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An optical sensor for pesticide determination based on the autoindicating optical properties of peroxidase

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ABSTRACT

During the enzymatic reaction of the heme-protein Horseradish peroxidase (HRP) with hydrogen peroxide there are changes in the molecular absorption spectra of HRP and its different oxidation states which can be used for quantitative determination of the substrate. One of these intermediate oxidation states is the HRPII, with iron as an oxyferryl. This compound is assumed to be responsible for the organophosphate pesticide degradation in the Fenton reaction. In this work, the enzymatic HRP–H_2O_2 reaction has been studied, based on the effect of different pesticides on the mechanism reaction; these modifications have been used for the quantitative determination of pesticides. A mathematical model has been developed relating to the analytical signal with the pesticide concentration. Three organophosphate pesticides (diazinon, trichlorfon and tetrachlorvinphos) and one sulfamide (dichlofluanid) have been used to demonstrate the viability of the methodology and the accomplishment fulfillment of the model. Tetrachlorvinphos was chosen as the pesticide model to develop the optical sensor film for continuous pesticide determination, consisting of HRP immobilized in a polyacrylamide gel. The sensor can be used for at least 15 days and responds linearly to tetrachlorvinphos concentrations in the range from 4.0×10^{-7} to 4.0×10^{-6} mol L^{-1}. The main advantage of the methodology is its reversibility in contrast to the irreversible Fenton reaction. The HRP–H_2O_2 methodology has been used to measure the pesticides in a waste water sample spiked with tetrachlorvinphos.

1. Introduction

Organophosphate pesticides are used as insecticides, nematicides, herbicides, fungicides, plasticizers, hydraulic fluids (in industry) and chemical weapons [1–3]. Due to their properties, these pesticides are easily absorbed in biological membranes (i.e. skin, mucous, etc.), can be stored in fatty tissues (leading to delayed toxicity), are volatile (being easily absorbed by inhalation), and degradable (undergoing hydrolysis in alkaline or biological media). These compounds are the main cause of pesticide intoxication and thus it is very important to study analytical methods for their quantitative determination or degradation. It is very well known that most existing methods for pesticides determination are based on chromatographic techniques [4–7] and that these methods present good sensibility and reproducibility. However, when pesticides monitoring is required, faster and reversible methods need to be developed. Most of the sensors available for pesticides determination are enzymatic and based on the inhibition effect of the analyte over the acetilcholinesterase with different detection techniques. Electroanalytical methods are mainly focused on increasing the selectivity [8,9] or looking for less contaminating electrodes [9–11]. In the case of optical detection, the use of more sensitive techniques, such us opto-acoustic resonande [12], Qdots [13,14], or AFM [15] are the main objective.

Despite the good analytical parameter associated with most of these methodologies it has been claimed that interference problems need to be carefully studied [16]; in addition most of them cannot act reversibly.

Fenton's reagent (a mixture of hydrogen peroxide and iron (II), H_2O_2–Fe(II)) has been used for organophosphate pesticide degradation due to its considerable oxidizing power [17]. The mechanism of this reaction has been extensively studied over time but only in recent years it has been demonstrated [18] that the key intermediate is the oxyferryl ion (FeO^{2+}) which is the real oxidant of the process. This intermediate oxidizes other species (i.e. pesticides) present in solution, leading to the Fenton reaction (FR). Although the reaction is simple and fast, the selectivity is poor, this being the reason for the limited analytical use of the Fenton reaction. One of these applications has been reported by Liu et al. [19] who have determined H_2O_2 by reacting the sample with sodium salycilate in the presence of Fe^{2+} to produce

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[2] Deceased.

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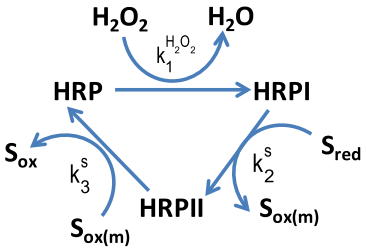
hydroxybenzoate, which is separated by HPLC and then detected the absorption of the compound at 310 nm. On the other hand, López Cueto et al. have used the FR for atrazine determination by applying kinetic methods and Partial Least Square (PLS) regression to the reaction profiles between 206 and 270 nm [20,21].

Horseradish peroxidase (HRP) is a heme-protein which catalyzes the oxidation of different substrates (S) in the presence of hydrogen peroxide [22]. This reaction is very useful in analytical chemistry as an indicator of other enzymatic reactions in which H_2O_2 is formed (for example, analyte oxidation by O_2 catalyzed with oxidase-type enzymes). This makes use of the spectroscopic properties of S (S_{red} or S_{ox}). However, the reaction can be analytically used in a different way. Kinetically speaking, the enzymatic reaction is conducted by the heme-group of the protein according to a three step process as follows:



(1)

H_2O_2 reacts with HRP to give HRPI and the regeneration to the native state is through the reducing substrates: first HRPI is reduced to HRPII ($S_{Ox(m)}$ being an intermediate of the substrate), and from this state to HRP. The values described in the literature for the kinetic constants indicate that the reduction of HRPII to HRP is the limiting process for most of the substrates [23–25]. The three peroxidase species (HRP, HRPI and HRPII) show differences on their UV–visible molecular absorption spectra. Based on these differences, and after appropriate management of the kinetic of the reaction, we have developed quantitative methods for the determination of H_2O_2 or substrates which have produced this compound in a previous enzymatic reaction [26,27].

In this paper we propose a different approach in which these molecular absorption properties and the mechanism of the Fenton reaction are combined to develop an analytical method for the determination of pesticides. The method is based on two main facts: 1) in the HRPII state, the iron is in the form of an oxyferryl and this compound is assumed to be responsible for pesticide degradation in the Fenton mechanism, so that pesticides can be used as substrates in Reaction (1); 2) the molecular absorption properties of the HRP/HRPII system are used. The method has been first optimized in batch and afterwards HRP has been immobilized in a polyacrylamide film as the base of an optical sensor film. Because of the regeneration of HRP the method could be described as a sustainable methodology for pesticide determination.

2. Material and methods

2.1. Apparatus

All measurements were performed with an Agilent 8453 diode-array spectrophotometer. A home-made flow cell described elsewhere [27] was used for the sensor film measurements.

For flow measurements, a 4-way Miniplus3-Gilson peristaltic pump and an Omnifit manual sample injection valve were used.

2.2. Reagents and solutions

A buffer solution of 0.1 mol L^{-1} phosphate at pH 6.0 (from H_2KPO_4 and HNa_2PO_4) was used during the study; peroxidase (HRP) from *Horseradish* 59 IU mg^{-1} of lyophilized solid was obtained from Sigma (P8250); hydrogen peroxide (Merck, www.vwr.com) was previously titrated; NNN′N′tetramethylethylenediamine (TEMED) solution was obtained from Bio-Rad (www.bio-rad.com) and used as received. All other reagents were of analytical grade and obtained from Sigma-Aldrich (www.sigma-aldrich.com). diazinon, trichlorfon, tetrachlorvinphos, and dichlofluanid, as analytical standards, were obtained from FLUKA (www.sigma-aldrich.com).

HRP and H_2O_2 stock solutions were daily prepared in the phosphate buffer.

2.3. Sensor film preparation

The sensor film was prepared according to the procedure described elsewhere [27] but with the following modifications: 16 mg of HRP (944 Units), 10 mg of N,N′-bis acrylamide and 150 mg of acrylamide were dissolved in 800 μL of a 0.1 mol L^{-1} phosphate buffer solution of pH 6. The mixture was bubbled with nitrogen for 5 min, and 4 μL of a 10% (w/v) $(NH_4)_2S_2O_8$ solution and 1 μL of the commercial TEMED solution were added.

2.4. Procedure for batch measurements

Batch absorption measurements were obtained by placing 2 mL of the phosphate buffer solution in a quartz cuvette, adding 0.5 mL of the HRP solution and registering the absorbance measurements at 400 and 424 nm. When the signal was stable, 100 μL of the corresponding H_2O_2 solution was added.

For pesticides determination, 200 μL of the corresponding pesticide and 1800 μL of the phosphate buffer solution were placed in the cuvette, 0.5 mL of the HRP solution was added and the absorbance was registered at 400 and 424 nm. When the signal was stable, 100 μL of a 6.7×10^{-5} mol L^{-1} H_2O_2 solution was added.

2.5. Procedure for sensor film measurements

The sensor film was placed in a homemade flow cell described elsewhere [27] and settled in the cuvette compartment of the spectrophotometer. A 5×10^{-5} mol L^{-1} tyrosine solution in phosphate buffer solution of pH 6 was flowed at 1 mL min^{-1} thorough the flow cell with a peristaltic pump. The variation in absorbance at 400 and 424 nm was registered and when the signal was stable, 1 mL of a 2.9×10^{-5} mol L^{-1} H_2O_2 solution was injected with a manual sample injection valve (Omnifit).

For pesticides determination, 1 mL of the corresponding mixture pesticide–H_2O_2 was injected.

2.6. Analytical parameter

In order to quantify the changes in absorbance and relate these to the pesticide concentration, the area (A) and the maximum height (H_{max}) of the signal measured at 424 nm was used as the analytical parameter.

3. Results and discussion

3.1. Origin of the analytical signal

As stated in the Introduction section, the different oxidation states of peroxidase show different molecular absorption properties. Fig. 1 shows the absorption spectra of HRP and HRPII. As can be seen, HRP shows a maximum at 400 nm, HRPII at 424 nm and they share an isosbestic point at 410 nm. The kinetic model (1) seems to indicate that when H_2O_2 is added to a solution containing

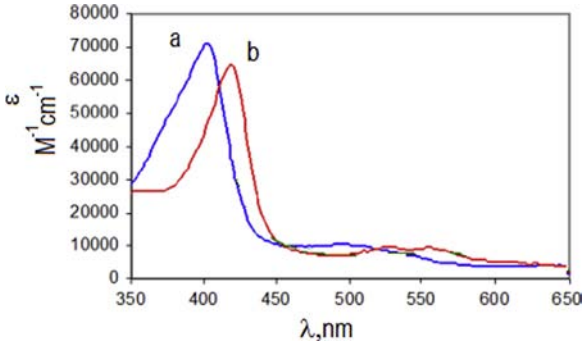
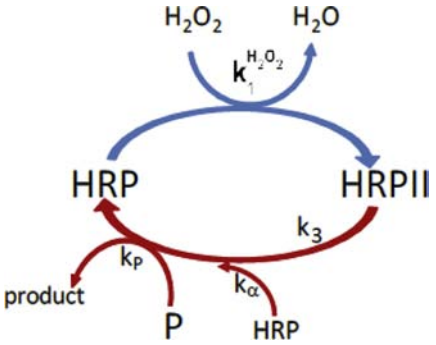


Fig. 1. Absorption spectra of the different oxidation states of peroxidase (2.9×10^{-5} mol L^{-1}, pH 6): a) HRP and b) HRPII.

HRP in the absence of a substrate (S_{rd}), HRPI is formed and should remain in this oxidation state. However, it has been demonstrated that the amino acids surrounding the heme-group [28] in the HRP molecule (intramolecular regeneration mechanism) or in other HRP molecules (intermolecular regeneration mechanism) can act as substrates, reducing HRPI to HRPII very quickly (species HRPI is barely observed) and then HRPII to HRP. Fig. 2a shows the behavior of HRPII: after H_2O_2 addition, the absorbance at 424 nm first quickly increases until a maximum is obtained and later decreases again up to the initial value (HRP is recovered); the absorbance at 400 nm follows the same variations but in the opposite direction. Both the area (A) regarding to the base line and the difference in absorbance at the maximum height (H_{max}) of the absorbance vs time representation are related to the H_2O_2 concentration.

According to our hypothesis, when a pesticide is added to a solution containing H_2O_2 and HRP, the intermediate oxidation states of HRP can then return to the initial state (HRP) not only by the inter- and intramolecular mechanisms but also by the pesticide action (HRPII can act as the Fenton reagent), and then the rate of the reduction increases resulting in a reduction of A or H_{max}. This is demonstrated in Fig. 2.

As a conclusion, the mechanism outlined in (1) for HRP in the presence of pesticides could be more suitably represented by that given in (2), where P represents the pesticide (k_p being the kinetic constant of the regeneration process), and k_2 and k_α being the kinetic constants of the intramolecular and the intermolecular regeneration mechanisms,



(2)

3.2. [HRP] and [H_2O_2] optimization

In the previous section it has been explained that because of the inter- and intramolecular regeneration mechanisms, the enzymatic reaction of HRP with H_2O_2 is actually reversible. We have previously found [22] that each HRP can give at least 17 regeneration cycles (i.e., each HRP molecule can react with 17 H_2O_2 molecules and regenerate by the intramolecular mechanism).

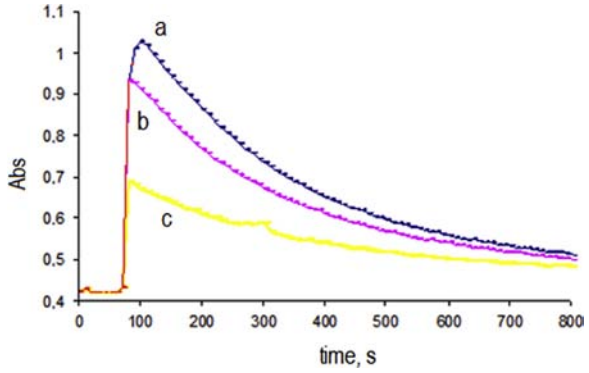


Fig. 2. Variation of the absorbance at 424 nm (2.9×10^{-5} mol L^{-1} HRP and 2.9×10^{-6} mol L^{-1} H_2O_2) for different diazinon concentrations: a) 0, b) 4.8×10^{-5} mol L^{-1}, and c) 9.9×10^{-4} mol L^{-1}.

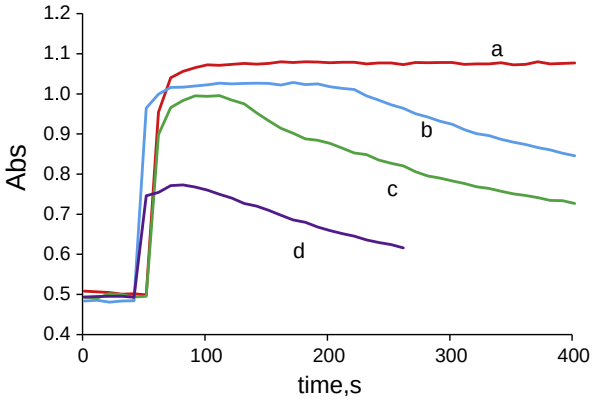


Fig. 3. Variation of Abs_{424} with different [HRP]:[H_2O_2] ratios. In all cases [HRP]= 2.9×10^{-5} mol L^{-1}: a) 1:1, b) 5:1, c) 10:1, and d) 20:1.

However, after each cycle the HRP activity diminishes, so the regeneration becomes slower and the available HRP concentration also diminishes. Considering this, it seems clear that to obtain a fully reversible method an excess of HRP over H_2O_2 should be used. Bearing this in mind, two important parameters need to be optimized: the [HRP] to [H_2O_2] ratio (i.e. the HRP excess) and the absolute [HRP] concentration. The quality parameters used for optimization were to obtain full and faster regeneration of HRP and as high analytical signal (A or H_{max}) as possible.

A systematic study was done using 4 different HRP concentrations with different HRP/H_2O_2 ratios (1, 2, 5, 10, 20 or 40). All the absorbance vs time representations obtained are compiled in the Supplementary material section. Fig. 3 shows the more relevant results. Regarding HRP/H_2O_2, the higher the ratio the faster the regeneration but the lower the A or the H_{max} obtained; the compromise solution chosen was 10. Working with different HRP concentrations maintaining HRP/H_2O_2 at 10, again, the higher HRP gave rise to larger H_{max} but the reaction time was longer. A 2.9×10^{-5} mol L^{-1} HRP (and then 2.9×10^{-6} mol L^{-1} H_2O_2) was considered as the optimum.

3.3. Analytical response to different pesticides: a general model

Before the characterization of the enzymatic reaction for pesticide determination, the pesticide–HRP and pesticide–H_2O_2 interactions were studied (see data in Supplementary material). First, the stability of a solution was considered containing the optimized concentration of HRP and increasing concentrations of the different pesticides. The absence of interaction between the pesticide and the HRP was demonstrated because no changes in the absorption spectra of the

HRP form were observed in any case. The possible interaction between the optimized concentration of H_2O_2 (2.9×10^{-6} mol L^{-1}) and the pesticides was also studied. Different solutions were prepared containing the optimized H_2O_2 concentration and a concentration 100 times higher (2.9×10^{-4} M mol L^{-1}) of the corresponding pesticide (maximum value at which the enzyme does not denaturalize). This solution was submitted to the measurement procedure after different H_2O_2/pesticide contact times. No changes in the reaction profile at 400 or 424 nm were observed even up to 360 min of contact time.

According to Scheme (2) it is possible to obtain a mathematical model relating to A and H_{max} with the pesticide concentration. Before the H_2O_2 addition, the solution only contains HRP ($[HRP]_0$ being the total peroxidase concentration) and P, so the absorbance at any wavelength is given by

$$Abs_{0,\lambda}=\varepsilon_\lambda^{HRP}\,[HRP]_0 \tag{3}$$

($\varepsilon_\lambda^{HRP}$ being the molar absorptivity of HRP at this λ)

When H_2O_2 is added, the HRP oxidation is produced and the absorbance at any time is given by (4), in which a mass balance to HRP has been applied ($[HRP]_0=[HRP]+[HRPII]$)

$$\begin{aligned}Abs_{t,\lambda}&=\varepsilon_\lambda^{HRP}[HRP]+\varepsilon_\lambda^{HRPII}[HRPII]=Abs_{0,\lambda}+(\varepsilon_\lambda^{HRPII}-\varepsilon_\lambda^{HRP})\,[HRPII]\\&=Abs_{0,\lambda}+\Delta\varepsilon_\lambda[HRPII]\end{aligned} \tag{4}$$

Combination of (3) and (4) gives the absorbance variation ($\Delta Abs_{t,\lambda}$) during the reaction at the chosen wavelength

$$\Delta Abs_{t,\lambda}=\Delta\varepsilon_\lambda\,[HRPII] \tag{5}$$

To relate [HRPII] with the pesticide concentration, the kinetic model (2) can be used:

$$\begin{aligned}\frac{d[HRPII]}{dt}&=k_1^{H_2O_2}[H_2O_2][HRP]-k_3[HRPII]-k_\alpha[HRPII][HRP]_0\\&\quad-k_P[HRPII][P]\end{aligned} \tag{6}$$

To solve Eq. (6), additional equations or conditions should be applied:

1) A differential equation for $[H_2O_2]$ can also be set out:

$$\frac{d[H_2O_2]}{dt}=-k_1^{H_2O_2}[H_2O_2][HRP] \tag{7}$$

2) Since the $[HRP]_0/[H_2O_2]_0$ ratio is 10, [HRP] is hardly consumed.

$$[HRP]\approx[HRP]_0 \tag{8}$$

3) When the pesticide concentrations are higher than $[H_2O_2]$, it can be considered that pesticide is hardly consumed and then

$$[P]=[P]_0 \tag{9}$$

After applying (8) and (9) in (6) and (7), and resolving the differential equation systems, the following equation for [HRPII] is obtained:

$$\begin{aligned}[HRPII]&=\frac{k_1^{H_2O_2}[HRP]_0[H_2O_2]_0}{k_3+(k_\alpha-k_1^{H_2O_2})[HRP]_0+k_P[P]_0}\\&\quad\times(e^{-k_1^{H_2O_2}[HRP]_0t}-e^{-(k_3+k_\alpha[HRP]_0+k_P[P]_0)t})\end{aligned} \tag{10}$$

To obtain H_{max}, the maximum condition is applied to (6), thus obtaining t_{max} which is later substituted into (10) again. A mathematically complex equation for H_{max} is obtained; however since the maximum appears at the beginning of the reaction (very small t) the polynomial simplification can be applied to the exponential function:

$$e^{-x}\approx1-x+\frac{x^2}{2} \tag{11}$$

And the final simplified equation for the model is derived

$$H_{max\,\lambda}=\Delta Abs_{t,\lambda}=\Delta\varepsilon_\lambda[HRPII]_{max}=\Delta\varepsilon_\lambda\frac{k_1^{H_2O_2}[HRP]_0[H_2O_2]_0}{2(k_3+k_\alpha[HRP]_0+k_P[P]_0)} \tag{12}$$

The area of the signal is obtained after integration of Eq. (10):

$$A_\lambda=\Delta\varepsilon_\lambda\int_0^\infty[HRPII]\,dt=\Delta\varepsilon_\lambda\frac{[H_2O_2]_0}{k_3+k_\alpha[HRP]_0+k_P[P]_0} \tag{13}$$

Eqs. (12) and (13) establish an inverse relationship between signal and pesticide concentration. Both equations can be linearized as follows:

$$\begin{aligned}\frac{1}{H_{max\,\lambda}}&=\left(2\frac{k_3+k_\alpha[HRP]_0+k_1^{H_2O_2}[HRP]_0}{\Delta\varepsilon_\lambda k_1^{H_2O_2}[HRP]_0[H_2O_2]_0}\right)\\&\quad+\left(2\frac{k_P}{\Delta\varepsilon_\lambda k_1^{H_2O_2}[HRP]_0[H_2O_2]_0}\right)[P]_0\end{aligned} \tag{14a}$$

$$\frac{1}{A_\lambda}=\left(\frac{k_3+k_\alpha[HRP]_0}{\Delta\varepsilon\lambda[H_2O_2]_0}\right)+\left(\frac{k_P}{\Delta\varepsilon_\lambda[H_2O_2]_0}\right)[P]_0 \tag{14b}$$

These equations justify that pesticides can be determined by the proposed methodology.

In order to test this, calibration graphs were produced with three pesticides: diazinon (Dz), tichlorfon (Tf) and dichlofluanid (Dc). Measurements were performed at 424 nm. Tables 1 and 2 show the analytical figures of merit for these pesticides using H_{max} and A, respectively. Several conclusions can be obtained from these results.

1) Regarding the H_{max} value, the intercepts obtained for the three pesticides are similar, as the model suggests (they only depend on the HRP and H_2O_2 concentrations used, which are the same

Table 1
Analytical figures of merit obtained for dichlofuanid (Dc), diazinon (Dz), trichlorfon (Tf) and tetrachlorvinphos (Tv) using H_{max} ($[HRP]=2.9\times10^{-5}$ mol L^{-1}, $[H_2O_2]=2.9\times10^{-6}$ mol L^{-1}, pH=6, λ=424 nm): calibration line, linear concentration range, relative standard deviation (RSD) ([Pesticide] used to obtain it: 2.0×10^{-4} mol L^{-1} for Dc, Dz and Tf; 2.0×10^{-5} mol L^{-1} for Tv), and the limit of detection (LOD).

Pesticide	H_{max}			
	Calibration line	Concentration range (mol L^{-1})	RSD (n=5)	LOD (mol L^{-1})
Dc	$1/H_{max}=1.78+6.10\times10^3$ [Dc, mol L^{-1}] $r^2=0.999$	5.0×10^{-5}–5.0×10^{-4}	2.4%	1.5×10^{-5}
Dz	$1/H_{max}=1.70+2.29\times10^3$ [Dz, mol L^{-1}] $r^2=0.994$	5.0×10^{-5}–1.0×10^{-3}	1.9%	1.5×10^{-5}
Tf	$1/H_{max}=1.78+4.09\times10^3$ [Tf, mol L^{-1}] $r^2=0.993$	5.0×10^{-5}–3.0×10^{-4}	2.1%	1.5×10^{-5}
Tv	$1/H_{max}=1.70+4.52\times10^4$ [Tv, mol L^{-1}] $r^2=0.995$	4.0×10^{-6}–8.0×10^{-5}	2.2%	1.0×10^{-6}

Table 2
Analytical figures of merit obtained for Dc, Dz, Tf and Tv using *A* (area) ([HRP]=2.9×10^{-5} mol L^{-1}, [H_2O_2]=2.9×10^{-6} mol L^{-1}, pH 6, λ=424 nm): calibration line, linear concentration range, relative standard deviation (RSD) ([Pesticide] used to obtain it: 1.0×10^{-4} mol L^{-1} for Dc, Dz and Tf; 4.0×10^{-6} mol L^{-1} for Tv), and the limit of detection (LOD).

Pesticide	*A*			
	Calibration line	Concentration range (mol L^{-1})	RSD (n=5)	LOD (mol L^{-1})
Dc	$1/A=0.0048+12.1$ [Dc, mol L^{-1}] $r^2=0.990$	5.0×10^{-5}–2.0×10^{-4}	3.0%	1.7×10^{-5}
Dz	$1/A=0.0050+19.1$ [Dz, mol L^{-1}] $r^2=0.990$	5.0×10^{-5}–2.0×10^{-4}	2.7%	1.7×10^{-5}
Tf	$1/A=0.0049+18.1$ [Tf, mol L^{-1}] $r^2=0.996$	5.0×10^{-5}–2.0×10^{-4}	2.4%	1.7×10^{-5}
Tv	$1/A=0.0047+290$ [Tv, mol L^{-1}] $r^2=0.992$	4.0×10^{-7}–4.0×10^{-5}	2.4%	1.5×10^{-7}

Table 3
Analytical figures of merit obtained for tetrachlorvinphos determination with the HRP optical sensors using *A* and H_{max} as analytical parameters (pH 6, λ=424 nm, $[HRP]_{film}$ 2.9×10^{-4} mol L^{-1}, [H_2O_2]=2.9×10^{-5} mol L^{-1}, FIA mode, 1 mL sample and 1 mL min^{-1} flow rate): calibration line, linear concentration range, relative standard deviation (RSD) (2.0×10^{-6} mol L^{-1}, Tv) and the limit of detection (LOD).

	Calibration line	Concentration range (mol L^{-1})	RSD (n=5)	LOD (mol L^{-1})
H_{max}	$1/H_{max}=12.7+2.79\times10^3$ [pesticide] $r^2=0.992$	4.0×10^{-7}–1.0×10^{6}	6.8%	2.3×10^{-7}
A	$1/A=0.0020+5.8\times10^3$ [pesticide] $r^2=0.993$	4.0×10^{-7}–4.0×10^{-6}	5.0%	2.0×10^{-7}

for the three compounds). Conversely, the slope depends on the pesticide to be used (k_P).

2) Regarding the area, the linear ranges scarcely change, the RSD was slightly worse than for H_{max} and the LOD were similar. In addition, in order to use this parameter it is necessary to wait the for measurement to finish (about 10 min, in some cases). For these reasons H_{max} is recommended. However according to the proposed model, it is possible to obtain the k_P value for each pesticide. Using $\Delta\varepsilon$=30,000 L mol^{-1} cm^{-1} (see Fig. 1) and [H_2O_2]=2.9×10^{-6} mol L^{-1} (see Table 2) from the slope of the calibration graphs k_P values of 1.1($\pm$0.3) s^{-1}, 1.7($\pm$0.5) s^{-1} and 1.6($\pm$0.3) s^{-1} for Dc, Dz and Tf respectively were obtained.

Assays were also carried out using tetrachlorvinphos (Tv). With this pesticide the sensitivity of both methods was about one order of magnitude higher than the other three (Tables 1 and 2). These results also fit with the general model described in (14a) and (14b) with k_P=25 ($\pm$3) s^{-1}, but considering the concentration range of the pesticide used, Condition (8) is not fully fulfilled. This seems to indicate that although the overall process of Tv is well represented by the general model (2), a different radical reaction mechanism, closer to the Fenton mechanism than in the case of the other three pesticides, drives the reaction.

3.4. Sensor film for tetrachlorvinphos determination in water

This method was then implemented in an optical sensor. A sensor film, prepared as described in the procedure section, which consisted of HRP (2.9×10^{-4} mol L^{-1}) entrapped in polyacrylamide was prepared. The film was placed in the homemade flow cell (see Material and Methods) and the change in absorbance at 424 and 400 nm was measured (see photographs in the Supplementary material section). All the assays were carried out using Tv as the analyte.

Several working methodologies were assayed. The most efficient consisted of injecting (in an FIA mode) a solution containing the 2.9×10^{-5} mol L^{-1} [H_2O_2] mixed with the Tv concentration to be tested; as in the batch measures, it was previously checked that the H_2O_2–tetrachlorvinphos solutions were stable for at least 24 h. Transient signals were observed and obviously the mathematical model previously developed (14) cannot be applied (it is necessary to include the mass transfer kinetic); however a relationship (see later) between the area (*A*) and the maximum height (H_{max}) of these profiles is still observed.

A problem with this methodology arises because the HRP cannot be fully regenerated during the time Tv is passing across the cell, so the intramolecular reduction mechanism is predominant. This means that the life time of the sensor film is reduced (only 7 measurements can be performed), the time for the autoregeneration is very long (about 30 min) and the sensitivity of the signal is continuously reduced. To avoid these problems, tyrosine was added to the carrier solution. This compound is also able to regenerate the HRP [27]. However, its concentration has to be optimized because the higher the concentration the faster the regeneration step but the lower the analytical signal. Finally, a 5.0×10^{-5} mol L^{-1} tyrosine concentration was proposed. Other parameters chosen were 1 mL min^{-1} carrier flow and 1 mL sample injection.

The analytical figures of merit obtained are compiled in Table 3. As can be seen, the linear response range is shorter than in the batch mode, which can be explained by both the tyrosine effect and the smaller optical pathlength of the sensor cell. As can be seen, *A* of the profile gives better analytical parameters than H_{max}. A primary study of the durability of the sensor has been carried out. Measuring two times a day, the life-time of the film is at least 15 days (the RSD of the 30 measurements was about 5%) and the regeneration time is about 10 min.

The HRP–H_2O_2 methodology has been used to measure the pesticides in a waste water sample spiked with Tv (1.0×10^{-6} mol L^{-1}, using *A* as the analytical parameter). The recovery obtained was 95% (RSD of 5%, n=5).

4. Conclusions

This paper demonstrates that the autoindicating properties of HRP can be used for pesticide determination as well as the base of an optical biosensor for continuous monitoring of these compounds. Future studies may be done in order to test the availability of other hemeproteins (hemoglobin, myoglobin, catalase and many others), because additional selectivity and higher sensitivity could be obtained.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.01.011.

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