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Hydrophobic nanoprecipitates formed by benzoylphenylureas and β-cyclodextrin inclusion compounds: synthesis, characterization and toxicity against *aedes aegypti* larvae

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ABSTRACT

The aim of this work was to synthesize and characterize the inclusion compounds formed by the complexation of β-cyclodextrin (βCD) with insecticides from the class of benzoylphenylureas (BPUs), named novaluron (NOV) and diflubenzuron (DIF), beyond evaluate their larvicidal activity against *Aedes aegypti* larvae. Solid state characterization by FTIR showed changes in the main peaks of BPUs and βCD, suggesting the formation of inclusion compounds in solid phase. DTA and TGA thermal analysis showed changes in temperatures of BPUs decomposition as result of molecular interactions. ^{1}H NMR experiments allowed to observe the occurrence of interactions in solution through changes in chemical shifts of BPUs aromatic hydrogens. However, the presence of H–H intermolecular correlations in 2D ROESY was found only for the DIF/βCD complex, suggesting different topology for each complex. Such hypothesis was corroborated by thermodynamic analysis using ITC, which showed different profile of titration curves, beyond endothermic and exothermic interactions for NOV/βCD and DIF/βCD complexes, respectively. DLS titrations of BPUs or BPUs/βCD DMSO solutions in aqueous solution demonstrated that the spontaneously formed hydrophobic nanoprecipitates (HNPs) have different profile of sizes depending on the BPU/βCD system, corroborating also with the hypothesis about the existence of different topologies for each complex. Finally, the HNPs of inclusion compounds showed to be more efficient than free BPUs, allowing proposing a new insecticide formulation.

1. Introduction

Benzoylphenylureas (BPUs) is an important class of low molecular weight insecticides, which are used to combat several arthropods, such as mosquitoes, cockroaches, fleas, ants and termites in wide world, including the *Aedes aegypti*, the vector of diseases as yellow fever, dengue, chikungunya and zika [1, 2, 3, 4].

These compounds belong to the group of insect growth regulators (IGRs), whose the mechanism of action is based on the inhibition of chitin synthesis during the insect larval stages, causing feeding difficulty, malformation (of pupae and adults), and complete or partial inhibition of molt [5]. Moreover, they are more selective than classical insecticides once they act on structures that do not exist in vertebrates, reducing thereby their toxicity to mammals [1]. The absorption of these compounds by the insect occurs mainly through ingestion. However, absorption cases by direct contact with the integument have been reported [6].

Among the main known BPUs (Fig. 1), the diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea) was the first one commercially used in agriculture for the control of flies in veterinary [1], with subsequent use

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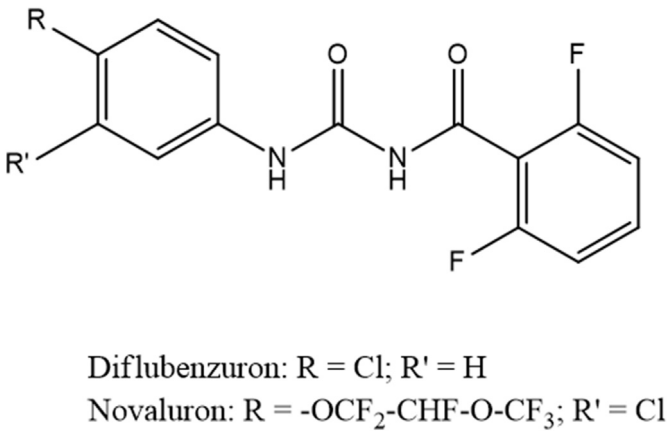

Diflubenzuron: R = Cl; R' = H
Novaluron: R = -OCF_2-CHF-O-CF_3; R' = Cl

Fig. 1. Structural formulas of the BPUs.

against the *Aedes aegypti*. After that, novaluron, its most potent analog ((±)-1-[3-chloro-4-(1,1,2-trifluoro-2-trifluoromethoxyethoxy) phenyl]-3-(2,6-difluorobenzoyl)urea), was recommended jointly with difubenzuron by the World Health Organization (WHO) as larvicidal, including the indication of use in drinking water [7, 8].

Some studies have demonstrated that IGRs are effective even in the control of vectors with a recognized resistance to conventional insecticides, due to their differential action mechanism. For this reason, BPUs have been widely used in Brazilian regions where the resistance to the organophosphate insecticides was detected [9, 10].

Recently, Benilato (2015) showed that upon the treating of successive *Aedes aegypti* generations with a predetermined dosages of diflubenzuron, it was identified a resistance development to this compound [11]. This result justifies the need for constant evaluation of the insecticides advocated by the WHO, as well as the need of continuous search for improvement of existing insecticides.

From the perspective of development of new formulations, the strategy of preparing controlled release systems for environmental purposes, via host/guest interactions, have been proposed [12]. In this context, complexation of insecticides with cyclodextrins (CDs) could be an important strategy to improvement of current formulations [13, 14, 15].

CDs (Fig. 2) are cyclic oligosaccharides composed usually of six, seven or eight units of glucopyranoses linked by α-1,4-type glycosidic bonds, being called of α-cyclodextrins (αCD), β-cyclodextrin (βCD) and γ-cyclodextrin (γCD), respectively (Fig. 2). As result of their conformations, such compounds acquire the truncated cone shape, with a central cavity that allows to accommodate hydrophobic portions of the guest molecules.

Among the natural CDs, βCD is the most used in formulations due to the diameter of its cavity (≈6.5 Å) and its best cost-benefit ratio when compared to other commercially available ones [16]. The formation of the complex between βCD and the guest molecule (inclusion compound) can cause changes in the physicochemical properties of the guest molecules (specially solubility, stability and reactivity), affecting their responses in biological systems [17, 18, 19].

Thus, aiming the developing of new formulations to overcome the problems about *A. aegypti* resistance against conventional insecticides, in this work we have synthetized and characterized the inclusion compounds formed between βCD and the benzoylphenylureas DIF and NOV, aiming to improve their activity against *Aedes aegypti* larvaes. Concerning the low aqueous solubility of these BPUs, even in presence of βCD, additionally we have invoked the simple concept of hydrophobic nanoprecipitates (HNP) [13, 20, 21] by using an organic co-solvent (DMSO) to promote the initial solubilization of compounds and subsequent precipitation of nanostructured material by mixture with water.

Initially, the inclusion compounds were synthetized by co-precipitation method followed by lyophilization. The stability of the compounds were investigated by thermogravimetric and differential thermal analysis (TGA and DTA) while the topology of the complex in solid state and in solution were evaluated by FTIR and NMR (^{1}H and 2D-ROESY) respectively. The stoichiometry of the complexes and thermodynamic parameters of complexation were determined by Isothermal titration calorimetry (ITC). The HNPs, prepared by mixture of DMSO solution of BPUs or their respective inclusion compounds in water were characterized by DLS measurements. Finally, the larvicidal activity of HNPs formed by BPUs or their respective inclusion compounds were evaluated against 4th instar larvae and emergence inhibition of *Aedes aegypti* mosquitoes.

2. Experimental

2.1. Materials

Novaluron (purity 95.20%) and diflubenzuron (purity 96.00%) were respectively extracted and purified from the commercial formulations (Mosquilon®-Bayer) and (Dimilin®-Chemtura). The purity of these products was determined by HPLC comparing with novaluron and diflubenzuron standards purchased from Sigma-Aldrich®. The βCD was purchased from Sigma-Aldrich® (St. Louis, MO, EUA). All other chemical reagents were purchased from reputable companies (Vetec, Synth and J.T. Baker) and used as received. During all experiments, Milli-Q® water was used.

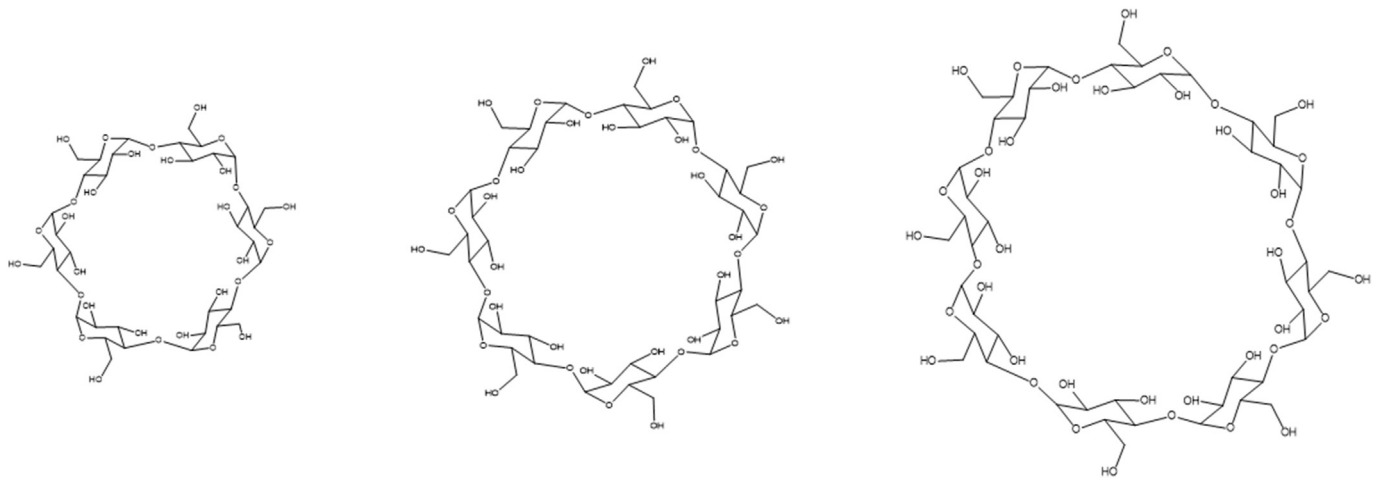

Fig. 2. Structural formulas of the natural cyclodextrins.

2.2. Preparation of the inclusion compounds

The inclusion compounds between BPUs and βCD were prepared by co-precipitation method followed by liophilization. Briefly, equimolar amounts of the host molecule (DIF or NOV) and βCD were separately solubilized in ethanol and Milli-Q water, respectively. This proportion was chosen after the determination of stoichiometry by ITC experiment, as showed below in section 2.4.2.

The ethanolic solution of the host molecule was poured into the aqueous βCD solution and submitted to stirring during 24 h. Finally, the suspension formed was placed in rotary evaporator to remove the ethanol, and then submitted to the freeze-drying process to obtain the desired inclusion compound (NOV/βCD or DIF/βCD).

2.3. Preparation of the hydrophobic nanoprecipitates

HNPs were prepared by dissolution of appropriate amounts of NOV, DIF, NOV/βCD or DIF/βCD in DMSO (as cossolvent) to promote the initial solubilization of compounds. Further, these solutions were mixed in water and subsequent precipitation of nanostructured material were spontaneously observed. These compounds were used in bioassays against *A. aegypti*.

2.4. Physical-chemical characterization

2.4.1. Solid-state characterization

The FTIR spectra of DIF, NOV, βCD, NOV/βCD, DIF/βCD, and physical mixtures (PM) at molar ratio of 1:1 were obtained using a Perkin Elmer Fourier transform spectrometer (Spectrum Two™ model). The spectra in KBr discs were recorded in range from 4000 to 400 cm^{-1} with a resolution of 2 cm^{-1} as the mean of 16 consecutive scans. The Perkin Elmer Spectrum ES program (application version: 10.03.08.0133) was used for the spectra acquisition and processing.

Differential thermal analysis (DTA) and thermogravimetric analysis (TGA) for DIF, NOV, βCD, DIF/βCD, NOV/βCD and physical mixtures (PM) at molar ratio of 1:1 were performed using a simultaneous TGA/DTA HITACHI-STA 7200 RV thermoanalytical module. The analytical conditions were: air atmosphere at 300 mL/min, with a temperature ramp of 35 °C–800 °C, a heating rate of 10 °C/min, and mass of ≈3.0 mg of the samples in a platinum crucible. As reference, the empty platinum crucible was used. The data were exported to Microcal Origin 9.0 for editing.

2.4.2. Isothermal Titration Calorimetry (ITC)

The thermodynamic parameters involved in the inclusion compounds formation between guest molecules and βCD in solution were determined using isothermal calorimetric titration at 25 °C. The titrant consisted of a DMSO:H_2O (9:1 v/v) solution of DIF or NOV at 30.0 mM, while the titrand consisted of a DMSO:H_2O (9:1 v/v) solution of βCD at 2.0 mM. The blank experiment consisted of DIF or NOV solutions in the solvent (DMSO:H_2O, 9:1 v/v solution) titration. The experiments were performed using a microcalorimeter VP-ITC (Microcal Company, Northampton, MA, USA). The following experimental parameters were used: 300 rpm rotation; 51 automatic 5.0 μL injections of titrant in 1.5 mL of the titrand; 2s injection time; and 5 min equilibration time. The first injection of 1.0 μL was discarded in order to eliminate the diffusion material effects from the syringe to the cell and *vice-versa*. After the experiments, the titration curves were subtracted from the respective blanks in order to mathematically eliminate the interaction effects of the guest molecules with the solvent. The data were treated using Microcal Origin 7.0 program for ITC.

2.4.3. Nuclear magnetic resonance (NMR)

NMR spectra were recorded at 27.0 °C on a Bruker DRX 400 - AVANCE spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 400 MHz, equipped with a 5 mm inverse probe with a z-gradient coil. ^1H NMR spectra were achieved using the WATERGATE technique for suppression of the residual water signal. The nuclear Overhauser enhancement spectroscopy (2D NOESY) experiments (mixing time 500 ms) were acquired using standard experiments from the spectrometer library. All NMR experiments were performed in the Laboratòrio de Ressonância Magnética Nuclear de Alta Resolução - LAREMAR - DQ/ICEx-UFMG.

2.4.4. Size determination of HNPs by dynamic light scattering (DLS)

DLS experiments were performed in a Malvern Zetasizer Nano ZS particle analyzer, using polyethylene square cells to measure the average hydrodynamic diameter (D_h) of NOV, DIF, NOV/βCD and DIF/βCD hydrophobic nanoprecipitates spontaneously formed by the mixture of their DMSO solutions in water. Initially, it was prepared solutions containing 1.0 mg of NOV, DIF or equimolar amounts of NOV/βCD or DIF/βCD in 0.5 mL of DMSO. Then, 25 injections of 20.0 μL of these solutions were titrated in 2.0 mL of Milli-Q water, with subsequent measurements of the HNPs hydrodynamic diameter by DLS. These samples were submitted to a monochromatic light (4 mW He–Ne laser, wavelength 633 nm) and the scattered light intensity was measured at an angle of 90°. The D_h were determined by the average of five independent measurements, each of them obtained as the mean of 10 counts, with intervals of 2 s each.

2.5. Biological analyzes

2.5.1. Bioassays involving larvae of the Aedes aegypti mosquito

The *Aedes aegypti* (PPCampos strains) eggs were donated by the Department of General Biology from Federal University of Viçosa, MG - Brazil. They were placed in plastic basins containing 2L of dechlorinated water, with a temperature of 26 ± 2 °C, RH> 70% and photoperiod of 12L:12D. After 24 h, the 1st instar larvae (L1) were transferred to another basin with 2 L of dechlorinated water and approximately 118 mg of food (Alcon GoldFish Colour Bits). Thereafter, the water was changed daily by replenishing the food until individuals reached the stage of development required for each test, 3rd instar larvae (L3) for Emergency inhibition assay or 4th instar larvae (L4).

The larvicidal activity of the obtained HNPs described in section 2.3, on 4th instar larvae, was evaluated using the method recommended by the World Health Organization with small modifications [22]. Briefly, twenty 4th instar larvae were added to a beaker (250 mL) together with 50 mL of dechlorinated water and food. Thereon, 50 mL of each sample stock solution was added to the beaker so that suspension reached the desired concentration of the compound and 1% DMSO in volume. The nominal concentrations used in experiments for each HNP were 10^2, 10^1, 10^0, 10^{-1}, 10^{-2} and 10^{-3} μmol/L.

Mortality of the larvae was determined after 24 h and 48 h of incubation at 26 ± 2 °C and RH > 70%. The larvae were considered dead when they did not demonstrate any response to the external stimulus. Three replicates were performed in three independent experiments. The mortality percentage curves *versus* the compound concentration logarithm were constructed using the GraphPad Prism 5.0 software. The lethal concentration value (LC_{50}) at 24 h and 48 h was calculated by Probit algorithm using the same software.

The emergency inhibition of *Aedes aegypti* mosquitoes in presence of HNPs of DIF, NOV, βCD, DIF/βCD and NOV/βCD was evaluated also using a method recommended by the World Health Organization with small modifications [22]. In this experiment, twenty 3rd instar larvae were added to a beaker (250 mL) together with 50 mL of dechlorinated water and food. Then, 50 mL of each sample stock solution was added to the beaker in order to obtain the desired concentration of the compound (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} μmol/L) and 1% of DMSO in volume. The mortality of the insects was verified at intervals of every 12 hours until the emergence of all adults in the control (nearly 10 days after the experiment beginning) [22].

During the experiment, the information about the development stage of insects were also collected. Three replicates were performed in three

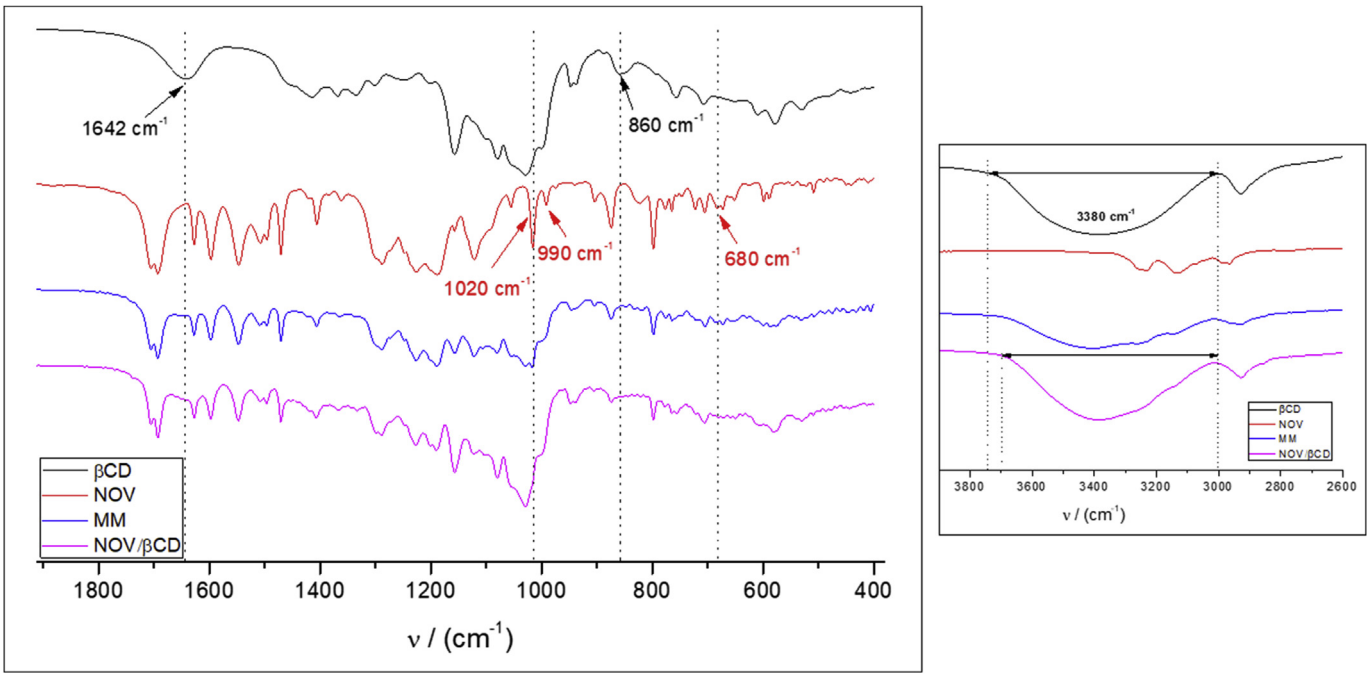

Fig. 3. Infrared spectra of NOV, βCD, NOV/βCD and PM. Spectra were recorded at the 4000-400 cm^{-1} range, in transmittance mode, using KBr as support.

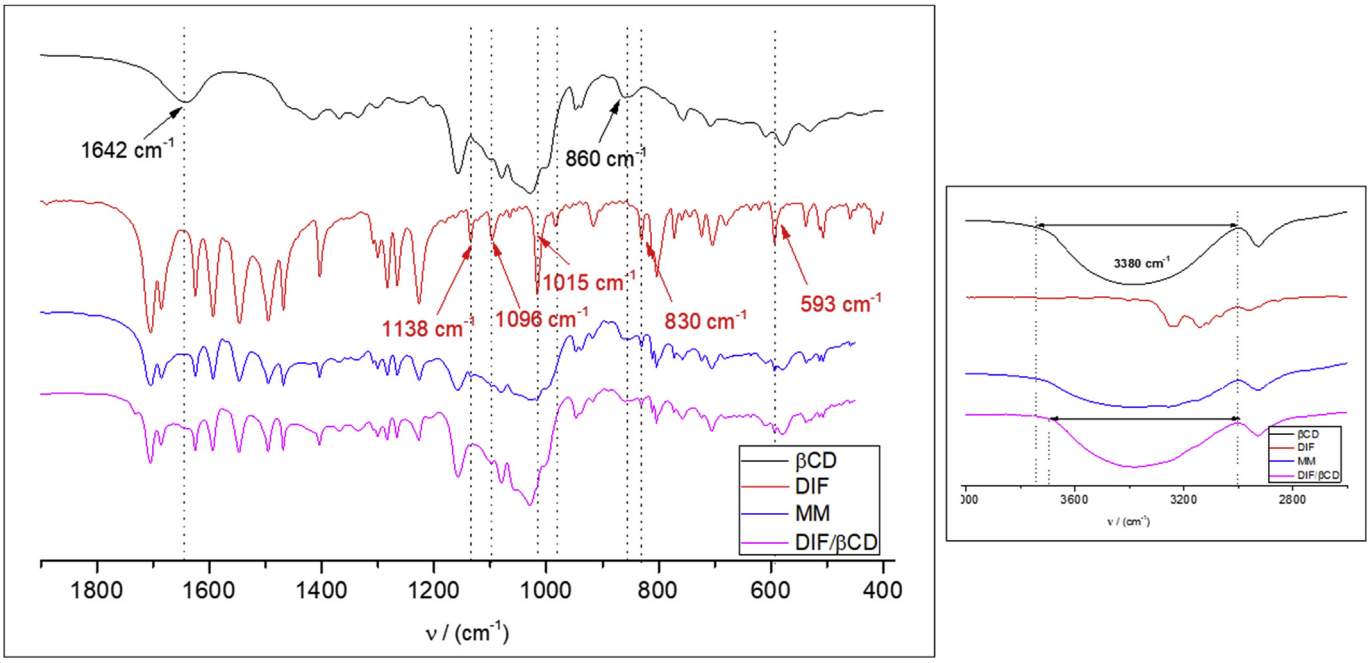

Fig. 4. Infrared spectra of DIF, βCD, DIF/βCD and PM. Spectra were recorded at the 4000-400 cm^{-1} range, in transmittance mode, using KBr as support.

independent experiments. The plot of mortality percentage *versus* experiment time and the curves of mortality percentage *versus* compound concentration logarithm were constructed using the GraphPad Prism 5.0 software. The results on last day of experiment were used to calculate the emergency inhibition to 50% of the population, IE_{50}.

3. Results and discussion

3.1. Characterization of the inclusion compounds

3.1.1. Solid-state characterization

The infrared spectra for DIF, NOV, βCD, DIF/βCD, NOV/βCD and physical mixtures (PM) at molar ratio of 1:1, in the 4000-400 cm^{-1} range, are shown in Figs. 3 and 4. In the βCD spectrum, bands centered at 3371, 1650, 1158, 1082, 1028 and 942 cm^{-1} attributable to ν(O–H), ν(C–H), δ(O–H), ν(C–C) and the skeletal vibration involving (α-1,4 linkage), respectively, were observed. These data are similar to those previously described in literature [23].

Due to the molecular similarities between NOV and DIF, both compounds showed FTIR bands or peaks at the same range: 3230 and 3120 cm^{-1}, attributed to the stretching of N–H bonds; 1706 and 1692 cm^{-1}, attributed to the stretching of C=O bonds; 1600-1400 cm^{-1}, attributed to vibrations of aromatic rings; and at 800-600 cm^{-1}, attributed to characteristics of the C–Cl bonds. The main difference between the two

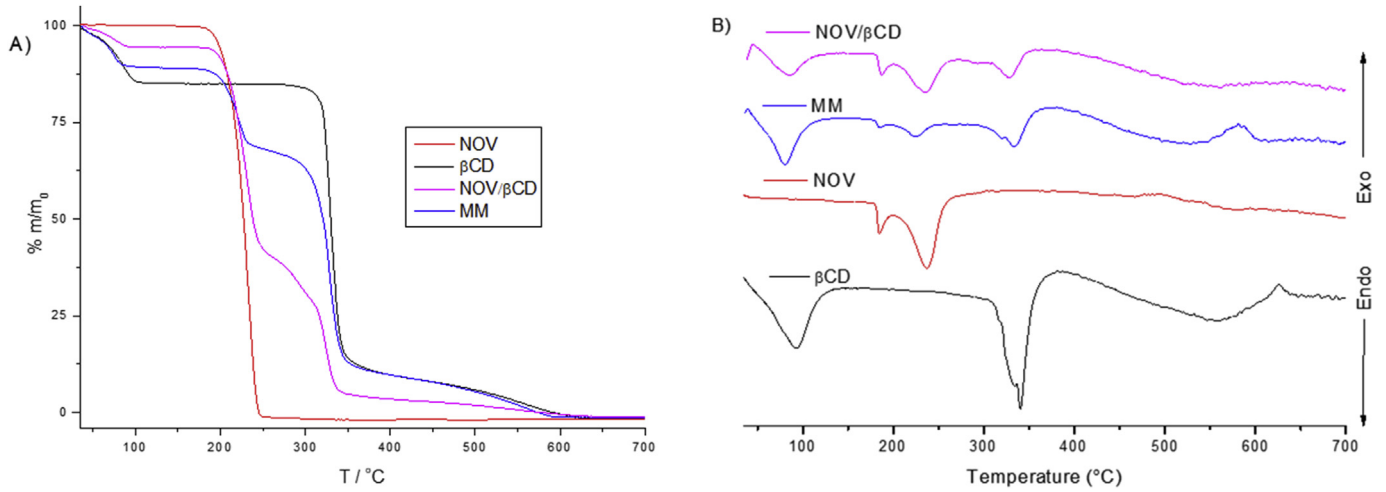

Fig. 5. A) TGA and B) DTA curves for NOV, βCD, NOV/βCD and PM.

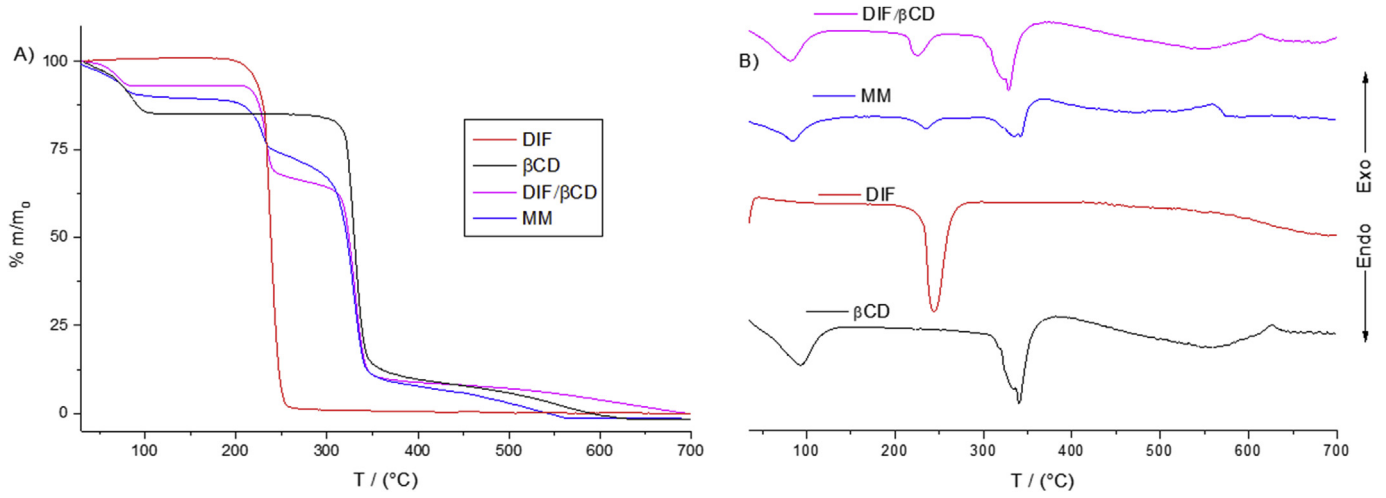

Fig. 6. A) TGA and B) DTA curves for DIF, βCD, DIF/βCD and PM.

spectra is in the range of 1400–1000 cm^{-1}, attributed to vibrations of CF_2 and CF_3 groups present only in the NOV molecule.

In the physical mixture's spectra, no change in the positions of the main bands was observed in relation to free benzoylphenylureas or free βCD. Moreover, the observed profile can be ascribed as the overlapping of bands of BPUs and βCD.

In the FTIR spectra of the inclusion compounds, significant changes in the shape and position of the vibrational bands were observed which in turn did not superimpose with the FTIR spectra of free species or their PMs. For the NOV/βCD and DIF/βCD complexes, the OH bands of βCD at 3386 cm^{-1} were sharper if compared with the free βCD, as result of βCD-βCD hydrogen bonds breakdown in solid state, after formation of new interactions. Moreover, it was observed disappearance of ν(C–H) and δ(C–H) bands of βCD, at 1650 and 860 cm^{-1}, confirming the existence of new vibrational profile for the cyclodextrins after interactions.

Angular deformations disappearance of aromatic C–F, C–H and C–Cl bonds of NOV, at around 1020, 990 and 680 cm^{-1} respectively suggest interactions of aromatic groups with the cavity of βCD. Other NOV or DIF bands, at 796, 778, 766, 722, 595, 539 and 500 cm^{-1}, had reduced or missed their intensities (Fig. 3). For the DIF/βCD system, it was observed reduction or disappearance of ν(C–N) peaks at 1138 and 1096 cm^{-1}, ν(C–F) at 1015 cm^{-1}, δ(C–H) at 830 and ν(C–Cl) at 593 cm^{-1} (Fig. 4).

Figures 5A-B and 6A-B show the TGA and DTA curves for NOV, DIF, βCD, DIF/βCD, NOV/βCD and their physical mixtures (PM) at molar ratio of 1:1.

Transitions observed for free βCD are consistent with those previously described in literature [13, 24].

In TGA curves of NOV and DIF, a single loss of mass event was observed in the range from 170 °C to 245 °C, which has been attributed to the one step decomposition process. The two curves similarity was result from the structural similarity. On the other hand, DTA curves showed some differences. For NOV, it was possible observe two endothermic peaks, being the first one attributed to its fusion, while the second one, attributed to its decomposition. For DIF, only a broad endothermic event, ascribed as the simultaneous fusion and decomposition of compound, has been observed.

In the TGA and DTA curves of PMs, there are four events related to βCD and benzoylphenylureas, with low differences if compared with free compounds. However, the results for NOV/βCD and DIF/βCD inclusion compounds showed greater differences in thermal profiles. The maximum of decomposition peak was reduced from 237 °C to 224 °C and from 244 °C to 235 °C for NOV and DIF respectively. Moreover, the fusion decomposition peaks for both insecticides, as well as the decomposition event of βCD, were all attenuated in the complexes, suggesting the amorphization of solid phase resulting from the formation of new interactions.

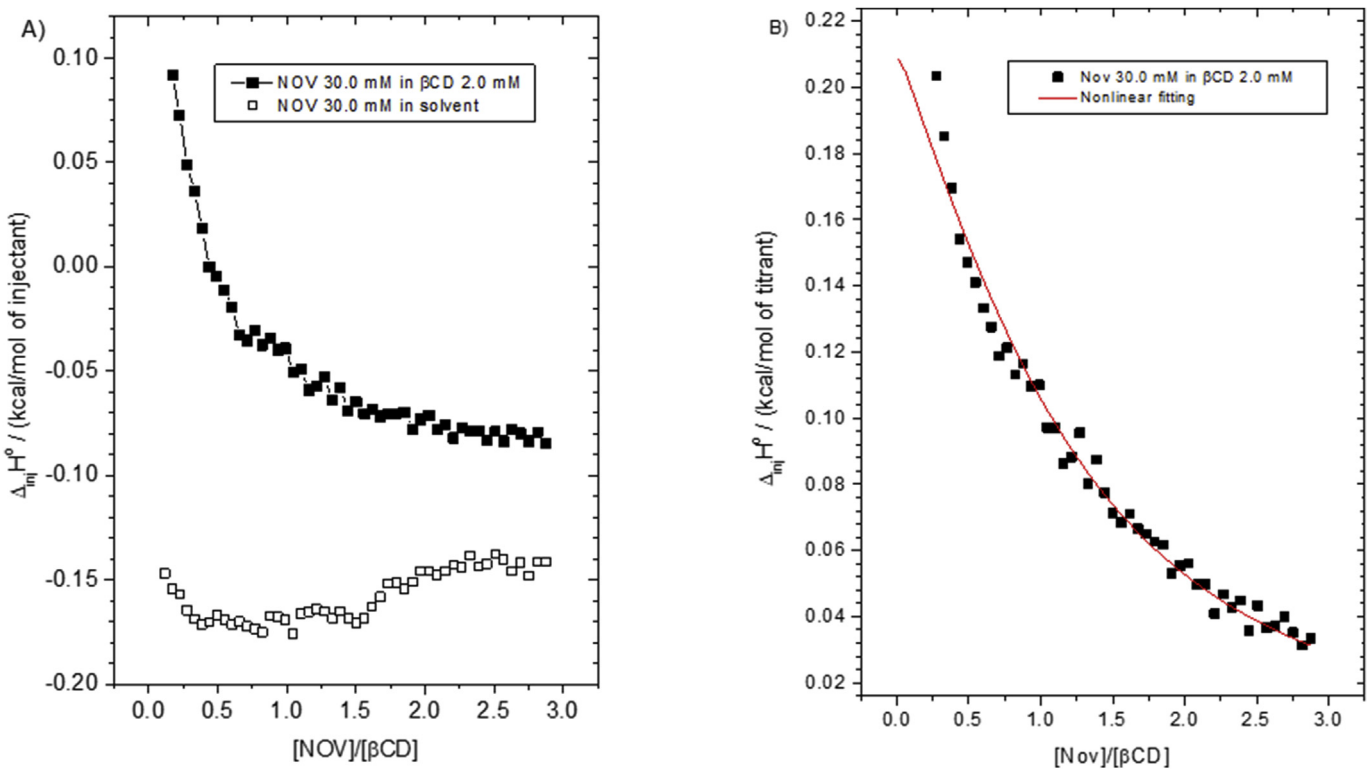

Fig. 7. A) ITC for (white square) NOV 30.0 mM in DMSO: H_2O (90:10, v/v) and (black square) NOV 30.0 mM in βCD 2.0 mM DMSO: H_2O (90:10, v/v) solution. B) (black square) ITC data after blank subtraction and (red line) non-linear fitting using the Wiseman Isotherm. Titrations performed at 298.15 K, with 51 injections of 5.0 μL of titrant in cell charged with 1.5 mL of βCD solution.

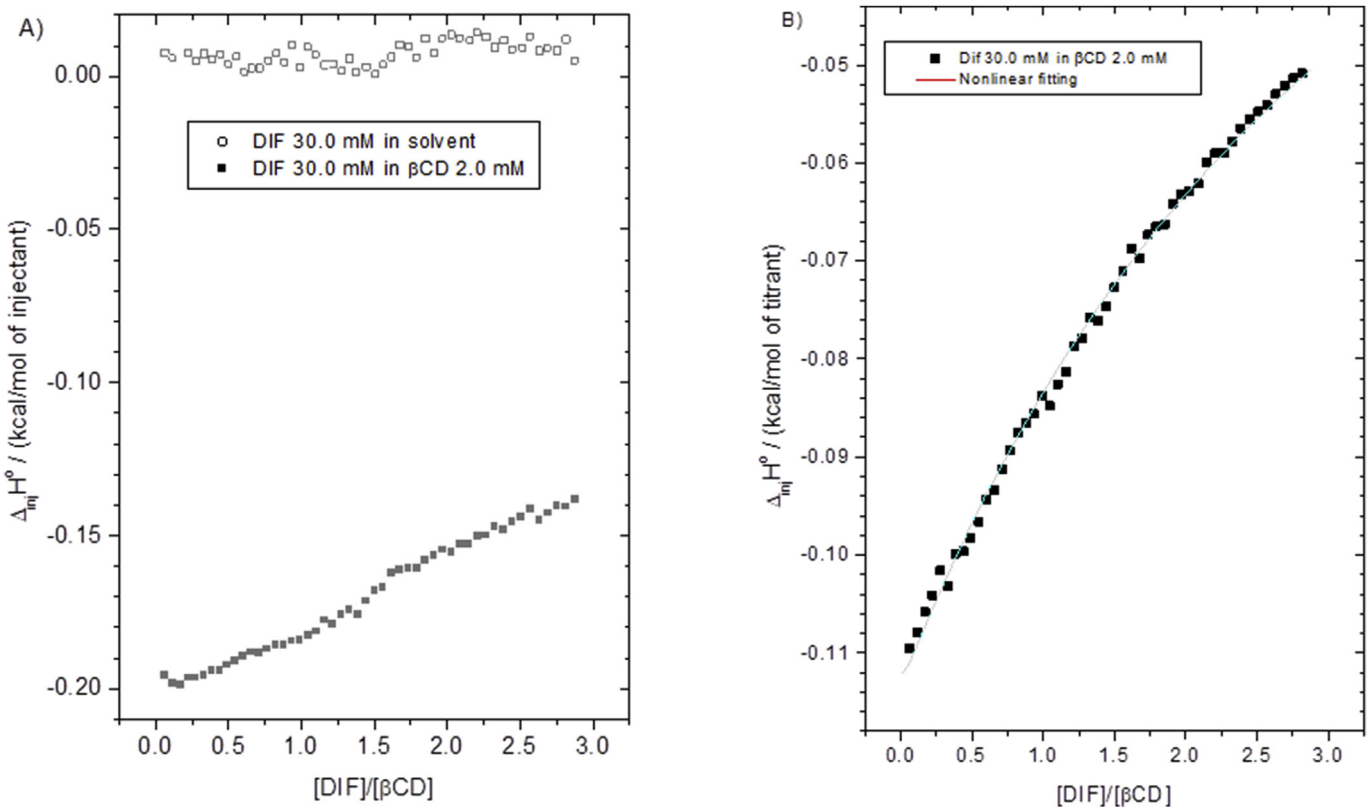

Fig. 8. A) ITC for (white square) DIF 30.0 mM in DMSO: H_2O (90:10, v/v) and (black square) DIF 30.0 mM in βCD 2.0 mM DMSO: H_2O (90:10, v/v) solution. B) (black square) ITC data after blank subtraction and (red line) non-linear fitting using the Wiseman Isotherm. Titrations performed at 298.15 K, with 51 injections of 5.0 μL of titrant in cell charged with 1.5 mL of βCD solution.

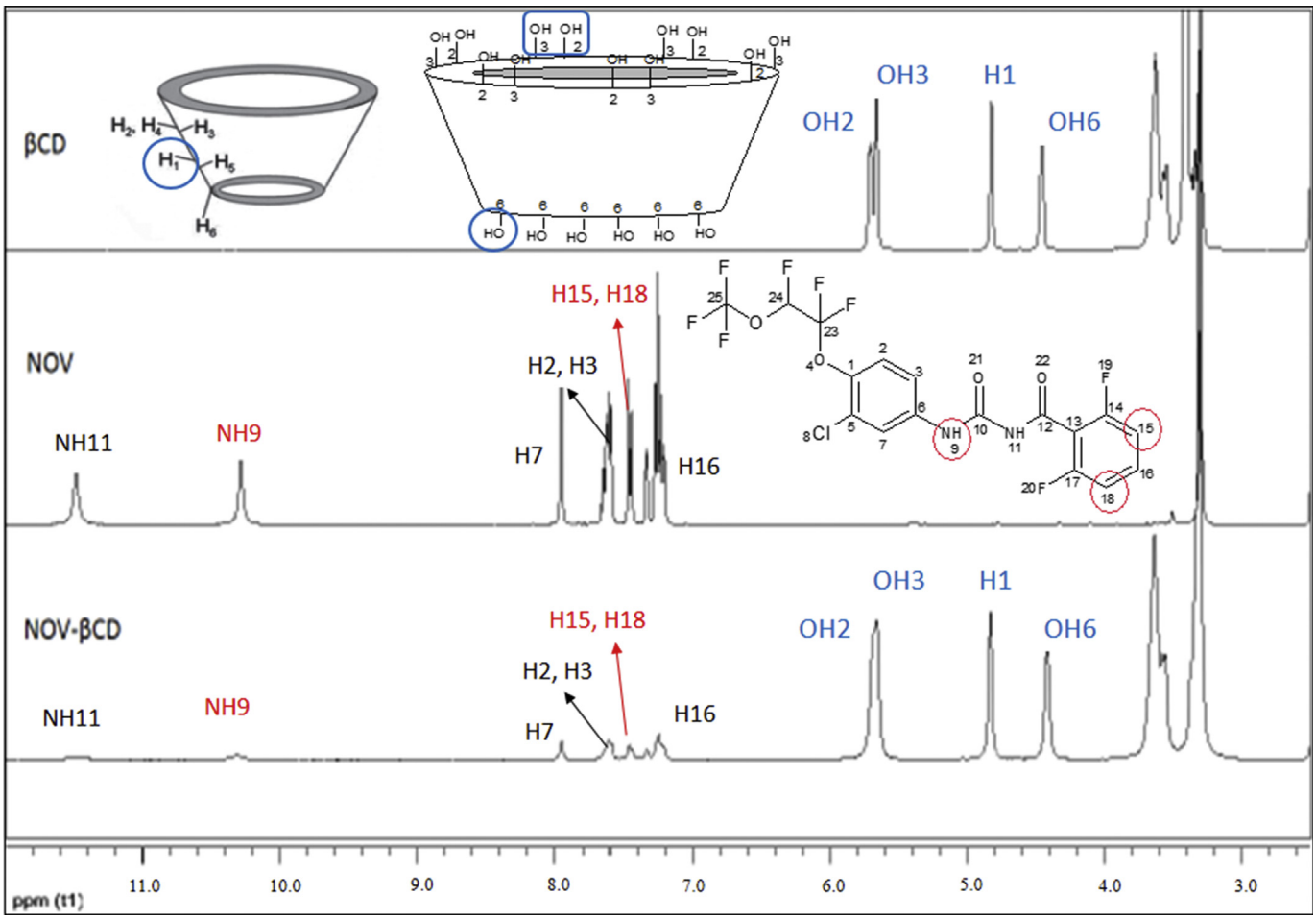

Fig. 9. ^{1}H RMN spectra for NOV, βCD and inclusion compound (NOV/βCD) at 400 MHz, DMSO-d^{6} and 27 °C.

3.1.2. Isothermal Titration Calorimetry

Changes in thermodynamic properties due to interactions between compounds can be properly measured by Isothermal Titration Calorimetry (ITC) [25], which allows to determinate the thermodynamic parameters of binding, as free energy of Gibbs ($\Delta_b G^o$), enthalpy ($\Delta_b H^o$) and entropy ($T\Delta_b S^o$). Besides, the binding constant (K_b) and the reaction stoichiometry (N) can be calculated using the Wiseman isotherm to model [26].

The titration curves of NOV and DIF, at 30.0 mM, into the ITC cell charged with βCD solution at 2.0 mM are shown in Figures 7A-B and 8A-B. βCD was chosen as titrand, since high concentrations of cyclodextrins in presence of low amounts of guest molecules can induce self-aggregation [27, 28, 29, 30, 31].

As can be seen in Figs. 7A and 8A, the heats of titration (injection of NOV or DIF solutions in βCD solution), were very different from the heats of dilution (injection of NOV or DIF solutions in solvent). These differences are indicative of interactions in solution, so that the heat measured in ITC experiments are resultant from establishment of new intermolecular interactions. After subtraction of blank experiments, Figs. 7B and 8B were obtained and used to calculate the stoichiometry and thermodynamic parameters by non-linear fitting with Wiseman Isotherm.

In both systems, the stoichiometric coefficient was close to unity ($N_{NOV/\beta CD} = 0.92 \pm 0.04$ and $N_{DIF/\beta CD} = 1.01 \pm 0.02$), indicating that the 1:1 stoichiometries are the most favored in the used solvent. Thus, it is justified the preparation of inclusion compounds with this molar ratio for biological experiments, using DMSO. In overall, the equilibrium constants determined for the systems were in the same magnitude as that found in literature for other inclusion compounds [21, 32, 33].

However, the equilibrium constants were very different for the two binding process, suggesting the occurrence of different molecular topology, in spite of the molecular similarity ($K_{NOV/\beta CD} = 510.0 \pm 31.8$ and $K_{DIF/\beta CD} = 65.1 \pm 1.2$). The formation of NOV/βCD complex was endothermic ($\Delta_b H^o = +1.81 \pm 0.04$ kJ/mol) and thereby, exclusively driven by entropy ($T\Delta_b S^o = 15.4$ kJ/mol), with a $\Delta_b G^o = -13.6$ kJ/mol. For the DIF/βCD system, the contribution of either enthalpy or entropy to the free energy of the process was approximately 40% and 60%, i.e. $\Delta_b H^o = -4.08 \pm 0.57$ kJ/mol and $T\Delta_b S^o = 6.3$ kJ/mol, leading to a $\Delta_b G^o = -10.4$ kJ/mol.

In both cases the entropy contribution suggests desolvation of precursors and releasing of solvent molecules upon inclusion. The solvent molecules must gain rotational and translational degrees of freedom, causing an increase in the entropy (disorder) of the system [34]. However, the explanation for the enthalpic differences relies on the fact that the 1,1,2-trifluoro-2-trifluoro-methoxyethoxy group makes NOV much more hydrophobic molecule than DIF (DIF is 27 times more soluble than NOV in water), increasing therefore, its affinity by the βCD. The presence of this fluorocarbon group explains the endothermicity of the NOV/βCD interaction, once is necessary an energy consumption for the partial desolvation of these groups during inclusion.

3.1.3. Structural characterization by NMR spectroscopy

It is well known in the literature that the formation of supramolecular complexes with cyclodextrins generates changes on the electron density of the hydrogen nuclei involved in the interactions, which is reflected in the NMR spectra as changes of chemical displacement or extension of the peaks. In Figs. 9 and 10 in supplementary material are shown the ^{1}H NMR spectra of NOV, DIF, βCD, NOV/βCD and DIF/βCD. The ^{1}H NMR spectra of NOV and DIF were similar to that provided by Sigma-Aldrich® while

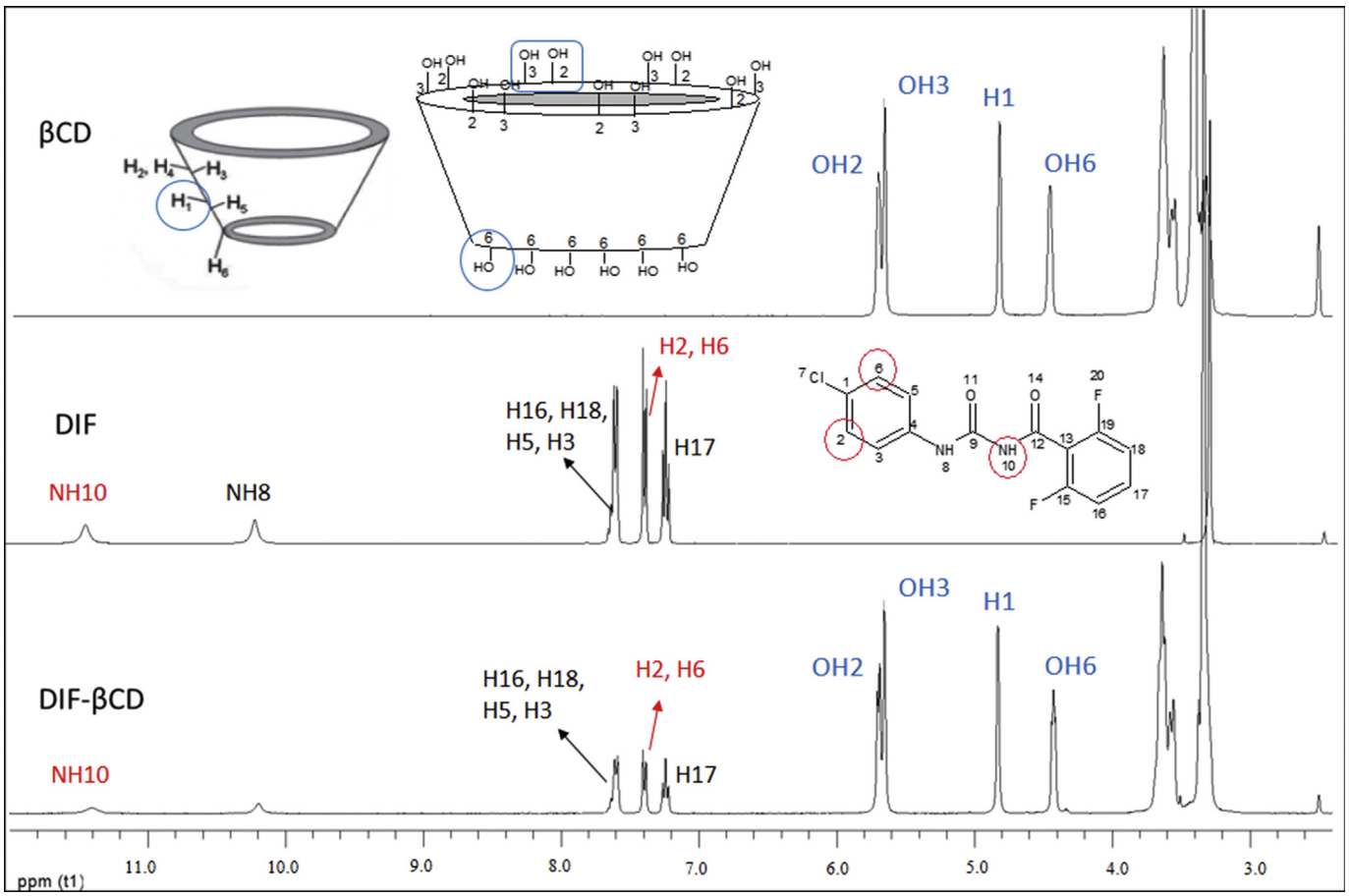

Fig. 10. ^{1}H RMN spectra for DIF, βCD and inclusion compound (DIF/βCD), at 400 MHz, DMSO-d^{6} and 27 °C.

the βCD spectrum was similar to others found in literature [21].

NOV/βCD complex showed changes in chemical shifts of OH(3), OH(6) and CH(1) hydrogen atoms of βCD, as well as of NH(9), CH(15) and CH(18) hydrogen atoms of NOV. In DIF/βCD complex it was observed changes in the chemical shifts of CH(1), CH(5), OH(2), OH(3) and OH(6) of the βCD and in NH(10), CH(2) and CH(6) hydrogen atoms of DIF. In addition, OH(2) hydrogen atom have been found to have better defined coupling.

The ROESY experiments were performed in order to identify the presence of dipolar correlation until 5Å between the hydrogen atoms and therefore confirm the interaction between the host and guest. Fig. 11 shows ROESY correlations between the OH(6) hydrogen atom of the βCD and the hydrogen atoms bound to the NH(8) hydrogen atom of the DIF, as well as correlations among the OH(2) and OH(3) of the βCD and NH(8) and NH(10) hydrogen atoms of DIF. Nevertheless, NOV/βCD system did not show any correlation in 2D spectrum (data not shown), in spite of the use of the same parameters in the two ROESY experiments. This suggests that the mode of DIF interaction with the βCD must be different from that observed for NOV/βCD system as proposed in Fig. 12 in Supplementary material.

A possible explanation for these differences is the inclusion mode of NOV by the fluorocarbon group, which is invisible to the ^{1}H NMR. This hypothesis explains the great difference in thermodynamic parameters as described in ITC experiments.

3.1.4. *Characterization of hydrophobic nanoprecipitates*

Considering that the insecticides described in the present work, even in presence of βCD, presented very low solubilities, it was proposed the strategy to use them in form of hydrophobic nanoprecipitates. Such kind of materials are formed when a low water soluble substance, previously dissolved in an organic solvent (such as DMSO), is added in water at low co-solvent concentration and volume [13, 21]. They can be stabilized in an aqueous environment by the appropriate concentration control of the solid phase and mixture ratio of solvent/cosolvent. Thus, in order to evaluate the ability of βCD modulate the self-aggregation of benzoylphenylureas in water/DMSO mixture, the size of the so formed aggregates was investigated by measurements of average hydrodynamic diameter (D_h).

Fig. 13 shows the DLS titrations of NOV, DIF, NOV/βCD and DIF/βCD DMSO solutions in ultrapure (Milli-Q®) water. It was observed in all systems that the particle size increased with concentration of titrant. This behavior may be associated with the gradual increase of collision probability between the particles, with subsequent coalescence.

Titration of NOV and NOV/βCD showed that at concentrations lower than 600 μmol/L, the inclusion compound presents lower values of D_h, suggesting higher colloidal stability for this nanocoprecipitate. As consequence from the size reduction, a larger surface area of the particle is exposed allowing a new pattern of interactions with the medium. These smaller particle size can be attributed to the presence of βCD molecules on the particles surface, favoring greater interactions with the solvent and reducing the particle-particle interaction.

For the DIF, it was observed that the D_h values were higher in presence of βCD, but with very lower polidispersity if compared with DIF/βCD titration. These data show once again that DIF must has a different profile of interaction with βCD if compared with NOV/βCD system. This difference was attributed to the absence of the 1,1,2-trifluoro-2-trifluoromethoxy-ethoxy group in the DIF molecule, which should generate a novel host-guest interaction mode, producing colloidal changes in the hydrophobic nanoprecipitates.

Based on these data and on the knowledge about the fast and

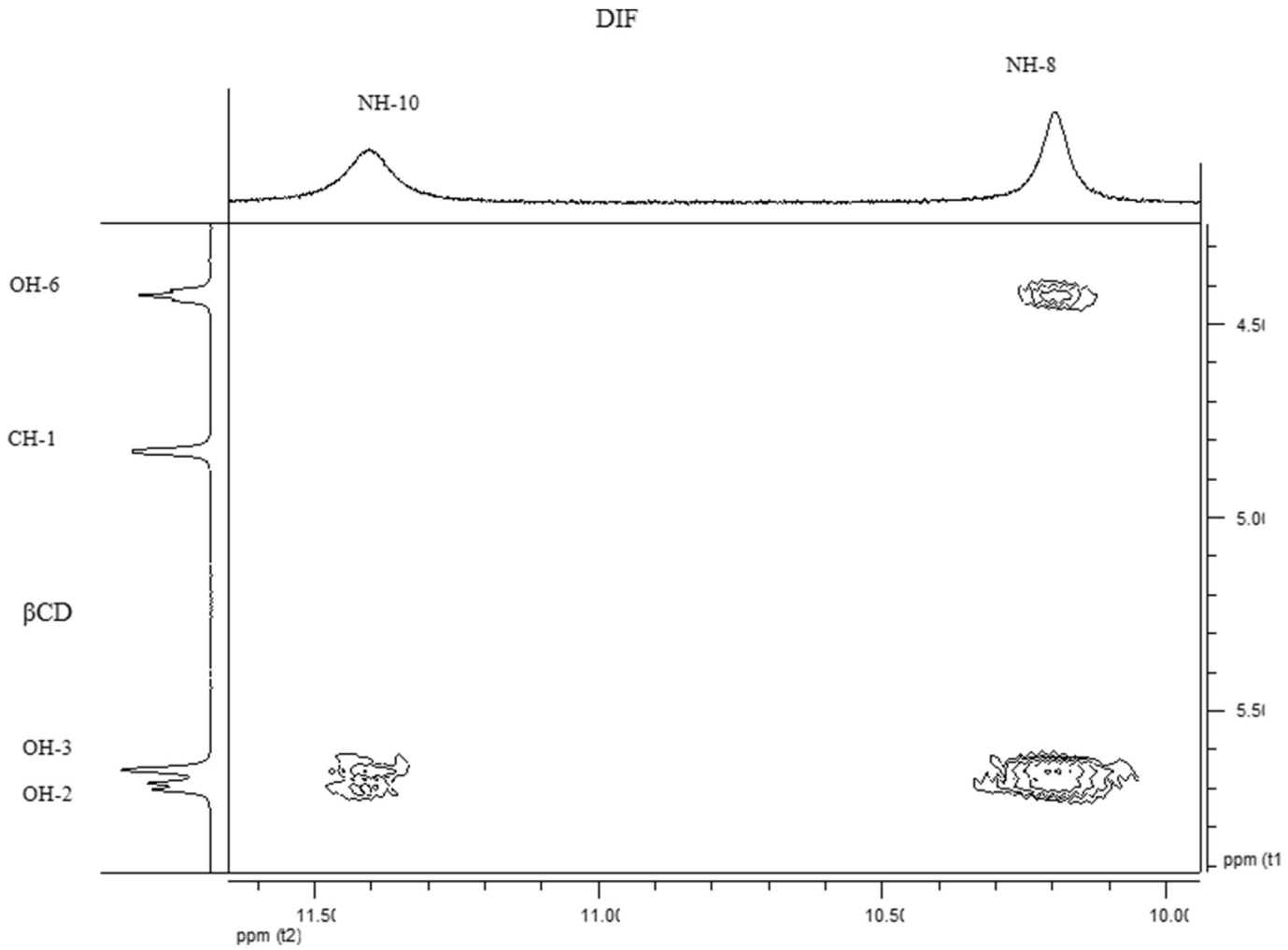

Fig. 11. 2D ^{1}H–^{1}H ROESY experiment for the DIF/βCD inclusion compound in DMSO-d_6, 400 MHz, 300 K and mixing time of 500 ms.

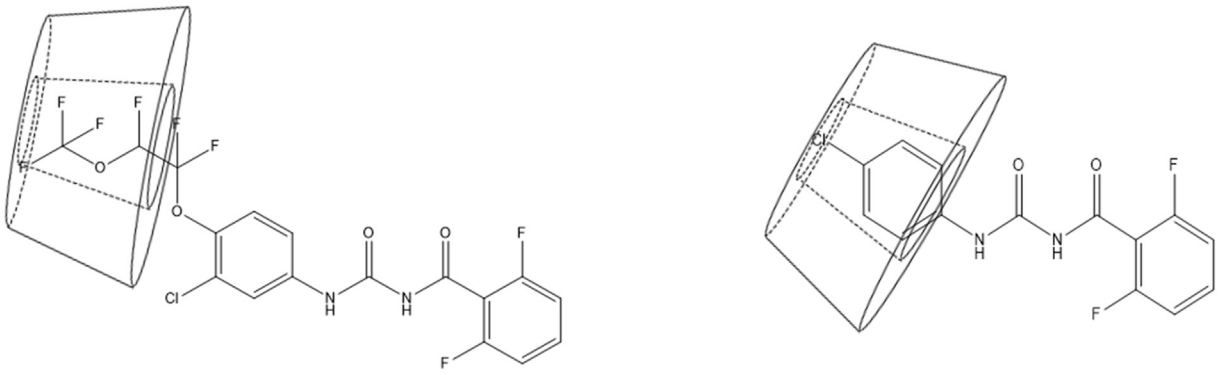

Fig. 12. Propose of structures for NOV/βCD and DIF/βCD inclusion compounds, based on NMR and ITC data.

indiscriminate feeding of larvaes in initial stages of their life, the presence of hydrophobic nanoprecipitates could be a way to attract them, actin as bait for larvaes.

3.2. Biological activities

3.2.1. Larvicidal assay in 4th instar larvae

According with recommendation from the WHO, the 4th instar larvae bioassay is the standard test for evaluate the larvicidal activity of new compounds or insecticide formulations, before their approbation to use in the environment. Thus, the HNPs of inclusion compounds, prepared in DMSO/water solvent were evaluated and compared with the HNPs of their BPUs precursors. In Fig. 14 are shown the curves of % mortality as a function of log concentration of the tested compound (NOV, NOV/βCD, DIF or DIF/βCD).

The obtained dose-response curves demonstrate that both free compounds and DIF/βCD have poor larvicidal activity in this bioassay. As mentioned above, the acute effect is not expected for compounds of this class, so that their mechanism of action are based on the inhibition of chitin synthesis during the insect larval stages. This can be confirmed by

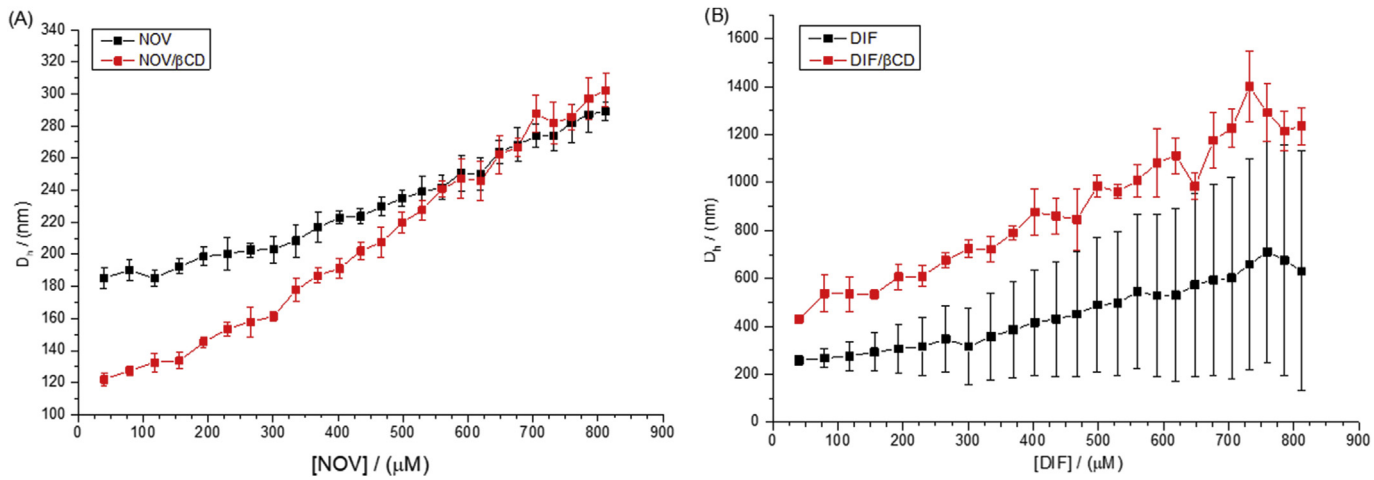

Fig. 13. DLS titrations for: A) NOV and NOV/βCD DMSO solutions; and B) DIF and DIF/βCD DMSO solutions; both in milli-Q® water, at 25 °C.

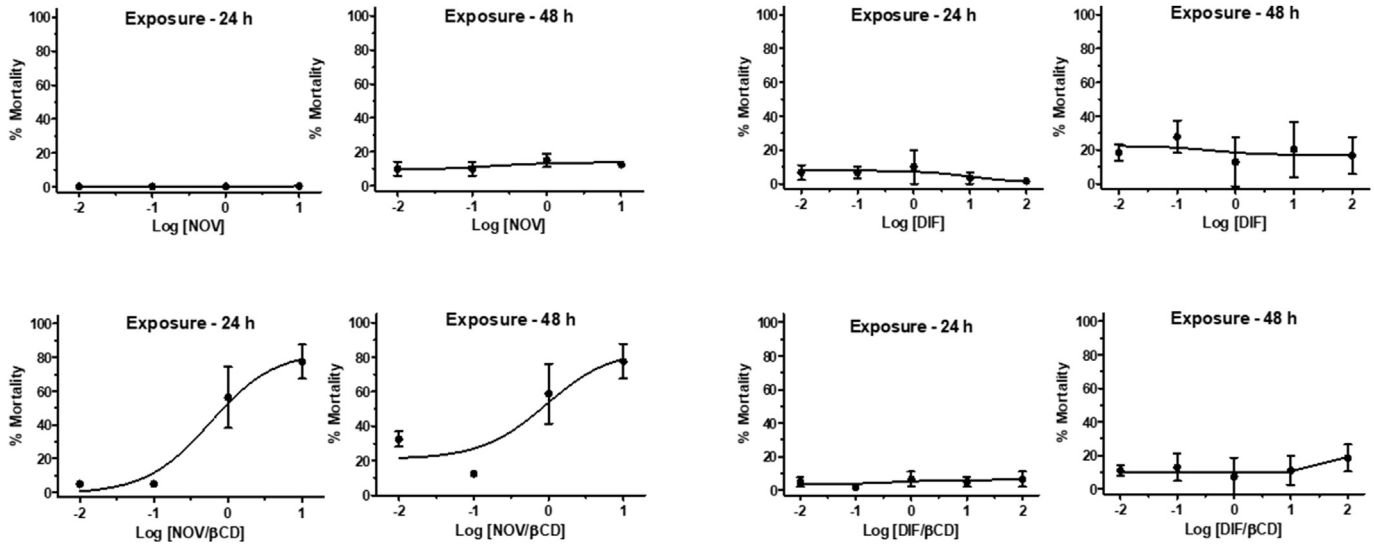

Fig. 14. Dose-response curves obtained by bioassay to evaluate the larvicidal activity of inclusion compounds (DIF/βCD and NOV/βCD) and free benzoylphenylureas (DIF and NOV). Three replicates were performed for each concentration.

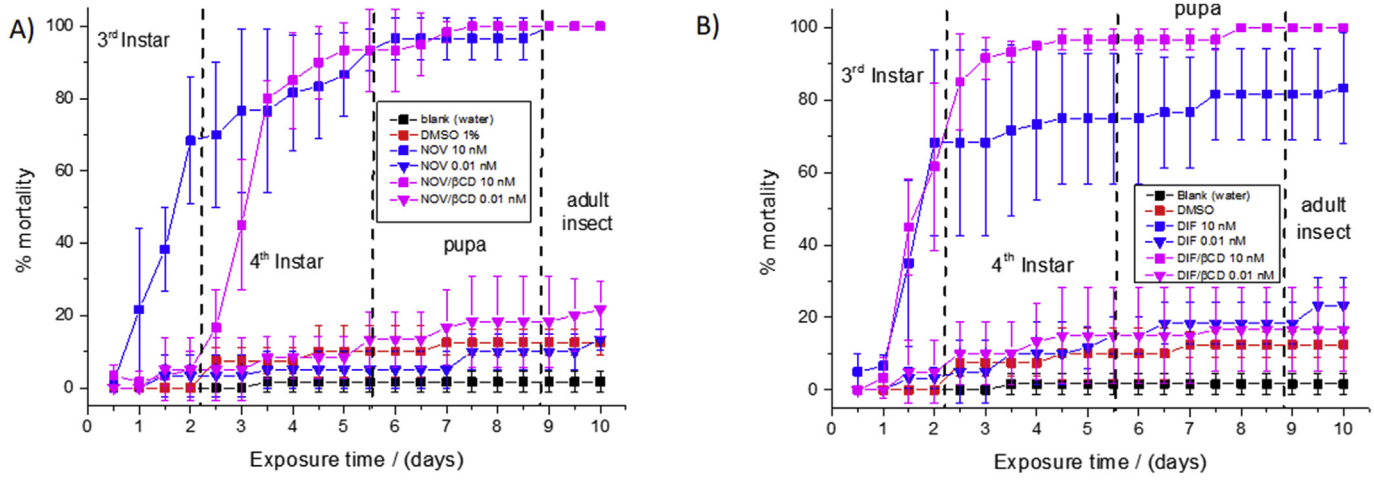

Fig. 15. Cumulative mortality curves for larvae/pupa as function of exposure time during larvae emergence for NOV, DIF and their inclusion compounds at 10 and 0.01 nmol/L. In dash lines represent the average time for ecdysis in control group. Nine replicates in three different days were performed for each concentration.

Table 1
Percentage of Emergency Inhibition (% IE_{50}) of *Aedes aegypti* mosquitoes for NOV, DIF and their inclusion compounds.

Compound	% IE_{50} in nmol L^{-1} (confidence interval at 95%)	Values in μg/L for comparison
NOV	0.877 (0.459–1.676)	0.432 (0.226–0.826)
NOV/βCD	0.490 (0.268–0.896)	0.242 (0.132–0.441)
DIF	5.688 (3.340–9.685)	1.767 (1.038–3.009)
DIF/βCD	3.772 (1.812–7.853)	1.172 (0.563–2.440)

the low mortality of subjects treated with the free compound at the studied concentration range. On the other hand, NOV/βCD was much more potent than NOV, with lethal concentration of 50% for larvae (LC_{50}) at 0.57 (0.11–2.98) μM at 24 h and 0.94 (0.09–9.56) μM at 48 h. This suggests that for NOV, the strategy of preparing the HNP of inclusion compound might have a new acute effect on dose reduction. However, new studies would be necessary to confirm this hypothesis.

3.2.2. Evaluation of the emergency inhibition

Compounds whose mechanism of action is related to changes in the process of growth and development of insects, such as BPUs, will require monitoring the development of the insect to verify its action. For this reason, the WHO advocated the implementation of the emergency inhibition test for compounds in which the mechanism of action is the regulation of insect growth [22].

Fig. 15 show the cumulative mortality curves as a function of exposure time during emergency inhibition bioassay for NOV, DIF, NOV/βCD and DIF/βCD HNPs. The negative controls used were dechlorinated water, 1% aqueous DMSO solution and aqueous βCD solution at the highest concentration used in the tests.

For all the compounds, no significant mortality was observed for larvae in the first 12 hours of experiment. However, after the ecdysis period, it was observed a significant mortality, mainly, at concentrations of 10 nmol/L for both pure and the complexated compounds, which matches with the action mechanism of compounds by growth regulation.

As can be seen in Fig. 15A, the NOV/βCD HNP is able to cause a delay in ecdysis if compared with free NOV, representing a new advantage. Moreover, Fig. 15B show a lower error bar of the points in mortality curve for the DIF/βCD HNP, indicating a more robust mortality profile in βCD presence.

In Table 1 are shown the necessary concentration to inhibit the emergence of 50% of the *Aedes aegypti* mosquito's insect population (% IE_{50}) for NOV, DIF, NOV/βCD and DIF/βCD HNPs.

The %IE_{50} values observed for the compounds were all in ppb magnitude. The values obtained for NOV and DIF are close to those presented by other works related in the literature, 0.135 ppb and 1.59 ppb [35], respectively. The data in Table 1 show that the inclusion compounds HNPs were more potent than free molecules. This demonstrates that the strategy of preparing HNP inclusion compound with BPUs was advantageous, leading to an increase of toxicity against *Aedes aegypti* larvae.

4. Conclusions

In the present work, two strategies were employed in order to develop new insecticide formulations based on benzoylphenylureas. The first one refers to complexation of BPUs with βCD in order to obtain their respective inclusion compounds, which were properly characterized. The second one was based on the production of hydrophobic nanoprecipitates through dispersion of DMSO solutions of these compounds in water. The results obtained during the characterizations confirmed the success in the preparation of the compounds, in spite of the differences in modes of inclusion for the two compounds, as showed by NMR and ITC experiments. These differences were attributed to the presence of 1,1,2-trifluoro-2-trifluoro-methoxyethoxy moiety in the NOV, which is responsible for reducing its solubility 27 times if compared with DIF, as described in literature. It is believed that this functional group is also responsible to give rise the drastic difference in the hydrophobic nanoprecipitates of the inclusion compounds, as demonstrated by DLS titrations. Results of larvicidal tests, both at the L4 stage and the emergency inhibition, showed that the inclusion compounds were more potent than their precursors, bringing other advantages as delaying in the ecdyses for NOV/βCD and becoming the mortality profile much more robust for DIF/βCD complex. The present results show that the preparation of new formulations from already used insecticides led to an increase in larvicidal potency after encapsulation, possibly producing an acute effect for NOV/βCD system.

These results are, at the best, tentative. Deeper research must be performed in order to bring out more conclusive results.

Declarations

Author contribution statement

Ângelo Márcio Leite Denadai: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

[1] J.F. Graf, The role of insect growth regulators in arthropod control, Parasitol. Today 9 (1993) 471–474.
[2] S.G. Salokhe, S.G. Deshpande, S.N. Mukherjee, Evaluation of the insect growth regulator Lufenuron (Match®) for control of Aedes aegypti by simulated field trials, Parasitol. Res. 111 (2012) 1325–1329.
[3] M.F. Moreira, A.S. dos Santos, H.R. Marotta, J.F. Mansur, I.B. Ramos, E.A. Machado, G.H.M.F. Souza, M.N. Eberlin, C.R. Kaiser, K.J. Kramer, S. Muthukrishnan, A.M.H. Vasconcellos, A chitin-like component in Aedes aegypti eggshells, eggs and ovaries, Insect Biochem. Mol. Biol. 37 (2007) 1249–1261.
[4] L.C. Farnesi, J. Brito, J. Linss, M. Pelajo-Machado, D. Valle, G. Rezende, Physiological and morphological aspects of Aedes aegypti developing larvae: effects of the chitin synthesis inhibitor novaluron, PLoS One 7 (2012).
[5] D. Doucet, A. Retnakaran, Insect chitin: metabolism, genomics and pest management, in: T.S. Dhadialla (Ed.), Advances in Insect Physiology, 43, 2012, pp. 437–511. Insect Growth Disruptors.
[6] A.C. Grosscurt, Diflubenzuron - some aspects of its ovicidal and larvicidal mode of action and an evaluation of its practical possibilities, Pestic. Sci. 9 (1978) 373–386.
[7] World Health Organization, Novaluron in Drinking-Water: Use for Vector Control in Drinking-Water Sources and Containers, Geneva, 2008.
[8] G.C. Cutler, C.D. Scott-Dupree, Novaluron: prospects and limitations in insect pest management, Pest Technol. 1 (2007) 38–46.
[9] E.P. Lima, A.M.d. Oliveira Filho, J.W.d.O. Lima, A.N. Ramos Júnior, L.P.d.G. Cavalcanti, R.J.S. Pontes, Resistência do Aedes aegypti ao temefós em Municípios do Estado do Ceará, Rev. Soc. Bras. Med. Trop. 39 (2006) 259–263.
[10] J.S. Prophiro, O.S. Silva, J.E.D. Luna, C.F. Piccoli, L.A. Kanis, M.A.N.d. Silva, Aedes aegypti and Aedes albopictus (Diptera: Culicidae): coexistence and susceptibility to temephos, in municipalities with occurrence of dengue and differentiated characteristics of urbanization, Rev. Soc. Bras. Med. Trop. 44 (2011) 300–305.
[11] T.A. Belinato, D. Valle, The impact of selection with diflubenzuron, a chitin synthesis inhibitor, on the fitness of two Brazilian *Aedes aegypti* field populations, PLoS One 10 (2015), e0130719.

[12] M. Kah, S. Beulke, K. Tiede, T. Hofmann, Nanopesticides: state of knowledge, environmental fate, and exposure modeling, Crit. Rev. Environ. Sci. Technol. 43 (2013) 1823–1867.
[13] E.G. Lanna, V.C.E. Bittencourt, A.M.S. Moreira, J.G. Da Silva, O.V. Sousa, A.M.L. Denadai, Physicochemical characterization and biological activities of the ethanol extract of Bryophyllum pinnatum (Lam.) Oken incorporated in beta-cyclodextrin, J. Incl. Phenom. Macro. 85 (2016) 247–259.
[14] S.V. Kurkov, T. Loftsson, Cyclodextrins, Int. J. Pharm. 453 (2013) 167–180.
[15] K.I.R. Teixeira, A.M.L. Denadai, R.D. Sinisterra, M.E. Cortes, Cyclodextrin modulates the cytotoxic effects of chlorhexidine on microrganisms and cells in vitro, Drug Deliv. 22 (2015) 444–453.
[16] M.M. Nitalikar, D. Sakarkar, P.V. Jain, Cyclodextrins: A review, Int. J. Curr. Pharm. Res. 10 (2012) 1–6.
[17] E.M.M. Del Valle, Cyclodextrins and their uses: a review, Process Biochem. 39 (2004) 1033–1046.
[18] A.R. Hedges, Industrial applications of cyclodextrins, Chem. Rev. 98 (1998) 2035–2044.
[19] T. Loftsson, M. Másson, M.E. Brewster, Self-Association of cyclodextrins and cyclodextrin complexes, J. Pharm. Sci. 93 (2004) 1091–1099.
[20] J.W. Chung, K. Lee, C. Neikirk, C.M. Nelson, R.D. Priestley, Photoresponsive coumarin-stabilized polymeric nanoparticles as a detectable drug carrier, Small 8 (2012) 1693–1700.
[21] A.M.D. Moreira, V.C.E. Bittencourt, F.L.S. Costa, M.E. de Lima, M.T.P. Lopes, W.S. Borges, G.F. Martins, C.S. Nascimento, J.G. da Silva, A.M.L. Denadai, K.B. Borges, Hydrophobic nanoprecipitates of beta-cyclodextrin/avermectins inclusion compounds reveal insecticide activity against Aedes aegypti larvae and low toxicity against fibroblasts, J. Agric. Food Chem. 66 (2018) 7275–7285.
[22] World Health Organization, Dept. of Communicable Disease Prevention, C.a.E., Scheme, W.P.E., Guidelines for Laboratory and Field Testing of Mosquito Larvicides. Geneva, 2005, pp. 2013–2039.
[23] O. Egyed, Spectroscopic studies on β-cyclodextrin, Vib. Spectrosc. 1 (1990) 225–227.
[24] J.M.L. Correa, A. Abrishamkar, J.G. Da Silva, J.R. Pereira, F.C. de Oliveira, A.M.L. Denadai, Modulation of size and viscosity of Ni/Zn ferrites: effect of doping with beta CD and chemical treatment with HNO3 and NaOH, J. Mol. Struct. 1100 (2015) 438–446.
[25] T. Wiseman, S. Williston, J.F. Brandts, L.-N. Lin, Rapid measurement of binding constants and heats of binding using a new titration calorimeter, Anal. Biochem. 179 (1989) 131–137.
[26] W.B. Turnbull, A.H. Daranas, On the value of c: can low affinity systems Be studied by isothermal titration calorimetry? J. Am. Chem. Soc. 125 (2003) 14859–14866.
[27] A.M.L. Denadai, M.M. Santoro, A.V. Texeira, R.D. Sinisterra, New insights regarding the cyclodextrin/AAS self-assembly: a molar ratio dependent system, Mater. Sci. Eng. C-Mater. Biol. Appl. 30 (2010) 417–422.
[28] F.B. De Sousa, A.C. Lima, A.M.L. Denadai, C.P.A. Anconi, W.B. De Almeida, W.T.G. Novato, H.F. Dos Santos, C.L. Drum, R. Langer, R.D. Sinisterra, Superstructure based on beta-CD self-assembly induced by a small guest molecule, Phys. Chem. Chem. Phys. 14 (2012) 1934–1944.
[29] T. Loftsson, M. Masson, M.E. Brewster, Self-association of cyclodextrins and cyclodextrin complexes, J. Pharm. Sci. 93 (2004) 1091–1099.
[30] M. Messner, S.V. Kurkov, M.E. Brewster, P. Jansook, T. Loftsson, Self-assembly of cyclodextrin complexes: aggregation of hydrocortisone/cyclodextrin complexes, Int. J. Pharm. 407 (2011) 174–183.
[31] M. Messner, S.V. Kurkov, R. Flavia-Piera, M.E. Brewster, T. Loftsson, Self-assembly of cyclodextrins: the effect of the guest molecule, Int. J. Pharm. 408 (1-2) (2011) 235–247.
[32] Á.M.L. Denadai, K.I. Teixeira, M.M. Santoro, A.M.C. Pimenta, M.E. Cortés, R.D. Sinisterra, Supramolecular self-assembly of β-cyclodextrin: an effective carrier of the antimicrobial agent chlorhexidine, Carbohydr. Res. 342 (15) (2007) 2286–2296.
[33] M.V. Rekharsky, Y. Inoue, Complexation thermodynamics of cyclodextrins, Chem. Rev. 98 (5) (1998) 1875–1918.
[34] M. Rekharsky, Y. Inoue, S. Tobey, A. Metzger, E. Anslyn, Ion-pairing molecular recognition in water: aggregation at low concentrations that is entropy-driven, J. Am. Chem. Soc. 124 (50) (2002) 14959–14967.
[35] E. Seccacini, A. Lucia, L. Harburguer, E. Zerba, S. Licastro, H. Masuh, Effectiveness of pyriproxyfen and diflubenzuron formulations as larvicides against Aedes aegypti, J. Am. Mosq. Control Assoc. 24 (3) (2008) 398–403.