CHAPTER V

HOLLOW TORUS BASED ON INCLUSION OF SPARINGLY SOLUBLE AMINO ACID INVESTIGATED BY SPECTROSCOPIC, MICROSCOPIC, CALORIMETRIC AND LIGHT SCATTERING TECHNIQUES

V.1. INTRODUCTION

Amino acids are organic compounds that combine to form proteins. Amino acids and proteins are the building blocks of life.[1-3] The human body uses amino acids to make proteins to help the body, e.g. break down food, grow, repair body tissue, used as a source of energy by the body, perform many other body functions.[4,5] Amino acids are classified into three groups: Essential amino acids, nonessential amino acids and Conditional amino acids. Out of these three categories conditional amino acids are usually essential in times of illness and stress. Few examples of conditional amino acids are arginine, cysteine, glutamine, tyrosine, glycine, ornithine etc.[6-9]

Cystine is the oxidized dimer of the amino acid cysteine. Under reductive conditions (in the cytoplasm, nucleus, etc.) cysteine is predominant as cystine is break down into former. So, cystine is also treated as conditional amino acids. [10-11]

L-Cystine is the least soluble of the naturally occurring amino acids, and cystine stones, caused by a genetic disorder, account for between 1± 4% of all urinary stones. [12-16]

In this work the host–guest interaction of an amino acid viz., L-Cystine as guest with α and β cyclodextrin (CYD) had carried out. To confirm the formation of inclusion complex many experimental techniques e.g., FTIR, SEM, DSC etc. were used. SEM EDS data was also used to get idea about compositional variation upon inclusion & rough

homogeneous mixture with respect to guest and host. Finally biological activities (antimicrobial activity assay) of the inclusion compounds were also performed to get idea about its application with two important gut micro flora.

V2. EXPERIMENTAL SECTION

V.2.1 MATERIALS

The amino acid L-cystine and CYDs of puriss grade were purchased from Sigma-Aldrich, Germany. The mass fraction purity of L-cystine, α-CYD and β-CYD were 0.97, 0.98, and 0.98 respectively. Double distilled water with specific conductance ~1.1 μScm^{-1}, pH ~6.9–7.0 was used for all experimental purposes. The source and purity of the chemicals have been given table S.1.

V.2.2 APPARATUS AND PROCEDURE

FTIR spectra were recorded in KBr pellets using PerkinElmer FTIR spectrometer (RX-1) operating in the region of 4000 to 400 cm^{-1} at ambient temperature. The software connected with the instrument was PerkinElmer precisely version 5.3 (PerkinElmer, Inc). The pellets formed manually. Humidity during experiments was approximately 45%. [17-18]

Scanning electron microscope (SEM) instrument used was of Jeol JSM-IT 100, connected with EDS compartment with detector input area of 20 mm^2. Microanalysis was performed by an Oxford INCA energy dispersive spectrometer (EDS) connected to the SEM. All photography was taken & initially analyzed by InTouchScope (Version 1.060) software. Samples of cystine, α-CD β-cyclodextrin, and their inclusion complexes were mounted onto aluminium stubs and sputter-coated with a gold layer of about few millimetres. These samples were analyzed by an energy dispersive X-ray spectroscopy. Experimental conditions involved 15 kV at low vacuum (30 Pa), current 8–10 nA, beam diameter 6 µm using a backscattered electron detector. [19]

In antimicrobial activity assay experiment, *B. subtilis* (gram positive), *E. coli* (gram negative), were considered as model organisms. Tests were done according to the Agar cup method. [20]

In short, organisms were inoculated by spread plate technique in Muller-Hinton agar and the compounds (Pure hosts, pure guest and host-guest complexes) were applied in agar cup at 1 mg/mL concentration in separate plates and incubated at 37 °C/310 K for 24 hrs. Double distilled water was used as the control. Antimicrobial activity was determined by means of the clear zone (zone of inhibition) surroundings agar cup. Each of the experiments was done in triplicate.

V.3. RESULTS AND DISCUSSION

V.3.1 FTIR STUDY ESTABLISHES INCLUSION

The formation of inclusion complex of L-Cystine with α and β-CYD in solid state is supported by reliable FT-IR study.[21,22] There are numerous changes in FT-IR spectra of solid ICs owing to the changes of bending plus vibrating peaks of the guest molecule also aroused due to the symmetrical along with anti-symmetrical stretching vibrations of the -COO^- grouping. The various frequencies of L-Cystine, α-CYD, β-CYD, (L-Cystine + α-CYD i.e. IC-1) and (L-Cystine + β-CYD i.e. IC-2) ICs were reported in (table 1. a. & 1. b). The -O-H frequency of both α & β-CYD are shifted to lower region probably due to participation of the -O-H groups of the host molecules in H-bonding molecule after complexation. The FTIR spectrum of L-Cystine with both host presented in figure 2. The spectra were measured in the solid state of the sample as a KBr disk. The following bands in (cm^{-1}) have been assigned in the table 1. b. Inclusion complexes formation due to strong bands caused by overlapping of -C-H stretching vibrations of methylene and m groups of the guest molecule with cyclodextrins. Moreover strong bands included in the tables and figures with the guest molecule. Furthermore the spectra of the two inclusion complexes are unalike to CYD. A lot of peaks of L-Cystine are absent or somewhere shifted which is due to the change in environment after inclusion in the cavity of α-CYD, these changes was more appropriately noticed in β-CYD than α-CYD. So, we conclude that the inclusion is better with β-CYD.

V.3.2. DIFFERENTIAL SCANNING CALORIMETRIC (DSC) STUDY

From the DSC study, various kind of information such as crystallization, thermal stability, melting etc. can be obtained of chemical compounds in their solid states. [23-24]

The peaks of guest molecule in the thermogram may be completely diminished or shifted to the different temperatures due to the formation of inclusion complexes with the respective host supramolecules. Thermograms of solid cys, α-CYD, β-CYD, and their ICs have been shown in the figure 3. a. DSC thermogram of cys shows a characteristic sharp distinct endothermic peak at 253.88 °C (ΔH=822.88 J/g) corresponding to its melting point while in its ICs with both α and β-CYD, a comparatively flat and broadened signals are observed along with a new small peak at 81°C and 97°C for IC-1 and IC-2 respectively. These broad signals refer that there is a high loss of the crystallinity of cys in its ICs, indicating a strong complexation with CYDs and the smaller peaks at quite lower temperature are probably due to the loss of water molecules adhered with ICs. From the figure 3. b. ,it has been clearly seen that the nature of the peak at 253.88 °C in the thermogram for IC-2 is more flattened compare to that for the IC-1 which indicates more complexation of cys with β-CYD rather than with α-CYD.

V.3.3. SCANNING ELECTRON MICROSCOPY (SEM) STUDY

SEM photographs of cys, α-CYD, β-CYD and their inclusion complexes are shown in Fig 4. a-d. Typical crystal of cys, α-CYD and β-CYD are found in many different sizes. Pure cys appears as irregular hexagonal-shaped crystal particles with large dimensions and β-CYD crystallizes in polyhedral form. This structural change may be due to conversion of homogeneous to heterogeneous interaction pattern during IC formation.

CYD. This data also generates a parallel conclusion from the DSC and FTIR data. Both weight percentage (%) and atomic percentage (%) of one the important constituent element of the guest into inclusion complex are very important supporting facts regarding the successful formation of inclusion complex (Table 2. a. & 2. b.; Fig 5-6). [25] Elemental composition of the sulphur is more than seven times (7) in the inclusion complex of the β-CYD compare to the α-

V.3.4. CYTOTOXIC ACTIVITY OF THE INCLUSION COMPLEXES

No zone of inhibition was observed in case of both the aerobic gram-negative bacterium and anaerobic gram-positive bacteria viz. bacillus subtilis & escherichia coli respectively. [26]

There was similar growth seen in compare to control (double distilled water). These results indicate that ICs don't have any antimicrobial activity. With that it also suggests that these ICs can't act on the normal gut microflora. So, it can be said that it is non-toxic for the body and further research can be done to explore their application inside physiological system (Fig. 7. i -ii). [27]

V.3.5. DLS STUDY

In Dynamic light scattering (DLS) the size distribution of microscopic particles is the property of importance. The distribution explains how much particle there is nearby of the different size "slices." In DLS, the local distribution is the distribution of concentration which indicates how much light is scattered from the various size "slices". In the past, a simpler forced single exponential fitting method has been used to find an overall mean size (by intensity) and an overall polydispersity. Traditionally, this overall polydispersity has also been converted into an overall polydispersity index (PDI) which is the square of the light scattering polydispersity. For a perfectly uniform sample ("monodisperse"), the PDI would be smaller than ~'0.1'. [28-32]

We had obtained the average PDI value of >0.7(> 0.9 in inclusion complex with average of 0.899) which indicate uniformity of the inclusion complex, supported by TEM.

This value may be due to presence of one kind of particle in inclusion complexes though in aqueous solution individual host and guest in dynamic equilibrium with each other. In the case of IC-1 we get various size particles whereas in the case of IC-2 only two distinct size peaks within usual size limit appear. So in the case of IC-2 i.e. with β-CYD inclusion complex formation was more successful than of α-CYD. (Fig. 8. a. to c.). Therefore light scattering technique support the formation of inclusion complex.

V.4. CONCLUSION

FT-NMR spectra, DSC study demonstrate that the natural amino acid L-Cystine forms host-guest inclusion complex with both CYDs, further put in the picture that inclusion complexation was more ideal with β-CYD compared to the α-CYD. DSC data also strengthen the idea more complexation β-CYD with L-Cystine compared to the α-CYD. SEM morphology and DLS size profile also suggest the same; moreover show size

variation on supramolecular complex formation. These two inclusion complexes should have many applications in the field of bio-chemistry and medical research as it is non-toxic for the body & also guts microorganism present inside the body.

TABLES

Table V.1: FTIR spectral assignments of (L-Cystine+ α-CYD) IC with compare to the pure cystine.

L-Cystine	
Wave number / cm^{-1}	**Group**
3446.57	**Stretching of -N-H**
3082-2875	**–C-H from methylene groups**
1634	**Streching for -C=O**
1560.01	**Symmetrical Stretching of $–COO^-$**
1399	**Anti-symmetrical stretching of –COO-**
α-CYD	
3446.15	**stretching of -O-H**
2922.50	**stretching of –C-H from –CH2**
1399.07	**bending of –C-H from –CH2 and bending of O-H**
1154.39	**bending of -C-O-C**
1029.36	**stretching of -C-C-O**
945.62	**skeletal vibration involving α-1,4 linkage**
L-Cystine+ α-CYD	
3416.45	**stretching of -N-H of L-Cys**
2922.50	**Symmetrical stretching of –C-H from –CH2 of L-Cys**
1634	**-C=O from D-NA**
1538.74	**Stretching of -COO- from D-NA**
1406.45 **1046.03**	**bending of –C-H from $–CH_2$ & of C-C-O of α-CYD respectively**
984.46	**stretching of -C-C-O of α-CYD**

Table V.2: FTIR spectral assignments of (L-Cystine+ β-CYD) IC with compare to the pure cystine.

wave number / cm^{-1}	Group
3446.57	Stretching of -N-H
3082-2875	-C-H from methylene groups
1634	Streching for -C=O
1560.01	Symmetrical Stretching of $-COO^-$
1399	Anti-symmetrical stretching of –COO-
β-CYD	
3349.23	stretching of -O-H
2919.12	stretching of –C-H from –CH2
1409.18	bending of –C-H from –CH2 and bending of O-H
1153.17	bending of -C-O-C
1033.02	stretching of -C-C-O
938.64	skeletal vibration involving α-1,4 linkage
L-Cystine+ β-CYD	
3441.74	stretching of -N-H of L-Cys
2922.50	Symmetrical stretching of –C-H from $–CH_2$ of L-Cys
1631	-C=O from D-NA
1538.74	Stretching of -COO- from D-NA
1399.07 1029.31	bending of -C-H from -CH2 & of -C-C-O of α-CYD respectively
984.46	stretching of -C-C-O of α-CYD

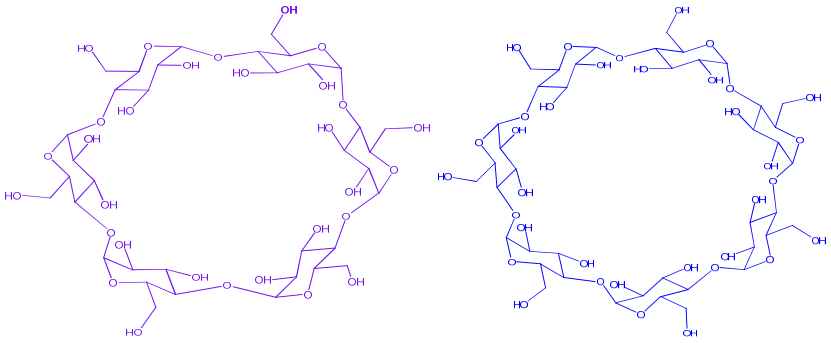
Table V.3. SEM EDS data in tabular form [α-CYD+ L-Cystine]

Element	App Conc.	Intensity Corrn.	Weight%	Weight% Sigma	Atomic%
C K	225.92	0.9780	52.01	0.48	59.36
O K	89.82	0.4314	46.88	0.48	40.17
S K	4.67	0.9412	1.12	0.04	0.48
Totals			100.00		

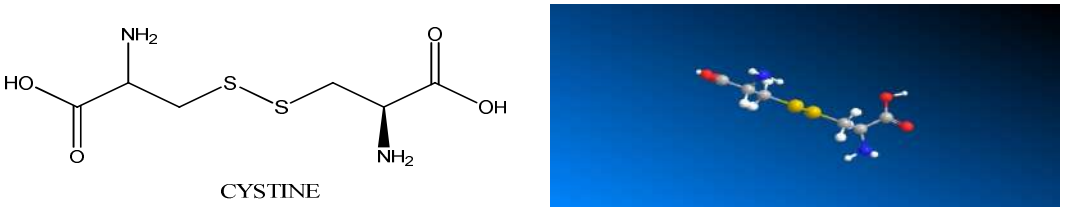
TableV. 4. SEM EDS data in tabular form [β-CYD+ L-Cystine]

Element	App Conc.	Intensity Corrn.	Weight %	Weight % Sigma	Atomic %
C K	38.34	0.5354	62.52	1.08	71.45
O K	10.67	0.3203	29.10	1.08	24.96
S K	9.39	0.9774	8.38	0.24	3.59
Totals			100.00		

FIGHURES

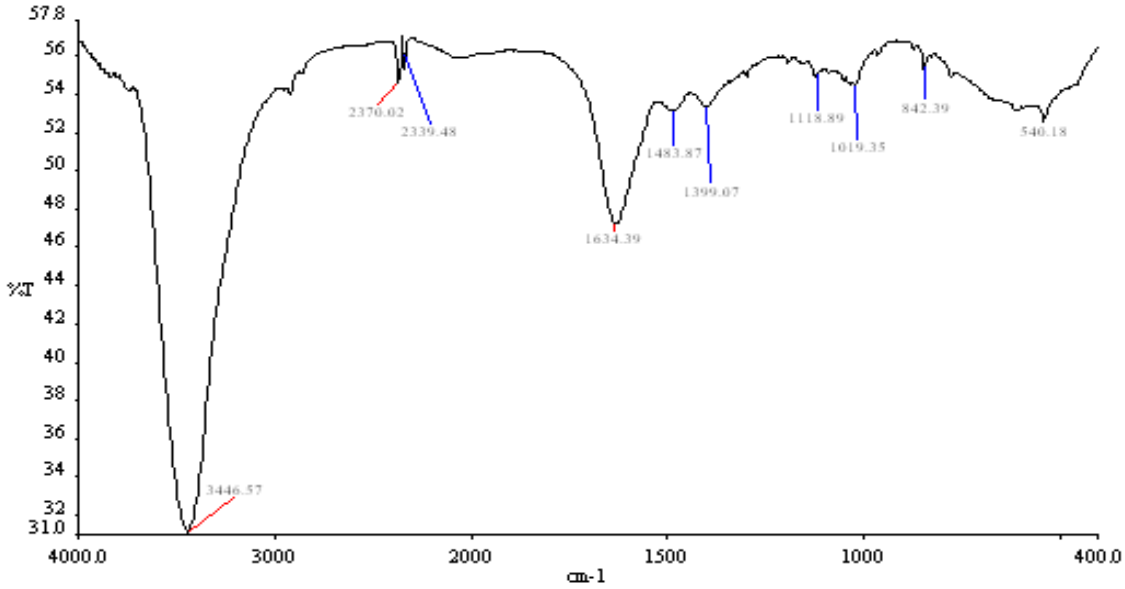


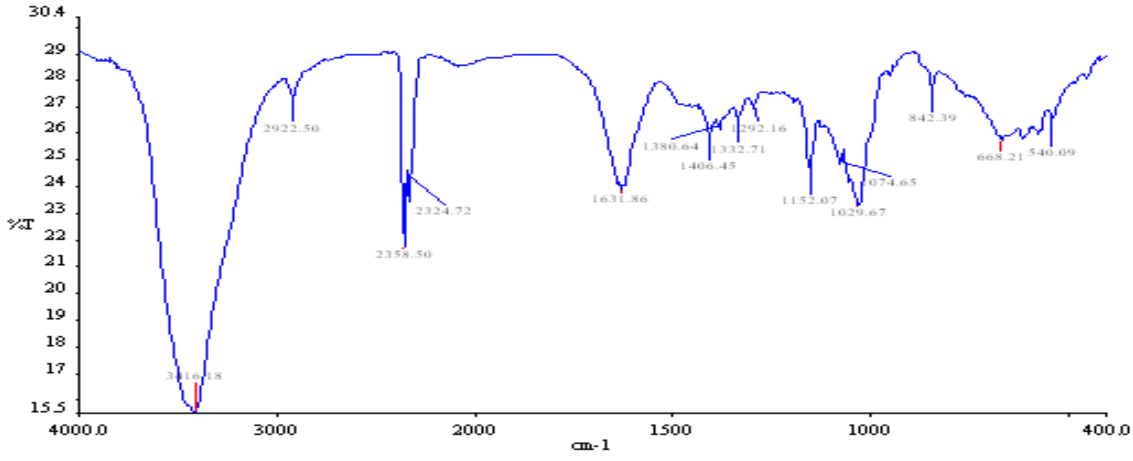
(A) (B)



(C) (D)

Figure V.1. Molecular structure of (a) α-CYD, (b) β-CYD, (c) Cystine, (d) 3D structure of Cystine





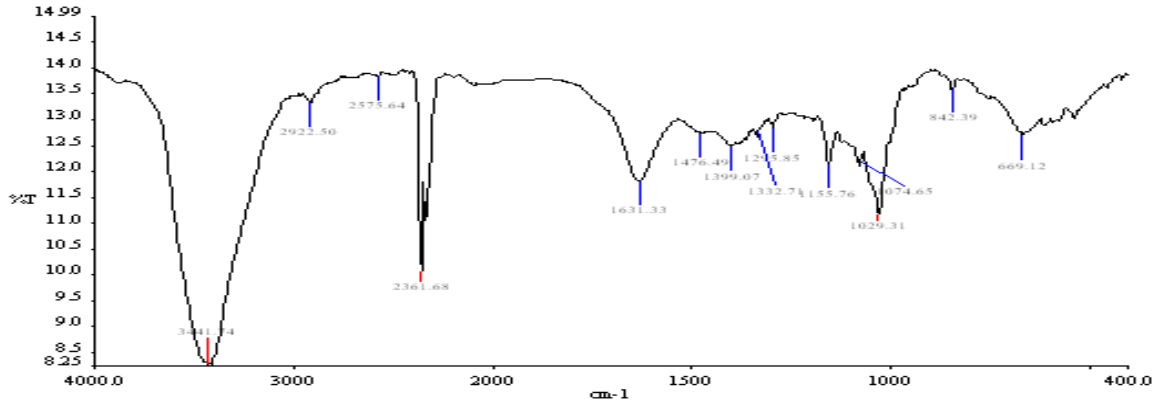


Figure V.2. (a) FTIR spectrum of pure L-Cystine (b) FTIR spectrum of (L-Cystine+ α-CYD) IC (c) FTIR spectrum of (L-Cystine+ β-CYD) IC.

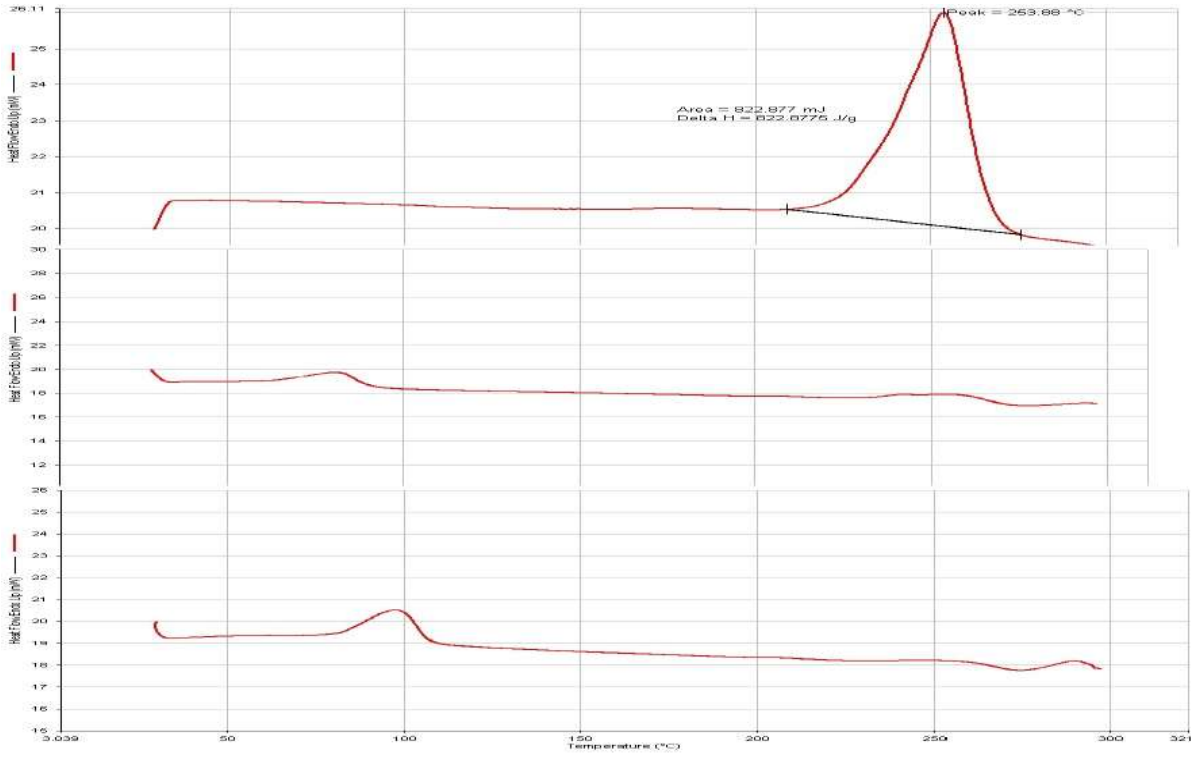


Figure 3a. Thermograms of solid cys and their ICs.

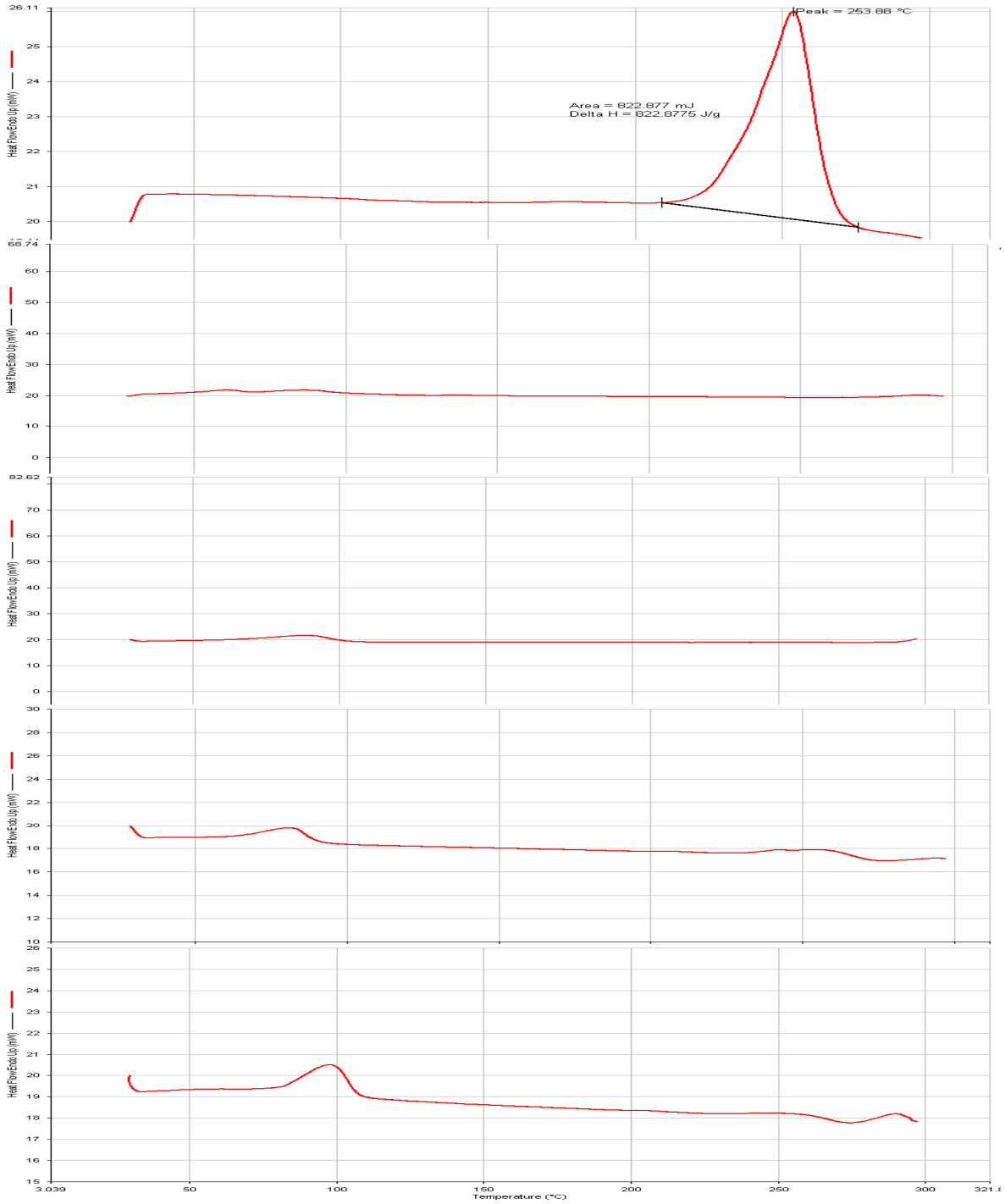


Figure V. 3b Thermograms of solid cys, α-CD, β-CD, and their ICs.

Figure V.4a. SEM images of the pure cystine at different resolution.

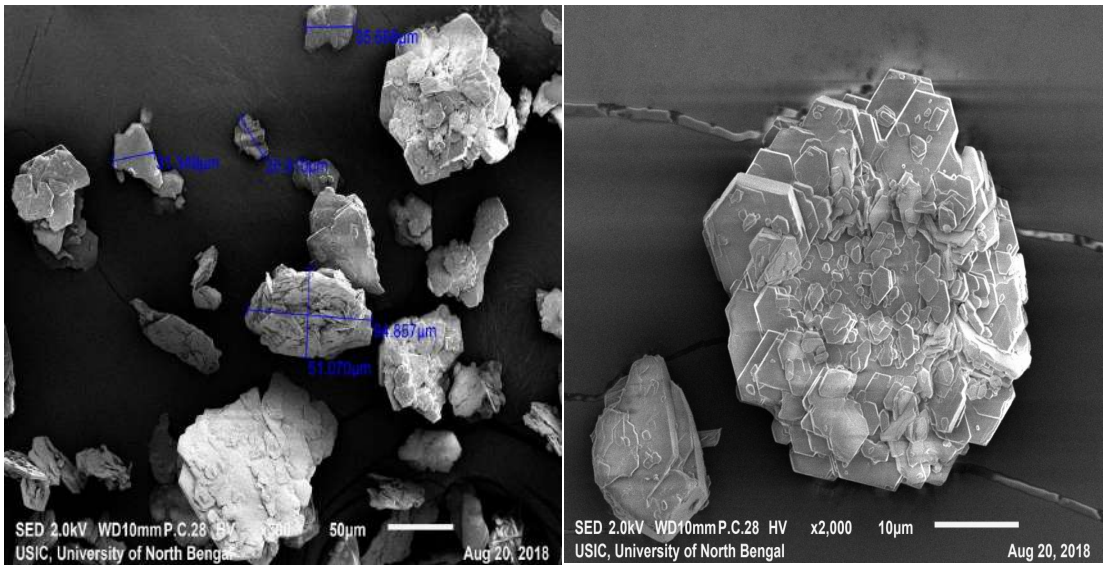


Figure V. 4. a. SEM images of the pure cystine at different resolution.

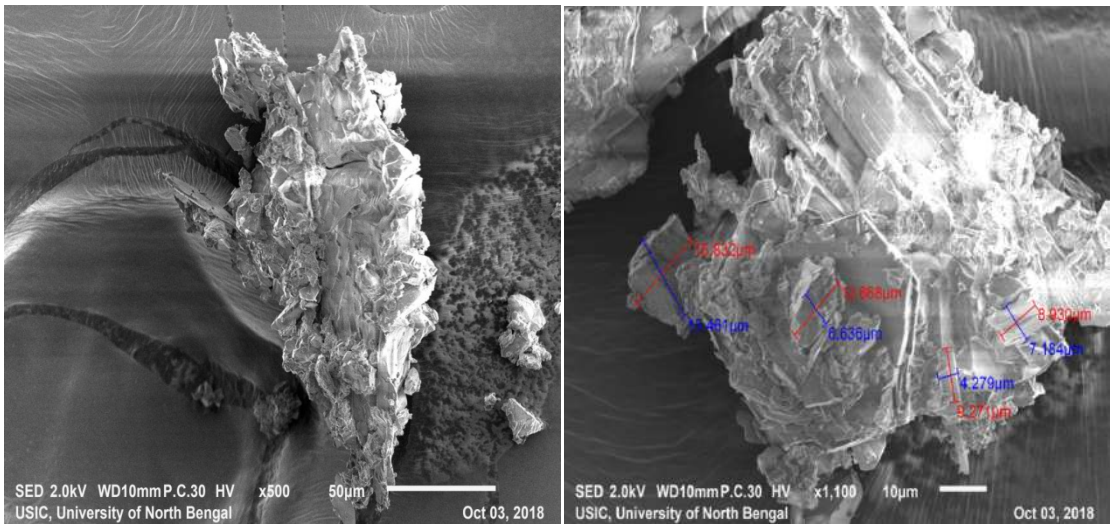


Figure V.4b. SEM images of the cystine +α-CyD inclusion complex at different resolution.

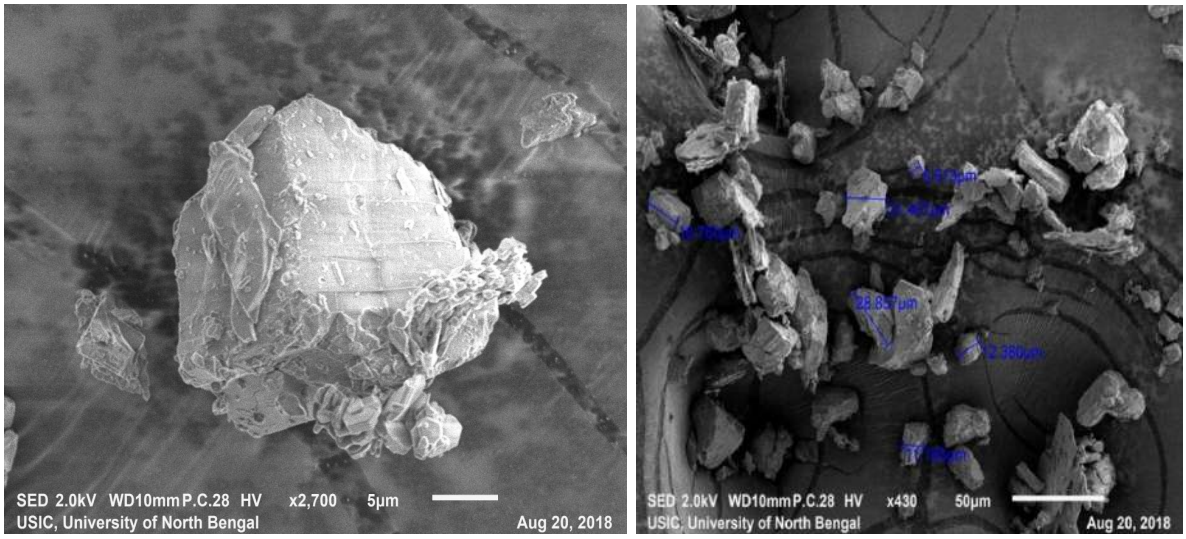


Figure V. 4. c. SEM images of the Cystine +α-CYD physical mixture at different resolution

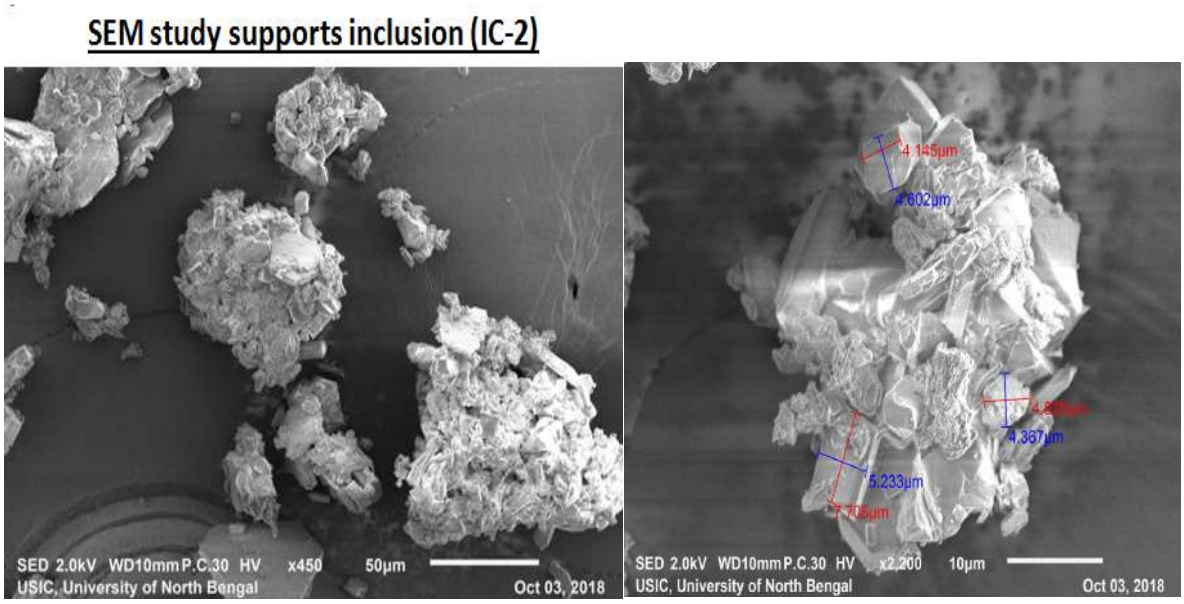


Figure V. 4. d. SEM images of the cystine +β -CYD inclusion complex at different resolution

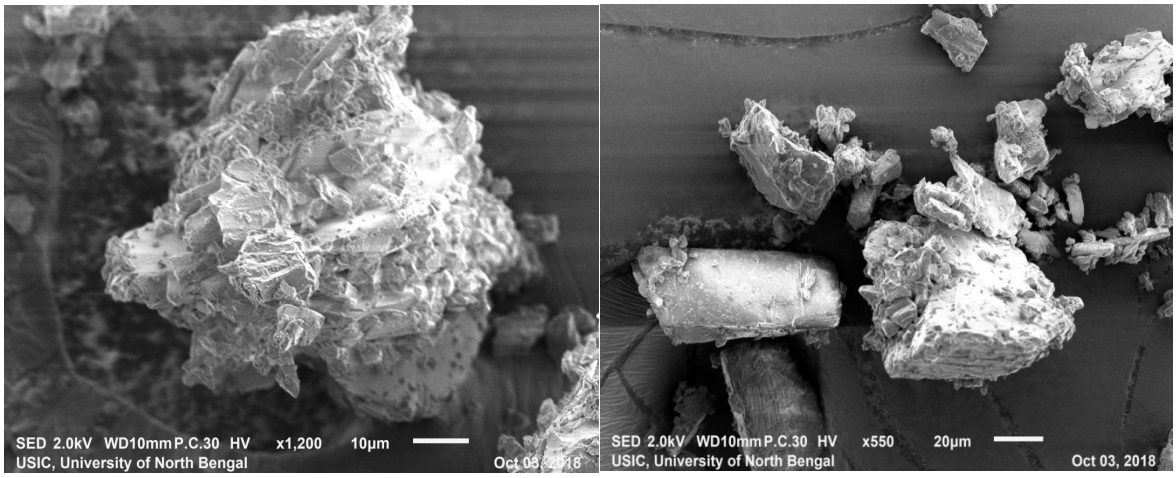
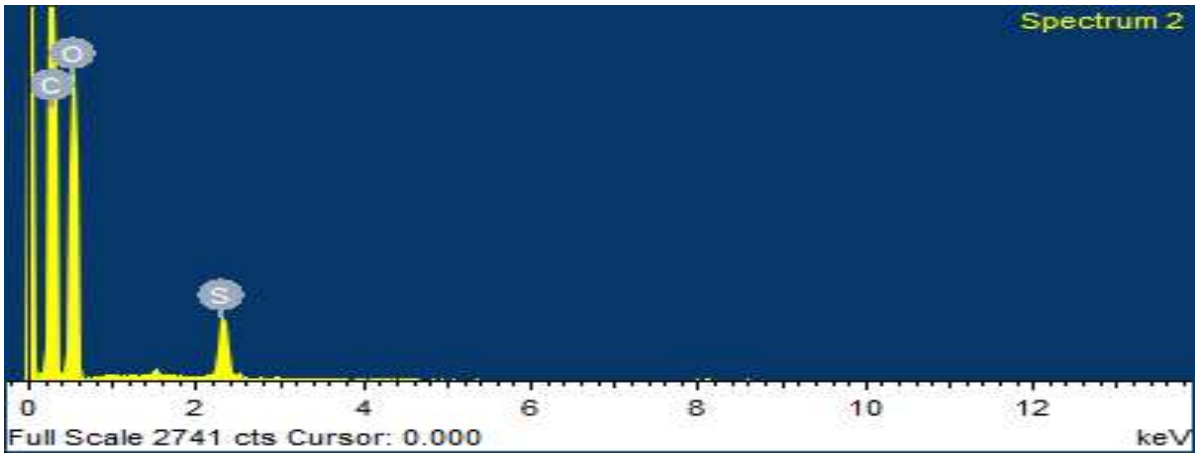
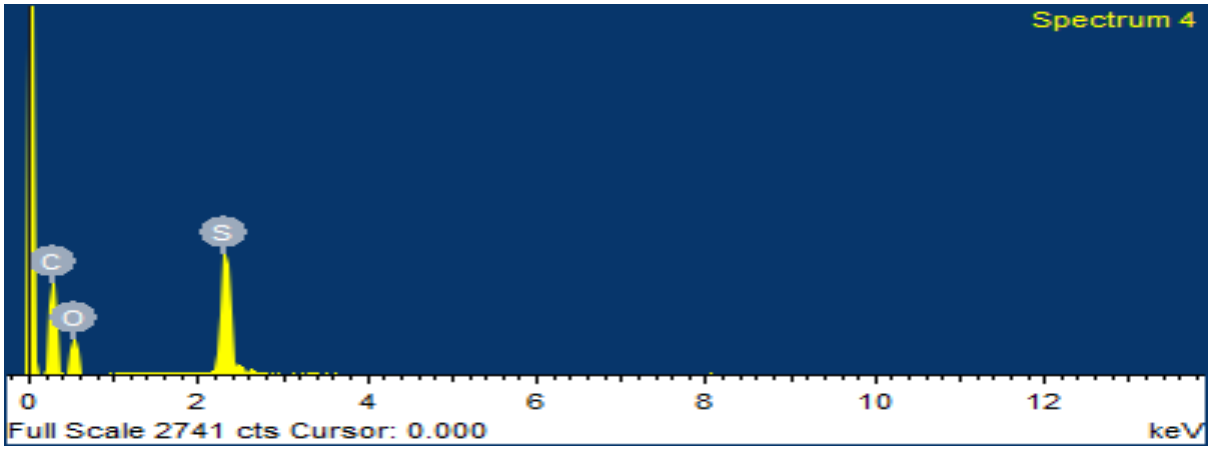


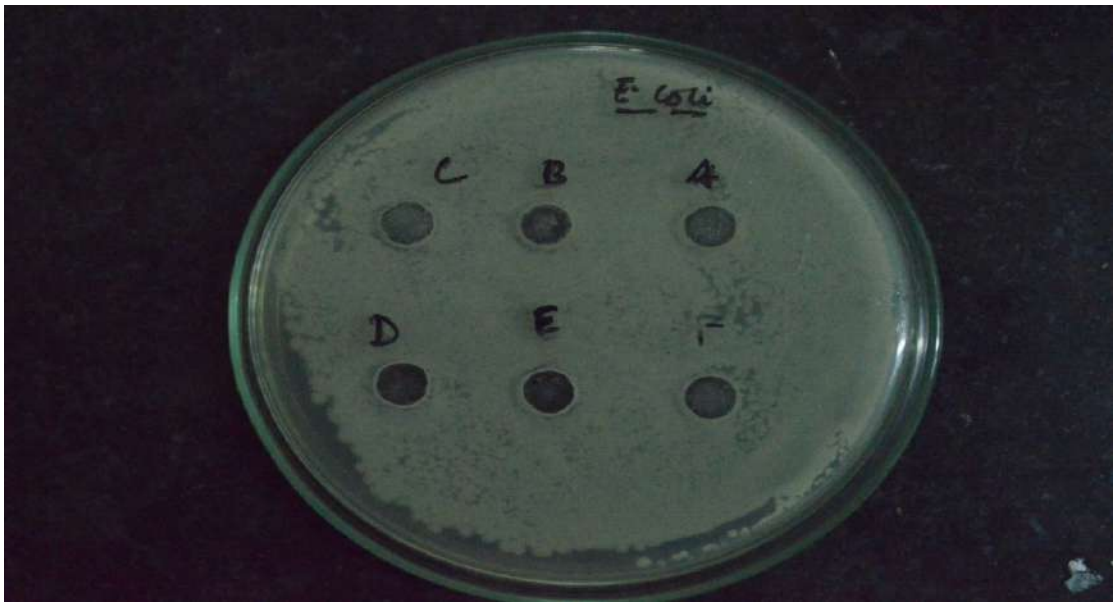
Figure V.5. SEM EDS spectrum [α-CYD+ L-Cystine]



FigureV.6. SEM EDS spectrum [β-CYD+ L-Cystine]

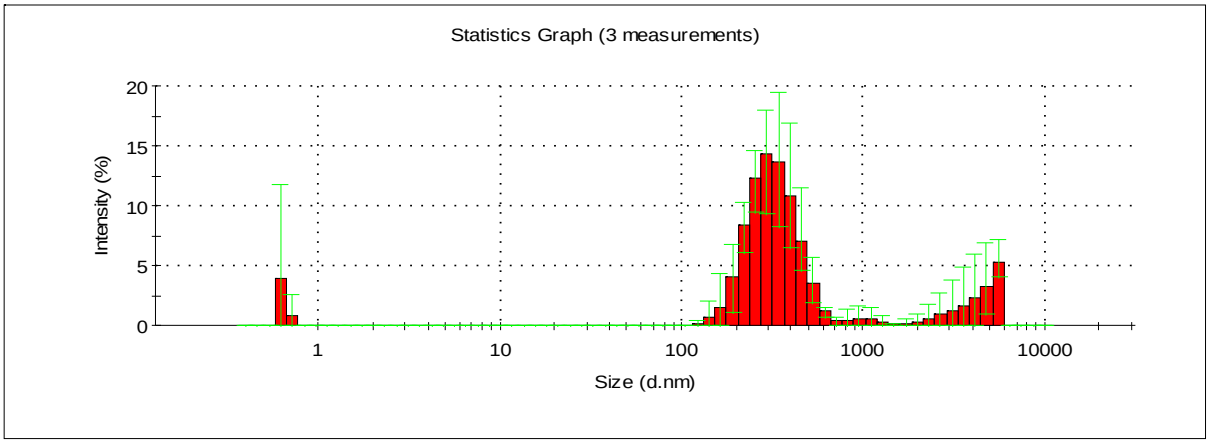


(I)



(II)

Figure V.7. (i) & (ii): Distinct but equivalent zone of inhibition for both the gram-negative and gram-positive bacteria viz. bacillus subtilis & escherichia coli respectively, A→ Control, B→ Pure Cystine, C → Pure α-CYD, D→ Pure β-CYD, E→ IC of α-CYD i.e. IC-1, F→ IC of β-CYD i.e. IC-2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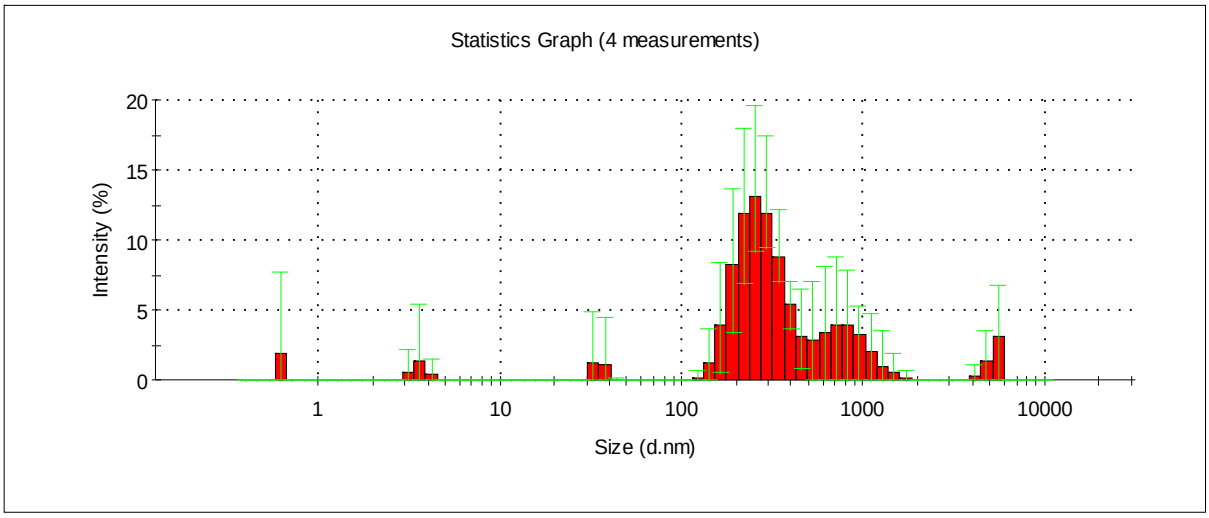
15
10
5
0
1
10
100
1000
10000
Size (d.nm)

(A)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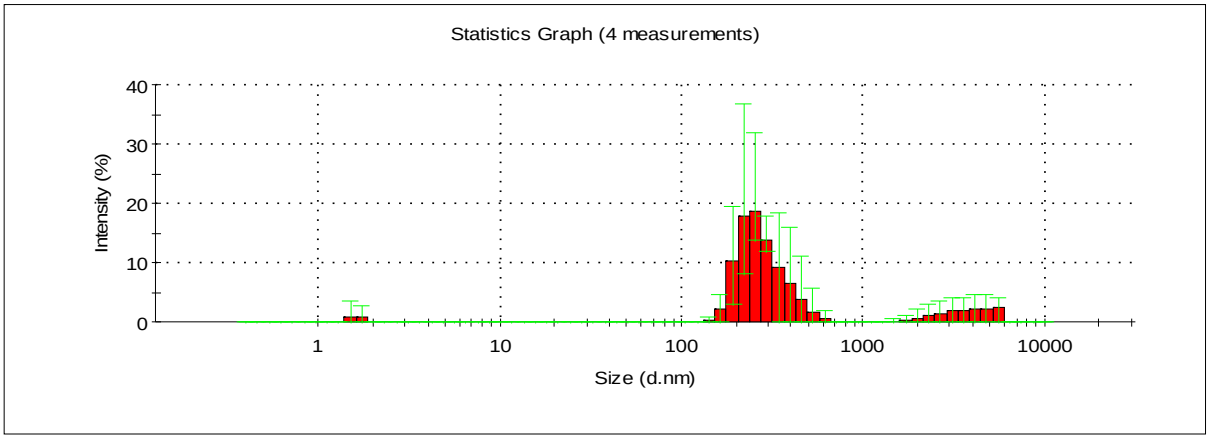
Size (d.nm)

(B)


Statistics Graph (4 measurements)
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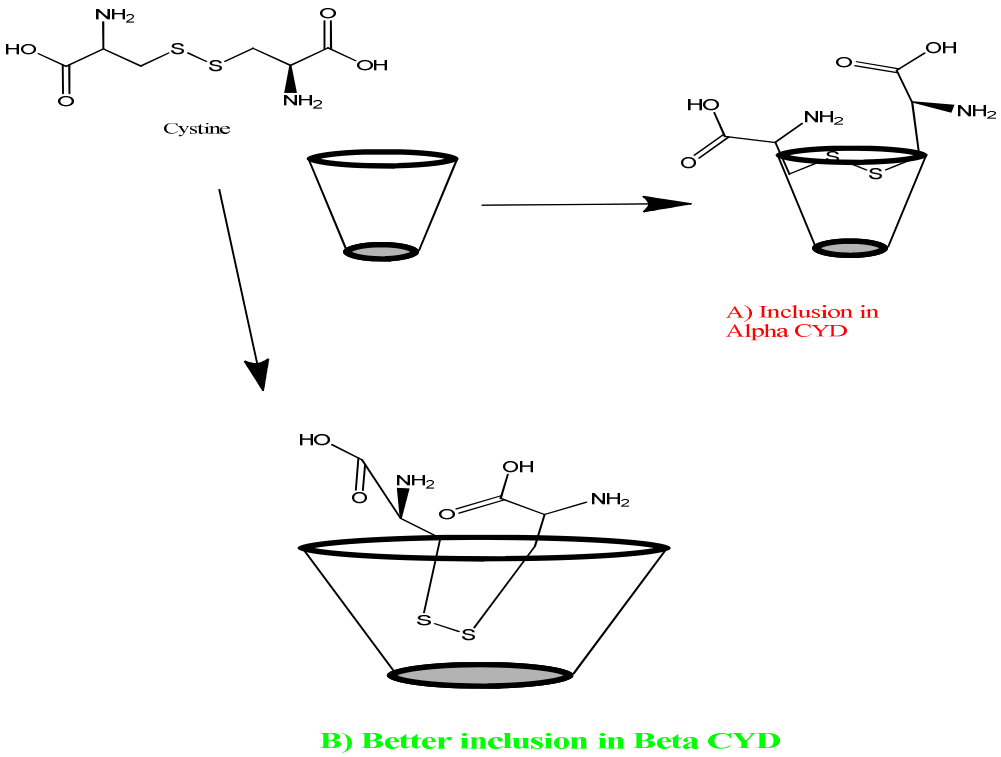
30
20
10
0
1
10
100
1000
10000
Size (d.nm)

(C)

Figure V. 8. (a) CYSTINE (b) IC-1 (α-CYD) SIZE from DLS (c) IC-2(β-CYD) SIZE from DLS

Scheme 1



Favourable Mode of inclusion In both α & β-CYDs.