the use of assay buffers prepared by diluting 10-fold concentrated PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.5), to cover a

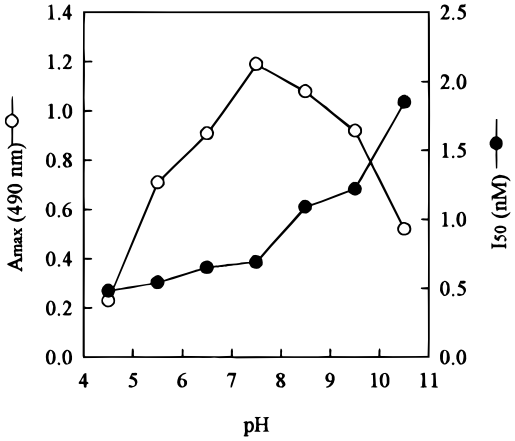

Figure 2. Influence of the pH of the assay buffer on the analytical characteristics of TCP competitive standard curve: (○) absorbance in the absence of TCP (A_{max}) and (●) value of I_{50} for TCP. The assay conditions were those previously selected as optimum for antibody coating (0.5 μg/mL), tracer concentration (10 ng/mL), and incubation time of the competition step (1 h). Standards and tracer were prepared in PBS and PBST, respectively, at the appropriate pH. Each point represents the average of four replicates.

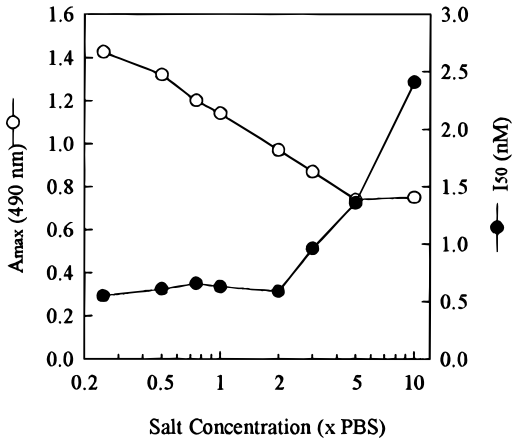

Figure 3. Influence of the salt concentration of the assay buffer on the analytical characteristics of TCP competitive standard curve: (○) absorbance in the absence of TCP (A_{max}) and (●) value of I_{50} for TCP. The assay conditions were those previously selected as optimum for antibody coating (0.5 μg/mL), tracer concentration (10 ng/mL), incubation time of the competition step (1 h), and pH (7.5). The assay solutions were prepared by dilution of 10-fold concentrated PBS, and salt concentration is represented as relative to that of PBS. Each point represents the average of four replicates.

wide range of salt concentrations. As for the pH study, the parameters of each competitive curve were estimated and plotted, in this case as a function of salt concentration. Results shown in Figure 3 indicate that the MAb ability to recognize the tracer (A_{max}) decreased gradually as buffer salt concentration increased. Concerning the MAb affinity to the analyte (I_{50}), similar values were estimated in the range 0.25×−2× PBS, while at higher salt concentrations the assay progressively lost the ability to properly recognize TCP. This behavior could be explained by considering the anionic nature of TCP at neutral pH and taking into account the strong participation of the negative charge of the pyridinolate ion in the antibody−analyte interaction, as discussed in the preceding paper (Manclús and Montoya, 1996a). Biochemical interactions with a predominant hydrophobic component are generally favored by increasing the ionic strength of the reaction medium, while this increase exerts a detrimental effect on interactions where ionic driving forces prevail (Jefferis and Deverill, 1991). Therefore, the interaction between LIB-MC2 and TCP, which has an important electrostatic component, could be adversely affected by high salt concentrations in the assay buffer. In the study of the 4-nitrophenol ELISA carried out by Li *et al.* (1991), the antibody affinity for this polar analyte was improved by increasing the buffer concentration. In this case, however, the participation of electrostatic bonds on the antibody−analyte interaction was not so evident. On the contrary, ELISAs developed for relatively nonpolar analytes as carbaryl and chlorpyrifos behave as expected for antibody−analyte interactions where hydrophobic bonds are considered important, *i.e.*, interactions favored by increasing the ionic strength (Abad and Montoya, 1996; Manclús and Montoya, 1996b).

Apart from the influence of the ionic strength on the maximum signal, a practical aspect was also considered to select the optimum buffer salt concentration within the range 0.25×−2× PBS. Due to the different origins and composition of water samples for which the TCP ELISA is intended to be applied, an assay buffer concentrated enough to bring the ionic strength of any water sample to that of standards was desirable. Therefore, although 0.25× PBS gave the highest A_{max}, 1× PBS, maintaining a good signal but providing a higher capacity of sample conditioning, was preferred as the assay buffer.

Detergent (Tween 20). Tween 20 is a nonionic detergent commonly used in ELISA to reduce nonspecific interactions. The usual concentration of Tween 20 used for most pesticide immunoassays is 0.05%; however, for several pesticides there is a marked effect of detergent concentration on ELISA performance (Stanker *et al.*, 1989; Lee *et al.*, 1995). Therefore, the influence of Tween 20 concentration on the analytical characteristics of the TCP immunoassay was examined. When competitive curves were prepared in buffers containing Tween 20 in the range 0.5−0.001%, no significant variations of assay characteristics were obtained. In these circumstances, the usual 0.05% Tween 20 concentration in the assay buffer was maintained. It has been reported that higher affinities can be achieved by decreasing or even omitting the detergent in the competition buffer in immunoassays to permethrin (Stanker *et al.*, 1989) and endosulfan (Lee *et al.*, 1995). This effect has been also found for immunoassays to carbaryl (Abad and Montoya, 1996) and chlorpyrifos (Manclús and Montoya, 1996b). The negative influence of Tween 20 in these cases may be related with nonspecific hydrophobic interactions between the detergent and nonpolar small organic molecules in an aqueous environment, interfering thereby with the specific analyte−antibody interaction. These nonspecific interactions are unlikely to occur with a highly polar compound such as TCP, which could explain the absence of a Tween 20 effect on the TCP immunoassay.

Evaluation of Matrix Effects. When analyzing field water samples, the antigen−antibody biochemical interaction can be affected by a variety of compounds, mainly inorganic salts, which can modify the assay characteristics. To evaluate these matrix effects, standards were prepared in several water samples collected from different sources and used to obtain competitive curves for TCP. As shown in Figure 4A, the family of curves obtained in water samples shifted to higher maximum absorbances than the PBS control curve. This behavior correlated with the measured conductivities

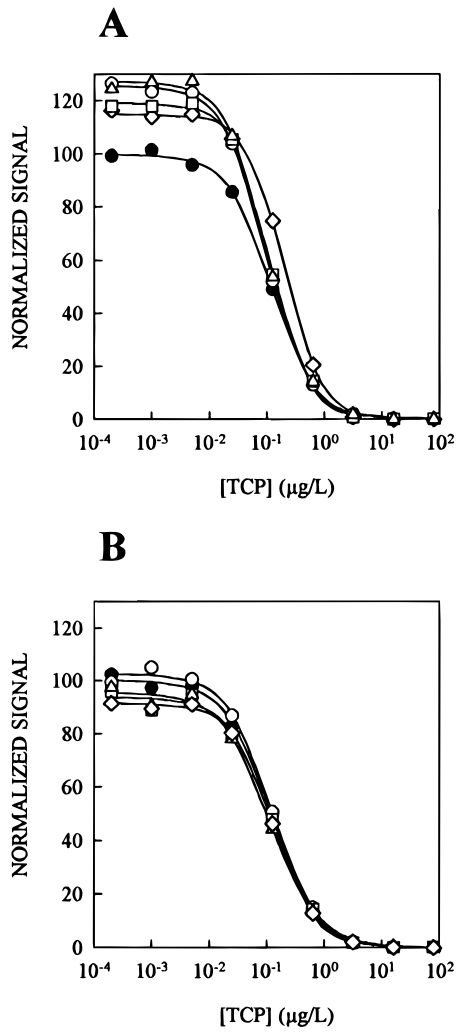

Figure 4. Comparison of TCP competitive curves obtained from standards prepared in nonconditioned (A) and conditioned (B) water from different sources: (○) bottled, (□) river, (△) well, (◇) tap, and (●) PBS. The assay conditions were those previously selected as optimum for antibody coating (0.5 μg/mL), tracer concentration (10 ng/mL), incubation time of the competition step (1 h), pH (7.5), and ionic strength (1× PBS). Each point represents the normalized signal with respect to the control curve in PBS, for the average of four replicates.

of the nonconditioned water samples, which were similar to each other but clearly lower than the conductivity of PBS (Table 1). Moreover, this increase in A_{max} may be directly related to a lower concentration of ionic species in the water tested, according to the influence of the ionic strength on the maximum absorbance shown in Figure 3. Therefore, to avoid these adverse effects, the ionic strength of water samples in the final ELISA wells should be adjusted as much as possible to that of PBS. With this aim, a simple operation to normalize samples was followed, consisting of the addition of 1 vol of 10× concentrated PBS to 9 vol of water sample, as reported by Gee *et al.* (1988) and Van Emon and Gerlach (1992). After this operation, the conductivity of all conditioned water samples was nearly the same as that of PBS (Table 1).

Standard curves for TCP were subsequently obtained using conditioned water samples and compared with the one obtained using PBS. As shown in Figure 4B, only minor differences were found, which can be attributed to interplate variability among standard curves. Therefore, sample conditioning minimized the effect on the TCP immunoassay caused by differences in the ionic composition of waters. Furthermore, the assay system evaluated can be considered robust enough to perform direct TCP determinations in the water samples tested, as other matrix effects were not observed.

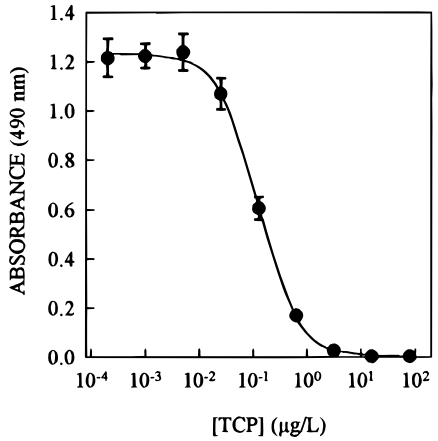

Figure 5. Representative standard curve for TCP obtained under the optimized assay conditions: immunoreagents, LIB-MC2 MAb (0.5 μg/mL) and HRP-**1** (10 ng/mL); assay buffer, 1× PBS containing 0.05% Tween 20, pH 7.5; competition step, 1 h at room temperature. Each point represents the mean ± SD for three replicates.

Analytical Parameters of the Optimized TCP Immunoassay. Assay analytical parameters were estimated from standard curves run in triplicate. A representative standard curve for TCP using the optimized ELISA system is shown in Figure 5. The assay affinity for TCP, represented by the I_{50} value, was 0.62 nM (0.12 μg/L). The specificity of LIB-MC2 MAb had been earlier evaluated in the indirect ELISA format (Manclús and Montoya, 1996a), indicating weak recognition of TCP-related compounds. When cross-reactivity was reexamined in the optimized direct format, the results confirmed that the assay was highly specific for TCP.

There is not a general agreement for the calculation of assay sensitivity and working range of competitive immunoassays (Sherry, 1992). For this assay, the limit of detection (LOD), calculated both as the concentration corresponding to the zero dose absorbance minus two standard deviations and as the concentration giving 10% inhibition, was almost the same, *i.e.*, 0.02 μg/L. The quantitative working range was established between the concentrations producing 20% and 80% inhibition, *i.e.*, 0.04−0.41 μg/L. Taking into account the dilution factors used, the practical analytical parameters can be calculated by multiplying by a 2.22 factor, *i.e.*, a 1.11 factor for the conditioning dilution and a 2 factor for the dilution in the competition step. Thus, the assay possesses a practical LOD of 0.04 μg/L and a working range of 0.09−0.91 μg/L in real water samples.

Analysis of Spiked Water Samples. Spiking matrix samples with several amounts of analyte is a common practice to perform a preliminary evaluation of analytical assay reliability. Accordingly, water samples from different sources were spiked at several concentrations of TCP covering the established working range (100, 200, 400, and 800 ng/L). Spiked samples were conditioned as described and analyzed without further treatment. Results of the analysis, expressed as the percentage of recovery, are shown in Table 4. The comparison between the amount of TCP estimated by ELISA and the amount added indicates good agreement for all waters tested, with mean recovery values ranging

Table 4. Recovery of TCP from Spiked Water Samples

	TCP recovered[a]							
	bottled water		well water		river water		tap water	
TCP added (ng/L)	mean ± SD[b] (ng/L)	recovery (%)	mean ± SD[b] (ng/L)	recovery (%)	mean ± SD[b] (ng/L)	recovery (%)	mean ± SD[b] (ng/L)	recovery (%)
100	122 ± 10	122	105 ± 20	105	100 ± 19	100	119 ± 7	119
200	185 ± 21	92	169 ± 10	84	245 ± 9	122	198 ± 10	99
400	383 ± 28	96	430 ± 16	107	375 ± 19	94	387 ± 16	97
800	809 ± 31	101	742 ± 35	93	762 ± 57	95	887 ± 42	111
mean		103		97		103		107

[a] Spiked water samples were conditioned for pH and ionic strength by adding 1 vol of 10× concentrated PBS to 9 vol of water and analyzed without further treatment. [b] Data obtained from five determinations performed in the same ELISA plate.

from 97% to 107%. The average intra-assay CV was 7.5%, ranging from 3.7% to 19%. These results confirmed the suitability of this immunoassay for the direct, accurate, and precise determination of TCP in environmental water samples.

CONCLUSIONS

A MAb with high sensitivity and specificity to TCP, as previously described (Manclús and Montoya, 1996a), was used in a direct competitive immunoassay format to determine TCP in water. First, the ELISA conditions were optimized to achieve the best assay performance. The highest sensitivity was afforded by a thorough selection of the immunoreagent concentrations. The influence of some physicochemical factors on the characteristics of the TCP ELISA is consistent with the polarity of the analyte, which determines the importance of electrostatic bonds in the analyte−antibody interaction. The optimization of these factors did not result in any significant sensitivity improvement.

To analyze polar compounds such as TCP by immunochemical techniques, special attention should be devoted to the pH and ionic strength of the final assay solutions (standards and samples), as these factors can markedly influence assay performance. These effects may be overcome with relative ease by adjusting pH and ionic strength of samples to values as close as possible to those of standards. In this study, the adjustment was successfully attained by a minimum dilution in a buffer with the appropriate ionic strength. In these conditions, preliminary evaluation of the assay reliability on spiked water samples from different sources showed the robustness of the ELISA to carry out precise and accurate determinations of TCP.

While an extensive sample preparation is required for an adequate GC determination of TCP in water (Edgell *et al.*, 1993), the highly sensitive TCP ELISA described herein has been proved not to require sample cleanup for the water samples tested. This offers considerable practical advantages in simplicity and rapidity over existing chromatographic techniques and allows this ELISA to be considered as a promising analytical alternative for the quantitative measurement of TCP in water. In this respect, LIB-MC2 MAb has already reached a practical application, having been recently incorporated into a commercial kit for TCP determination in environmental samples.

ABBREVIATIONS USED

CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; GC-ECD, gas chromatography-electron capture detector; HRP, horseradish peroxidase; MAb, monoclonal antibody; I_{50}, concentration giving 50% inhibition of maximum response; PBS, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.5; PBST, PBS containing 0.05% Tween 20; TCP, 3,5,6-trichloro-2-pyridinol.

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