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Complexation of Several Fungicides with β-Cyclodextrin: Determination of the Association Constants and Isolation of the Solid Complexes

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The formation of inclusion complexes with β-cyclodextrin was studied for several popular fungicides of different types: prochloraz, 2-phenylphenol, thiophanate methyl, 8-hydroxyquinoline, and benalaxyl. Phase solubility diagrams showed that in all cases complexation takes place, leading to an important increase of water solubility in prochloraz and benalaxyl. Equilibrium association constants could be determined from the phase solubility data and from NMR titrations in the case of 2-phenylphenol. Because of the low solubility of the complex formed between 8-hydroxyquinoline and β-cyclodextrin, the corresponding association constant could not be determined. The solid complexes of fungicide−cyclodextrin were prepared and isolated by different methods. The isolation of real complexes and not physical mixtures was confirmed in the cases of prochloraz, 2-phenylphenol, and benalaxyl by differential scanning calorimetry.

KEYWORDS: Cyclodextrins; fungicides; inclusion complexes

INTRODUCTION

Nowadays, it is known that fungi are the most important cause of diseases in plants, and to prevent their unwanted growth, fungicides must be added to any culture medium. To achieve a good biological activity, fungicides usually must be used dissolved in water. That involves in most cases preparation of saltlike derivatives of the fungicides and, if this is not feasible, the use of organic solvents. In many cases, it would be interesting to produce alternative formulations with the active compounds, which show higher water solubility than the pure fungicides. In this work, we investigate the possibility to obtain such formulations by using CDs, which could form inclusion complexes with the fungicides. CDs are widely used in the pharmaceutical and the agriculture industries since complexation usually increases the water solubility of the guest molecule, enhancing the bioavailability of poorly soluble drugs (*1*, *2*). Moreover, a synergistic effect of the CDs has been demonstrated for several drugs that further increases their biological activity (*3*).

In an earlier work (*4*), the complexation of several benzimidazole type fungicides with CDs was studied. Those fungicides are widely used due to their easy penetration in plants through the roots and the leaves but must be alternated with other types of fungicides to avoid the appearance of resistance. Therefore, in this work, we have studied five popular fungicides, which are used alternately to benzimidazole derivatives: prochloraz, 2-phenylphenol, thiophanate methyl, 8-hydroxyquinoline, and benalaxyl (**Figure 1**). They are systemic fungicides with both protective and curative action against a wide range of diseases in plants (*5*). All five substances show quite low water solubility, especially thiophanate methyl, and the aim of this work is to investigate the possible increase of their solubility by complexation with CDs and the feasibility of preparation of solid complexes of fungicide−CD.

To study the complexation in solution, solubility isotherms were measured for each of the fungicides and the selected CD, β-CD. NMR titrations could only be performed for 2-phenylphenol and 8-hydroxyquinoline due to their higher solubility. DSC was used to confirm the isolation of the solid complexes.

MATERIALS AND METHODS

Materials. The β-CD used in this work was kindly supplied by Roquette and was purified by recrystallization from water. 8-Hydroxyquinoline and 2-phenylphenol were purchased from Sigma. 8-Hydroxyquinoline was recrystallized three times from petroleum ether and 2-phenylphenol from carbon tetrachloride. Prochloraz, thiophanate methyl, and benalaxyl were purchased from Riedel-de Haën as analytical standards (Pestanal). All solvents used, both in the recrystallizations and in the synthesis of the solid complexes, had at least high-performance liquid chromatography (HPLC) purity. The deuterated water used in the solutions for NMR measurements was purchased from SDS.

Phase Solubility Diagrams. Samples were prepared by adding the same amount of the solid fungicide to 10 mL of aqueous solutions of

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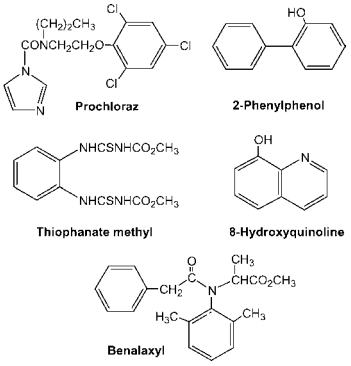

Figure 1. Molecular structures of the five fungicides under study.

Table 1. Results of the Quantitative Analysis of Phase Solubility Diagrams and NMR Data[a]

fungicide	S_0 (10^{-4} mol dm^{-3})	S_{10mM} (10^{-4} mol dm^{-3})	ΔS (%)	K_{11} (mol^{-1} dm^3)
prochloraz	2.1 ± 0.3	18.9 ± 0.6	800	$(9.6 \pm 1.5) \times 10^{2}$[b]
2-phenylphenol	87.0 ± 0.6	109.0 ± 0.9	25	25.5 ± 1.3[b]
				$(1.29 \pm 0.25) \times 10^{3}$[c]
thiophanate methyl	0.829 ± 0.004	1.521 ± 0.006	83	84.1 ± 0.7[b]
8-hydroxyquinoline	49	8.9	−82	
benalaxyl	1.06 ± 0.06	5.7 ± 0.2[d]	440[d]	718 ± 42[b,e]

[a] Fungicide solubilities in water (S_0) and in a 10 mM solution of β-CD (S_{10mM}) (obtained from the linear fits of the increasing region in phase solubility diagrams), increments of fungicide solubility in 10 mM β-CD solution with respect to water ($\Delta S = (S_{10mM} - S_0)/S_0 \times 100$), and association constants for 1:1 complex formation. All values are at 25 °C. [b] Association constants obtained by analysis of phase solubility diagrams. [c] Association constant obtained by analysis of NMR data. [d] Values corresponding to the maximal solubility at a concentration 6.6×10^{-3} mol dm^{-3} of β-CD. [e] Apparent association constant (see text).

β-CD of different concentrations between 0 and 15 mM. The amount of solid fungicide was calculated so that it exceeded several times the fungicide water solubility (see **Table 1**): 4 mg of prochloraz, 30 mg of 2-phenylphenol, 2.2 mg of thiophanate methyl, 15 mg of 8-hydroxyquinoline, and 4.4 mg of benalaxyl. The samples were kept with constant stirring at 25 °C for 1 week in order to achieve solubility equilibrium. The supernatants were then separated, and after 10 or 100 times dilution, their absorption spectra were measured. Using a previously measured calibration curve, the concentration of fungicide−CD complex was determined for each sample. For each guest molecule, a calibration curve was determined by measuring the absorption spectra of aqueous solutions of different fungicide concentrations. The use of such a calibration curve to determine the concentration of complex involves the assumption that the absorption spectrum of the fungicide does not change by complexation. This was checked by comparing the absorption spectra of each pure fungicide and the fungicide in the presence of 0.010 M β-CD. All absorption spectra were measured with a Varian Cary100 spectrophotometer. In the solubility experiments with 8-hydroxyquinoline, a phosphate buffer of pH 7.5 (total phosphate concentration of 0.10 mol dm^{-3}) was used to keep the aqueous fungicide in its neutral form.

NMR Measurements. NMR spectra were recorded in a Bruker AC300 spectrometer, with working frequencies of 300 mHz and 75 MHz for ^{1}H and ^{13}C, respectively. NMR titrations were used to study the complexation of 2-phenylphenol and 8-hydroxyquinoline with β-CD. These mixtures were prepared after the continuous variation method (*6*), i.e., with a constant sum of host and guest concentrations, which was 4.90 mM for 8-hydroxyquinoline and 3.80 mM for 2-phenylphenol. Phosphate buffer was also used in the NMR experiments with 8-hydroxyquinoline. All NMR experiments were carried out in D_2O at 25 °C.

Preparation of Complexes. To obtain the solid fungicide−β-CD complexes, different methods were followed.

Complex of Prochloraz. Equimolar amounts (1.6×10^{-4} mol) of prochloraz and β-CD were dissolved in water under continuous stirring at 55 °C. The mixture was first cooled to room temperature and then with liquid nitrogen, and the solid was freeze-dried; yield, 96%.

Complex of 2-Phenylphenol. Suitable amounts of 2-phenylphenol and β-CD were dissolved in water under continuous stirring at 80 °C for 1 h, yielding equimolar concentrations (10 mM) of the two compounds. The mixture was then cooled slowly, and a solid precipitated. This solid was filtered and dried in a vacuum oven at 45 °C; yield, 66%.

Complex of Thiophanate Methyl. Equimolar amounts (1.7×10^{-4} mol) of thiophanate methyl and β-CD were dissolved in a mixture of 250 mL of water and 100 mL of ethanol. The mixture was kept under continuous stirring at 50 °C for 48 h. Once the ethanol was removed at that temperature, the reaction product was isolated by freeze-drying; yield, 77%.

Complex of 8-Hydroxyquinoline. Forty milliliters of a 4.9 mM aqueous solution of 8-hydroxyquinoline was mixed with 60 mL of a 14 mM aqueous solution of β-CD. The mixture was left in an ultrasound bath for 70 min and then for 24 h in a water bath at 25 °C. The solid appearing in the solution was filtered and dried in a vacuum oven at 45 °C; yield, 85%.

Complex of Benalaxyl. Fifty-five milligrams of benalaxyl was added to 125 mL of a 10.2 mM aqueous solution of β-CD. The mixture was left in an ultrasound bath for 1 h and then kept under continuous stirring at 35 °C for 72 h. After this time, the solution was turbid but no precipitate appeared. The reaction mixture was cooled in a refrigerator for 24 h and then filtered and dried; yield, 80%.

DSC. To characterize the solid complexes, DSC experiments were performed, where the thermograms of pure fungicide, pure CD, physical mixture fungicide−CD, and complex were measured under the same experimental conditions. Thermograms were obtained in a differential scanning calorimeter of Mettler Toledo Thermal Analysis Systems using vented aluminum pans and under nitrogen flux (18 cm^3 min^{-1}). Typical conditions were as follows: temperature range, 25−300 °C; scanning rate, 10 °C min^{-1}; sample weight, 4 mg.

RESULTS AND DISCUSSION

Complex Stoichiometry and Association Constants. Figure 2 shows the phase solubility diagrams, i.e., plots of total concentration of dissolved fungicide (total fungicide solubility, S_t) vs total CD concentration, obtained for the five fungicides with β-CD. The fungicides prochloraz, 2-phenylphenol, and thiophanate methyl show a linear increase of the total fungicide solubility when increasing β-CD concentration, yielding phase solubility diagrams of type A_L, after the classification of Higuchi and Connors (*7*). This kind of diagram is due to the formation of soluble complexes of first order in the complexing agent. In consequence, the solubility of these three fungicides increases with addition of β-CD, and this can lead to an enhancement of their biological activity. As shown in **Table 1**, prochloraz shows the most important increase of solubility, about 800% at a β-CD concentration of 10 mM.

In the case of benalaxyl (**Figure 2e**), the phase solubility diagram obtained is of the type B_S, which corresponds to systems where insoluble complexes are formed (*7*). Three regions can be distinguished in this kind of diagram: an increase due to the formation of one or more complexes with higher solubility than the free fungicide; a plateau where the total fungicide solubility is constant since one of the complexes has achieved its maximal solubility and precipitates while the solid fungicide dissolves to maintain the equilibrium in solution; and a decrease that begins when the excess of solid fungicide is completely dissolved and the complex precipitates at the expense of the free fungicide in solution. Although the maximal solubility of benalaxyl is already achieved at about 6.6 mM β-CD, this β-CD concentration causes a solubility increase of about 400%

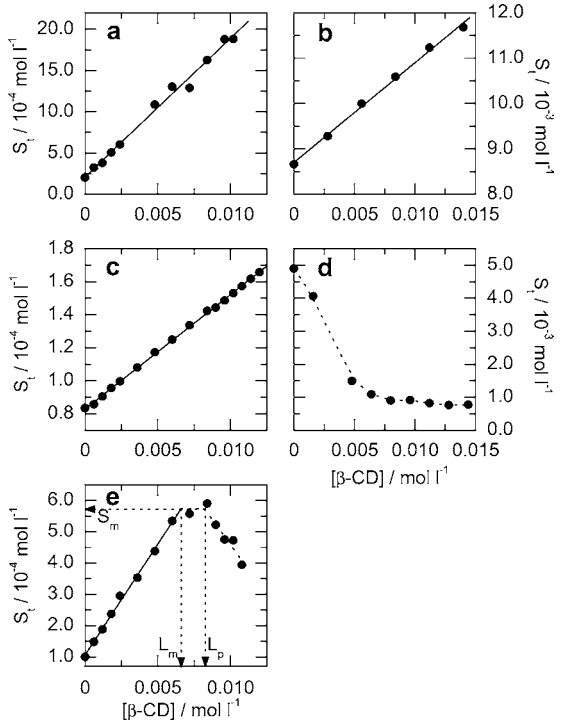

Figure 2. Phase solubility diagrams for (**a**) prochloraz, (**b**) 2-phenylphenol, (**c**) thiophanate methyl, (**d**) 8-hydroxyquinoline, and (**e**) benalaxyl with β-CD at 25 °C. Solid lines are the results of linear regressions to the experimental data.

with respect to pure benalaxyl (**Table 1**), proving that the complexation is very effective in the solubilization of this fungicide.

The phase solubility diagram of 8-hydroxyquinoline (**Figure 2d**) is of a rarely encountered type. It is closely related to type B diagrams. An immediate and progressive decrease of the total fungicide solubility is observed when increasing β-CD concentration. The explanation given by Higuchi and Connors (*7*) for this kind of diagram is that a very insoluble complex is formed that mixes with the solid fungicide leading to a single solid phase. At a β-CD concentration of 10 mM, the relative decrease of solubility of 8-hydroxyquinoline is about 80% and practically does not change when the β-CD concentration is further increased. Therefore, in the case of 8-hydroxyquinoline, complexation with β-CD causes an effect opposite to that desired, i.e., a significant decrease of the fungicide solubility in water.

Quantitative analysis of diagrams of types A_L and B_S allows the determination of the stoichiometry of the complexes formed and estimation of the corresponding association constants. However, this is not possible in the case of 8-hydroxyquinoline with β-CD, since the corresponding diagram has not been quantitatively explained until now.

Linear regression applied to the three experimental A_L diagrams yielded values of the slopes, which are smaller than unity. This indicates that the complexes formed are also of first order in fungicide, i.e., of stoichiometry 1:1 fungicide:β-CD (*7*). For such complexes, the total fungicide solubility varies linearly with the total CD concentration following the equation:

$$S_t = S_0 + \frac{K_{11}S_0}{1 + K_{11}S_0}[\mathrm{CD}]_0 \qquad (1)$$

where S_0 is the fungicide solubility in the absence of CD and K_{11} is the equilibrium association constant. From the values of intercept and slope of the straight lines fitted to the experimental data (**Figure 2a−c**), the association constants of prochloraz, 2-phenylphenol, and thiophanate methyl with β-CD were determined. The values are shown in **Table 1**. As expected from the huge increase of solubility, the association constant of prochloraz with β-CD is high, about 10^3 mol^{-1} dm^3, and proves a great tendency of this fungicide to enter into the β-CD cavity. Thiophanate methyl has a much smaller association constant than prochloraz, which is in the limit at which the complexation is considered to be effective. In the case of 2-phenylphenol, the value of K_{11} obtained from the phase solubility diagram is very small. Nevertheless, this value will be compared later with that obtained from NMR titrations, since the relative high solubility of this fungicide allows the performance of such studies.

Phase solubility diagrams of type B_S have been extensively analyzed in a recently published article (*8*), where all three regions of the diagram are used to determine individual complex formation and solubility product constants. The complex equilibria involved over the entire range of a B_S diagram are those corresponding to the formation of 1:1 and 1:2 solute:solubilizer complexes, characterized by the association constants K_{11} and K_{12}. In the range of solubilizer concentrations corresponding to the rising portion of the diagram, the two complexes are soluble and the plot of the total solute solubility against concentration of solubilizer is usually not linear but has a positive curvature. Under these conditions and when excess of solid solute is present, a linear plot proposed by Zughul and Badwan (*8*) can be used for a rough estimation of K_{11} and K_{12}. Such a plot for benalaxyl shows a very poor linearity. The corresponding linear fit yields a negative slope, which leads to a negative value of the association constant K_{12}. As reported by Zughul and Badwan, this result is typically obtained when the rising portion of the diagram is practically linear due to imprecision of the experimental data. In such cases, only an apparent association constant can be obtained by applying a linear regression to the experimental data in this region based on eq 1. For benalaxyl, an apparent association constant of about 700 mol^{-1} dm^3 was obtained (see **Table 1**), which is expected to be a rough estimation of K_{11}.

The second region in B_S diagrams, the plateau, appears when one of the complexes formed has achieved its maximal solubility and is observed so long as solid fungicide is left. The intersection of the rising portion with the plateau defines the value of the solubilizer concentration L_m at which the solute solubility remains constant with a value S_m. The end point of the plateau, with a solubilizer concentration L_p, is determined from the intersection with the decreasing portion of the diagram. With those values, it can be determined which of the complexes precipitates in the plateau region. In the case of precipitation of the 1:1 complex, the plateau is observed for values of S_t following the constraint:

$$S_t \geq S_m + (L_t - L_m);\ L_t > L_m \qquad (2)$$

whereas precipitation of the 1:2 complex is subjected to the constraint

$$S_t \geq S_m + 0.5(L_t - L_m);\ L_t > L_m \qquad (3)$$

From the phase solubility diagram of benalaxyl, the following values are obtained using linear regressions for both the rising and the decreasing portion of the diagram (see **Figure 2e**): $S_m = (5.7 \pm 0.2) \times 10^{-4}$ mol dm^{-3}, $L_m = (6.6 \pm 0.3) \times 10^{-3}$ mol dm^{-3}, and $L_p = (8.2 \pm 3.2) \times 10^{-3}$ mol dm^{-3}. Substituting the values of S_m and L_m in eqs 2 and 3, it is obtained that the plateau

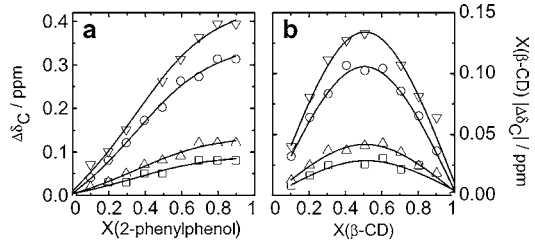

Figure 3. (**a**) Variations of the chemical shift displacements of carbons C3 (□), C4 (○), C5 (△), and C6 (▽) of β-CD vs molar fraction of 2-phenylphenol. (**b**) Job plots corresponding to the data in panel **a**. Lines are the curves fitted to the experimental data (see text).

comes to end at a solubilizer concentration of 7.4×10^{-3} mol dm^{-3} for precipitation of the 1:1 complex and 8.2×10^{-3} mol dm^{-3} for precipitation of the 1:2 complex. The latter concentration is totally in agreement with the value obtained for L_p, indicating that the 1:2 complex precipitates in the plateau region.

Finally, the decreasing portion of B_S diagrams could be analyzed to obtain good estimations of the association constants K_{11} and K_{12}. Nevertheless, such an analysis requires more and more precise experimental points in this region than we could achieve in the case of benalaxyl.

For those substances with higher solubility in water, 2-phenylphenol, and 8-hydroxyquinoline, NMR titrations could be performed, where the displacements of the peaks in the ^{13}C spectra (chemical shift displacement, $\Delta\delta_C$) of fungicide−β-CD mixtures with respect to pure CD were measured. In both cases, an increase of the chemical shift displacement of the CD carbon atoms with increasing molar fraction of the fungicide is observed, indicating the existence of interactions between the fungicides and the β-CD. This is shown in **Figure 3a** for 2-phenylphenol, where four of the CD carbon atoms were selected, those with the largest displacements. On the other hand, the data obtained for 8-hydroxyquinoline had very poor quality, showing great deviations from that general tendency. This can be explained on the basis of the significant solubility decrease of this fungicide in the presence of β-CD (see **Figure 2d**), which would cause precipitation of the fungicide in the sample tubes.

The observed variations of chemical shift displacement with the molar fraction of 2-phenylphenol must be attributed to the formation of one or more complexes between this fungicide and the β-CD. The stoichiometry of the complexes formed can be deduced from the corresponding Job plots (*6*), which are shown in **Figure 3b**. For all four carbon atoms, these plots are symmetric and have maxima at a β-CD molar fraction of about 0.5, showing that only a complex of stoichiometry 1:1 is formed between 2-phenylphenol and β-CD. This is in agreement with the results of phase solubility diagrams discussed above. Furthermore, quantitative analysis of the NMR data allows determination of the complex association constant, K_{11}. In this analysis, we use an equation derived elsewhere (*9*), which relates the chemical shift displacement to the initial concentrations of fungicide ($[H]_0$) and CD ($[CD]_0$):

$$\Delta\delta_C = \frac{\Delta\delta_{max}}{2[CD]_0}\left\{\frac{1}{K_{11}} + [CD]_0 + [H]_0 - \sqrt{\left(\frac{1}{K_{11}} + [CD]_0 + [H]_0\right)^2 - 4[CD]_0[H]_0}\right\} \quad (4)$$

where $\Delta\delta_{max}$ is the maximal chemical shift displacement, i.e., the displacement of the peaks of CD carbon atoms in the complex with respect to free CD. A global nonlinear least squares fit of this equation to all experimental data (*10*) yields

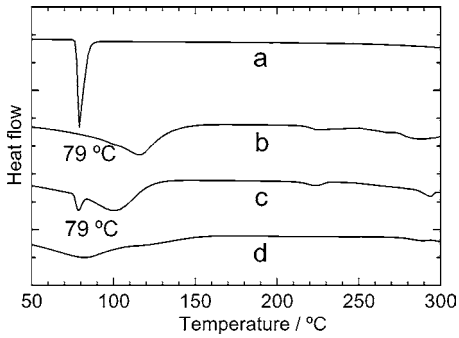

Figure 4. DSC thermograms of (**a**) benalaxyl, (**b**) β-CD, (**c**) a physical mixture of benalaxyl and β-CD, and (**d**) solid product isolated as a complex of benalaxyl and β-CD. The thermograms have been displaced along the *y*-axis to facilitate comparison.

a value for the K_{11} association constant of 2-phenylphenol with β-CD of about 1300 mol^{-1} dm^3 (**Table 1**). This value does not agree at all with that obtained from the analysis of the phase solubility diagram. Nevertheless, the result of NMR titrations is more reliable since those measurements were performed within several hours whereas solubility experiments took 8 days. The known tendency of phenols to oxidize, increased in 2-phenylphenol due to the charge donor properties of the phenyl group, could lead to the degradation of this fungicide during the solubility experiments; therefore, the obtained result for K_{11} from the phase solubility diagram should be discounted.

Characterization of the Solid Complexes. As described in the Experimental Section, solid products were isolated from mixtures of each fungicide with β-CD. The first step to characterize these solids was to check whether they contain the two compounds, fungicide and β-CD, and whether any degradation of these molecules has occurred. This was done by measuring the ^1H NMR spectra of the products dissolved in D_2O. In all cases, NMR spectra showed the peaks due to the fungicide protons and the β-CD protons. Furthermore, the observed chemical shifts coincided with those expected for the fungicides and the β-CD. These results show that the products isolated contain both fungicide and β-CD and that the two compounds maintain their molecular structure.

Further experiments were performed in order to check whether the isolated solid products are inclusion complexes between fungicide and β-CD or simple physical mixtures of the two compounds. DSC was the technique chosen for this purpose. The thermograms of pure fungicide, pure CD, physical mixture fungicide−CD, and isolated product, all measured under the same experimental conditions, were compared for each fungicide. The thermogram of a simple physical mixture must be a combination of the thermograms of pure fungicide and pure β-CD, whereas the thermogram of a real complex must clearly differ from them.

In the cases of prochloraz, 2-phenylphenol, and benalaxyl, the thermogram of the isolated product does not show the endothermic peak of the fungicide fusion, which is per contra present in the thermogram of the physical mixture. **Figure 4** shows the thermograms obtained for benalaxyl as an example of the behavior of these compounds. It can therefore be deduced that the solid products isolated from mixtures of prochloraz, 2-phenylphenol, and benalaxyl with β-CD are not simple physical mixtures. Taking into account the ability of these molecules to form inclusion complexes with β-CD in solution, the solids prepared should then be considered to be real inclusion complexes between each fungicide and β-CD. On the contrary, the solid obtained by mixing thiophanate methyl and β-CD is

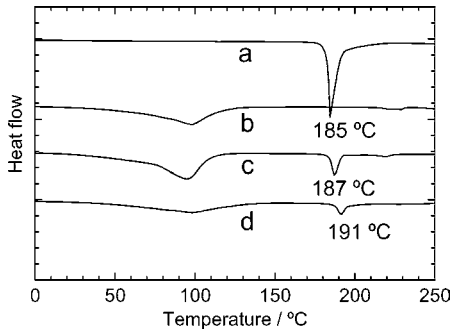

Figure 5. DSC thermograms of (**a**) thiophanate methyl, (**b**) β-CD, (**c**) a physical mixture of thiophanate methyl and β-CD, and (**d**) solid product isolated as a complex of thiophanate methyl and β-CD. The thermograms have been displaced along the *y*-axis to facilitate comparison.

probably a physical mixture, since the endothermic peak of the fungicide fusion clearly appears also in the thermogram of this product (**Figure 5**). In the case of 8-hydroxyquinoline, it is not clear from the DSC data whether the isolated solid is a complex, since its thermogram is different from that of the physical mixture, with a new endothermic peak at 180 °C, but shows the endothermic peak due to the fusion of the fungicide. A possible explanation of this result is that the isolated product is not the pure complex but contains certain amounts of free fungicide and free CD.

Each of the fungicides studied in this work show different behaviors in their complexation with β-CD, and no general features can be established. Therefore, the potential increase of fungicide solubility by encapsulation with CDs must be investigated in each individual case with suitable experimental techniques.

ABBREVIATIONS USED

CD, cyclodextrin; β-CD, β-cyclodextrin; DSC, differential scanning calorimetry.

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