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Probing Inclusion Complex of a Dye (ISD) with Cyclic Oligosaccharide for Minimizing Harmful Effects

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Abstract: Indigo is a colouring agent used widely in various fields. The synthetic indigo has many adverse effects when it is consumed with foods and beverages. Cyclodextrin (CD) is known to have special chemical characteristics and biological activities and has a suitable cavity that can include molecule of suitable diameter. In our present study, we have outlined different modes of characterization of the inclusion complex (IC) formation between poorly water soluble dye Indigosulfonic Acid Dipotassium Salt (ISD) and β -Cyclodextrin with the help of FTIR Spectroscopy, UV-Visible spectroscopy, fluorescence spectroscopy, ¹H NMR study, 2D NOESY, Isothermal Titration Calorimetric study and SEM analysis. ¹H-NMR study and other spectroscopic analysis clearly revealed the successful formation of the (IC) which is supported by cross-peaks formed in 2D-NOESY spectrum. Comparable association constants and thermodynamic parameters obtained from both UV-Visible study and ITC study confirmed the higher stability of the IC. The solubility of the IC was found higher than the pure ISD.

Keywords: Association constant, β -Cyclodextrin, Fluorescence study, Inclusion complex, Job plot.

1. INTRODUCTION

In the era of globalisation, host-guest inclusion chemistry based research has become a matter of utmost importance because of their substantial applications in the area of industrial and biomedical research [1]. Now days, cyclodextrins (CDs) are being widely used for its excellent ability to form inclusion complexes with various biologically as well as industrially important compounds [2]. Such phenomenon which is known as inclusion brings about certain modifications in both physical and chemical properties of the complex formed [3]. Inclusion complexes (ICs) with CDs are being widely used as biomimetic systems and as unique media for various types of reactions [3-6]. The most common CDs belong to cyclic oligosaccharide category having distinctive truncated cone-shaped structure with a hydrophobic cavity and hydrophilic rim. The hydrophilic wider rim contains primary and narrower rim contains secondary -OH groups. The specific structural features lead CD molecules to form inclusion complexes with a variety of hydrophobic and amphiphilic species in both the aqueous and mixed solvent medium [7]. Commonly there are three types of cyclodextrin molecules namely α -CD, β -CD and γ -CD basing on the number of glucopyranose units. α -CD, β -CD and γ -CD are made up of six and seven and eight glucopyranose units respectively with cavity diameter of 4.7 Å and 6.0 Å and 8.3 Å respectively. As the CD molecule has no free rotation about the glucopyranose linkage, the cyclodextrins are not perfectly cylindrical in shape rather toroidal or cone shaped [8-9]. Thus, a hydrophobic cavity is formed within the molecule and the outer surface remains hydrophilic due to presence of -OH groups. The hydrophobic void of CD can trap the hydrophobic portion of the guest molecule to form a stable inclusion complex and the system is stabilised by different types of non-covalent interactions, such as van der Waals interactions, hydrogen bonding interactions etc. [10].

The ICs with cyclodextrin molecules have been reported to have diverse applications in scientific literature which comprises enhanced solubility, bioavailability, increased stability, masking of awkward taste and odour, decrease of volatility, probability of controlled drug delivery system and many more. However, ICs have special importance in the field of education and industry [11-14]. ICs can be used to create stimuli responsive supramolecular substances. Here, various external stimuli for enzyme activation, photo sensing, thermal dependence, variations in pH/ redox environments, competitive binding are used to control the release of guest molecules from the Host-Guest complexes [15-18]. Researchers paid importance on molecular sensing, release of anticancer drug and gene transfection in the past few years [19-22].

The host molecules are chosen for the formation of ICs because of their cyclic-constrained conformations, which is beneficial for the molecular selectivity [23]. Due to the amphiphilic nature CDs are able to form self-assemble in aqueous medium to form various well defined systems such as nano tubes, nano sheets and nano rods, micelle, vesicles which have been found applicable in various fields of drug delivery as well as cell imaging systems and nano devices [23-27]. Sophisticated probes are being designed and applied for various systems such as molecular switches and machines, chemo sensors, transmembrane channels, molecular logic gates, and other interesting host-guest systems [28-30].

Dyes and their derivatives are used mostly as indicators but they also have vast applications in colouring foods, cosmetics, solvents, drugs, papers, waxes, plastics etc. Dyes as colouring agent are widely used to colour food and beverages as well as pharmaceutical products. This procedure is to enhance the attractiveness of products and also to help people to distinguish different pharmaceutical products. A number of dyes, especially organic colouring agents, sometimes show negative effect on the human body [31]. Synthetic dyes are highly coloured, toxic, and carcinogenic in nature [32,33]. The colour of food and beverages are released inside our body when consumed. Indigo is known to be a natural dye extracted from plants *Isatis Tinctoria* and *Indigofera Tinctoria*. Because of having dark

blue colour, they have wide spread applications in the textile industries and food technology. Due to the presence of intra and intermolecular H-bonding network the dye molecule becomes more stable which reflects in its high melting point 390-392⁰ C^[34]. Indigosulfonic Acid Dipotassium Salt is known to be a derivative of naturally occurring dye Indigo. It has low solubility in water in pure form^[35]. ISD becomes harmful for our respiratory tract when it is swallowed. It also acts as a skin and eye irritant. Dye may be inserted inside of the β -CD molecule and therefore be used to increase the stability of the dye on surface as well as may get control released inside human body to reduce the severity of its adverse effect^[36]. Thus, inclusion of the colouring agent inside CD can be safe and significant as its release will be controlled and hence it will be less harmful^[37]. In this work, we studied the inclusion of ISD inside the hydrophobic cavity of β -CD in both solution and the solid phase.

2. EXPERIMENTAL SECTION

2.1. Materials Used

2.1.1. Source and Purity Of Samples: Indigosulfonic Acid Dipotassium Salt was purchased from TCI chemicals (INDIA) Pvt. Ltd. having mass purity > 90.0% and alpha, beta cyclodextrins have been purchased from Sigma Aldrich Germany having mass purity fraction \geq 98% and used without further purification (Table 1).

Table 1: Details of Chemicals used

Name of chemicals	Molecular Weight (g/mol)	Source	CAS number	Purification method	Mass purity
β -Cyclodextrin	1134.98	Sigma Aldrich Germany	7585-39-9	Used as purchased	\geq 97%
Indigosulfonic Acid Dipotassium Salt	498.57	TCI Chemicals Pvt.Ltd. India	13725-33-2	Used as purchased	>90%
Distilled Water	18	Self-made	-	-	-

2.2. Apparatus and Procedure: The mother solutions of ISD and β -CD were prepared by mass using Mettler Toledo AG-285 (Uncertainty \pm 0.1 mg) at 298.15 K and other solutions of required strengths were prepared by mass dilution.

Fourier transform infrared spectra (FTIR) were recorded on a Perkin Elmer 8300 FT-IR spectrometer (Shimadzu, Japan) using KBr disk technique at a resolution 4 cm⁻¹. Samples were prepared as thin KBr disks with minute amount of sample at room temperature. The range of scanning was kept at 4000–400 cm⁻¹.

Isothermal titration calorimetry was used to determine the binding stoichiometry, association constant at 298.15 K using a MicroCal VP-ITC isothermal titration calorimeter (Microcal now Malvern instrument). At first, thermal equilibration was established at room temperature, followed by 120-s delay initially and then 28 subsequent injections of ISD into Beta CD solution. (The duration of each injection was 10s with a spacing time of 180s.) An enthalpy generation curve was produced in each injection (in micro calories per second versus time in minutes). The association affinity and thermodynamic properties of the binding phenomenon were determined by fitting the integrated heats

of binding to the one-site binding model to give the association constant (K_a), stoichiometry (N^C), binding enthalpy (ΔH^0) and entropy (ΔS^0). ^1H NMR and 2D NOESY were performed in D_2O medium using BRUKER AVANCE 400 MHz instrument.

UV-Visible Spectroscopy was performed in Agilent 8453 spectrophotometer (uncertainty $\pm 2\text{nm}$, 1cm path quartz cell was used) and to control the temperature a digital thermostat was attached with the UV instrument. For fluorescence measurement, we used Quanta Master 40 spectrofluorometer. The scanning electron micrographs were determined by JEOL JSM-IT 100 scanning electron microscope model.

2.3. Synthesis of the Inclusion Complex: The inclusion complex of ISD and β -CD was prepared by co-evaporation method. ISD (dye) and β -CD were accurately weighed according to their 1:1 molar ratio. Here, 0.2 mM of ISD was dissolved in 15 ml 20% ethanol-water solution. Then, it was added drop wise to 25 ml 0.4mM aqueous solution of β -CD. The solution was stirred continuously with the help of a magnetic stirrer for 12 hours at 60°C following the filtration of the mixture using filter paper and the precipitate obtained was washed with 50% ethanol. The crude residue was then air dried at room temperature for the next 6 hours and final dry inclusion compound was stored in a desiccator at room temperature.

3. RESULTS AND DISCUSSION

3.1. Job Plot for The Determination of Stoichiometry of the Host-Guest Inclusion Complex: UV-Vis spectroscopic study is used in the field of host-guest inclusion complexation to understand the subsistence of the IC as well as the stoichiometry. While entering from the highly polar bulk solution to the hydrophobic cavity, the guest molecule experiences a variation of the molar extinction coefficient ($\Delta\epsilon$)^[38] and thus change in the absorption pattern took place. We found the absorption maxima for the guest molecule at 289 nm. (Fig.1. absorption pattern)^[39].

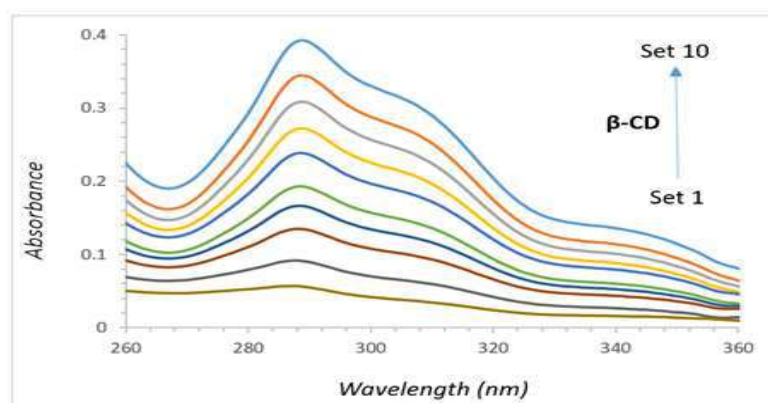


Fig.1: Absorption spectra of intensity against wavelength obtained from UV-Visible spectroscopy at varying concentration of β -Cyclodextrin keeping the guest concentration (Indigo sulfonic acid dipotassium salt) constant. The different lines represent absorption pattern at a particular concentration of β -CD.

The continuous variation method, in other words, Job's plot method was applied to determine the stoichiometric ratio of the host and guest molecule. The sample solutions of different concentration ratios of β -CD and Guest were prepared and mixed in such a way that the total volume of host and

guest molecule remains constant. The changes in absorption spectra were recorded and shown in the **table 2**. We plotted $\Delta A \times R$ against R [where ΔA is the difference in absorbance of ISD without and with CD i.e. (ISD+ β -CD) and $R = [\text{ISD}] / ([\text{ISD}] + [\beta\text{-CD}])$] to determine the stoichiometric ratio. We found the R_{max} value near 0.5^[40] (**Fig. 2**). In general; $R = 0.5$ stands for 1:1 or 2:2 G:H (guest: host) complexes; $R = 0.33$ for 1:2 G:H complexes^[41]. Thus the value of R , obtained experimentally, indicates successful inclusion of one guest molecule inside the hollow cavity of one molecule of β -CD promising the 1:1 host-guest inclusion.

Table2: Data for Job Plot performed by UV-Vis spectroscopy for ISD- β -CD system at 298.15K^a

Guest conc. [ISD] (μM)	β -CD (μM)	$R = [\text{ISD}] /$ $([\text{ISD}] + [\beta\text{-CD}])$	A @ λ_{max} 289 nm	ΔA (0.39195-A)	$\Delta A \times [\text{ISD}] /$ $([\text{ISD}] + [\beta\text{-CD}])$
100	0	1	0.39195	0	0
90	10	0.9	0.34407	0.04788	0.043092
80	20	0.8	0.30812	0.08383	0.067064
70	30	0.7	0.27243	0.11952	0.083664
60	40	0.6	0.23841	0.15354	0.092124
50	50	0.5	0.19298	0.19897	0.099485
40	60	0.4	0.16663	0.22565	0.09026
30	70	0.3	0.1345	0.25745	0.077235
20	80	0.2	0.09087	0.30108	0.060216
10	90	0.1	0.05604	0.33591	0.033591
0	100	0	0.01112	0.38083	0

^aStandard uncertainties in temperature: $\pm 0.01\text{K}$, Pressure: $\pm 10\text{kPa}$

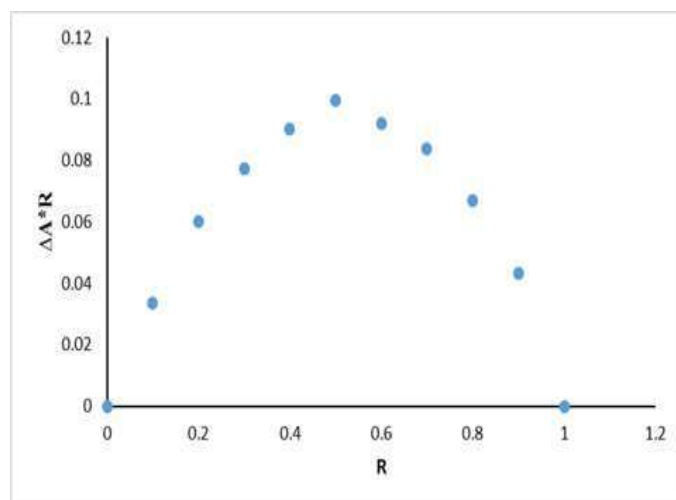


Fig.2: Job plot of β -Cyclodextrin (β -CD) and Indigosulfonic Acid dipotassium salt (ISD) system at λ_{max} (Indigosulfonic Acid Dipotassium Salt) = 289 nm

3.2. Association constants and thermodynamic parameters: UV-Visible spectroscopy being the most consistent method to calculate the association constant (K_a) for the formation of IC^[42]. Incorporation of the guest (act as a chromophore) molecule inside the hydrophobic cavity of the CD molecule indulges some variations of the chemical environment^[43, 40].

The guest molecule binds to the host molecule by means of hydrophobic interactions. We recorded the UV-Visible spectra of the complexes at different concentrations of the host molecule keeping the concentration of the guest molecule fixed (**Fig.1**). The changes in the values of absorbance (at $\lambda_{\text{max}} = 289\text{nm}$) were noted at three distinct temperatures. A graph of $1/\Delta A$ against $1/[CD]$ was plotted to calculate the association constant using Benesi–Hildebrand equation (See supporting information **Table 3, 4, 5 and Fig. 4**)[40,44] equation (1).

$$\frac{1}{\Delta A} = \frac{1}{\Delta \varepsilon [ISD] K_a} \times \frac{1}{[CD]} + \frac{1}{\Delta \varepsilon [ISD]} \quad (1)$$

The higher positive values of K_a at three different temperatures signify the increasing feasibility of the process (**Table 6**). The values of the K_a were used to determine the change in the enthalpy (ΔH^0) and entropy (ΔS^0) of the inclusion process. We plotted $\log K_a$ against $1/T$ following van't Hoff equation (2) (**Fig. 5**). The values of ΔH^0 and ΔS^0 found are given in the table 6 and from these values ΔG^0 was calculated[2,42].

$$2.303 \log K_f = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (2)$$

In this experiment, the values of ΔH^0 and ΔS^0 were found to be negative. This signifies that the formation of the IC is an exothermic process and is entropy-restricted (**Table 6**)^[2]. This may be due to the molecular association of the CD and ISD molecules. As a result of association, the entropy is decreased, which is contrary for a process to be spontaneous. However, the restriction due to negative ΔS value is overcome by the highly negative value of ΔH making the entire inclusion process thermodynamically favourable.

The negative Gibb's free energy change (ΔG^0) of a process is the measure of the spontaneity of that process. Thus, ΔG^0 of the process of inclusion was calculated using the values of thermodynamic parameters ΔH^0 and ΔS^0 from the following equation (3) at 298.15K.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (3)$$

It is seen that the value of ΔG^0 is negative (**Table 6**). This, in fact, concludes that the formation of the ICs is feasible and the process is an exergonic one. This is due to the effective association of the guest ISD molecule inside the suitable cavity of CD molecule.

Table 3: Data for the Benesi-Hildebrand double reciprocal plot performed by UV-VIS spectroscopic study for ISD- β -CD complex at 293.15 K

	[ISD] (μM)	[CD] (μM)	A_0	A_1	ΔA	$1/\Delta A$	$1/cd$
ISD+ β -CD	50	10	0.21546	0.22035	0.00489	204.499	100000.00
	50	20	0.21546	0.22473	0.00927	107.8749	50000.00
	50	30	0.21546	0.22804	0.01258	79.49126	33333.33
	50	40	0.21546	0.22924	0.01378	72.56894	25000.00

50	50	0.21546	0.23439	0.01893	52.8262	20000.00
50	60	0.21546	0.23559	0.02013	49.6771	16666.67
50	70	0.21546	0.23681	0.02135	46.83841	14285.71
50	80	0.21546	0.23951	0.02405	41.58004	12500.00
50	90	0.21546	0.24102	0.02556	39.12363	11111.11
50	100	0.21546	0.24447	0.02901	34.47087	10000.00

Table 4: Data for the Benesi-Hildebrand double reciprocal plot performed by UV-VIS spectroscopic study for ISD-β-CD systems at 303.15 K

	[Drug] (μM)	[CD] (μM)	A ₀	A ₁	ΔA	1/ΔA	1/[CD]
ISD-β-CD	50	10	0.20213	0.20707	0.00494	202.4291	100000
	50	20	0.20213	0.21126	0.00913	109.529	50000
	50	30	0.20213	0.21561	0.01348	74.18398	33333
	50	40	0.20213	0.21906	0.01693	59.06675	25000
	50	50	0.20213	0.22258	0.02045	48.89976	20000
	50	60	0.20213	0.22429	0.02216	45.12635	16667
	50	70	0.20213	0.22641	0.02428	41.18616	14286
	50	80	0.20213	0.22656	0.02443	40.93328	12500
	50	90	0.20213	0.22879	0.02666	37.50938	11111
	50	100	0.20213	0.23637	0.03424	29.20561	10000

Table 5: Data for the Benesi-Hildebrand double reciprocal plot performed by UV-VIS spectroscopic study for ISD-β-CD systems at 313.15 K

	[Drug] (μM)	[CD] (μM)	A ₀	A ₁	ΔA	1/ΔA	1/[CD]
ISD+β-CD	50	10	0.19368	0.19822	0.00454	220.26432	100000
	50	20	0.19368	0.20315	0.00947	105.59662	50000
	50	30	0.19368	0.20616	0.01248	80.128205	33333
	50	40	0.19368	0.20761	0.01393	71.787509	25000
	50	50	0.19368	0.21313	0.01945	51.413882	20000
	50	60	0.19368	0.21394	0.02026	49.358342	16667
	50	70	0.19368	0.21496	0.02128	46.992481	14286
	50	80	0.19368	0.21971	0.02603	38.417211	12500
	50	90	0.19368	0.22884	0.03516	28.441411	11111
	50	100	0.19368	0.23792	0.04424	22.603978	10000

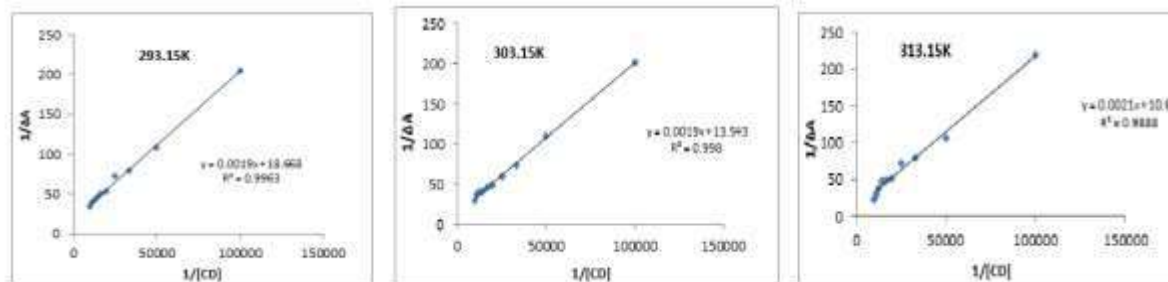


Fig.4: Benesi-Hildebrand double reciprocal plot for the effect of β -CD on the absorbance of ISD at three different temperatures 293.15 K, 303.15 K and 313.15 K.

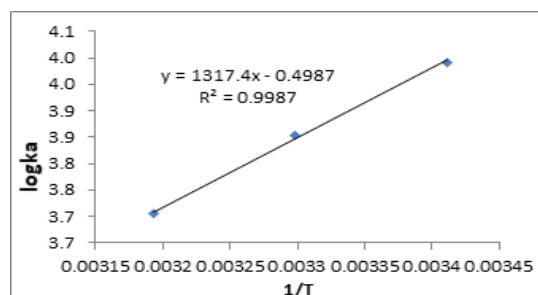


Fig.5: Plot of $\log K_a$ vs $1/T$ for the interaction of β -CD with ISD.

Table 6: Association constants obtained by the Benesi–Hildebrand method (K_a) from UV-Visible study, Fluorescence study and ITC study along with corresponding thermodynamic parameters of Indigosulfonic Acid dipotassium Salt- β -cyclodextrin inclusion complexes at 293.15K^a, 303.15K^a and 313.15K^a.

Method	$K_a(10^3M^{-1})$			ΔG^0 (kJmol ⁻¹)	ΔH^0 (kJ mol ⁻¹)	ΔS^0 (Jmol ⁻¹) K ⁻¹
	293.15K	303.15K	313.15K			
UV-Vis Spectroscopy	9.82	7.13	5.07	-2.38	-5.23	-9.55
Fluorescence Spectroscopy	9.51	-	-	-	-	-
ITC study	8.70±1.98			-3.203	-1.60±0.23	5.38

3.3. Fluorescence study: With the help of fluorescence study we can evaluate the binding constant by observing the reasonable change in the fluorescence emission spectrum due to some sort of interactions between β -CD and ISD.

The modified Stern-Volmer equation (equation 4) was employed to determine the extent of interaction (association constant K_a) between the host and guest molecule used in this experiment,

$$\frac{F_0}{\Delta F} = \frac{1}{F_q \cdot K_a \cdot [Q]} + \frac{1}{F_q} \quad (4)$$

Where, ΔF is the difference in fluorescence at a concentration $[Q]$ in absence and presence of cyclodextrin. K_a , the quenching constant, is equivalent to the association constant. F_q is the fraction of fluorescence accessible to the quencher (here cyclodextrin). From the plot of $F_0/\Delta F$ vs $1/[Q]$ the binding constant was calculated^[44-46].

The concentration of ISD was kept constant at 0.5 μ M while the concentrations of β -CD varied from 0.2 μ M to 0.8 μ M. (Table7, Fig.6). From fluorescence study, the association constant was found to be 9.51 $\times 10^3$ at 298.15K, which is comparable to the association constant obtained from the UV-Visible spectroscopy at 293.15K (Table7).

Table 7: Data for calculation of Association Constant using fluorescence spectroscopic study

F ₀	F	ΔF= F ₀ -F	1/[β-CD] /M-1	1/ΔF	Intercept	Slope	K _a /M ⁻¹
987921.44	1028281.44	40360.00	50000.00	2.48E-05			
987921.44	1038976.82	51055.38	33333.33	1.96E-05			
987921.44	1052248.39	64326.95	25000.00	1.55E-05	3.8E-06	4E-10	9512.25
987921.44	1067024.89	79103.46	20000.00	1.26E-05			
987921.44	1080453.77	92532.33	16666.67	1.08E-05			
987921.44	1092249.02	104327.58	14285.71	9.59E-06			
987921.44	1101122.40	113200.96	12500.00	8.83E-06			

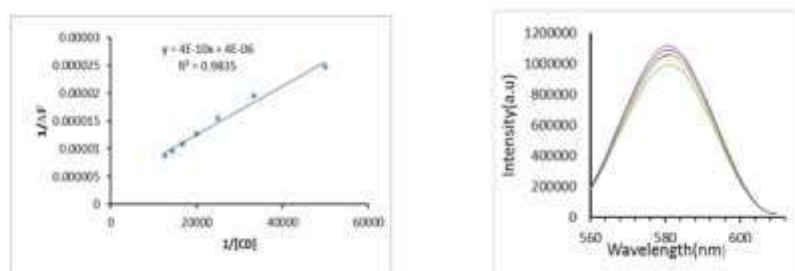


Fig.6: Plot of $1/\Delta F$ (ΔF is the absorbance difference of guest in presence and absent of CD) Vs $1/[CD]$ ($[CD]$ is the concentration of CD) from the Stern-Volmer equation and fluorescence spectra of Indigosulfonic Acid Dipotassium salt and β -Cyclodextrin at different molar concentration.

3.4. Calorimetric characterization of complexation: In order to calculate the weak binding constants and the corresponding thermodynamic parameters for the host-guest inclusion complex with high accuracy, we have applied Isothermal titration calorimetry (ITC) method. This analytical method is used to determine binding constants ranging from 10^8 to 10^2 M^{-1} [47]. It has become an effective method for directly determining the thermodynamic parameters instead of the previously used van't Hoff equation methodology, ITC method is more precise in terms of the determination of thermodynamic parameters [48]. The ITC diagrams of ISD binding to β -CD are given in **Fig. 7**.

From the plot of ITC, of ISD and Beta CD, the upper graph denotes heat release upon each injection of ISD to the sample cell i.e. in Beta CD [49]. The heat release is generally due to complexation of the guest molecule with the host until saturation is achieved that means guest molecules are getting associated to the specific number of the host molecules. It has been observed that the process of inclusion of ISD inside the β -CD is exothermic in nature and the magnitude of the heat evolved during the inclusion process decreases gradually with each injection until complete saturation is achieved.

The extent of the host-guest inclusion is supported by the structural orientation of cyclodextrin molecule which makes the non-polar part of the guest molecule to enter into the hollow cavity of CD molecule, thus the inclusion complex gets stability [42]. Another driving force for inclusion process can be attributed to the fact of releasing the water molecules from the hydrophobic cavity into the bulk and hence the total entropy of the system gets increased [50]. The incorporation of the guest molecule takes place from the wider rim of the β -CD molecule, as evident from NOESY spectrum.

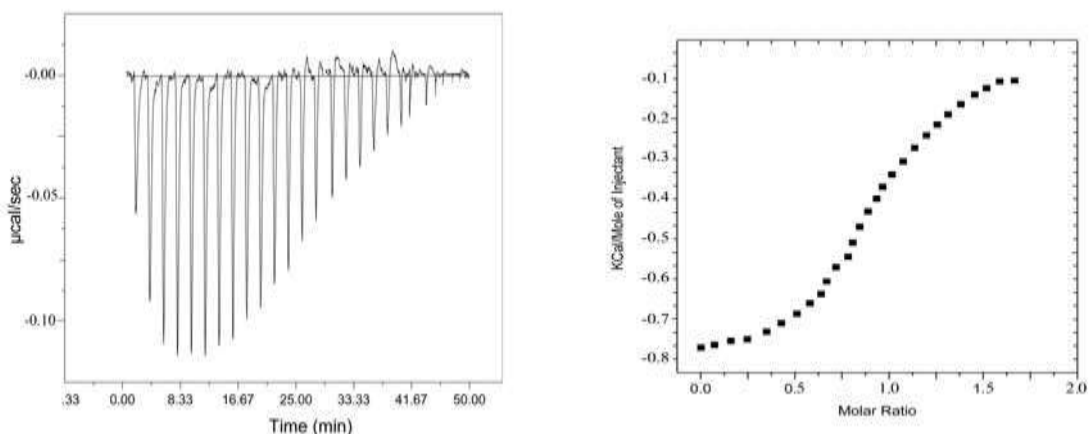


Fig.7: Representative ITC(Isothermal Titration Calorimetry) profiles for the titration of ISD(Indigosulfonic Acid Dipotassium salt) (500mM) with β -CD (50 mM) at 298.15 K. Figure above represent the raw data for the continuous injection of β -CDs(β -Cyclodextrin) into the ISD(Indigosulfonic Acid dipotassium Salt), after correction of heat of dilution. Figure below is the binding isotherm fitted to the raw data and the bottom panels show the integrated heat data after correction of heat of dilution.

The heats of binding were plotted as a function of the molar ratio of [CD/ISD]. The binding parameters like the stoichiometry of binding (N^c), the equilibrium binding constant (K), enthalpy of complexation (ΔH^0) and standard changes in free energy (ΔG^0) and entropy (ΔS^0) were estimated from ITC data on the basis of fitting the data according to the independent binding model. The values of thermodynamic parameters, particularly ΔH^0 and ΔS^0 (**Table 6**), shows that of the complexation deals with non-covalent forces in solvent medium, e.g. electrostatic, hydrophobic, van der Waals, and H-bonding interaction. The complexation process was found to be exothermic ($\Delta H^0 < 0$) and spontaneous ($\Delta G^0 < 0$) with positive entropic contribution. The negative value of ΔH^0 indicates heat evolution during the inclusion phenomenon while positive entropy changes (ΔS^0) usually arise from the translational and conformational freedoms of host and guest upon complexation.

Negative Gibbs energy indicates that inclusion process is a spontaneous one under experimental conditions; $-T\Delta S^0$, denotes that inclusion of ISD in the CDs is accompanied by displacement of water molecules from the CD cavity. On the other hand, comparatively it has been observed that smaller $-T\Delta S^0$ terms are associated with larger CD cavities ^[51]. Every peak shown in the binding isotherm indicates a single injection of the guest molecule into the host solution. The exothermicity of the calorimetry peaks (**Fig. 7**) arises because of the considerable interaction between ISD and β -CD molecule. The stoichiometry (N^c) of the inclusion phenomena using ITC analysis is determined from the value of number of binding sites. We obtained the value very close to 1 (Table 8), which clearly indicates 1:1 stoichiometry, which is in good agreement with the 1:1 stoichiometric ratio obtained from the Benesi-Hildebrand plot analysis of the UV-Visible spectroscopic data.

The principal forces involved are van der Waals and hydrophobic interactions. Hydrophobic interactions are entropy driven ($|H^0| < |T\Delta S^0|$), whereas van der Waals interactions are essentially enthalpy driven processes, ^[52, 53]. From the data obtained, it has been found that the binding of ISD with β -CDs are entropy driven as the enthalpy value of the interaction is smaller with respect to the entropy of the interaction ($|H^0| < |T\Delta S^0|$). This indicates hydrophobic interactions predominate over major van der Waal's interactions in this case.

Table 8: Value indicating the Binding sites obtained from Isothermal Titration Calorimetric study.

Temperature(K)	Number of binding sites(N ^c)
298.15	0.954±0.0693

3.5. FT-IR SPECTROSCOPY

During inclusion procedure, if the guest molecule gets inserted into the hollow cavity of CD molecule some changes in stretching frequencies of the concerned spectral bands will take place, in other words either the spectral bands will get shifted from their previous positions or two spectral bands get merged or widening of bands happen. From the FTIR spectral pattern, we found some bands were absent and some got shifted from its earlier positions in the inclusion complex (**Table 9, Fig.8**). In the IC the bands due to the O-H stretching, stretching of -C-H from -CH₂, bending of C-O-C of β-CD are found shifted from 3406.34 cm⁻¹ to 3458.65 cm⁻¹, 2944.46 cm⁻¹ to 2934.45cm⁻¹, 1163.56cm⁻¹ to 1152.45cm⁻¹. On the other hand, band at 1631.15 cm⁻¹ for the Stretching due to conjugated C=O was found shifted to 1661.53 cm⁻¹ and stretching due to C-N was shifted from 1420.43 cm⁻¹ to 1464.32 cm⁻¹. In pure CD bending of -C-H from -CH₂ and bending of O-H at 1403.38 cm⁻¹ were found absent. In the guest molecule, stretching due to aliphatic N-H bond at 3446.17 cm⁻¹, out of plane C-H bending at 820.27cm⁻¹ and broad band due to bending of N-H bond at 674.07 cm⁻¹ were found absent in the FT-IR spectra of ISD-CD IC.

Table 9: Data obtained from FT-IR spectroscopic study of β-CD, ISD and β-CD+ISD.

Groups	Wave number(Cm ⁻¹)		
	β-CD	ISD	ISD-β-CD
stretching of O-H	3406.34		3458.65
stretching of -C-H from -CH ₂	2944.46		2934.45
bending of -C-H from -CH ₂ and bending of O-H	1403.38		
bending of C-O-C	1163.56		1152.45
vibration involving α-1,4 linkage	958.23		957.34
Stretching due to Aliphatic N-H bond		3446.17	
Stretching due to conjugated C=O		1631.15	1661.53
Stretching due to C-N		1420.43	1464.32
Out of plane C-H bending		820.27	
Broad band due to bending of N-H bond		674.07	

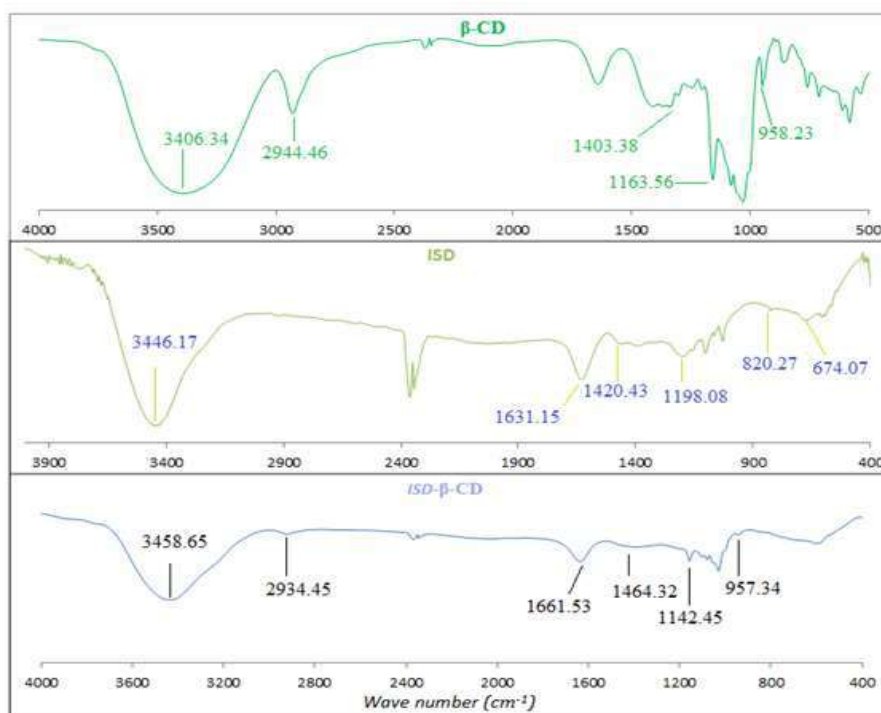


Fig.8: FT-IR spectra of β -Cyclodextrin, pure Indigosulfonic acid dipotassium salt and ISD- β -Cyclodextrin inclusion complex.

3.6. ^1H NMR AND 2D NOESY spectral analysis of solid inclusion complex: The inclusion complex formation between β -CD and ISD can be established with the help of ^1H NMR study. The change in chemical shift values of the protons of inclusion complex is of main interest here. The H3, H5 protons of β -CD situated in the interior hydrophobic cavity where H3 is at the wider rim and H5 is at the narrower rim. The chemical shift values obtained from the ^1H -NMR spectroscopic analysis were recorded (table 10 and Fig.9).

Table 10: The chemical shift values of β -CD, pure ISD and ISD- β -CD IC obtained from ^1H -NMR spectroscopy.

β -CD(400 MHz, Solvated in D_2O) δ /ppm	3.48–3.53 (6H,t, $J = 9.2$ Hz), 3.56–3.59 (6H, dd, $J = 9.6, 3.2$ Hz), 3.73–3.78 (18H,m), 3.87–3.92 (6H, t, $J = 9.2$ Hz), 5.00–5.01 (6H, d, $J = 3.6$ Hz).
ISD	6.85-6.87(2H,d, $J=6.8\text{Hz}$),7.65-7.67(2H,d, $J=7.6\text{Hz}$),7.92(2H,s)
ISD- β -CD IC	3.42-3.48 (6H, t, $J = 9.2$ Hz), 3.51-3.57 (6H, dd, $J = 9.6, 3.2$ Hz), 3.53-3.59 (18H, m), 3.63-3.67 (6H, t, $J=9.2$ Hz), 5.01-5.02 (6H, d, $J = 3.6$ Hz), 0.95(3H, s);6.87-6.90(2H,d, $J=6.8\text{Hz}$),7.69-7.72(2H,d, $J=7.6\text{Hz}$),7.98(2H, s).

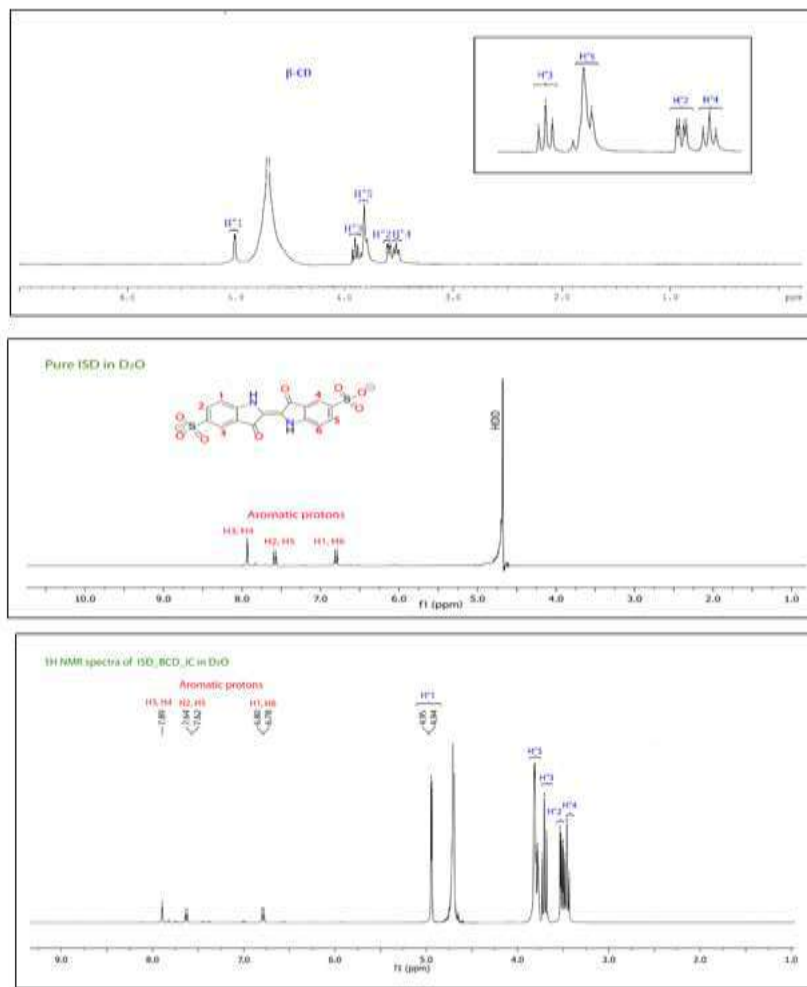


Fig.9: ¹H NMR spectra of β-Cyclodextrin, pure Indigosulfonic acid dipotassium salt and β-CD-ISD inclusion complex.

From the data tabulated in the **table 10** it is seen that H3 proton experiences more interaction compared to H5 proton. The more up field shift of the H3 proton of β-CD in the inclusion complex than the H5 proton clearly suggest that insertion occurs through the wider rim (**Table 11**). The other protons are at the same position as in the pure β-CD. So, from the chemical shifts values of the interacting protons (mainly H3 and H5) we can come to the conclusion that the guest molecule is entering towards the hydrophobic cavity from the hydrophilic environment^[54,55]. This, in fact, supports the association constant values obtained from UV-Vis, fluorescence and ITC studies.

Table 11: Change in chemical shifts (ppm) of the H3 and H5 protons of β-cyclodextrin molecule in host-guest complexes in D₂O.

Protons of CD	ISD+β-CD
H3	0.24
H5	0.19

In NOE spectroscopy the two protons at 0.4 nm apart in space can cause nuclear overhauser effect [36]. It is an imperative method to interpret the extent of interaction among the host (β -cyclodextrin) and guest (ISD) molecules when two protons are in close proximity (3-5Å). Then the appearance of an NOE cross peak can be detected among the relevant protons in the NOESY spectrum. The existence of the host-guest interaction in ISD- β -CD inclusion complex can be proved by Nuclear Overhauser Effect measurements (NOESY spectrum) in D₂O medium. The NOESY spectrum of the IC shows significant NOE cross-peaks between the H''3 and H''5 protons of cyclodextrin and the aromatic protons (H1, H2, H3, H4, H5, H6) of ISD molecule (**Fig.10**). These results obtained satisfactorily coincide with the tentative complex structure given in **fig.11**, indicating the partial inclusion of dye molecule into the cavity of Cyclodextrin. Such observation strongly supports the existence of the non-covalent type of interaction in the complexation of ISD by the β -CD molecule.

The NOE cross-peaks between aromatic protons of ISD (circled in the **Fig. 10**) and H''5, H''3 protons of CD was observed implying complete insertion of non-polar part of ISD into the β -CD cavity. The NOESY spectrum also specified the insertion of the non-polar part of the ISD into the β -CD cavity by the interaction between H1, H2, H3 protons of the guest molecule and H''3, H''5 from the CD, which completely supports the shift of H''3 and H''5 protons in the ¹H-NMR spectroscopic study.

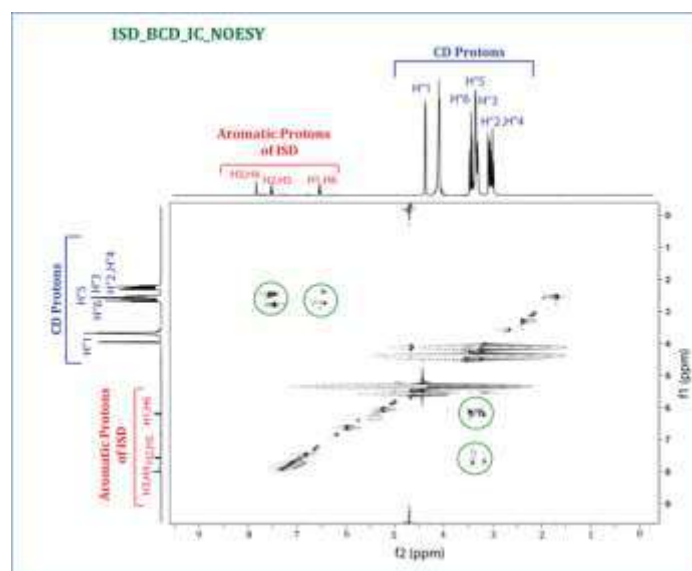
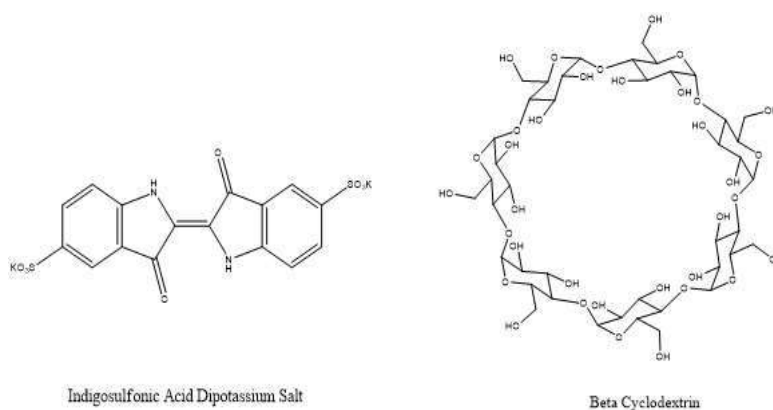
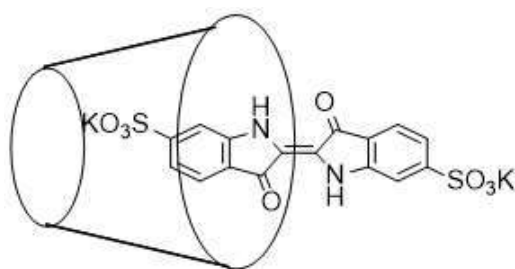


Fig.10:2D NOESY NMR spectra of ISD- β -Cyclodextrin inclusion complex.





Inclusion Complexation through the wider rim

Fig 11: Structures of Indigosulfonic Acid Dipotassium Salt and β -Cyclodextrin and their schematic representation of inclusion.

3.7. Structural Effect of Cyclodextrin: The formation of host-guest IC is mainly focused on the structural combination of both the molecules. ICs are formed only when there is the stronger association between the guest and host molecules over other forces. The complexation strength depends on the factors such as the size of the guest molecule, the van der Waals interactions, the release of water molecules, hydrogen bonding, charge transfer interactions, hydrophobic interactions, the release of conformational strain etc. Here, the hydrophobic part of the dye molecule enters inside the hydrophobic cavity of the CD molecule during the formation of the inclusion complex and it is stated that no covalent bond forms or breaks in the system of IC^[8, 56].

The interesting fact is that the cavity of the CD molecule is blocked by water molecules but this is unfavourable. When the hydrophobic part of the guest molecule enters inside the hollow cavity of CD, the water molecules are easily replaced. It is clear that the hydrophobic interaction predominates here. However, entropy of the system increases due to the elimination of the water molecules to the bulk as seen from the ITC experiment, which helps the process of formation of the ICs to be spontaneous. The size of the guest molecule or more specifically the hydrophobic part of the guest molecule, which enters into the CD cavity, is another determining factor for the formation of the IC. The hydrophobic part of ISD fits better inside β -CD by relieving the ring strain of the CD as well as lowering the energy of the system. Encapsulation of a single ISD molecule sterically blocks the side of the wider rim inhibiting other molecule to enter inside the cavity, which is reflected in the UV-Vis study of Job plot.

3.8. SEM Study: Scanning Electron Microscope study is a qualitative method to figure out the morphological changes of the starting compound and the inclusion complex attained by the complexation by host β -CD molecule. **Fig.12** shows the micro images of A, B and C. The surface morphology of the three components namely β -cyclodextrin (host), ISD (guest) and their inclusion complex were studied by scanning electron microscope. The surface morphology of β -CD appears as flat configuration having a regular arrangement of fine lines whereas pure ISD is found to have thread/flakes like morphology. ISD. β -CD inclusion complex appears like irregular shaped crystals [the surface looks like crystals (though no real crystals are there) here only the surface morphology is considered], in which the original morphology of both of the starting compounds gets disappeared. Those changes on the surface structure of the isolated compounds indicate the establishment of interactions with a new phase formation.

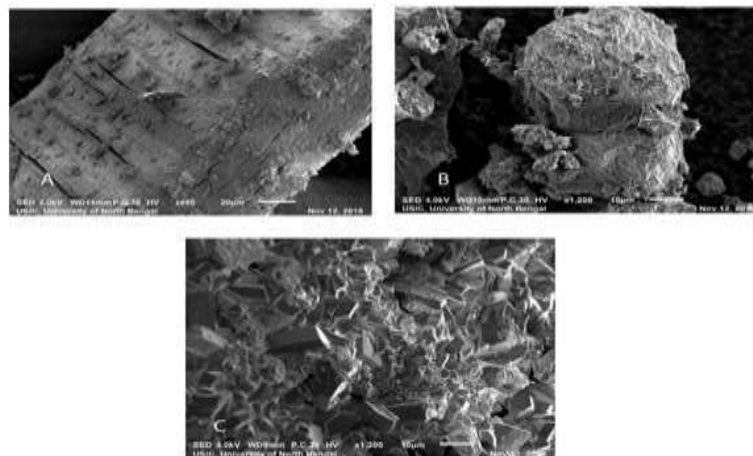


Fig.12: SEM images of (A) pure β -Cyclodextrin, (B) pure Indigosulfonic acid Dipotassium Salt and (C) ISD. β -CD inclusion complex.

CONCLUSION

In this study we have successfully characterised the inclusion of the dye molecule Indigosulfonic Acid Dipotassium Salt inside the naturally occurring oligosaccharide β -CD molecule. The IC was characterised using various thermodynamic as well as spectroscopic methods. The negative value of ΔG of the process, the high association constant from UV-Vis and ITC study of the inclusion show that the IC formed is more stable compared to the pure ISD. The higher stability may be the main reason for the controlled release of the ISD. The inclusion complex found to have higher solubility in water compared to the free ISD. Thus it may reduce the toxic effect generated from colouring foods and drugs. Moreover its controlled release inside the body may reduce the toxicity. As cyclodextrins have very low toxicity, it might be anticipated that the inclusion process makes the overall moiety (IC) of very low toxicity. This study may further open scopes for scientific and industrial research in future.

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