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THERMODYNAMIC PARAMETERS FOR THE MOLECULAR INCLUSION REACTION OF DULCIN WITH β-CYCLODEXTRIN. SPECTROFLUORIMETRIC DETERMINATION OF DULCIN

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Summary—The inclusion of dulcin in α- and β-cyclodextrin has been studied by fluorescence spectroscopy. To quantitatively describe complex formation between the β-cyclodextrin and dulcin, an association constant of 290 M^{-1} at 21° was obtained. The thermodynamics associated with the complex formation between dulcin and β-cyclodextrin in aqueous solution has been studied. The obtained value of $\Delta G^0 = -13.7$ kJ/mole at 21°, together with $\Delta H^0 = -33.6 \pm 2.3$ kJ/mole and $\Delta S^0 = -67.2 \pm 8.3$ Jmole^{-1} K^{-1} indicate that dulcin has a very marked tendency to associate with β-cyclodextrin in water. The inclusion complex of dulcin in β-cyclodextrin has been used to determine dulcin in the range 0.13–5 μg/ml, the method has been applied to determine dulcin in soft drinks.

Dulcin [(4-ethoxyphenyl)urea] is a non-nutritive sweetener, about 250 times as sweet as cane sugar. In 1950 the Food and Drug Administration prohibited the use of dulcin as a sweetener because of the liver adenoma and lipidic infiltrations associated with its intake. In the 1950's contradictory communications had been found on acute and chronic toxicity. However, since 1968 the Food and Agriculture Organization/World Health Organization have included it among the additives which can cause liver tumors and is non-recommended for use until deeper investigations had been done.

Methods for its determination include liquid chromatography with photometric detection at 200 nm,[1] direct photometry at 440 nm[2] and redox volumetry.[3] To increase sensitivity, fluorimetric methods[4,5] have been developed; one of them[4] is based in the fluorescence at 440 nm, of dulcin reaction product by sodium nitrite. This method is laborious and required a long preparation and reaction time, about two hours. Recently, a direct, more sensitive fluorimetric method[5] has been proposed, based in the measure of fluorescence at 332 nm of dulcin in 100% acetonitrile. However, analytical methods in aqueous medium are preferable since they avoid the use of toxic organic media.

Cyclodextrins tend to include guest molecules in their cavities, because the hydrophobic character of the cyclodextrin cavity[6–8] means more apolar molecules are preferred for inclusion and substances which are not very soluble in water become more soluble in the presence of cyclodextrins. Because of the organizing ability of cyclodextrin media, the molecules in the internal cavity are isolated from the surrounding environment, their excited states shielded from extinction processes and luminescent phenomena are enhanced. In this manner cyclodextrins aqueous media increase the aqueous solubility of substances sparingly soluble in water and simultaneously enhance fluorescence. This has been exploited in the determination of a variety of organic compounds, mainly pesticides and drugs.[9–14]

To study the inclusion of dulcin in cyclodextrins fluorescence spectroscopy has been used. Qualitative evidence for host-guest binding comes from a study of changes in fluorescence spectra. Quantitative description of the complex formation between dulcin and β-cyclodextrin is obtained through the association constant and thermodynamic parameters associated with the process. The inclusion complex in β-cyclodextrin has been used to determine dulcin and the method has been applied to the determination of dulcin in soft drinks.

EXPERIMENTAL

Materials and methods

Dulcin α- and β-cyclodextrin were obtained from Sigma. A stock solution of $5.50 \times 10^{-3}M$ dulcin was prepared in ethanol. β-cyclodextrin was purified by recrystallization once from boiling water and $10^{-2}M$ aqueous solutions were prepared. All solvents used were of analytical-reagent grade and were obtained from Merck. The water was distilled and de-mineralized.

Emission measurements were made with a Perkin-Elmer LS-50 luminescence spectrometer. Information is sent via the RS232C interface of the fluorescence instrument to an external computer. Instrumental parameters are controlled by Fluorescence Data Manager (FLDM) software. Graphical print-out is by a Kyocera F-1000 laser printer connected to the spectrofluorimeter.

Procedures

General procedure. Place in a 10-ml standard flask an aliquot of dulcin solution in ethanol to give a final concentration between 0.1 and 5 μg/ml. Slowly evaporate to dryness by a nitrogen stream and add 10 ml of $10^{-2}M$ β-cyclodextrin solution. Sonicate these samples for 20 min and measure the relative fluorescence intensity at $\lambda_{exc} = 250$ nm and $\lambda_{em} = 340$ nm using slit widths of 2.5 nm and 5 nm for excitation and emission respectively, against a reagent blank.

The relative fluorescence intensity (RFI) is converted into units of concentration by applying the corresponding regression equation or calibration graph.

Determination of dulcin in soft drinks. Degas the samples by immersion for 30 min in an ultrasonic bath before analysis. Pipette volumes of samples, below 0.5 ml, containing dulcin in the range 0.1–5.0 μg/ml and dilute to 3 ml with $10^{-2}M$ β-CD. Sonicate these solutions for 20 min and measure fluorescence intensities as described in the general procedure.

RESULTS AND DISCUSSION

Relative fluorescence intensity was used as a qualitative measure of the complexing ability of dulcin with cyclodextrins. Separate experiments were conducted to study the behavior of dulcin in water, α- and β-cyclodextrin solutions. In Fig. 1 the fluorescence emission spectra in these media are presented. A fluorescence intensity enhancement in cyclodextrin media compared to water is observed because cyclodextrins offer a protective, more constrained microenvironment to an electronically excited lumiphor and so the resulting fluorescence is enhanced. The maximum fluorescence intensity is obtained from a molecule which is totally encapsulated inside the cyclodextrin cavity.[9,10] The more a molecule is subjected to the aqueous medium surrounding the cyclodextrin, the lower is the fluorescence intensity. So, the greater enhancement with β-cyclodextrin probably results from a better fit of the dulcin molecules in the larger β-cyclodextrin cavity which have a cavity diameter of 7.8 Å. In contrast, the diameter of the α-cyclodextrin cavity (5.7 Å) is too small to include totally the dulcin molecule and so the fluorescence intensity is lower.

In addition to size, hydrophobicity of the molecule establishes the strength of the complex, more hydrophilic species form weaker complexes producing faint emission signals. To describe quantitatively complex formation between the β-cyclodextrin and dulcin the binding constant was obtained by the Benesi-Hildebrand method,[15,16] using the expression:

$$\Delta F^{-1} = (\alpha K C_0[\beta - \mathrm{CD}])^{-1} + (\alpha C_0)^{-1}$$

where CD is cyclodextrin, ΔF is the change of fluorescence intensity upon addition of β-cyclodextrin, C_0 is the initial concentration of dulcin (mol/l.) and α is a proportionality constant. The linear relationship between ΔF^{-1} and $[\beta\text{-CD}]^{-1}$ gives K, the host-guest association

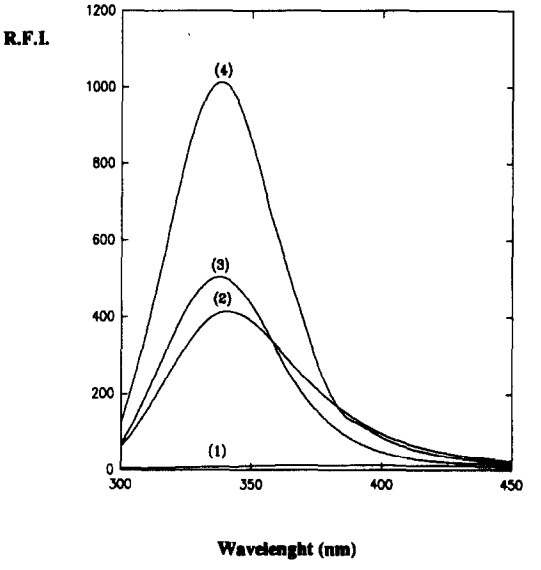

Fig. 1. Fluorescence emission spectra of (1) β-CD and of dulcin in (2) water, (3) α-CD and (4) β-CD. [dulcin] = $5.55 \times 10^{-6}M$; [α-CD] and [β-CD] = $10^{-1}M$, $\lambda_{exc} = 250$ nm.

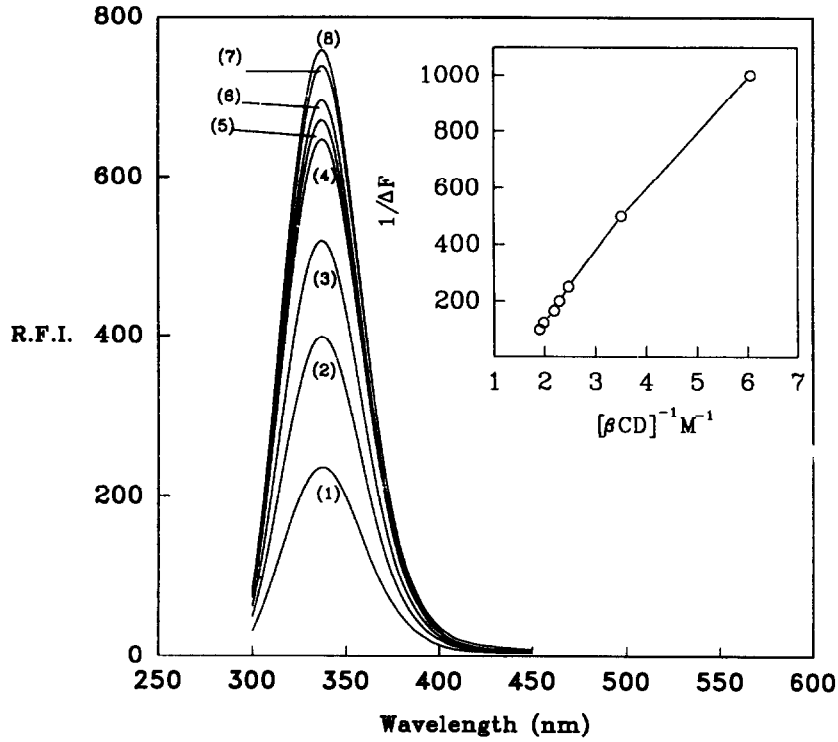

Fig. 2. Fluorescence emission spectra of dulcin solutions ($5.55 \times 10^{-6}M$) at various concentrations of β-cyclodextrin: (1) $0M$, (2) $1 \times 10^{-3}M$, (3) $2 \times 10^{-3}M$, (4) $4 \times 10^{-3}M$, (5) $5 \times 10^{-3}M$, (6) $6 \times 10^{-3}M$, (7) $8 \times 10^{-3}M$. Inset: Linear dependence between ΔF^{-1} and $[\beta\text{-CD}]^{-1}$.

constant. Figure 2 shows this linear dependence over the β-CD concentration range studied (10^{-3}–$10^{-2}M$). The binding constant was determined at $5.55 \times 10^{-6}M$ initial concentration of dulcin over the temperature range between 15 and 44°. The results obtained are shown in Table 1. The results indicate that there is a significant temperature dependence for K. The magnitude of the association constant reported for the dulcin β-cyclodextrin complex (290 at 21°) shows the trends of the polar guests which are bound less strongly to cyclodextrin than the apolar guests.[17]

Table 1. Association constants and thermodynamic parameters for the inclusion reaction of dulcin with β-cyclodextrin

Temperature (°C)	K* (*l./mol*)
15.0	370 ± 25
21.0	290 ± 10
31.0	247 ± 25
34.0	140 ± 20
41.0	113 ± 10
44.0	109 ± 20
ΔH^0 (*kJ/mol*)	−33.6 ± 2.3†
ΔS^0 (*Jmol*$^{-1}$ *K*$^{-1}$)	−67.2 ± 8.3†
ΔG^0 (*kJ/mol*)	−13.7 (at 21°)

*Uncertainties are standard deviation estimates calculated from the scatter of points about least-squares Benesi-Hildebrand plot lines.

†Uncertainties are standard deviation estimates calculated from the scatter of points about least-squares van't Hoff lines.

The substituents in dulcin are a hydrophobic ethoxy group and urea a weak base, only protonated below pH 0.[18] So, dulcin is an uncharged molecule at pH above 0 and neither protonation or dissociation takes place in aqueous solution of dulcin or in the α- or β-cyclodextrin complexes. To confirm this, the effect of pH on fluorescence intensity of dulcin solutions in water, α- and β-cyclodextrin has been studied but no effect has been observed. So the association constant is not affected by the pH of the

Table 2. Analytical parameters

Analytical sensitivity, s_A ($\mu g/ml$)	5.58×10^{-5}
Detection limit, C_L ($\mu g/ml$)	0.04
Quantitation limit, C_Q ($\mu g/ml$)	0.13
Linear dynamic range ($\mu g/ml$)	0.13–5
Amount taken ($\mu g/ml$)	1.00
Amount found ($\mu g/ml$)	1.04
Relative standard deviation, (%) ($n = 8$)	5.67
Error, $100ts/xn^{\frac{1}{2}}$ (%)	4.75

Table 3. Results of interference study at 1 μg/ml dulcin

Interferent	Dulcin:interferent weight ratio	Recovery of dulcin, %
Sodium cyclamate	1:1	105
	1:10	104
	1:20	95
	1:25	79
Saccharin	1:1	102
	1:3	82
	1:4	75
Sunset Yellow	1:1	126
	1:2	104
	1:3	84
	1:4	82
	1:5	67
β-carotene	1:1	121
	1:2	124
	1:3	128
	1:4	124
Benzoic acid	1:1	93
	1:5	108
	1:10	99
	1:20	77
Citric acid	1:1	97
	1:5	98
	1:10	107
Tartaric acid	1:1	97
	1:5	97
	1:10	98
	1:20	105
Phosphoric acid	1:1	95
	1:5	102
	1:10	80

medium, which in turn does not affect the distribution of charge in the dulcin molecule.

The interior of the cyclodextrin cavity is a region of low dielectric constant compared with that of the bulk solvent. To place an uncharged molecule such as dulcin within the cavity, entails moving it from a region of high to low dielectric constant, which is a favorable process. This hypothesis can be confirmed by determining the thermodynamics associated with the inclusion process. The thermodynamic parameters, ΔH^0, ΔS^0, and ΔG^0, for the inclusion reaction of the guest molecules were determined from the temperature dependence of the association constant. Van't Hoff plots of RT ln K *vs.* T and R ln K *vs.* $1/T$ were linear and from the slopes obtained enthalpies and entropies of complexation. The results are shown in Table 1 and indicate that dulcin has a very marked tendency to associate with β-cyclodextrin in water.

Several chemical phenomena have been proposed[19] as driving forces for complexation. Hydrophobic interaction essentially involves a favorable positive entropy ($\Delta S > 0$), together with a slightly positive enthalpy change ($\Delta H > 0$). However, thermodynamic parameters determined for the formation of cyclodextrin complexes showed that the inclusion process is more governed by a negative enthalpy change ($\Delta H < 0$) than by a positive entropy change.[20] The enthalpy stabilization can be explained by the expenditure of less energy in the guest desolvation step. Similar thermodynamic data[21] to those in Table 1 have been explained in terms of the release of high energy water from the cyclodextrin cavity. The expulsion of these enthalpy-rich molecules into bulk water upon substrate inclusion results in a negative enthalpy change, together with a negative entropy change. This hypothesis is consistent with the results obtained because a large negative enthalpy change (33.6 kJ/mole) was measured. However, the importance of this binding force is still controversial.[22]

Quantitative analysis

The maximum relative fluorescence intensity (RFI) is achieved in $10^{-2}M$ solutions of β-CD. A slight modification to this concentration has little effect on fluorescence intensity (Fig. 2). Thus, a final β-CD concentration of $8 \times 10^{-3}M$ decreases the fluorescence intensity by only 3%. Ethanol also has little effect on the fluorescence, 10% ethanol decreases the RFI by 5%, and 30% ethanol decreases RFI by 10%. Therefore, although low proportions of ethanol are permitted, absence of the solvent gives maximum sensitivity.

A linear calibration graph was obtained by plotting the fluorescence intensity against standard dulcin concentration between 0.1 and 5 μg/ml. The calibration graph obtained by the least-squared treatments is

$$I_f = 179.0[\text{Dulcin}] + 20.8 \quad (r = 0.9996,\ n = 8)$$

where I_f is the relative fluorescence intensity, r the correlation coefficient and [Dulcin] the dulcin concentration in μg/ml.

The sensitivity of the method is reported as the analytical sensitivity $s_A = s_2/m$ (s_s is the standard deviation of the analytical signal (eight measurements) and m is the slope of the calibration graph). The detection limit was calculated as $3s_{bk}/m$ in which s_{bk} is the standard deviation of the blank signals. The limit of quantification was calculated as $10s_{bk}/m$ and is employed to establish the lower limit of the linear dynamic range. To evaluate the reproducibility, eight replicates of dulcin at the 1.0 μg/ml level were measured. The results obtained are given in Table 2.

Interferences

To evaluate the selectivity of the proposed spectrofluorimetric method, the effect of some other food additives on the determination of 1 μg/ml of dulcin was studied. The additives selected were other sweeteners usually found in food, (saccharin and cyclamate) coloring (Sunset Yellow and β-carotene) a preservative (benzoic acid) usually found in carbonated beverages and substances such as the acids, citric, tartaric and phosphoric used in soft drinks as pH regulators. Various volumes of stock solutions of the different potential interferents were added to a dulcin standard solution in order to obtain different interferent to analyte ratios in the final solution. The solutions were treated as described under Experimental. The results obtained are given in Table 3, where it can be seen that acceptable recoveries are obtained, except for that corresponding to β-carotene. However, it can be easily separated from dulcin because of the different solubilities; β-carotene is soluble in apolar solvents such as hexane and dulcin in polar solvents such as ethanol or acetonitrile.

Application to the determination of dulcin in soft drinks

The usefulness of the method developed was evaluated by application to the determination of dulcin in soft drinks. To determine recoveries at different sample volumes in a 3-ml standard flask, different amounts of dulcin were added to give a final concentration of between 0.1 and 5 μg/ml, after checking for the absence of dulcin.

Table 4. Determination of dulcin in soft drinks

Sample volume (μl)	Dulcin added (μg/ml)	Dulcin found (μg/ml)* CWS†	Dulcin found (μg/ml)* MOSA‡	Recovery (%) CWS	Recovery (%) MOSA	MOSA curve (n = 4)
Soft drink						
40						147.6[D] + 43.6 (r = 0.9985)
	0.5	0.46 ± 0.04	0.43 ± 0.05	92.0	86.0	
	0.7	0.71 ± 0.02	0.71 ± 0.02	101.7	101.4	
	1.0	1.05 ± 0.01	1.08 ± 0.01	105.0	108.0	
	3.0	2.59 ± 0.06	2.98 ± 0.07	86.4	99.3	
80						156.9[D] + 31.2 (r = 0.9991)
	0.5	0.46 ± 0.01	0.46 ± 0.01	92.0	92.0	
	0.7	0.66 ± 0.01	0.69 ± 0.01	93.8	98.6	
	1.0	1.06 ± 0.01	1.07 ± 0.01	106.0	107.0	
	3.0	2.68 ± 0.03	2.99 ± 0.03	89.3	99.6	
100						149.3[D] + 21.8 (r = 0.9998)
	0.2	0.17 ± 0.02	0.19 ± 0.02	83.3	95.0	
	0.5	0.43 ± 0.03	0.51 ± 0.03	87.0	102.0	
	0.7	0.59 ± 0.04	0.69 ± 0.05	84.3	98.6	
	1.0	0.84 ± 0.10	0.99 ± 0.12	84.5	99.8	
Soft drink (flavour lemon)						
40						153.1[D] + 20.1 (r = 0.9989)
	0.2	0.16 ± 0.01	0.19 ± 0.01	78.3	95.0	
	0.5	0.43 ± 0.01	0.51 ± 0.01	86.0	102.0	
	0.7	0.61 ± 0.01	0.72 ± 0.01	87.0	102.8	
	1.0	0.84 ± 0.01	0.98 ± 0.01	84.3	98.5	
80						156.5[D] + 18.8 (r = 0.9955)
	0.2	0.17 ± 0.02	0.19 ± 0.02	83.3	99.0	
	0.5	0.39 ± 0.01	0.49 ± 0.01	78.7	99.6	
	0.7	0.56 ± 0.00	0.65 ± 0.00	80.0	92.1	
	1.0	0.96 ± 0.01	1.02 ± 0.01	96.0	102.2	
100						143.1[D] + 16.9 (r = 0.9991)
	0.2	0.16 ± 0.01	0.18 ± 0.01	80.0	90.0	
	0.5	0.39 ± 0.03	0.51 ± 0.04	78.7	102.0	
	0.7	0.53 ± 0.01	0.68 ± 0.01	75.5	97.1	
	1.0	0.73 ± 0.00	0.99 ± 0.00	73.0	99.2	

*Mean ± standard deviation (n = 3)
†Calibration with standards
‡Method of the standard additions

Volumes of the aqueous samples must be below 0.5 ml to ensure a β-CD final concentration above $8 \times 10^{-3}M$. The samples were sonicated for 20 min. Table 4 gives the results obtained by means of calibration with standards (CWS). In order to minimize the detected matrix effects when the concentration of dulcin is obtained by means of a calibration with standards, the method of standard additions (MOSA) was applied. The MOSA curves were obtained for each soft drink and soft drink (flavour lemon) at sample sizes of 40, 80 and 100 μl and the results found are shown in the MOSA column of Table 4. The MOSA technique provides an *in situ* normalization of the proportional error. Correction for the constant error is provided by an adequate blank correction.

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