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Alkaline Phosphatase Inhibition-Based Amperometric Biosensor for the Detection of Carbofuran

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An indirect amperometric biosensor was designed for the detection of carbofuran exploiting inhibition of the enzymatic activity of alkaline phosphatase immobilized on a carbon nano-powder paste electrode (CNPPE). Measurements were performed by chronoamperometrical monitoring on the inhibition of the enzyme activity. Experimental parameters were investigated and optimized. The biosensor showed optimum performance at an operation potential at +0.75 V vs Ag/AgCl at pH 8.5 (stirred phosphate buffer solution, 0.1 mol/L). The calibration curve had a dynamic range of 10 - 97 µg/L carbofuran with a detection limit at 10 µg/L. The repeatability was 2.3 % (3 measurements, 50 µg/L), the reproducibility was 3.4 % (3 sensors). The determination of carbofuran in spiked water and chili samples using the proposed biosensor was satisfactory when compared to a spectrophotometric reference method. The results showed no significant difference at 95 % confidence level with t-test statistics.

Keywords: Alkaline phosphatase, Amperometric biosensor, Enzyme inhibition-based biosensor, Carbon paste electrode, Indirect method, Carbofuran

1. INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is one of the most toxic carbamate pesticides; it is frequently used for the control of insects in agriculture and food production because it inhibits cholinesterase. Nevertheless it exhibits significant toxicity to humans and wildlife animals causing nerve disorder and immunological respiratory diseases [1-3]. Commonly used analytical methods for the determination of carbofuran are high performance liquid chromatography, gas chromatography coupled to mass spectrometry, and spectrophotometry [4-6].

These conventional techniques are sensitive, reliable and precise. However, they have some disadvantages such as complex and time consuming analytical processes, relatively high costs, and requirement of highly trained personnel. Alternative assays to detect carbofuran were devoted to the implementation of biosensors in environmental analysis owing to some advantages, such as rapidity, use of small, portable and low cost instruments, fast signal response and low detection levels for quantitative detection.

Most biosensors for detecting toxic substances are relying on the inhibition of the activity of an enzyme [7-13]. Indirect biosensors for herbicides or pesticides have been mainly designed with acetylcholinesterase [14-23].

For the determination of carbofuran alkaline phosphatase has been used in a few cases [24-27]. An electro-inactive substrate releases a product which can be electrochemically detected at the electrode surface. Usually phenolic phosphate esters are employed which deliver the free phenol upon enzymatic hydrolysis; the latter is electrochemically oxidized to a quinone subsequently. With a constant substrate concentration inhibition leads to a decrease in the activity of the enzyme and as a consequence to a decrease of the response signal which is proportional to the amount of inhibitor in the sample.

In this work, a new designed biosensor based on a carbon nanopowder paste is proposed to fabricate alkaline phosphatase inhibition-based amperometric biosensor for the detection of carbofuran. The designed biosensor is based on alkaline phosphatase cross-linked with bovine serum albumin and glutaraldehyde immobilized on the surface of a carbon nanopowder paste electrode. Preliminary studies showed that carbon nanopowder paste electrodes provide good response with low current background currents, a wider window potential range and long term stability compared to the ordinary carbon paste electrode. The performance of this new type of biosensor was evaluated by cyclic voltammetry and hydrodynamic amperometry. Details on the practical design and its applications of this biosensor will be discussed.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Alkaline phosphatase (ALP, P6772- 2 KU), bovine serum albumin (BSA), carbon powder (size < 50 nm) and all other chemicals were purchased from Sigma-Aldrich. Deionized water was refined with a purification system (Milli-Q, Millipore) and had a specific resistivity of 18.2 MΩ cm; it was used throughout the experiments. The supporting electrolyte was a phosphate buffer solution (PBS, 0.1 M) prepared by mixing aqueous NaH_2PO_4 and Na_2HPO_4 solutions (0.1 M) to achieve the desired pH (usually 8.5). The disodium phenyl phosphate stock solution (DSP, 10 % m:v) and carbofuran standard solution (100 mg/L) were prepared freshly in phosphate buffer solution pH 8.5 according to the needs.

2.2 Apparatus

For electrochemical measurements a computer-controlled electrochemical workstation (Potentiostat/Galvanostat, Metrohm PGSTAT12) was used to perform cyclic voltammetric measurements; a corresponding software (GPES) was used to register signals and evaluate the data. pH was measured with a pH-meter from Sartorius (pH Meter PP-50). Spectrophotometric measurements were done with a Lambda 25 spectrophotometer from Perkin Elmer.

2.3 Biosensor design

Carbon paste (CE) was prepared by thoroughly mixing carbon nanopowder (1.00 g) and paraffin oil (0.30 g) until homogenous consistency was achieved; afterwards it was left to stand overnight before use. The electrode holder was a plastic syringe tube (diameter 5.8 mm, length 40 mm) and was filled with the carbon paste. Electrical contact to the paste was established by inserting a copper wire into the back of the paste. The CPE electrode surface was polished with weighing paper until a smooth surface by optical inspection was obtained.

The alkaline phosphatase solution for casting the bio-film was prepared by mixing bovine serum albumin (0.040 g) with 40 μL of an aqueous solution of alkaline phosphatase (2 KU/mL), 20 μL of glutaraldehyde solution (2.5 %) and 20 μL of glycerol and adjusting the final volume to 500 μL with phosphate buffer solution (pH 8.0). The mixture was gently agitated by a vortex shaker at room temperature for 15-20 min. The biosensor was fabricated by dropping 20 μL of the enzyme casting solution onto the surface of the carbon paste electrode (28 mm^2). The electrode was dried at room temperature and then stored at +4 ^oC in a refrigerator until use.

2.4 Sample pretreatment

Water sample

1 L of water sample from a natural reservoir was passed through a filter paper (Whatman, grade 1) and an aliquot (100 mL) of the filtrate was spiked with an appropriate concentration of carbofuran. The spiked sample was transferred into a separatory funnel for extraction with 100 mL dichloromethane by shaking for 10-20 min. Then, the extract was evaporated to a residual volume of 1-2 mL with a rotary *evaporator* (water bath temperature 40 ^oC). The remaining liquid was dissolved in methanol (5 mL), transferred to a volumetric flask (10 mL), and made up to the mark with deionized water. The blank solution was likely prepared by using deionized water instead of natural water (modified from [28]).

Chili samples

Bird's eye and yellow chili samples were purchased at a local fresh market in the north eastern part of Thailand. The peppers were initially homogenized in a blender; afterwards, 20.0 g of each sample were weighed into Erlenmeyer flasks and 80 mL of hexane (saturated with acetonitrile) were added and agitated for 5-10 min. The extract was filtered and transferred into a separatory funnel. The extraction was performed by adding 50 mL of acetonitrile (saturated with hexane) by vigorously

shaking for 15-20 min. The acetonitrile phase was isolated and evaporated to a volume of 1-2 mL by a rotary *evaporator;* the residue was carefully transferred into a volumetric flask (5 mL) and made up to the mark with methanol. The blank solution was analogously prepared by omitting the chili peppers (modified from [28]).

2.5 Electrochemical measurement

The electrochemical cell was composed of a Ag/AgCl (3 M KCl) reference electrode, a platinum counter electrode, and a modified carbon paste working electrode (ALP/CPE). All potentials mentioned in the text refer to the silver-silver chloride reference electrode.

If not mentioned otherwise PBS (0.1 mol/L, pH 8.5) was used as supporting electrolyte.

Cyclic voltammorgrams were recorded from -0.1 to +1.5 V with a scan rate of 10 mV/s.

All chronoamperometric measurements were carried out in stirred phosphate buffer solutions (approx. 300 rpm) at room temperature with an operation potential of +0.75 V.

The current response was recorded with 500 mg/L disodium phenyl phosphate as the standard substrate concentration. Signals were evaluated as the difference of the registered steady state current to the current obtained before addition of the analyte.

2.6 Spectrophotometric analysis

Spectrophotometry was chosen as a reference method modified from the report of Chu and Fan [28]. Carbofuran was in a first step hydrolyzed to the corresponding phenol in alkaline medium. Afterwards p-nitroaniline was diazotized by the aid of sodium nitrite in acidic solution forming the p-nitrobenzene diazonium salt, which couples to the aromatic ring of 2,3-dihydro-2,2-dimethylbenzofuran-7-ol to yield p-nitroaniline-5-amino-2,3-dihydro-2,2-dimethylbenzofuran-7-ol which is a wine-red azo dye with an absorption maximum at 520 nm. The standard addition method was chosen to determine carbofuran in the sample solutions using a concentration range of 0-5 mg/L carbofuran.

3. RESULTS AND DISCUSSION

3.1 Cyclic voltammetry

Cyclic voltammetry uncovers that under the chosen experimental conditions phenol is oxidized electrochemically at the nano-powder carbon paste at around 0.7 V (Figure 1). As oxidation products finally quinoid structures are formed after primary oxidation to o- or p-dihydroxybenzene. When employing the biosensor with phenyl phosphate similar electrochemical behavior is found (Fig.1, curve c) which clearly demonstrates that the enzyme is catalytically active retaining fast kinetics; thus the intermediate phenol is reasonably rapidly formed to be monitored electrochemically by the carbon paste electrode.

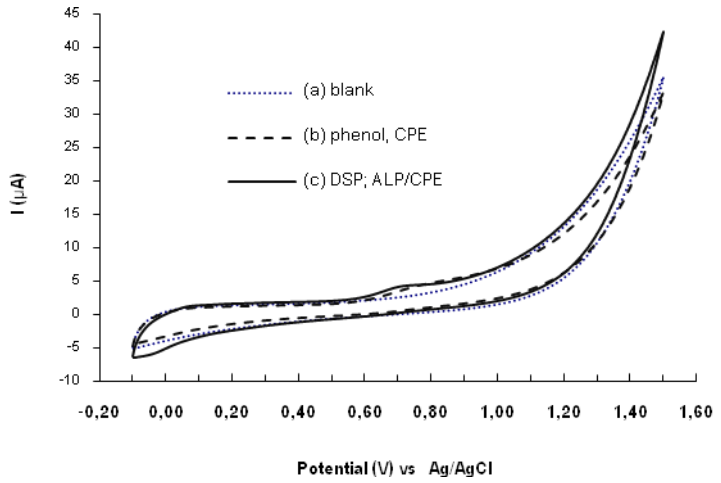

Figure 1. Cyclic voltammograms of phenol with a CPE and of DSP with the biosensor; (a) blank, CPE (dotted line); (b) 1000 mg/L phenol, CPE (broken line); (c) 500 mg/L DSP, ALP/CPE (solid line); supporting electrolyte PBS 0.1 M, pH 8.5; scan rate 10 mV/s

Figure 2 summarizes the overall electrode reactions occurring at the biosensor.

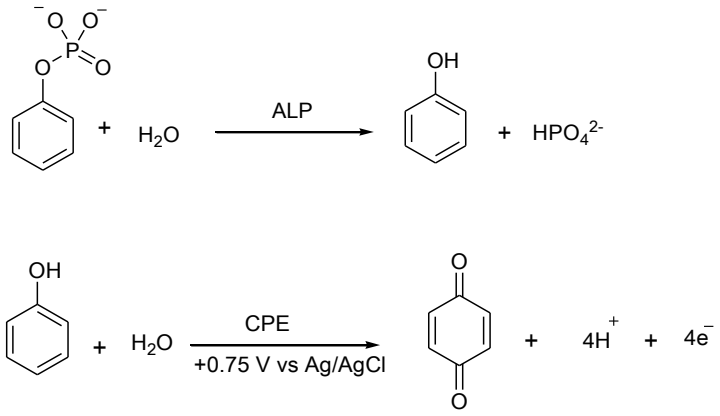

Figure 2. Measurement principle of the ALP/CPE biosensor

3.2 Effect of operating potential

In hydrodynamic amperometry (amperometric measurements with agitated liquids) a crucial parameter is the operating potential. In the present study the dependence of the applied potential on an ALP/CPE exposed to a stirred solution of the supporting electrolyte containing phenyl phosphate was investigated (Fig.3).

The electrode shows an almost linearly increasing current with an increasing potential up to +0.75 V; beyond this the signal decreases again the reason for which is not completely clear. One possibility could be the formation of oxidized products other than benzoquinone or of polymers which are sorbed on the surface and reduce its active area. Another explanation is the occurrence of possible

potential-dependent sorption processes of oxidation products. For further studies a potential of 0.75 V was chosen as optimum value.

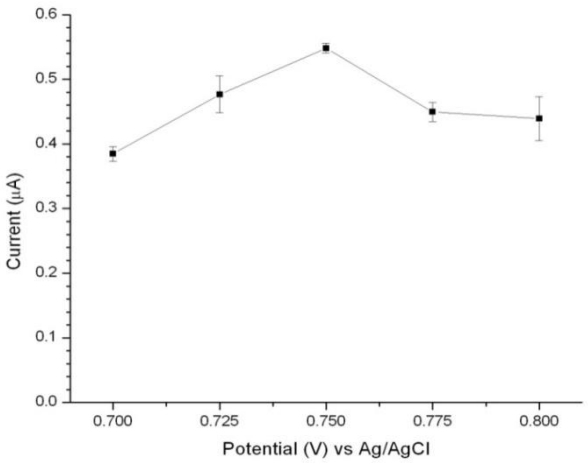

Figure 3. Dependence of the current of ALP/CPEs on the operating potential; stirred phosphate buffer solution, pH 8.5; 500 mg/L disodium phenyl phosphate

3.3 Influence of pH

The effect of the pH on the performance of the biosensor is of great importance, because the three-dimensional structure of the enzyme and the electrochemical response of the biosensor were depended on the different pH values. For the effect of pH, the biosensor responses with different pH value from 7.0 to 9.0 were investigated.

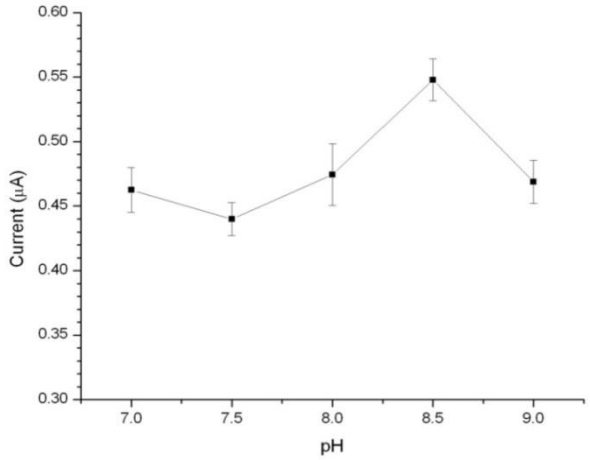

Figure 4. Effect of pH on the amperometric response of ALP/CPEs; phosphate buffer 0.1 M; 500 mg/L disodium phenyl phosphate; operating potential +0.75 V

Additionally the electrochemical reaction may be dependent on this parameter in case that hydronium ions are consumed or released in the course of the electron transfer process, which is valid indeed for the oxidation of phenol (Fig.2).

To optimize the measurement medium its pH was varied within 7.0 – 9.0 (Fig. 4). The highest activity was obtained at pH 8.5. Alkaline phosphatases show a pH-optimum *in vitro* between 7.3 and 9.2 depending on the source and also on the substrate concentration [29]; the bovine variety (used in this work) has an optimum at around pH 8.5 [30] which has been exactly found in our study. Therefore, this value which is also similar to experimental conditions described by Pingarron's group [31] was chosen for further investigations.

3.4 Enzyme inhibition-based biosensor

For the indirect determination of analytes by inhibition of an enzyme the substrate concentration is held constant, and the decrease of the response signal is monitored after addition of the inhibitor. This principle was applied to the determination of carbofuran with an ALP/CPE biosensor using disodium phenyl phosphate as a substrate (Fig.5). The response current drops when carbofuran is present in the test solution; the extent of inhibition is proportional to the concentration of carbofuran. The mechanism of the inhibition of the enzyme by carbofuran is its strong binding to zinc and magnesium which are essential cofactors of alkaline phosphatase close to its active center [32, 33]. Thus, the determination of carbofuran can be realized according to the inhibition degree of the enzyme. A typical chronoamperogram of an ALP/CPE biosensor for successive additions of the same amount of carbofuran under optimal experimental conditions can be seen in Figure 6. The anodic current decreases due to the presence of increasing amounts of the carbofuran causing inhibition of the activity of alkaline phosphatase immobilized on the CPE. In this work, the concentration of disodium phenyl phosphate solution was kept constant as 500 mg/L, whereas the concentration of the inhibitor was increased stepwise by adding defined amounts of carbofuran (Fig. 6). The relative inhibition, expressed in percents, was evaluated as $(I_0 - I) / I_0 \times 100$, where I_0 and I are the current response in the absence and in the presence of the inhibitor, respectively; its dependence on the inhibitor concentration is shown in Fig. 7. The dynamic range for the indirect method was evaluated from 10 to 97 µg/L carbofuran.

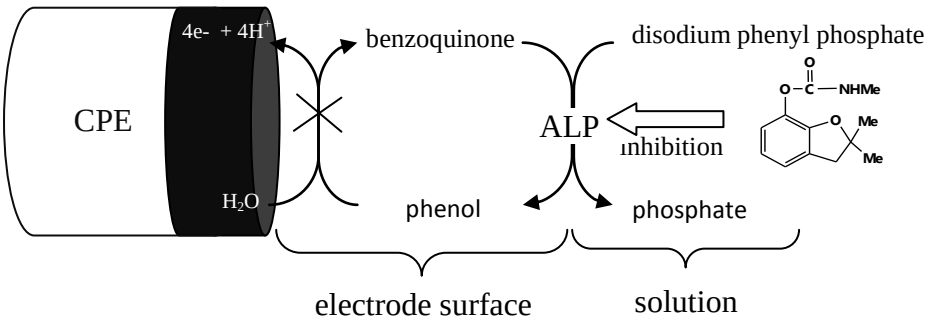

Figure 5. Inhibition of the ALP/CPE biosensor with carbofuran

Below 10 µg/L of carbofuran the calibration plot deviates from linearity with an estimated practical detection limit of about 5 µg/L. Above 100 µg/L the graph levels off (not shown). For 50 µg/L carbofuran the repeatability was enumerated as 2.32 % (n = 3 measurements). The reproducibility was determined as 3.38 % (n = 3 sensors).

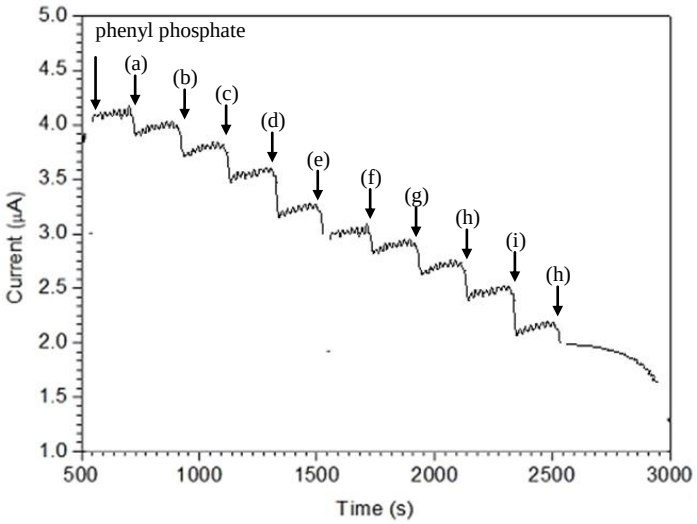

Figure 6. Amperometric response of a ALP/CPE to the addition of carbofuran; phosphate buffer (0.1 M, pH 8.5), 500 mg/L disodium phenyl phosphate; operation potential +0.75 V; carbofuran concentrations: (a) 10, (b) 20, (c) 30, (d) 40, (e) 50, (f) 60, (g) 70, (h) 80, (i) 90 and (h)100 µg/L

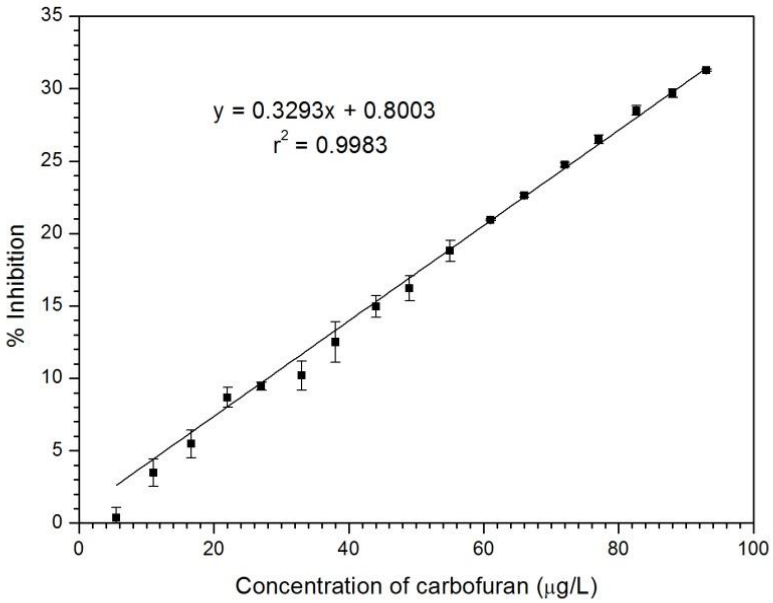

Figure 7. Relative inhibition of a ALP/CPE biosensor by carbofuran of concentration 5 - 97 µg/L; 0.1 M phosphate buffer solution pH 8.5 containing 500 mg/L disodium phenyl phosphate; operation potential at + 0.75 V; the data represent mean values and standard deviations of three measurements

3.5 Selectivity

Selectivity is an important aspect for the performance of an inhibition-based enzyme catalytic process. In this study fenobucarb and carbaryl (two other carbamate insecticide) as well as some metal ions were investigated on their possible interfering effect on the determination of carbofuran under the same experimental conditions. The results show that Fe(III), Cu(II), Cd(II), Mn(II), Pb(II) and Cr(VI) practically exert no inhibition on the enzyme even at concentrations of up to 100 mg/L. As expected carbaryl and fenobucarb which belong to the same chemical class of insecticides have a similar effect as carbofuran at even low concentration. Therefore the method is applicable to the determination of carbamate insecticides as a summation parameter.

3.6 Detection limit

The detection limit was evaluated as the lower end of the linear dynamic range of the sensor, i.e., as 10 µg/L carbofuran.

3.7 Stability

The long-term stability of the biosensor was monitored during storage at +4 ^{o}C in a refrigerator. The biosensor's response to 500 mg/L disodium phenyl phosphate as a function of the storage time yielded a loss in activity approximately 50 % during 2 weeks but was still useful for performing inhibition experiments of carbofuran.

The biosensor presented here shows high sensitivity combined with a low detection limit compared to previous works (LOD 0.02 mg/L) [25, 26], a wider linear range with otherwise similar result to the works of Mazzie et.al. and Thavarungkul et.al. [24, 25]. The biosensor described in this paper consumes less time for the measurement due to omittance of incubation [24-27]. We may conclude that these advantages could be achieved by the use of carbon nanopowder to produce the carbon paste rather than micro-particles. It may be expected that CNPPEs could improve the performance of many other analytical procedures employing conventional CPEs. Moreover, the biosensor presented here is easy to construct and operate, high precise and exhibits longer stability than ALP-biosensors described before.

3.8 Application of biosensor to samples

The biosensor based on ALP/CPE was applied to natural water as a sample matrix and chili samples. A concentration of about 10 mg/L carbofuran was spiked to the water sample (which did not show any inhibition of the biosensor without spiking), and the recovery was determined. The standard addition method was chosen for all sample measurements to exclude matrix effects. The results were compared with data obtained by spectrophotometric analysis (Table 1). The analysis of the statistically significant difference of the two techniques showed that the results obtained with the biosensor were in

satisfactory agreement with data from the reference method obtained at 95% confidence level using the t-test model. It can be seen from the results that the biosensor provides slightly lower concentrations than the reference method; nevertheless the obtained data underline that the method is applicable for a quick determination of carbofuran in chili pepper samples.

Table 1. Determination of carbofuran in spiked water and chilli samples with the biosensor and with spectrophotometric method

Sample	Biosensor	Spectrophotometry
Natural water	n.d.	-
Spiked natural water	9.18 ± 0.10 mg/L	-
Bird's eye chili	5.55 ± 0.07 mg/Kg	5.93 ± 0.14 mg/Kg
Yellow chili	5.52 ± 0.06 mg/Kg	5.62 ± 0.07 mg/Kg

n.d. = not detected

4. CONCLUSION

Inhibition of the enzymatic activity of alkaline phosphatase immobilized onto the surface of carbon nanopowder paste electrode was exploited for the determination of carbofuran. The data show that the performance of the biosensor in terms of detection limit, dynamic range, stability, precision is satisfactory facilitating qualitative and quantitative determination of carbofuran. The biosensor is inexpensive, simple to operate and shows short response time. The biosensor seems promising for being applied in environmental analysis.

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