Journal of Pharmacy and Pharmacology 6 (2018) 395-402
doi: 10.17265/2328-2150/2018.04.010

Analysis of Carbamates Pesticides: Immunogical Technique by Local Development of Enzyme-linked Immuno-Sorbent Assay

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Abstract: Carbamates insecticides are used increasingly in agriculture as a replacement for environmentally more persistent organochlorine insecticides for the control of insect pests. It was the most used pesticides in Loukkous-Morocco. Conventional methods employed to detect/analyze carbamates residues are time consuming and require sophisticated equipment only available in well-equipped laboratories. In addition, the conventional methods usually require a lot of complex pre-treatment of samples. Therefore, convenient and rapid pesticide detection system is urgently needed. In this order, we aimed to develop a rapid ELISA (enzyme-linked immuno-Sorbent Assay) for detection of some carbamates such as carbendazim [methyl 2-benzimidazole carbamate] and carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranylmethyl carbamate]. To develop an immunoassay for carbendazim and carbofuran we have synthesized molecules with acid function (haptens with 4 and 5 carbons) that are coupled with BSA protein and injected to the rabbits, collected antibodies are used for the achievements of the immunoanalytical assay.

Key words: Carbamates, polyclonal antibodies, ELISA.

1. Introduction

Pesticides are defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest [1]. They are used to protect agriculture and horticultural corps against damage and also used as domestic insecticides [2], excessive use of pesticides affects human's health, animals and environment. They have many different modes of action, but in general cause biochemical changes which interfere with normal cell functions.

The carbamate family of pesticides is registered for use on several crops in South American and European Countries, and in the USA. It was the most used pesticides in Loukkous-Morocco [3]. Its use for pest control has increased progressively in recent years, together with the OPs (organophosphorus), as alternatives to OC (organochlorine) insecticides. Owing to their broad spectrum of biological activity, carbamates can be used as insecticides, miticides, fungicides, nematocides, and molluscicides [4]. Some are suspected carcinogens and mutagens [5].

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranylmethyl carbamate) (Fig. 1) is a pesticide widely and effectively used to control insects, but it is a potent cholinesterase inhibitor and thus exhibits a high toxicity to human beings and wildlife. Because of its widespread use carbofuran residues are potentialair, soil, water and food pollutants [6, 7].

Carbendazim (methyl- 1H-benzo- [d]-imidazol-2-yl-carbanate) (Fig. 2)is used as fungicide to control a broad spectrum of diseases on arable corps, fruits and vegetables, usable also in post-harvest food storage and as a seed pre-planting treatment. Toxic effects of carbendazim in humans and animals have been reviewed [8].

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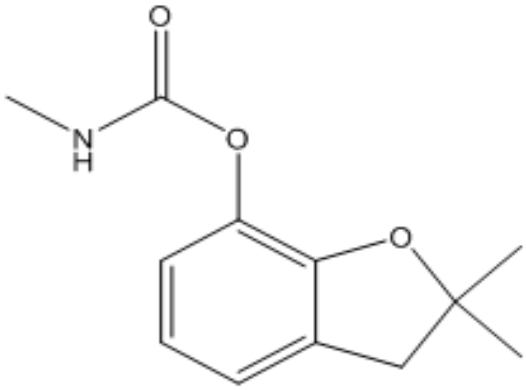

Fig. 1 Structure of carbofuran (2,3-dihydro-2, 2-dimethyl-7-benzofuranylmethyl carbamate).

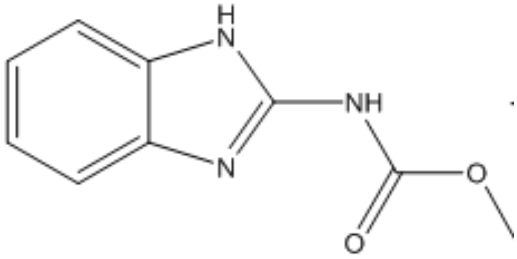

Fig. 2 Structure of carbendazim (methyl- 1H-benzo-[d]-imidazol-2-yl-carbanate).

Several analytical methods have been proposed for the separation and monitoring of these carbamates residues in food samples. The carbamate pesticides are thermally labile and not amenable to GC (gas chromatography): the use of LC (liquid chromatographic) techniques is preferable. One of the bottlenecks in the analysis of carbamates in food matrices is the time involved in classical sample preparation and this can limit the number of samples that can be analyzed. In addition, the amounts of chemicals and toxic solvents that are used often present a risk greater than that of the pesticide residue to be determined [9]. These disadvantages show clearly the need for developing fast, easy-to-use, robust, sensitive and cost-effective techniques that are suitable for field analysis. Instrumental techniques that combine some or several of these characteristics are slowly starting to appear for pesticide residue analysis. In this respect, we should mention IA (immunoassay) technique.

In this article, we present an overview of development of rapid ELISA (enzyme-linked immunosorbent assay) for detection of carbendazim and carbofuran.

The development of an immunoassay requires the production of antibodies to the analyte. Since, pesticides are small molecules, pesticide derivatives, namely haptens, must be synthesized and coupled to carrier proteins to induce anti- body production.

In this study we have synthesized molecules with acid function (haptens with 4 and 5 carbons) that are coupled with BSA protein and injected to the rabbits; collected antibodies are used for the achievements of the immunoanalytical assay. The developed ELISA method exhibited the potential to develop kits for a rapid detection of carbendazim and carbofuran for human health and environmental safety.

2. Material and Methods

2.1. Reagents Used

Carbamates pesticides including carbendazim and carbofuran were purchased from Sigma Aldrich.

All the other products (solvents, chemical and biological reagents) were obtained from Fluka, Sigma-Aldrich, Scharlau, or Reidel-de Haein.

2.2. Synthesis of Haptens

The synthesis of haptens used for immunization and antigen coating is presented in Figs. 3 and 4, the synthetic route for Haptens is the PTC (phase transfer catalysis) by the condensation between 5-bromovaleric acid or 4-bromobutanoic acid and carbendazim or carbofuran, this reaction is carried out in dry DMF (dimethylformamide) in the presence of carbonate of potassium and bromotetrabutylammonuim (as a catalyst).

- Haptens of carbendazim

To obtain haptens of carbendazim (Fig. 5) we used the following procedure:

In 30 mL of DMF, 7.5 mmoles of carbendazim, 8

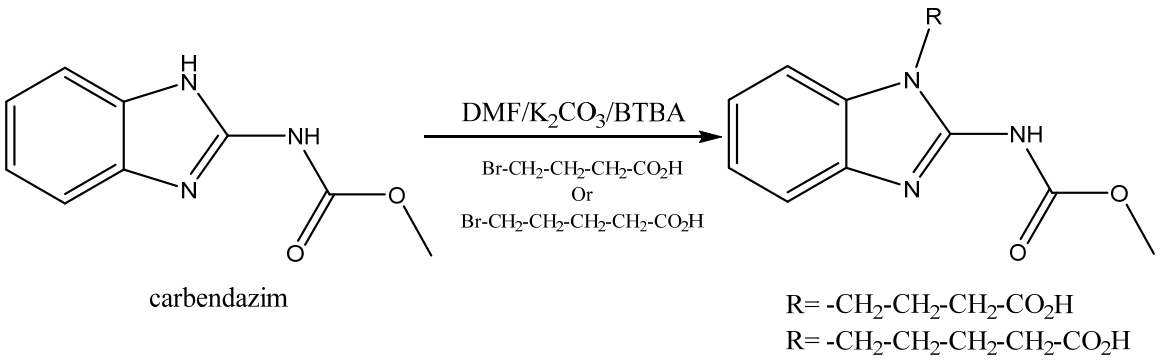

Fig. 3 Synthesis of carbendazim haptens.

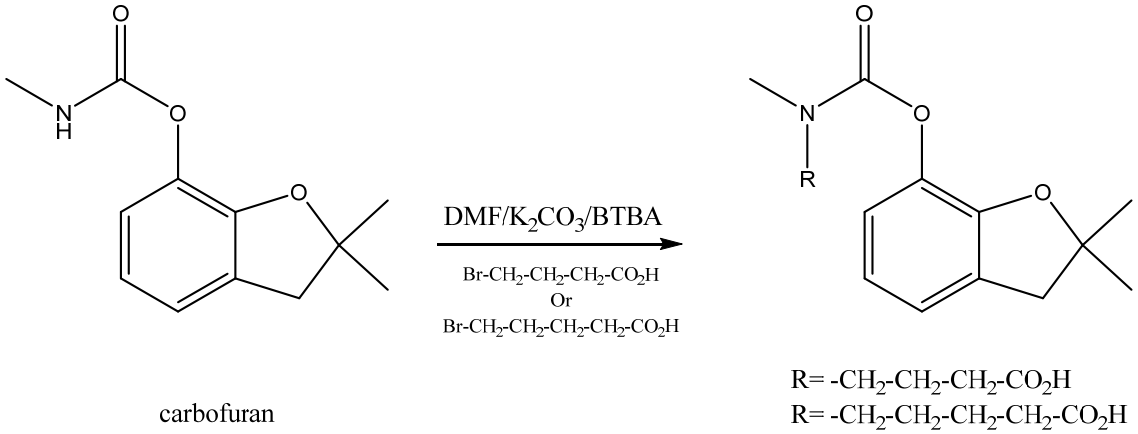

Fig. 4 Synthesis of carbofuranhaptens.

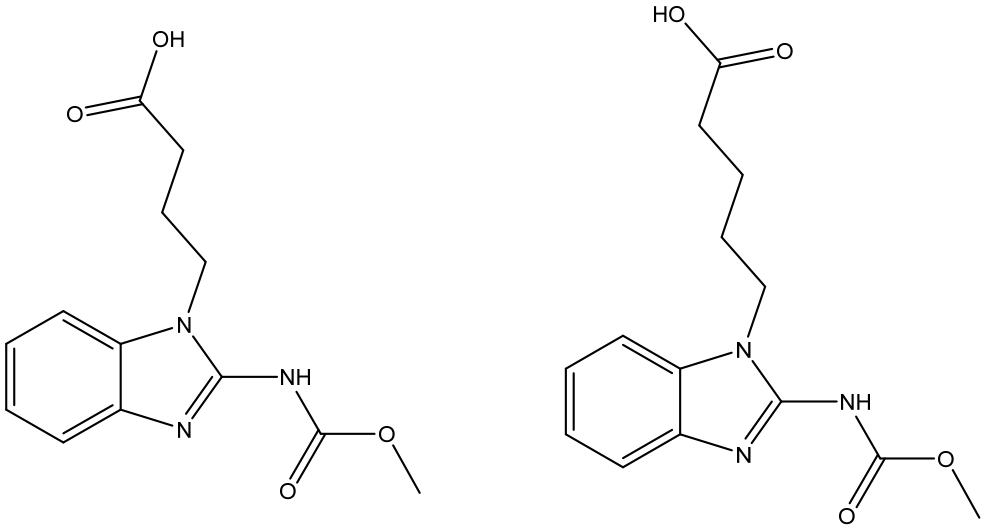

Fig. 5 Haptens of carbendazim: 4-(2-((methoxycarbonyl)amino)-1H-benzo[d]imidazol-1-yl) butanoic acid and5-(2-((methoxycarbonyl)amino)-1H-benzo[d]imidazol-1-yl) pentanoic acid.

mmole of potassium bicarbonate and 8 mmole of 5-bromopantanoic acid (or bromopentanoicacid) and the BTBA (few grains) bromotetre butylammonium are dissolved. The reaction mixture is left stirring for 1 hour at room temperature. After removal of the solvent under reduced pressure and purification by chromatography on silica gel (Eluent: Hexane/AcOEt 8/2) the product was recrystallized in ethanol to obtain

the final product.

- Haptensof carbofuran

To obtain haptens of carbofuran (Fig. 6) we used the following procedure:

To a solution of 2.26 mmol carbofuran in 20 mL of N, N-dimethylformamide DMF was dissolved 2.26 mmol of 4-bromovaleric acid or 5-bromovaleric acid, 5.6 mmol of potassium bicarbonate and some grain of BTBA. The mixture is stirred for two hours at room temperature, filtered to remove the salts, and then evaporated to remove the solvent; the aqueous phase is extracted with CH_2Cl_2. The organic phase is washed successively with a 10% hydrochloric acid solution and a $NaHCO_3$ solution, then dried over $MgSO_4$ and evaporated to dryness. Purification by chromatography on a silica column (eluent: Hexane/ethyl acetate 7/3) makes it possible to obtain the acid.

2.3. Preparation of Hapten-Protein Conjugates

Haptens of carbendazim and carbofuran spaced than 4 carbons were covalently attached to bovine serum albumin BSA to be used as immunogens.

The others haptens spaced than 5 carbons attached to HAS (human serum albumin) were used as the coating antigens for competitive assays. The method of conjugation used was the active ester method described by Aurora N. et al. [10] (The haptens were reacted with NHS (N-hydroxysuccinimide) and DCC to obtain active esters and reacted with proteins).

The ester-activated pesticide derivative prepared in solution is added to 8 mL of a solution containing (1.2 μmol) of (BSA or HAS) in sodium borate buffer (0.05 M, pH 8) and maintained in a solution in ice bath. The reaction mixture is then incubated overnight at 4 °C with stirring.

The "pesticide derivative-protein" conjugate obtained is dialyzed against 2l of 0.17 M TBS (tris-buffered saline) pH 7.5 overnight at 4 °C.

2.4. Immunization of Rabbits

We observed a trend in our test animals that female rabbits exhibited stronger specific antibody responses than males. For this reason, two females New Zealand white rabbits were immunized with haptens of carbendazim and carbofuran spaced than 4 carbons coupled to BSA following the short-term immunization protocol of Harlow and Lane [11]: 2 mg of immunogens and prepared in 500 μL of PBS are mixed with 500 μL of incomplete Freund's adjuvant. The mixture is stirred for 10 minutes (until a viscous white liquid is obtained) and then injected subcutaneously into each rabbit.

After 21 days, a second booster injection with 1 mg of immunogens in 1 mL of physiological saline (NaCl 0.09% w/v) is performed. Seven days later, 60 mL of blood are collected per rabbit.

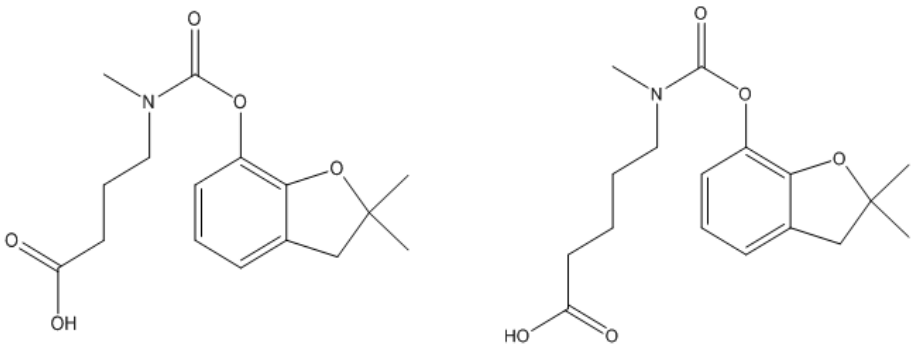

Fig. 6 Haptens of carbofuran 4-((((2,2-dimethyl-2,3-dihydrobenzofuran-7-yl) oxy) carbonyl)(methyl)amino) butanoic acid and 5-((((2,2-dimethyl-2,3-dihydrobenzofuran-7-yl)oxy)carbonyl)(methyl)amino)pentanoic acid.

The recovered blood is left to clot for 2 hours at room temperature and then overnight at 4 °C. Then the antiserum is recovered by centrifugation at 4,000 rpm for 15 min, placed in Falcon tubes labeled with 0.02% sodium azide and stored at -20 °C.

2.5. Dot Blot Test

Immunodot is a simple and effective assay for the detection of antibodies in serum.

The immunoblot is carried out as follows: On a nitrocellulose membrane, 1 μg of the pesticide coupled to HAS is deposited, 1 μg of BSA, 1 μg of HAS are also deposited as controls. The membrane is incubated in 3% (w/v) milk powder supplemented with 0.02% sodium azide overnight at 4 °C.

The membrane is then incubated in the presence of anti serum diluted at 1/500 in TBS with stirring overnight at 4 °C. After 4 washes with TBST (TBS containing 0.05% Tween 20 (v/v)), the membrane was incubated a second time with the secondary antibody coupled to peroxidase at 1:1,000 dilution in TBST for 1 h. Then, the membrane was washed 4 times with TBST and once with TBS before revealing it in the dark in a solution composed of 12 mg of 4-chloro-1-naphthol in 4 mL of methanol, 16 mL of TBS and 100 μL of 30% H_2O_2. As soon as dark spots appear, the reaction is stopped by washing with running water then the membrane is dried between two Wattman papers.

2.6. Competitive Indirect Enzyme-Linked Immunosorbent Assay

To develop an antigen coated (indirect) ELISA, antigen coating and antibody concentration for competitions assays were optimized. Microtiterplates were coated with haptens–HAS (100-10,000 mg/ml, 100 μL/well) in carbonate buffer (50 Mm, pH 9.6) by overnight incubation at 4 °C. The plates were washed fore time with PBS pH 7.4 and were blocked by incubation with milk 3% on PBS (300 μL/well) for overnight at 4 °C. After another washing step, 100 μL/well of antisera previously diluted with PBS (1/1,000 to 1/10,000) was added. After incubation at room temperature for 2 h, the plates were washed five times with PBST (PBS containing 0.05% of Tween 20) and 100 μL/well of a diluted (1/2,000) goat anti-rabbits IgG-horseradish peroxidase was added. The mixture was incubated at room temperature for 1 h and the plates were washed for time with PBST and with additional wash with PBS, then 100 μL of a p-nitrophenyl phosphate OPD (1 mg/mL) dissolved in 50 mL of citrate acetate buffer pH 5.5 containing 1% H_2O_2 were added at room temperature.

The reaction was stopped after 10 min by adding 50 μL of 3 M HCl and absorbance was read at 492 nm.

The procedure for the competition assay was follows. The microtiter plats coated and blocked with milk as described above, 50 μL/well of serial dilutions of the analyte in PBS was added, flowed by 50 μL/well of a previously determined antiserum dilution. After incubation at room temperature for 2 h antibody binding was assessed as described above. Competitive curves were obtained by plotting absorbance against the logarithm oh analyte concentration. Sigmoidal curves were fitted to a four-parameterlogistic equation [12] from which I_{50} values (concentration at witch binding of the antibody to the coating antigen in inhibited by 50%) are determined.

3. Results and Discussion

The detection of low-molecular-mass analytes (haptens) such as pesticides in solution must employ competitive ELISA formats. This method employs an immobilized hapten-carrier conjugate on the solid phase to which analyte and antibody are added. Antibody binds to the free analyte or to the immobilized hapten in certain ration of the reactants concentration. A secondary labeled antibody may be used to detect the bound antibody. In these competitive immunoassay formats, the signal is inversely proportional to the amount of free analyte in the sample.

3.1 Synthesis Result

Challenging step in pesticide immunoanalysis is the development of specific antibodies, since most pesticides are small molecules (haptens) that should be conjugated through a suitable active chemical group (COOH; -NH_2, -OH, -SH, etc.) to a carrier protein in order to elicit an immune response [13, 14]. In our study, we use a spacer arm consisting of a single hydrocarbon chain not too long or too short between two and five carbon atoms [15] (4 and 5 carbons containing an acid), the role of this spacer arm is to distance the hapten from the protein to ensure a better presentation also, it must not have functional groups recognizable by antibodies.

The haptens: 4-(2-((methoxycarbonyl)amino)-1H-benzo[d]imidazol-1-yl)butanoicacid, 5-(2-((methoxycarbonyl)amino)-1H-benzo[d]imidazol-1-yl) pentanoicacid, 4-((((2,2-dimethyl-2,3-dihydrobenzofuran-7-yl)oxy)carbonyl)(methyl)amino) butanoic acid and 5-((((2,2-dimethyl-2,3-dihydrobenzofuran-7-yl)oxy)carbonyl)(methyl)amino) pentanoic acid were synthesized by following the protocol cited before (Figs. 3 and 4). We obtained yellow and white powder, their NMR (nuclear magnetic resonance) spectra were further confirmed by Proton-NMR (1H NMR) and carbon NMR (13C NMR) using $CDCl_3$as the solvent.

3.2 Screening of Antisera

Female New Zealand white rabbits were immunized by the haptens coupled to BSA. The presence of carbofuran antibodies was checked by dot blotting. Antisera obtained from a rabbit injected with carbofuran-BSA (Spaced than 4 carbons) recognized carbofuran -HAS (Spaced than 5carbons) banded in nitrocellulose membrane (Fig. 7). It is the same for antisera anti-carbendazim.

Various dilutions of antisera were titrated against variety amounts of the coating antigens (carbofuran-HAS, carbendazim-HAS) were used to measure reactivity of antibodies and to select appropriate concentrations of coating antigens and antibodies.

For indirect competitive assay the optimal concentrations of antigens coating were 65 ng/well and 100 ng/well for carbofuran and carbendazim respectively. The optimal dilutions of antisera were 1/7,000 and 1/2,000 for carbofuran and carbendazim respectively.

3.3 Indirect ELISA

In recent years, the number of ELISA methods for determination of small molecules has increased, but there is still a lack of these methods for pesticide residue determination. ELISA can provide complementary and/or alternative approaches to reducing analysis time and the use of costly, sophisticated equipment, whilst still maintaining reliability and sensitivity. Direct ELISA tests for carbofuran using monoclonal antibodies were developed by Yang et al. [16]. We aim to develop a local indirect ELISA of these pesticides using polyclonal antibodies in rabbits, since they are inexpensive and quick to produce.

Fig. 8 shows a typical inhibition curve obtained after optimization of coated antigen and dilution of antiserum for carbofuran and carbendazim. The I_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) and the detection limits are determined. The I_{50} value of carbofuran was 20.2 ng/mL, with detection limit about

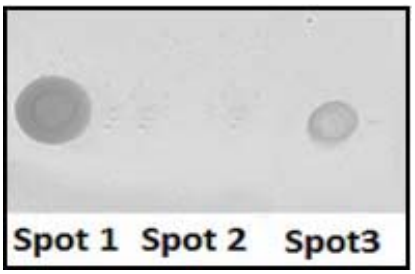

Fig. 7 Dot blot analysis using a produced antiserum (anti carbofuran-BSA): Spot 1: BSA, Spot 2: HAS, Spot 3: Hapten-5 carbons-HAS. Carbofuran-HAS were immunostained.

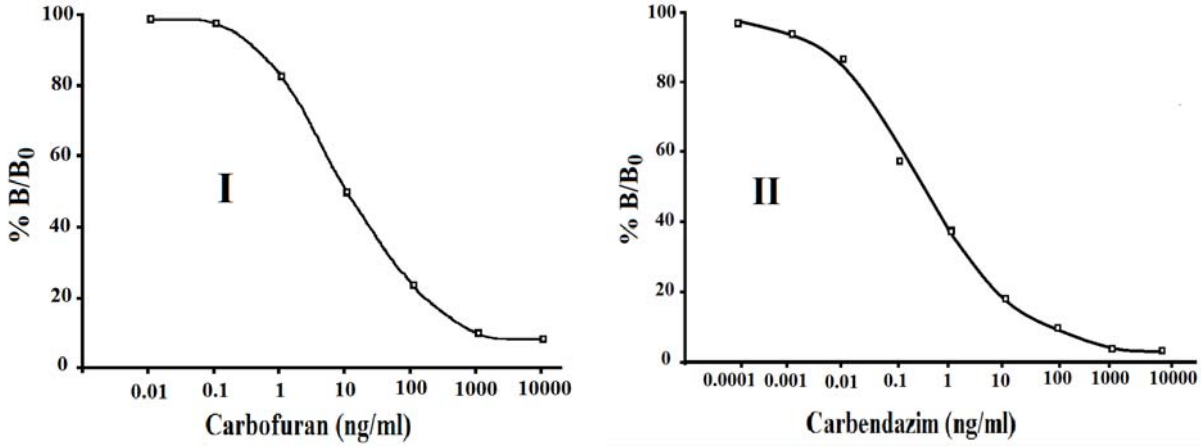

Fig. 8 Incubation curve by indirect competitive ELISA. I: Carbofuran using rabbit antiserum anti-pesticide-BSA diluted at 1/7,000 the coating antigen hapten-HAS (65 ng/well). II: carbendazim using rabbit antiserum anti-pesticide-BSA diluted at 1/2,000 the coating antigen hapten-HAS (100 ng/well). $\%B/B_0 = (A-A_{xs}/A_0-A_{xs}) \times 100$, where A is the absorbance, A_0 is the absorbance at zero dose of the analyte, and A_{xs} is the absorbance at an excess of the analyte.

0.19 ng/mL. This level of sensitivity is comparable with those observed in direct ELISA using monoclonal antibodies for carbofuran (I_{50} value and detection limit 18.49 ng/mL and 0.11 ng/mL) [16]. I_{50} and detection limit for carbendazim were 0.63 ng/mL and 0.026 ng/mL respectively.

4. Conclusions

We have succeeded to product anti-carbofuran and anti-carbendazim antibodies, and their reactivity was tested against haptens coupled to HAS spaced from 5 carbons. We have developed a conjugate-coated indirect competitive ELISA for detecting carbofuran and carbendazime.

The established ICA method did not require intensive work or expensive equipment could provide an alternative tool for a very high sensitivity of detection, fast and convenient pesticides carbamates.

We are currently testing the cross reactivity of anti-carbofuran and anti carbendazim with other carbamates pesticides. Then, the validation of the developed ELISA and correlation with others classical assays (HPLC, GC/MS ...) well be studied to ensure its reliability.

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