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Talanta 65 (2005) 586–592
Talanta
www.elsevier.com/locate/talanta

Mathematical model for the analytical signal of an herbicide sensor based on the reaction centre of *Rhodobacter sphaeroides*

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Received 2 March 2004; received in revised form 31 May 2004; accepted 20 July 2004
Available online 1 September 2004

Abstract

This paper introduces a mathematical model which makes it possible both to determine the concentration of photosynthetic herbicides and to obtain a quantitative parameter in order to compare their activity using a previously described sensing system. The working principle involves the changes in absorption properties at 860 nm of the reaction centre (RC) isolated from the bacteria *Rhodobacter sphaeroides* when photosynthetic herbicides are present. The method has been used for the determination and activity comparison of five photosynthetic herbicides: diuron, atrazine, terbutryn, terbuthylazine and simazine. Detection limits obtained were 2.2, 0.75, 0.046, 0.25, and 1.4 μM, respectively. The resulting order for the different herbicides according to their action on RC was: terbutryn > terbuthylazine > atrazine > simazine > diuron.

Keywords: Herbicides; Sensor; Reaction centre; Optical fibre; *Rhodobacter sphaeroides*

1. Introduction

Approximately 10% of all plant species are weeds, and some of these cause serious economic losses in crop production. For this reason, herbicides (chemicals designed specifically to kill weeds) account for an important percentage of all pesticide sales [1].

The first chemicals used in weed control were inorganic compounds. Brine and a mixture of salt and ashes were both used by the Romans to sterilize the soil as early as biblical times. Some of the inorganic herbicides used during the last century were copper sulphate, sodium arsenite, arsenic trioxide, ammonium sulfamate, sodium tetraborate, sodium borate and sodium chlorate. Several of these are still useful in weed and brush control, but are rapidly being replaced by organic herbicides.

Herbicides can kill weeds by means of different actions. Of these, the most important are growth regulation, amino acid synthesis inhibition, lipid synthesis inhibition, seedling growth inhibition, photosynthesis inhibition, cell membrane disruption, and pigment inhibition. About one half of the herbicides used at present in agriculture inhibit photosynthesis, mostly by targeting the photosystem II complex. Based on the chemical structure and binding properties, most of these herbicides act by competing with natural plastoquinone for the binding place in the D1 protein (located in the reaction centre of the photosystem II) and by avoiding photosynthetic electron transport [2]. Photosynthesis inhibitors include the following herbicide families: triazines, phenylureas, uracils, benzothiadiazoles and nitriles.

Herbicides can be highly toxic for human and animal health, and the enormous increase in the application of herbi-

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doi:10.1016/j.talanta.2004.07.026

cides in agriculture during recent decades has resulted in the herbicide pollution of both soil and water. Traditionally, high performance liquid chromatography and gas chromatography have been used to detect trace quantities of pesticides in water [3–5]. However, while chromatographic techniques allow high sensitivity herbicide detection, they also require expensive equipment, organic solvents, and the prior purification of the sample. This implies a limitation on the number of samples that can be analysed and the impossibility of using them as rapid screening methods. Different attempts have been made to introduce biological detection systems in order to overcome these problems. Several immunoassays have been developed, mainly enzyme-linked immunosorbent assays (ELISAs) [6–8] and immunosensors [9–12]. Most of them involve monoclonal antibodies which are specific to one or a few compounds having a similar chemical structure. Recently, an optical immunosensor able to detect several pesticides at the same time by using different fluorescent labels for each specific antibody has been described [13]. Other detection systems (mainly biosensors) for photosystem II herbicides that imitate the natural way of action of those herbicides have also been proposed [14–19]. Unlike ELISA methods, photosystem based sensors are not selective, but make it possible to determine all the compounds that affect a light-induced electron flow.

Some authors have proposed using the isolated photosynthetic reaction centre (RC) of purple bacteria, mainly *Rhodobacter sphaeroides*, as an optical transducer for the detection of photosystem II herbicides [20–24]. Due to the simplicity of material preparation (cultivation, extraction and purification), the purple bacterial RCs may be regarded superior materials to the photosynthetic proteins of plants for the development of herbicide detectors [23].

RC is a trans-membrane protein complex in which small organic cofactors are responsible for photon absorption and the subsequent electron transfer (Fig. 1). These cofactors are arranged along two almost symmetrical branches.

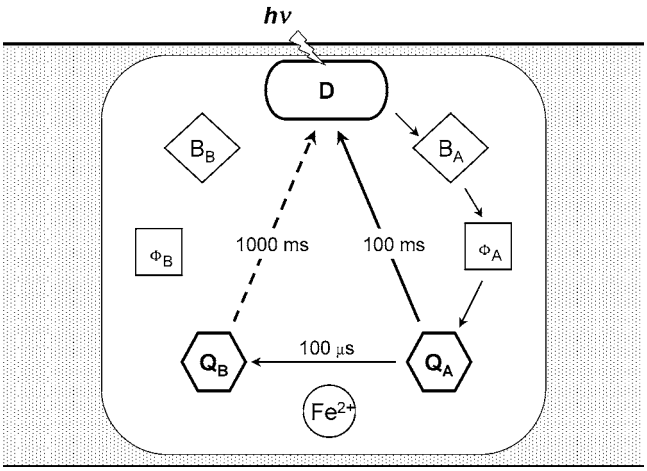

Fig. 1. Electron transfer processes between cofactors of the RC of *Rhodobacter sphaeroides* after light excitation.

However, the electron transfer is restricted to only one branch because of the structural engineering and specific environmental effects [25]. In substance, the absorption of a photon promotes a bacteriochlorophyll dimer (D) to its excited state. Consequently, an electron is transferred very rapidly, through a molecule of bacteriochlorophyll (B_A) and a molecule of bacteriopheophytin (Φ_A), to the first ubiquinone (Q_A), which is located in a hydrophobic pocket of the protein [20]. The electron is then rapidly transferred (about 100 μs) from Q_A to a secondary ubiquinone molecule (Q_B), which is located in a relatively polar protein domain. The return to the stationary state takes place at a charge recombination rate of about 1000 ms (dashed arrow). Moreover, Q_B is loosely bound to its pocket, and can be displaced from its binding site by competitive inhibitors, such as herbicides. If the Q_B binding site is empty or occupied by herbicides, the only possible recombination path is directly from Q_A (solid arrow), with a lifetime of about 100 ms.

The absorption spectrum of the RC complex shows different absorption bands corresponding to the different cofactors. The bacteriochlorophyll dimer contributes with a band at 860 nm that has the characteristic of being less intense for the excited form than for the fundamental state of the protein. Therefore, by following recombination kinetics by means of absorption at 860 nm, it is possible to learn the availability of the secondary ubiquinone and, consequently, the concentration of herbicides, since these can replace the latter ubiquinone.

In general, excitation of the RC is carried out with a flash lamp or a laser, and the subsequent 'time-resolved' absorption is then monitored [20,21]. Jockers et al. [22] describe a measuring method in which recombination processes are initiated and followed by continuous irradiation with weak actinic light.

Also in a previous work reported by the authors, simultaneous excitation and time-resolved absorption measurement are used by employing a light-emitting diode [24]: the development of the sensing system is described, with special emphasis on the design of the optoelectronic system which makes it possible to monitor the temporal changes in absorption following optical excitation.

The already described new sensing system [24] has been used in the present work for an in-depth analysis of the behaviour of the RC complex in the presence of different herbicides. According to the different published works based on RC as an optical transducer for the detection of photosystem II herbicides, the most original and relevant contribution to this work is the introduction of a mathematical model which makes it possible both to determine herbicide concentration and to obtain a quantitative parameter in order to compare the activity of different photosynthetic herbicides. This model has been validated by using standard solutions of five photosynthetic herbicides: diuron, atrazine, terbutryn, terbuthylazine, and simazine.

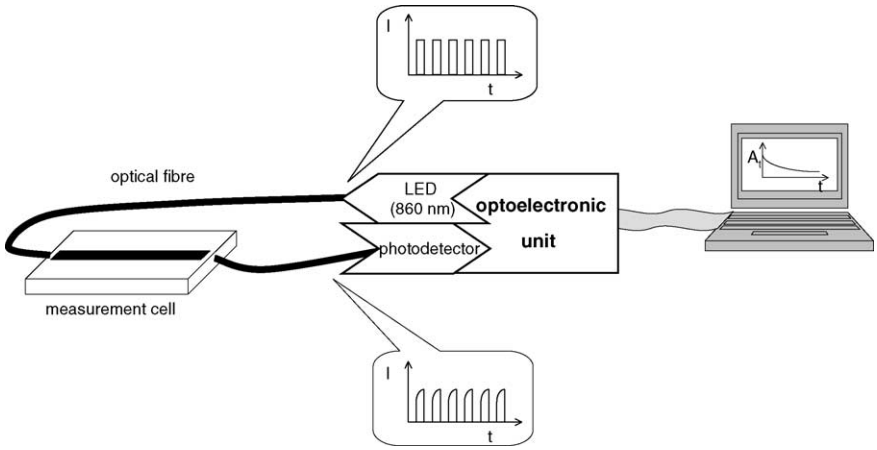

Fig. 2. Sketch of the optoelectronic system used to measure the time-resolved absorption.

2. Experimental

2.1. Materials

2.1.1. The optoelectronic unit

A previously described optoelectronic unit was used [24]. This device makes possible excitation (860 nm) and time-resolved absorption measurements at the same time (Fig. 2). The system was connected to the measurement cell by two optical fibres (200 μm). A National Instrument DAQ card 1200 data acquisition board allows the connection of the optoelectronic unit with a laptop. Lab View software was implemented to drive the optoelectronic unit and process all the collected data.

2.1.2. Reagents

Buffer solution: 0.02 M Tris–HCl solution of pH 8.0 and 0.08% (w/v) *N*,*N*-dimethyldodecylamine N-oxide (LDAO).

Herbicide solutions: The desired quantity of diuron (SIGMA, D-2425), atrazine (Supelco 49085), terbutryn (Supelco 49091), terbuthylazine (Supelco PS413) and simazine (Supelco 49089) was dissolved in methanol. Appropriate dilutions were carried out with doubly distilled water.

2.1.3. RC preparation

R. sphaeroides strain R-26 was kindly provided by Professor H. Scheer of the University of Munich (Germany). The bacteria were grown anaerobically in parafilm-sealed bottles filled with Codgel medium, and were exposed to a tungsten lamp until the absorbance at 660 nm was about 1 (around 6 days). Cells were separated from the grown reagents by means of centrifugation (10 min at 5000 × *g*). RC was isolated from *R. sphaeroides* in accordance to a previous work [26]. This consisted of the repeated solubilization of chromatophores with LDAO (dissolved in 0.02 M Tris buffer at pH 8 and 4 °C). RC was the purified by a column chromatography on diethyl-amino-ethyl-cellulose.

The purity of the RC was checked by measuring the ratio of the absorbance at 280 nm and 802 nm. In fact, SDS-PAGE electrophoresis indicates that when A_{280}/A_{802} = 1.21 ± 0.03 the purity of the protein is at least 95%. The concentration of RC was determined spectrophotometrically by the absorbance at 802 nm (ε_{802} = 288 $mM^{-1}\,cm^{-1}$) [26]. The value of the ratio A_{865}/A_{760} is usually adopted as integrity check for RC, which must be 1 [27].

Reconstitution of Q_B was required, because part of it was lost during the RC isolation and purification. The reconstitution of Q_B was accomplished with the natural ubiquinone (UQ_{10}) by incubating 1 ml of RC solution (A_{864} = 1, Tris–HCl 20 mM, pH 8, 0.08% LDAO) with 30 μl UQ_{10} (SIGMA c-9538) in EtOH/DMSO 1:1 (10 mg ml^{-1}) for 4 h at 4 °C. After incubation, the solution was centrifuged for 15 min at 6000 × *g* at 4 °C.

The RC was stored at −20 °C. Prior to use, dilution to a 2 μM RC concentration was made by using buffer solution.

2.2. Procedure

Time-resolved absorption was measured with the optoelectronic unit previously described. In the cell, 350 μl of RC solution and 15 μl of herbicide or buffer (blank) were added and mixed. Each measurement was carried out by repeating 50 times a cycle that consisted of turning the LED on for 2500 ms and off for another 2500 ms (time enough for the RC to return to its fundamental state). The evaluation of the time-resolved absorption was carried out by measuring what is called transient absorbance (A_t). Transient absorbance is defined as $A_t = \log(I_0/I_t)$, where I_0 and I_t are the intensities of light that are detected at the beginning and at time *t* of the pulse, respectively. Therefore, transient absorbance gives the change in absorbance with respect to its value in the fundamental state. Fig. 3 shows intensity and transient absorbance changes for the RC during the pulse for different atrazine concentrations. Transient absorbance data (containing A_t values for every 2 ms, that is 1251 values of A_t) were fitted to a two-exponential model (Eq. (1)) by using the software SigmaPlot 8.02 (iterations = 100; step size = 0.1; tolerance = 1×10^{-6}). Together with the equation this program showed the results of several statistic tests. Fitting was accepted when $r > 0.998$ and

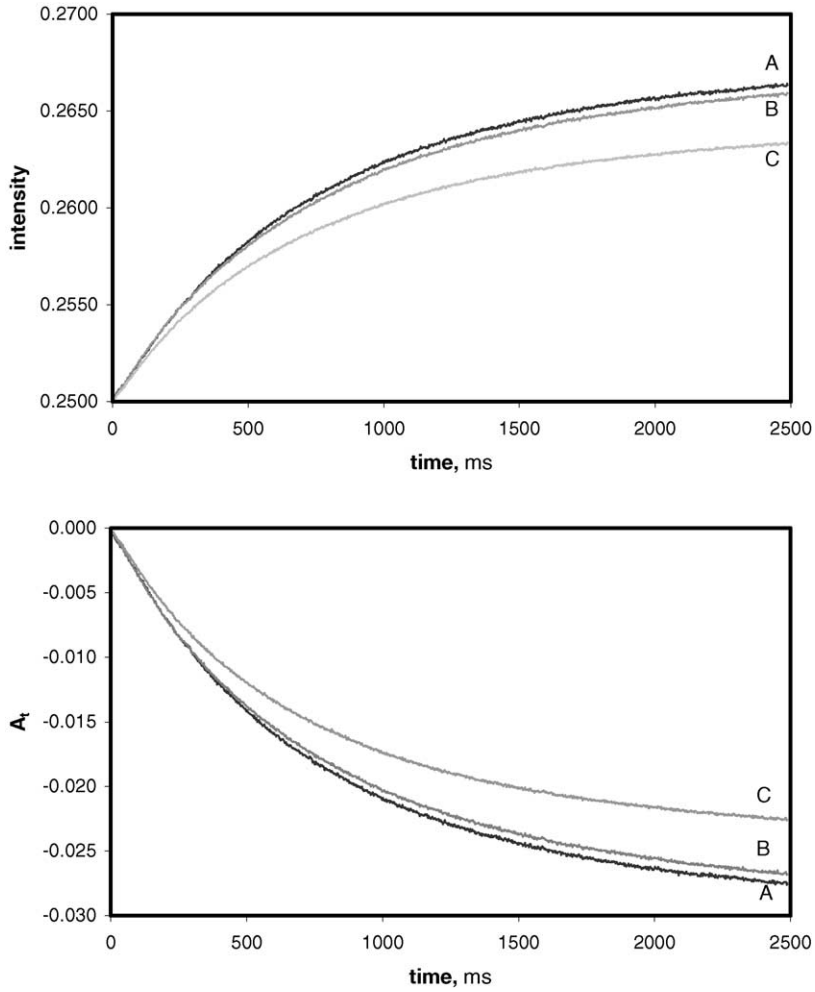

Fig. 3. Intensity and transient absorbance changes for RC (2 μM) during the pulse for different atrazine concentrations: (A) [atrazine] = 0 μM; (B) [atrazine] = 1 μM; (C) [atrazine] = 10 μM.

normality and constant variance tests, Kolmogorov–Smirnov and Leven Median tests respectively, were passed. The analytical parameter used was $1/A_1$ (for the definition of A_1, see next section).

3. Results and discussion

3.1. Signal explanation

At the beginning of the pulse, all the RC was in the fundamental state, with I_0 being the intensity of light that was not absorbed by the RC. During the pulse, the excited form of the RC was accumulated progressively; therefore, less light absorption occurred, and an increase in intensity was observed. After some time, equilibrium between the excited and the fundamental state form of RC was established, and a constant value of intensity was observed (Fig. 3). When herbicide was present (curve B and C in Fig. 3), Q_B was displaced from its binding site, and the short recombination (directly from Q_A) increased with respect to the absence of herbicide (curve A in Fig. 3). This implied that the concentration of the fundamental state form of RC in the equilibrium increased, and that higher light absorption took place. Consequently, a decrease in intensity was observed.

Transient absorbance depends on the time according to a two-exponential model [21]:

$$A_t = A_1(e^{-t/\tau_1} - 1) + A_2(e^{-t/\tau_2} - 1) \quad (1)$$

where τ and A are the time constants and the amplitude of the return to the fundamental state from Q_B (subscript 1) or Q_A (subscript 2), respectively. The experimental values obtained for τ_1 and τ_2 were 856 (±30) and 130 (±7) ms, respectively. A_2 depends on both RC containing Q_B and also RC not containing Q_B in the hydrophobic pocket since short decay is possible from both. However, A_1 is only related to RC with Q_B, because long decay is not possible for RC without Q_B. Fig. 4 shows the A_1 value for different total RC concentration. It should be noted that, even after the reconstitution step (see Section 2.1.3), there was some RC without Q_B being present. The solution therefore contained an unknown proportion of RC with and without Q_B, but one that was fixed for all measurements. As can be seen, A_1 is

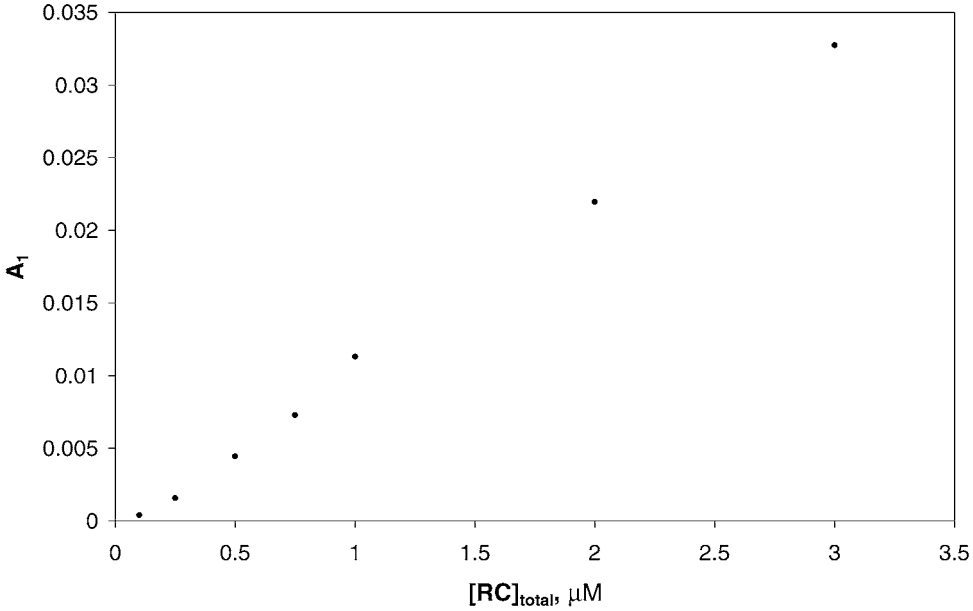

Fig. 4. Dependence of the A_1 value on RC concentration.

directly proportional to the RC concentration, indeed, to the RC fraction containing Q_B. Thus,

$$A_1 = C_1[\mathrm{RC}_{\mathrm{with\,Q_B}}] \quad (2)$$

Fig. 5 shows changes in A_1 and A_2 for different terbutryn concentrations. As can be seen, the higher the herbicide concentration, the lower the A_1 value and the higher the A_2 value. Although both amplitudes changed with the concentration of herbicide, A_1 was actually more sensitive than A_2. Therefore, A_1 was used to study herbicide effect.

3.2. Mathematical model

A mathematical model was developed in order to obtain an analytical parameter for evaluating the effect of herbicide on the transient signal of RC.

The reaction between RC and herbicide can be expressed as:

$$\mathrm{RC}_{\mathrm{with\,Q_B}} + \text{herbicide} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{RC}_{\mathrm{without\,Q_B}}$$

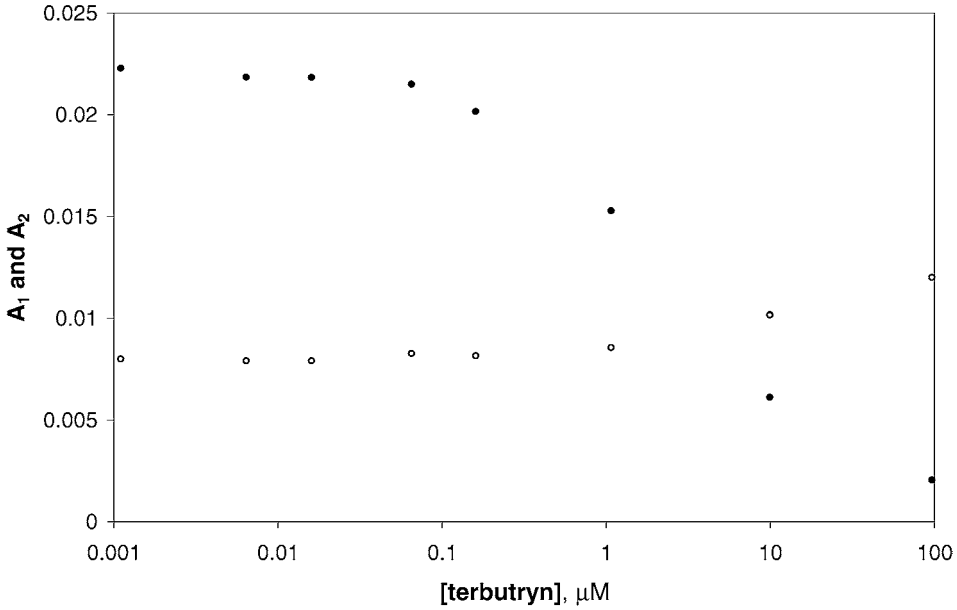

Fig. 5. Dependence of A_1 (●) and A_2 (○) on terbutryn concentration ([RC] = 2 μM).

Table 1
Calibration curves and detection limits for atrazine at different RC concentrations

[RC] (μM)	Calibration curve	Detection limit (μM)
1	$1/A_1 = 85.9 + 1.37$[atrazine] ($r = 0.995$)	1.9
2	$1/A_1 = 45.5 + 0.782$[atrazine] ($r = 0.99998$)	0.75
3	$1/A_1 = 31.2 + 0.557$[atrazine] ($r = 0.9991$)	0.44

Thus,

$$k_1 = \frac{[\mathrm{RC}_{\mathrm{without\,Q_B}}]}{[\mathrm{RC}_{\mathrm{with\,Q_B}}][\mathrm{herbicide}]} \quad (3)$$

By considering the mass balance:

$$[\mathrm{RC}]_{\mathrm{total}} = [\mathrm{RC}_{\mathrm{with\,Q_B}}] + [\mathrm{RC}_{\mathrm{without\,Q_B}}] = \mathrm{constant} \quad (4)$$

By combining Eqs. (3) and (4):

$$k_1 = \frac{[\mathrm{RC}]_{\mathrm{total}} - [\mathrm{RC}_{\mathrm{with\,Q_B}}]}{[\mathrm{RC}_{\mathrm{with\,Q_B}}][\mathrm{herbicide}]} \quad (5)$$

By combining Eqs. (2) and (5):

$$k_1 = \frac{[\mathrm{RC}]_{\mathrm{total}} - (A_1/C_1)}{(A_1/C_1)[\mathrm{herbicide}]}$$

which after reorganisation is:

$$\frac{1}{A_1} = \frac{1}{C_1[\mathrm{RC}]_{\mathrm{total}}} + \frac{1}{C_1[\mathrm{RC}]_{\mathrm{total}}} k_1[\mathrm{herbicide}] \quad (6)$$

Eq. (6) can be expressed as:

$$\frac{1}{A_1} = \alpha + \alpha k_1[\mathrm{herbicide}] \quad (7)$$

where α depends on the total RC concentration and on the proportionality constant between A_1 and RC with Q_B.

Eq. (7) establishes a linear relation between the herbicide concentration and $1/A_1$. This linear relationship was observed experimentally. It could thus be concluded that Eq. (7) can be successfully used as a calibration curve for herbicide determination.

3.3. Optimisation of RC concentration

The effect of the RC concentration can be seen in Table 1, which shows calibration curves and detection limits (according to IUPAC definition) for atrazine at different RC concentration. As can be seen, high RC concentration results in better detection limits but in lower sensitivity, in agreement with the mathematical model purpose (Eq. (6)). A RC concentration of 2 μM was chosen for further studies by taking into account both analytical characteristics.

3.4. Herbicides detection

The method described was applied in order to detect five different herbicides: diuron, atrazine, terbutryn, terbuthylazine and simazine. Transient absorbance curves were obtained for different concentrations of each of these herbicides. The A_1 value was obtained by using the two-exponential model (Eq. (1)) for each curve. Each measurement was repeated three times, and the A_1 mean values were then calculated. Fig. 6 shows the A_1 value for different concentrations of every herbicide. $1/A_1$ values were fitted to Eq. (7) in order to obtain the calibration curve for each herbicide by using least squares method (confidence interval = 95%). Equilibrium constants of the reaction between the

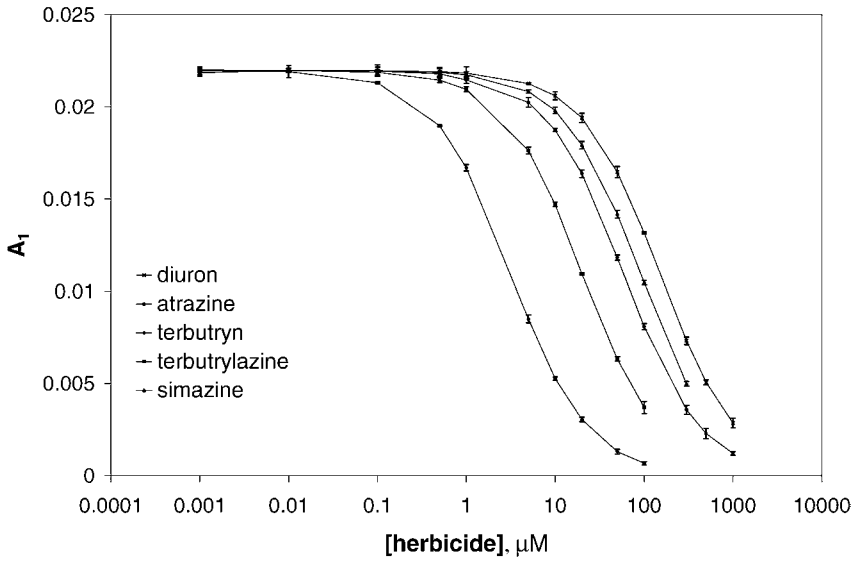

Fig. 6. Effect of the concentration of five different photosynthetic herbicides on the A_1 value ([RC] = 2 μM).

Table 2
Results for different herbicides: diuron, atrazine, terbutryn, terbuthylazine and simazine

Herbicide	Studied range (μM)	Calibration curve	k_1 (μM^{-1})	Detection limit (μM)	Linear range (μM)
Diuron	0.01–1000	$1/A_1 = 45.4(\pm0.3) + 0.304(\pm0.022)$[diuron] ($r = 0.998$)	0.00670 ± 0.00049	2.2	7.3–1000
Atrazine	0.01–1000	$1/A_1 = 45.5(\pm0.3) + 0.782(\pm0.033)$[atrazine] ($r = 0.99998$)	0.0172 ± 0.0007	0.75	2.71–500
Terbutryn	0.001–100	$1/A_1 = 44.8(\pm0.6) + 14.2(\pm0.7)$[terbutryn] ($r = 0.9997$)	0.317 ± 0.016	0.046	0.15–50
Terbuthylazine	0.001–100	$1/A_1 = 46.6(\pm0.5) + 2.30(\pm 0.16)$[terbuthylazine] ($r = 0.998$)	0.0494 ± 0.0035	0.25	0.95–100
Simazine	0.001–300	$1/A_1 = 47.1(\pm0.4) + 0.518(\pm 0.031)$[simazine] ($r = 0.9997$)	0.0110 ± 0.0007	1.4	4.6–300

RC and herbicides, k_1, were experimentally calculated by dividing the slope value of the calibration curves by their y intercept value. It is important to stress that k_1 is a quantitative parameter for comparing the activity of various herbicides. All the results are shown in Table 2. The resulting order for the different herbicides according to their action on RC was: terbutryn > terbuthylazine > atrazine > simazine > diuron. These results agreed with those of other authors using an RC of *R. sphaeroides*: terbutryn > atrazine > diuron [22]. The detection limits were established according to the IUPAC definition.

4. Conclusions

A mathematical model for analytical signal behaviour has been developed. This provides the basis for the use of RC as an optical transducer in the determination of photosynthetic herbicide concentration. Moreover, the mathematical model makes it possible to obtain a quantitative parameter in order to compare the activity of the different herbicides.

The sensor is a rapid, portable and inexpensive system, which could be useful as an alarm sensor for photosynthetic herbicide activity in groundwater in agricultural regions. It must be taken into account that improvements in detection limits can be obtained by increasing the RC concentration. In addition, a 30 times greater sensitivity to the herbicide *o*-phenantroline has been obtained by means of the mutation of the RC of *Rhodopseudomonas capsulatus* [28]. Therefore, with the help of a detailed structural knowledge of the Q_B site and the availability of genetic manipulation, in future it should be possible, to increase sensitivity with regard to herbicides. Moreover, the simplicity of RC preparation makes it suitable of commercialization.

Acknowledgements

This work was sponsored by Programa Europa-CAI and by a research grant (DGA B100/2000). The authors would like to thank Claudio Domenici and Domenica Masci for their helpful collaboration.

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