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Sensitive chemiluminescent immunoassay of triclopyr by digital image analysis

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ABSTRACT

An image based detection of chemiluminescence enzyme-linked immunosorbent assay (CL-ELISA) for the quantification of triclopyr has been developed. The immunoassay was an indirect competitive immunoassay with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP). Chemiluminescence was produced by the luminol/H_2O_2/HRP reaction, detected by a monochrome video CCD camera and digitized with an Imagraph IC-PCI frame grabber using a custom program developed in C^{++} (Microsoft Visual C^{++} 6.0). Two main improvements are reported in the data processing software: the implementation of a circular mesh covering the perimeter of each well, eliminating diffuse light from the neighboring wells, and the use of volume (the integration of light intensity of all pixels that define a well) as an analytical signal instead of CL intensity or area (as usual in commercial plate readers) to improve precision for normalization of the total light output. The standard curve was produced for 0.01–10 ng/L triclopyr. The limit of detection was 0.8 ng/L and the variation coefficient was 3.07% (n=10, P=0.05).

1. Introduction

Triclopyr is a selective post-emergency herbicide, widely used for weed control in cereals and other crops, it is extensively used in wheat cultivation and rice, mixed with 2,4-dichlorophenoxy acetic acid (2,4-D). The herbicide triclopyr is of low acute toxicity in mammals, it raises environmental concerns due to its effects on aquatic organisms and other adverse impacts. The heavy use of this herbicide leads to contaminated sites, especially water environments.

Both EU drinking water (DWD) (98/83/EC) [1] and water framework directives (WFD) (2000/60/EC) [2] establish low limits of permissible pesticide residues as well as encourage the development of analytical methods showing lower Detection Limit levels. Current analytical methods require a previous step to concentrate the samples in order to reach the constituent's concentration above the detection limit level of the method. Significant methods used for determination of triclopyr include gas chromatography equipped with electron capture detector [3] and immunoassays [4]. Immunoassay methods show high sensitivity and selectivity, characteristics that are pivotal when analyzing traces. In addition, the new method is low cost, avoids the previous step of concentration that is usual in other methods and allows the analysis of several samples simultaneously. Among the immunoassay methods, enzyme-linked-immunosorbent technique (ELISA), is one of the most versatile analytical tools for biosensing, which employs a label enzyme and a substrate to produce an amplified signal.

Among CL reactions, the most widely used system is the luminol-H_2O_2-horseradish peroxidase, frequently associated to a phenol or derivative compound acting as enhancer of the CL reaction [5].

Digital image analysis is appropriate when examining chemical processes with very weak light signals. Digital image analysis comprises two steps: the acquisition and the analysis of a digital image. The acquisition step delivers a matrix X, with n files and p rows, each element $x(i,j)$ is called a pixel. Cameras equipped with charge-coupled devices (CCD), are highly sensitive and can detect over 80% of incidents photons. They use a semiconductor sensor that converts incident photons in an electric signal, which is digitized by a frame grabber or digital port using sampling and quantification. The analysis process allows the quantification of relevant characteristics of the image with an objective interpretation. Digital images can be processed in a computer to reduce noise, to remove irrelevant parts, to increase quality and to quantify signal values.

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Image based detection analytical methods have been used in different applications: gel electrophoresis [6,7], thin layer chromatography [8,9], immunoassay on well plate [10,11] or DNA sequencing blots [12] among others, being especially useful in [13] screening assays.

In this work, we focus on the chemiluminescence enzyme-linked immunsorbent assay (CL-ELISA) with an anti-rabbit secondary antibody conjugated to peroxidase (goat anti-rabbit-IgG-HRP) of triclopyr by digital image analysis to detect lower concentrations. The format of the immunoassay was an indirect competitive immunoassay. Two interesting improvements were introduced in the data processing: the implementation of a circular mesh covering the perimeter of each well that allow us to eliminate the diffuse light appearing out the border of the wells, improving signals and avoiding contamination between wells, and the use of a virtual volume measurement (the integration of light intensity of all pixels that define a well) instead maximum CL intensity or area as analytical signal to improve precision because normalize the total light output.

2. Material and methods

2.1. Chemicals, materials and instrumentation

Triclopyr: [(3,5,6-tricloro-2 pyridinyl)oxy]acetic acid, triclopyr-2-butoxyethyl ester and (±)-2-(2,4-dichlorophenoxy)propanoic acid (dichlorprop) were provided by Dr. Ehrenstoffer Laboratories (Augsburg, Germany), 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) were from Riedel-de-Haen (Seelze, Germany). PIP (p-iodophenol), DCC (N,N′-dicyclohexylcarbodiimide), HGG (human γ globulin), NHS (N-hydroxysuccinimide) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were from Sigma-Aldrich, Spain. All other chemicals used were from Merck (Darmstadt, Germany). Goat anti-rabbit-IgG HRP conjugates were provided by Amersham International (Amdex conjugates). The protein (peroxidase) concentration is approximately 1 mg/mL. Antisera of immunized rabbits were obtained previously, the stages of preparing the immunogens and obtaining antisera against triclopyr are described in detail elsewhere [4].

Stock standards of triclopyr (5000 and 1000 μg/mL) were prepared in acetone (solubility: 782 g/L), 2,4,5-T, 2,4-D and triclopyr-2-butoxyethyl ester (all 10000 μg/L) were prepared in ethanol and stored in the dark at 4 °C. Buffer solutions were prepared from sodium phosphate (0.1 M) pH 7.5 supplemented with 0.1% Tween 20 (washing solution) and with 0.05% Tween 20 (dilution solution), carbonate (50 mM) pH 9.6 (coating buffer) and Tris-HCl (0.1 M) pH 8.5 (chemiluminescent substrate solution). Polystyrene microtiter plate of 96-wells "FluoroNunc™MaxiSorp™" were provided by Nunc.

2.2. Apparatus

Chemiluminescence images of the microtiter plate were acquired in the dark, with a National Electronics, Inc. model T-390 C monochrome video CCD camera [12 mm F/1.2 lens, 0.01 lx] and digitized with an Imagraph IC-PCI frame grabber using a custom program developed in C++ (Microsoft Visual C++ 6.0). We acquire a pack of 256 integer images 30 s after the beginning of the CL reaction and we go on acquiring such images until the chemiluminescence signal practically disappears (about 30 min). Quantitative data are provided for the detected wells on the image such as maximum intensity, area and volume of each well in the microtiter. The volume of each well is a measurement of the total light detected from each well. This parameter has been used for standard curve representation.

2.3. Standard curves

Aliquots of triclopyr covering the range 0.0001–10000 ng/mL were preincubates with antiserum 1:1000 for 1 h at 4 °C, and then incubates for 15 min with previously coated HGG-triclopyr. Free triclopyr was then quantified by its competition with the coated triclopyr for binding to anti-triclopyr (antibody). In the sandwich antibody assay, the antibody bound to the triclopyr-HGG is detected by goat anti-rabbit-IgG HRP which reacts with the CL substrate. In this method, HRP is conjugated to a secondary antibody and its concentration on the wells is inversely proportional to triclopyr concentration. A plot of CL reading V/V_0 (V is the volume: the integration of the light intensity of all pixels in each well, and V_0 is the volume for the well containing 0 ng/mL of triclopyr) against concentration (ng/mL) of the standard triclopyr in logarithmic scale. As can be seen, a very large dynamic range (about seven order of magnitude of analyte concentration) is a non-frequent characteristic of CL calibrations. Probably this fact can be related to the shortness of the first incubation step (15 min at 4 °C).

2.4. Coating of solid surfaces with HGG-triclopyr conjugate

Triclopyr was conjugated to HGG according to a procedure described by Fleeker [14]. 100 μmol of DCC (0.0206 g), 100 μmol triclopyr (0.0256 g) and 200 μmol of NHS (0.023 g) were dissolved in 1 mL of dimethyl formamide and agitated for 1 h at room temperature. The solution was filtered through 0.2 μm nylon filters to remove the precipitate of dicyclohexylurea. The intermediate triclopyr dihydrosuccinimide ester (25 μmol) was added to 0.25 μmol of HGG (0.0375 g) in 2 mL of 0.13 M $NaHCO_3$. The solution was left to stand overnight at 12 °C. The resulting solution was dialysed against eight changes of water at 4 °C. To estimate the amount of triclopyr bound to protein, the number of free amino groups in the conjugate was determined by 2,4,6-trinitrobenzenesulfonic acid (TNBS) titration [15]. The number of amino groups substituted by the hapten in each carrier molecule (HGG) was 3. For coating titer, Cantarero recommended adding about 500 ng of protein per 6.5 cm^2 of polystyrene, which appears to give a monolayer of protein [16].

2.5. ELISA procedure

The immunoassay was an indirect competitive immunoassay ELISA. Polystyrene microtiter plates were coated by adding, to each well, 200 μl of HGG-triclopyr (at 3 μg/mL) dissolved in 50 mM carbonate buffer at pH 9.6 and incubated for 24 h at 4 °C. The plates were emptied and washed three times with washing solution. Diluted antiserum (1:1000) in phosphate buffer, pH 7.5, supplemented with 0.05% of Tween 20 was preincubated for 1 h with triclopyr at concentrations covering the range 10000–10^{-4} ng/mL (this is the sufficient time for free antigen (triclopyr) antibody interaction, this is to say that the specific recognition of the free antigen is done by the antibody); 200 μL of these preincubated mixtures was transferred to the wells of the microtiter plate and incubated for 15 min at 4 °C (this is the most favorable time for competition between free antigen and immobilized antigen (HGG-triclopyr) for antibody binding sites). One column of the plate received no triclopyr and no antiserum, to determine non-specific binding of the secondary antibody-labeled horseradish peroxidase enzyme in the following step. Another column received no triclopyr to determine the maximum chemiluminescence reading. After 15 min the plates were emptied and

washed as described above. The immobilized antibodies were detected by labeled anti-Ig antibodies: goat anti-rabbit-horseradish peroxidase conjugate (1:160) was added (200 μl well) to the plates. The plates were incubated for 1 h at 4 °C with the secondary antibody, emptied and washed as before.

2.6. Chemiluminescence assay

The CL system used consist of the chemiluminescent reagent luminol (82.9 μL, 0.01 M), H_2O_2 (165.9 μL, 0.1 M) as oxidant, *p*-iodophenol (107.7 μL, 0.01 M) as chemiluminescence enhancer, Tris-HCl buffer (197.6 μL 0.1 M pH 8.5) because the maximum enhancement is about pH 8.5, and the enzyme HRP as a catalyzer (HRP is conjugated to a secondary antibody and its concentration in each well is inversely proportional to the amount of free antigen, triclopyr, in the sample). The reaction with the HRP bound to secondary antibody begins when 200 μL of CL mixture were transferred to each well. Within limits, the emitted CL intensity is directly proportional to the concentrations of luminol, H_2O_2, and catalyst (HRP). Thus, measurements of the CL signal can be used to quantify the amount of secondary antibody bound to peroxidase (goat anti-rabbit-IgG-HRP) bound to the bottom of each well which is inversely related to the free antigen or analyte.

2.7. Image analysis

An analog CL video sequence of the microtiter plate was acquired in a dark closure, and digitized with an IC-PCI 10 bit/pixel and $1024=2^{10}$ gray levels frame grabber using a custom program developed with Microsoft Visual C^{++} 6.0. The dark closure avoids light contamination from ambient illumination, reducing acquisition noise and the custom software performs integration or averaging of the video sequence signals to capture extremely low signals. A video camera, instead of a photo camera, is used because weak light signals are very sensitive to background noise (the main problem here) and a very high sensitive camera is also very expensive. In this way, it can exploit the dynamic nature of the signal and, as an additional advantage (not exploited here), it can study the dynamics of the CL process. Frame grabber is a method for providing short-term storage in the computer memory, which store one or more images and can be accessed rapidly, usually at video rates [17]. Digital images were processed with Matlab v 5.3 to obtain quantitative results. The overall imaging algorithm used can be splitted in several steps:

a. Creation of a model image of the wells, according with microtiter geometry.
b. Wells pattern recognition algorithm that comprises:
   i. Conversion to pixels of dimensions of the plate.
   ii. Extraction of 2D periodicity in the image (Δx,Δy) using signal information.
   iii. Estimation of the well radius.
   iv. Computation of the covariance function between model and real images to determine the true position of each well within the image.
   v. Definition of a mask for each well to reduce interference between adjacent wells.
c. Determination of background noise light signal.
d. Quantification of light signal for each well.

The use of an image model that is matched to a microtiter is a key feature of the proposed method to obtain high sensitivity because it reduces the contribution of light from the neighboring wells. This is especially important in the vicinity of high illuminated wells, where diffuse light can be accounted by the next well. Fig. 1 depicts the images evolution after processing. Fig. 1A shows the digital image obtained without pattern recognition. As it can be seen, very low light emission was obtained. Fig. 1B shows the normalized image with adjusted gray levels. In Fig. 1C is shown a detected circular mesh over the wells to delimit the area of measurement. Fig. 1D is a false color of the same wells plate, used to improve subjective interpretation. It also can be shown that some light signal is captured from unused wells in the microtiter because of the limitation of quantum noise of the video camera. A mean value of this signal is computed to determine the detection limit of the adquisition, defining a minimum signal to noise ratio, and to calibrate the used wells light measurements. Image data from the detected wells on the image can be processed to extract quantitative features, such as maximum intensity, defined as the maximum light signal in a particular well, area or volume of each well. The area is the spatial integration of the light signal detected in each well, obtained with a single image in the video sequence, whereas the volume is the time integration of the area feature. Volume is used as a feature to construct the standard calibration curve. The obtained

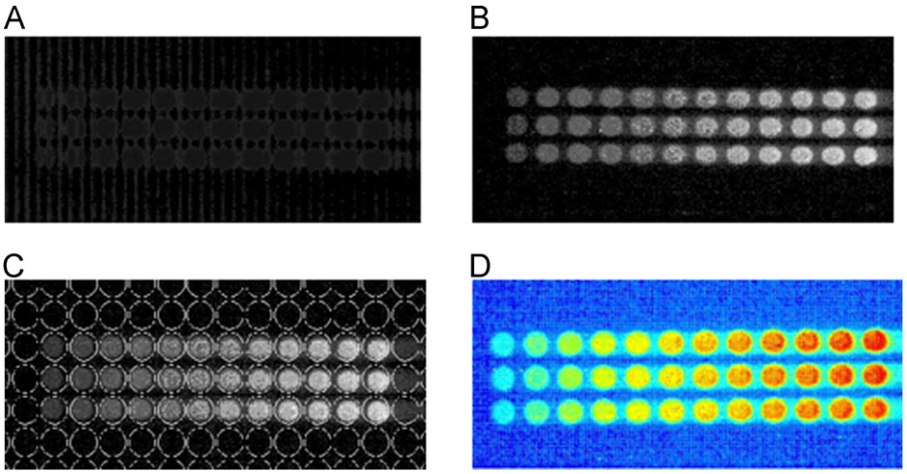


Fig. 1. Raw image well plate with 12 different concentrations of triclopyr, by triplicate (1A). Normalized image with adjusted gray levels (1B). Circular mesh delimiting the area of measurement (1C). False color version of Fig. 1B (1D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

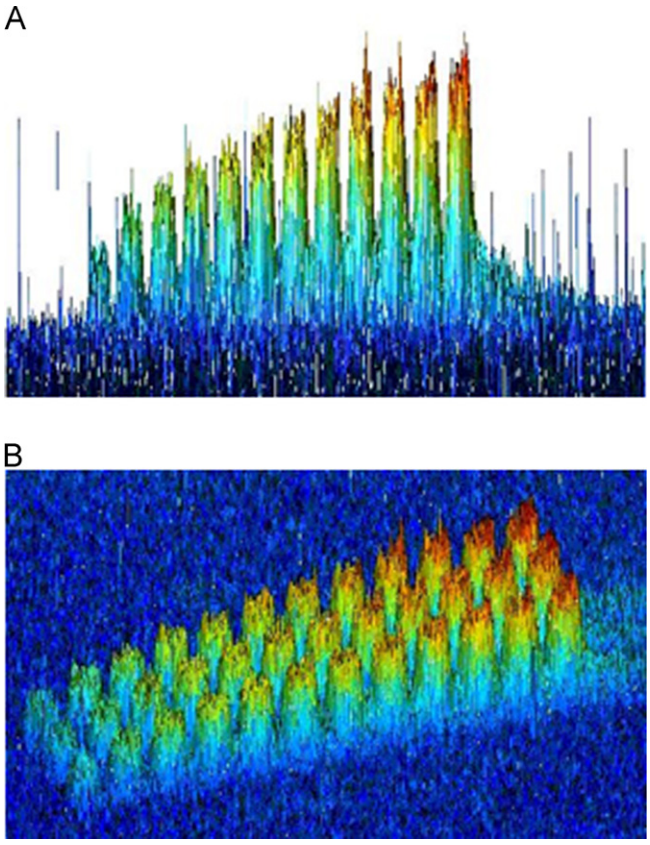


Fig. 2. Bi- (A) and three-dimensional (B) graphs of the wells plate showing several points (pixels) escaping from the general pattern.

signals were plotted as $\mathbf{V_{[\ ]}-V_{blank}/V_0-V_{blank}}$ against log [triclopyr] using a RIA four parameters logistic function to adjust the data. $\mathbf{V_{[\ ]}}$ is volume of a well for a concentration, $\mathbf{V_{blank}}$ is the volume of blank of the plate (no CL reaction) and $\mathbf{V_0}$ is the volume of the well that contains no triclopyr. Using *V* signals instead of intensity (the feature used when digital image techniques are not used) or area, a higher signal to noise ratio is obtained which is useful for extremely weak signals. Thus, the risk of a pixel to escape from the general standard emission profile is suppressed by the averaging of pixels. As can be seen in Fig. 2A and B, a one-dimensional section across the central row of microtiter wells can produce severe errors due to background noise. Thus, volume used as analytical signal reduces the noise and improve precision.

3. Results and discussion

The experimental conditions were optimized by means of the univariate approach, to obtain the best CL signal to be detected by the CCD. Different concentrations of coating agent were tested (3.5 and 7 μg/mL) with different dilutions of antiserum (1:1000, 1:2000, and 1:3000). Previously, each well were overnight incubated at 4 °C with 0.1 and 1000 ng/mL of triclopyr, and the emission were recorded for 5 min after the CL substrate was added as described in the ELISA procedure. The best CL signal was obtained with 15 μg/mL of coating agent and a dilution 1:1000 of primary antibody, and selected for further experiments. Previously studied was the kinetic of CL emission for 30 min, and observed that in the first 4 min the CL signal obtained were the best ones decreasing up to 20 min, as those were the selected 5 min for recording the assay. The time needed to prepare the wells plate ingredients and start image acquisition is near 2 min. CL signal shows high dependence of the pH, reached a maximum at pH 8–8.7 for the assay [4]; a pH of 8.5 was chosen as optimum. In the proposed method, the effect of Tris-HCl concentration was studied in the range 0.01–0.2 M. The maximum CL signal was observed at a Tris-HCl concentration of 0.1 M; therefore, Tris-HCl buffer (0.1 M and pH 8.5) was selected and used for subsequent studies. The highest CL signals with no triclopyr are obtained for antibody dilution 1/1000 and coating 3 μg/mL. These quantities are also the optima to distinguish between two next concentrations of triclopyr.

The limit of detection (LOD) is 0.0008 ng/mL and was calculated by substituting the average response (V/V_0) minus the relative standard deviation of three blank samples and then the concentration is calculated by the calibration equation. The lower the triclopyr concentration the higher the CL signal, so the measurements' precision determine the minimum and the background signal determine the maximum amount detectable of triclopyr. The precision was determined by measuring the CL of three well row with 10 samples containing 1000, 1 and 0.001 ng/mL triclopyr each one; variation coefficients expressed as %, were 1.66, 2.57 and 5.00, respectively. The medium relative variation coefficient was 3.07%. Calibration curve is shown in Fig. 3 and Table 1 shows the analytical parameters of the standard curve of this method for triclopyr and those obtained by measuring polarization fluoroimmunoassay (PFIA) [4]. It is observed that the CL-ELISA is more sensitive and slightly less precise at low concentrations than the fluorescence polarization method.

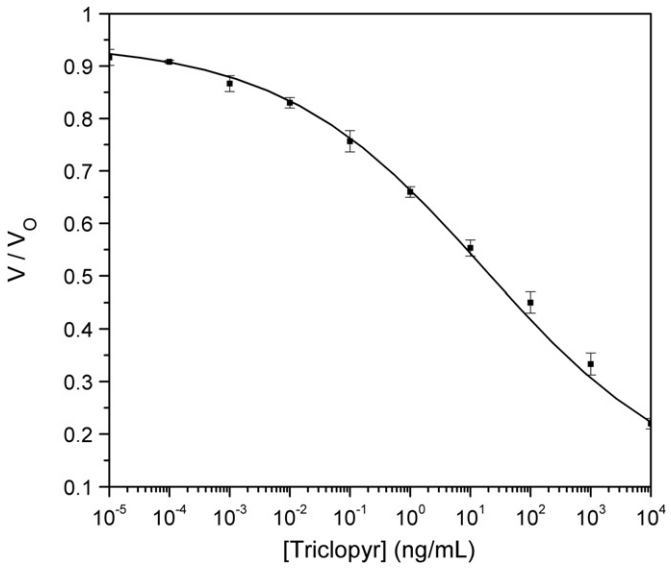


Fig. 3. RIA four parameters plot of the standard curve. Carbonate buffer pH=9.6; Ab 1:1000; peroxidase conjugated secondary Ab 1:160.

Table 1
Analytical parameters of standard curves.

	PFIA (Curve 1[a])	PFIA (Curve 2 [b])	CL-ELISA/ AI[c]
MDC (ng/mL)	861	0.7	0.0008
Dynamic range (ng/mL)	861–100000	0.7–100000	0.0008–10000
I_{50} (ng/mL)	2280	28	8.59
Slope (m)	1.604	0.233	0.167
Ψ^2	$8.6.10^{-6}$	$6.8.10^{-5}$	$7.1.10^{-4}$

[a] Obtained curve with $[Ag^*]=10^{-6}$ M and antibody dilution 1:600 by PFIA.
[b] Obtained curve with $[Ag^*]=6\times10^{-8}$ M and antibody dilution 1:1000 by PFIA.
[c] Obtained curve with 3 μg/mL Ag coating and dilution 1:1000 by CL-ELISA.

3.1. Assay specificity

Pesticides (2,4,5-T, triclopyr-2-butoxyethyl ester, 2,4-D and dichlorprop) with very similar structures to triclopyr or usually found in formulations were tested to characterize the specificity of the assay. Cross-reactivity (CR) in immunoassay is defined as the ratio of competitive binding between two or more structurally similar ligands with the antibody bonding sites. The CR was calculated by using the 50% displacement method. In this method the doses of triclopyr and structurally related compounds necessary to displace 50% of the bound labeled tracer are compared. Displacement curves were prepared by incubation for 1 h at room temperature of various doses of cross-reactant (10000–0.1 ng/mL) with a constant amount of antibody (dilution=1:1000) and a fixed amount of antigen coating (3 μg/mL), followed by separation of bound and unbound fractions, and quantification of the cross-reactant at each dose. These conditions were chosen because in this way, we can study the CR for a lineal range of lower concentrations. A displacement curve for each one of the different cross-reactant studied was constructed (Fig. 4). After fitting the curves by the four-parameter logistic model the doses of cross-reactant that give 50% displacement were determined and the CR of a compound was determined as

$$\%CR_{50\%} = \frac{Triclopyr_{50\%}}{CR_{50\%}} \times 100$$

where $triclopyr_{50\%}$ is the concentration of triclopyr at the assay midpoint ($V/V_0=0.63$) and $CR_{50\%}$ is the concentration of cross-reactant at the triclopyr assay midpoint. Table 2 gives formulas, $CR_{50\%}$, and I_{50}-values (concentration giving 50% inhibition of maximum response; it is a measure of the affinity of the antibody for the interferent) for the compounds studied. The doses of triclopyr and potentially interferents necessary to displace 50% of the bound antigen labeled are compared. A displacement curve for each one of the different cross-reactant was constructed. The compound that most affects the specificity of the assay is triclopyr-2-butoxyethyl ester, because to the similar structure to the triclopyr, mostly the pyridine ring with three –Cl groups.

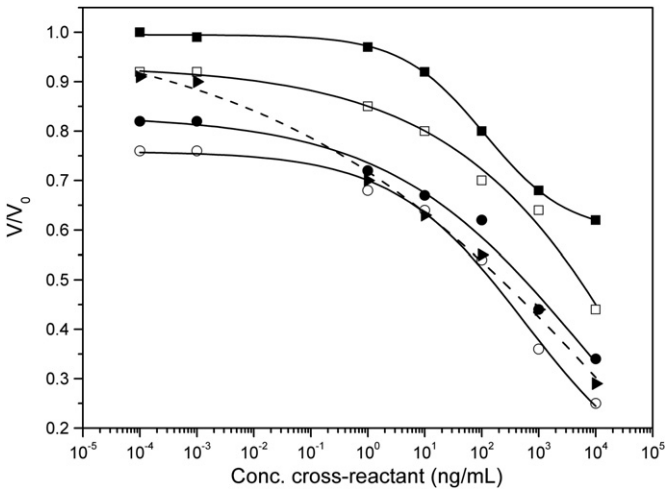


Fig. 4. Displacement curves of triclopyr (►), and the potential interferents triclopyr-2-butoxyethyl ester (○), 2,4-D (□), 2-4-5-T (●) and dichloprop (■).

Table 2
Cross reactivity of triclopyr and structurally related compounds.

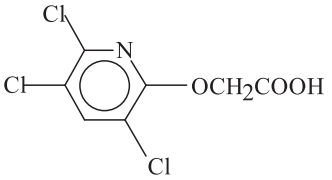
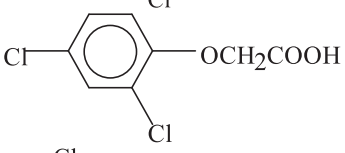
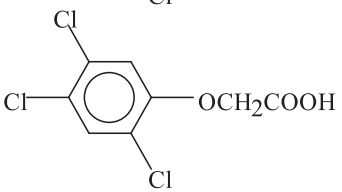
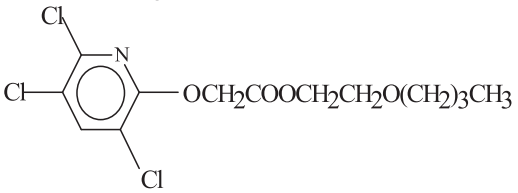
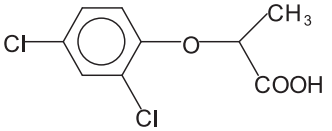
Formula	Compound	Cross reactivity
	Triclopyr	I50=8.6 %CR50%=100.0
	2,4 D	I50=611.1 %CR50%=1.4
	2,4,5 T	I50=39.6 %CR75%=21.6
	Triclopyr-2-butoxietil ester	I50=11.6 %CR50%=73.9
	Dichlorprop	I50=4326.7 %CR50%=0.2

Table 3
Mean recoveries percentages, RSD values and triclopyr concentration added on the CL-ELISA method for drinking water analysis.

Added ng/mL	Recovery[a]	RSD (%)
Level 1 (0.01)	118.7	5.66
Level 2 (0,1)	104.3	3.47
Level 3 (1)	98.3	1.84

[a] Mean of five measures ($n=5$), 1000 mL packaged drinking water.

3.2. Analysis of packaged drinking water samples

The applicability of the developed method was assessed by a recovery study at three spiking concentration levels of packaged drinking water, without the necessity of a previous extraction/concentration step. All experiments were carried out in quintuplicate at each level and the obtained results were ordered in Table 3. As can be seen very good recovery values were obtained, probably because no extraction/concentration step provides few sample manipulation.

4. Conclusion

This image based detection CL ELISA for triclopyr determination offers good analytical characteristics. These achievements are because: (1) the long CL signal (approximately 30 min), due to the use of the enzyme HRP as a catalyst, the intense light emission, due to the use of *p*-iodophenol as an enhancer, and low background and (2) the digital image processing which allows low price camera's data acquisition to examine low-level light emitting processes on microtiter plates.

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