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Research Paper

β–CAROTENE-β–CYCLODEXTRIN INCLUSION COMPLEX: TOWARDS ENHANCED AQUEOUS SOLUBILITY

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Abstract

β-carotene is a nutraceutic with limited aqueous solubility which renders its provitamin A activity diminished. Inclusion complexes have emerged as a promising approach towards enhancing the bioavailability of valued lipid like molecules, and β -cyclodextrins are favored hosts for the same. The present work was undertaken with an underlying objective to enhance the aqueous solubility of β-carotene. For this, inclusion complex of β-carotene with β-cyclodextrin was prepared and characterized. Phase solubility diagram was constructed for selection of 1:1 stoichiometric ratio. The complexes was prepared by co-precipitation and extended co-precipitation, and characterized by FTIR and NMR spectroscopy, followed by TEM imaging and quantification of the guest molecule into the host cavity. Studies revealed that extended co-precipitation method was a successful endeavor for inclusion complex formation, affording double times the β-carotene inclusion in comparison to simple co precipitation. Also, the possible structures of the inclusion complex formed were proposed suggestive of an interaction from the open mouthed wide end of β –cyclodextrin which were reiterated from ^{1}H-NMR analyses. Hydrophobic interactions within the cavity were observed in FTIR, exemplifying docking of the guest into the host cavity.

Key words: *β-carotene, β-cyclodextrin, inclusion complex, co-precipitation, nutraceutics*.

INTRODUCTION

β-carotene is a provitamin A carotenoid pigment found in many fruits and vegetables such as carrots and pumpkins. Vitamin A is formed by cleavage at the central double bond of β-carotene which acts as its precursor [1]. β-carotene has potent antioxidant activity as it scavenges free radicals and reactive oxygen species thus protecting cells from oxidative damage [2,3]. Consumption of β-carotene in sufficient amounts helps in preventing many chronic diseases such as cancer (by inhibiting the proliferation of pre-malignant cells), cardiovascular diseases, arteriosclerosis, diabetes, vision impairment, cataracts and other age-related disorders [2,4-7]. In addition to its nutraceutic property, it also has immunomodulatory activity being capable of enhancing the immune system [8]. The major limitation of using β-carotene as a nutraceutic is its aqueous insolubility. It is also sensitive to light and oxygen and thus has low absorption and bioavailability. This limits its use in pharmaceutics, cosmetics and food applications [9,10].

Solubility of hydrophobic compounds can be increased by various techniques such as particle size reduction, solid dispersion, nanosuspension technology, supercritical fluid technology, cryogenic techniques, floating granules, and inclusion complex formation [11,12].

Inclusion complexes are a promising approach for increasing the aqueous solubility of hydrophobic molecules. These are formed by the inclusion of the guest molecule into the cavity of the host molecule. Cyclodextrins are the most popular host molecules for inclusion complexes. They are also Generally Recognized as safe (GRAS) by US-FDA [13]. These are cyclic oligosaccharides composed of α-D glucopyranose rings linked together by α(1→4) glycosidic linkage. Among the three common forms of cyclodextrins- α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin, β-cyclodextrin is used commonly as a host for inclusion as it has a hollow bucket structure into which the guest molecule can dock. Moreover, it is preferred due to its high availability, low cost and non-toxic nature [14]. It has a hydrophobic cavity due to oxygen atoms of glycosidic bond at C1 and C4 and a hydrophilic exterior due to free hydroxyl groups on the outside [15]. The interactions between the guest and the host are non-covalent interactions such as van der Waals forces and hydrophobic forces [16]. Therefore, the solubility, stability and controlled release of guest molecules can be improved by using cyclodextrins as host molecules [17,18]. Inclusion complexes of β-cyclodextrins have been reported with drugs [18-22].

In this study, β-carotene is dock into the β-cyclodextrin to make it more bioavailable. To do so, inclusion complexes are made by two methods i.e. co-precipitation and extended co-precipitation. These two were then compared for loading of the guest molecule. The inclusion complexes were characterized by UV-Vis spectroscopy, FTIR, ^{1}H-NMR and TEM to check loading and possible interactions between the host and guest molecules.

2. MATERIALS AND METHODS

β-carotene, β-cyclodextrin, DI water of Milli-Q quality, ethanol, hexane, acetone, chloroform, butylated hydroxytoulene (BHT). All chemicals used were of analytical grade unless otherwise specified.

2.1. Phase solubility diagram for selection of stoichiometric ratio

Construction of phase solubility diagram was done to determine the stoichiometric proportions of β-carotene with β-cyclodextrin and to determine the stability constant, K_c. β-carotene (0.01 M) was added to different concentrations (0.001 M, 0.005 M, 0.01 M, 0.05 M, 0.1 M) of β-cyclodextrin dissolved in 10 mL of ethanol: water 25:75 (v/v) solution. The contents were agitated at 150 rpm for 2 h at 37ºC, after which the samples were centrifuged at 3000 rpm for 10 min. The supernatant was collected and conc. of β-carotene was determined from the standard curve. From the conc. of β-carotene, phase solubility diagram was constructed to observe the solubility profile. The stability constant K_c was calculated from the slope and the intrinsic solubility (S_o) according to Higuchi and Connors, 1965 [23].

$$K_c = \frac{Slope}{So\ (1 - slope)\ Kc}$$

The standard curve of β-carotene was made by dilution of the stock solution (500 µg/mL) in the range 20-80 µg/mL, 0.6 mL of acetone was then added to each dilution and its final volume was made up to 10 mL with 9% acetone in hexane. The absorbance was measured at 450 nm and standard curve was plotted.

2.2. Preparation of inclusion complexes

The β-carotene–β-cyclodextrin inclusion complex were prepared by co-precipitation and extended co-precipitation.

2.2.1. Co-precipitation

Equimolar concentrations of β-carotene (0.01 M) and β-cyclodextrin (0.01 M) were mixed with 5 mL of solvent ethanol: water 25:75 (v/v) to get a solution which was ultrasonicated under cold conditions with parameters: ultrasonication frequency = 20 KHz; ultrasonication time = 15 min; pulse cycle = 30 second on and 15 second off; amplitude = 80%. The obtained suspension was crystallized at 4ºC for 1 h, after which the solvent was removed by vaccum evaporation. The crystals were then dried at 37ºC to a dry mass [22,24].

2.2.2. Extended co-precipitation

β-cyclodextrin (0.01 M) was dissolved in 5 mL of water. β-carotene (0.01 M) was dissolved in 5 mL of ethanol and this solution was added drop wise to β-cyclodextrin solution under cold conditions and continuous stirring for 15 min, after which it was ultrasonicated with parameters same as in co-precipitation. The obtained suspension was refrigerated overnight at 4ºC for crystallization to occur, after which solvent was removed by vaccum evaporation. The crystals were then dried at 37ºC until dry mass was obtained.

UV-Vis spectroscopy was done to determine the spectral changes during complexation. The inclusion complexes were dissolved in ethanol: water 25:75 (v/v) for co-precipitated complex and 50:50 (v/v) for extended co-precipitated complex and filtered to remove free β-carotene. The spectrum was recorded in the range 200-500 nm.

2.3. Characterization of inclusion complexes

The inclusion complexes were characterized by FTIR spectroscopy, ^{1}H NMR spectroscopy and imaged by TEM.

2.3.1. FTIR spectroscopy

FTIR was done to determine the host-guest interactions. Spectra of the samples were obtained in the range 400-4000 cm^{-1} using Perkin Elmer spectrum 400 FTIR spectrophotometer.

2.3.2. NMR spectroscopy

^{1}H NMR studies were done to determine the penetration of β-carotene, the guest molecule into the cyclodextrin cavity. The high resolution ^{1}H NMR spectra of the prepared samples and that of pure β-cyclodextrin were recorded with a Bruker Avance II 400 NMR spectrometer in D_2O.

2.3.3. Transmission Electron Microscopy

The inclusion complex was dissolved in water and imaged by TEM Hitachi 7500 at an accelerating voltage of 90kV. The sample was dried on a carbon grid and examined.

2.4. Quantification of β-carotene in inclusion complexes

To determine the loading of β-carotene into the inclusion complex, the complex was dissolved in water and filtered to remove free β-carotene. To retrieve β-carotene in the filtrate, the complex was disintegrated. For this, 1 mL of filtrate, 2 mL of ethanol was added followed by 3 mL of hexane. The β-carotene was partitioned into hexane and quantified spectrophotometrically.

3. RESULTS AND DISCUSSION

3.1. Phase solubility diagram for selection of stoichiometric ratio

Phase solubility diagrams have been classified based on solubility of the substrate and are indicated by phase solubility profiles [24]. According to this classification, there are 2 types of phase solubility profiles, the A type and the B type as shown in Fig.1. A-type solubility profiles indicate increase in solubility of substrate with increasing ligand concentration. These type of solubility profiles are further classified as A_L, A_P and A_N type where A_L type indicate that complex is of first order with respect to substrate and ligand (1:1 soluble complex), A_P type indicate first order with respect to substrate and second order with respect to ligand (1:2 soluble complex) and A_N type depicts negative deviation from linearity. B-type solubility profiles indicate complexes with limited solubility where B_S type indicates soluble complexes with limited solubility whereas B_I type indicates insoluble complexes.

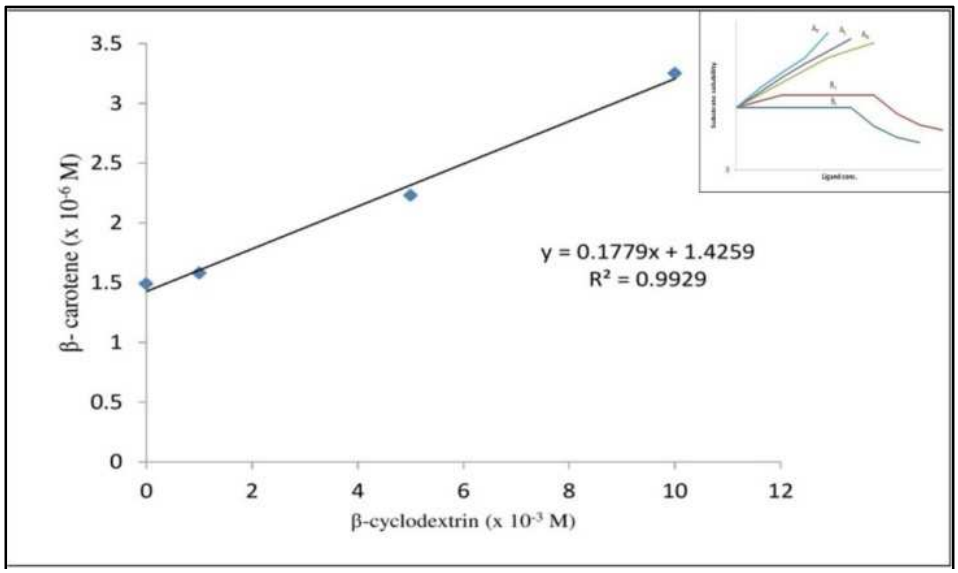

Figure 1. Phase solubility diagram of inclusion complex.

Phase solubility diagram for β-carotene - β-cyclodextrin complex is shown in Fig. 1 which indicates linear increase in solubility of β-carotene with increase in β-cyclodextrin concentration and thus classified as A_L type with a stability constant K_c of 125.3 M^{-1}. A 1:1 β-carotene–β-cyclodextrin stoichiometric ratio was selected for inclusion complex preparation from the phase solubility diagram with co-precipitaion method. This value of K_c i.e.125.3 M^{-1} is higher than the one reported with γ-cyclodextrin of 43 M^{-1} [25] suggesting β-cyclodextrin is a better host molecule than γ-cyclodextrin. Based on the phase solubility, it is clear that the complex is of first order with a high stability constant.

3.2. Preparation of inclusion complexes

The spectral changes upon complexation by different methods are shown in Fig. 2. It reveals docking of the guest molecule within the host cavity rendering a shift in absorption. Shifts in the absorption spectra are used to detect inclusion complex formation.

3.2.1. Co-precipitation

A UV-Vis spectrum of inclusion complex prepared by co-precipitation is shown in Fig. 2(a), which reveals a shift in absorbance of complex to 285 nm when compared with absorbance of β-carotene at 450 nm, suggesting inclusion of the same into the host molecule. The inclusion complexes by co-precipitation have not been reported before for β-carotene, although they have been prepared with astaxanthin and meloxicam [22,24].

3.2.2. Extended co-precipitation

UV Vis analysis of complex prepared by extended co-precipitation is shown in Fig. 2(b), which shows absorbance peaks at 220 nm and 300 nm. The maximum absorbance of complex shifts to shorter wavelengths when compared to that of pure β-carotene suggesting inclusion of the guest molecule.

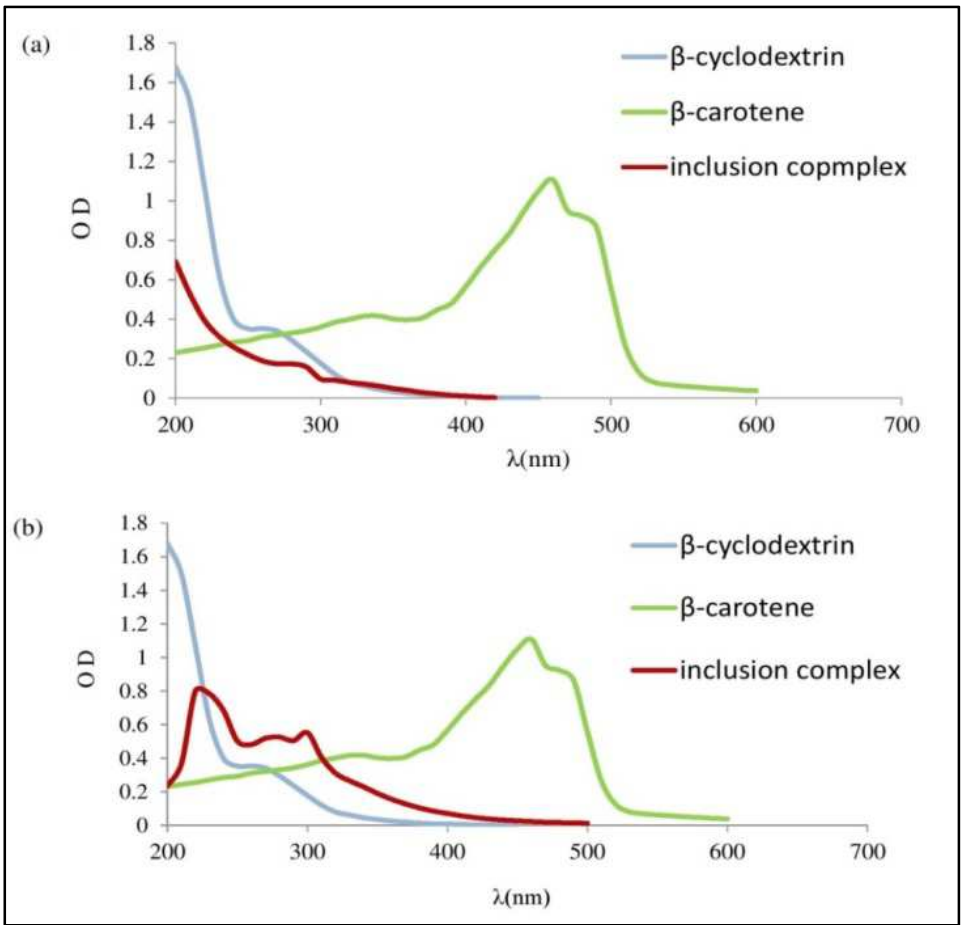

Figure 2. UV-Vis spectra of inclusion complex prepared by (a) co-precipitation and (b) extended co-precipitation.

The hypsochromic shift in the absorption maxima of β-carotene suggests its inclusion into the β-cyclodextrin cavity. However, the shift as well as intensity of maxima in the complex prepared by co-precipitation is less as compared to the maxima of complex prepared by extended co-precipitation, suggesting a better inclusion by extended co precipitation method. Based on the UV-Vis spectral analysis, it is clear that 1:1 β-carotene – β-cyclodextrin inclusion complexes are formed. To examine the extent of inclusion, FTIR and ^{1}H-NMR studies were done.

3.3. Characterization of inclusion complexes by FTIR spectroscopy

FTIR spectroscopy determines the functional groups of the guest and the host molecules involved in the inclusion complex formation. FTIR spectra of β-cyclodextrin, β-carotene, co-precipitated complex and extended co-precipitated complex are shown in Fig. 3, and the corresponding assignment of absorption bands is given in Table 1.

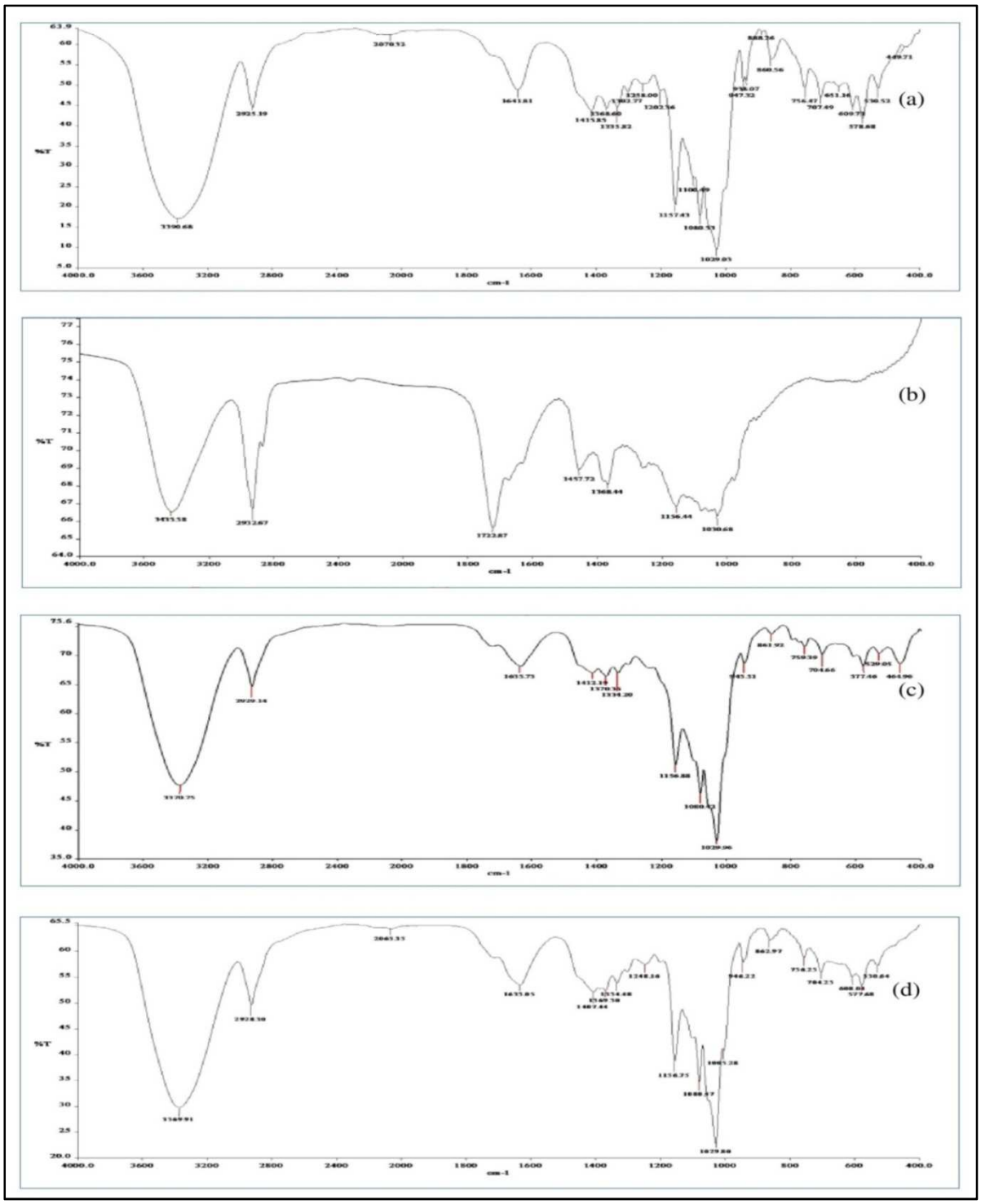

Figure 3. FTIR spectra of (a) β-cyclodextrin (b) β-carotene (c) co-precipitated complex (d) extended co-precipitated complex

Table 1. Absorption peaks (in cm^{-1}) obtained by FTIR spectroscopy for β-cyclodextrin, β-carotene, co-precipitated complex and extended co-precipitated inclusion complexes.

	β-cyclodextrin	**β-carotene**	**Co-precipitated inclusion complex**	**Extended co-precipitated inclusion complex**
OH stretch	3390	3435	3370	3369
C-H stretch	2925	2932	2929	2928

OH bend	1641	-	1635	1633
C-H deformation	1415, 1368 1335, 1302 1258	1457,1368	1412, 1370 1334	1407, 1369 1334, 1248
C-O-C and C-OH stretch	1157, 1080 1029	-	1156, 1080 1029	1156, 1080 1029
C-H out of plane bending	860	-	861	862
Pyaranose ring Vibration	756	-	759	756

An overall shift to lower wave numbers is observed upon complex formation. The peak at 3390 cm^{-1} in β-cyclodextrin is due to OH stretching which shifts to 3370 cm^{-1} and 3369 cm^{-1} in case co-precipitated and extended co-precipitated complex, respectively. The peak at 2932 cm^{-1} of β-carotene which is due to C-H stretching shifts to a lower wavenumber of 2929 cm^{-1} and 2928 cm^{-1} in co-precipitated and extended co-precipitated complex, respectively which indicates C-H stretching vibrations are restricted due to complex formation. The peak at 1641 cm^{-1} in β-cyclodextrin is due to O-H bending of water molecules within the cyclodextrin cavity [19]. This band is shifted in both the complexes with peaks at 1635 cm^{-1} and 1633 cm^{-1}, respectively. This shift in absorption to lower wavenumber indicates removal of water and inclusion of β-carotene into the cyclodextrin cavity. The peak at 1457 cm^{-1} in β-carotene due to C-H deformation shifts to 1412 cm^{-1} in co-precipitated and 1407 cm^{-1} in extended co-precipitated complex. This indicates that C-H vibrations are restricted due to complex formation.

3.4. Characterization of inclusion complex by ^{1}H-NMR spectroscopy

^{1}H-NMR spectroscopy provides the direct evidence for inclusion complex formation. Fig. 4 shows ^{1}H-NMR spectra of pure β-cyclodextrin, co-precipitated complex and extended co-precipitated complex.

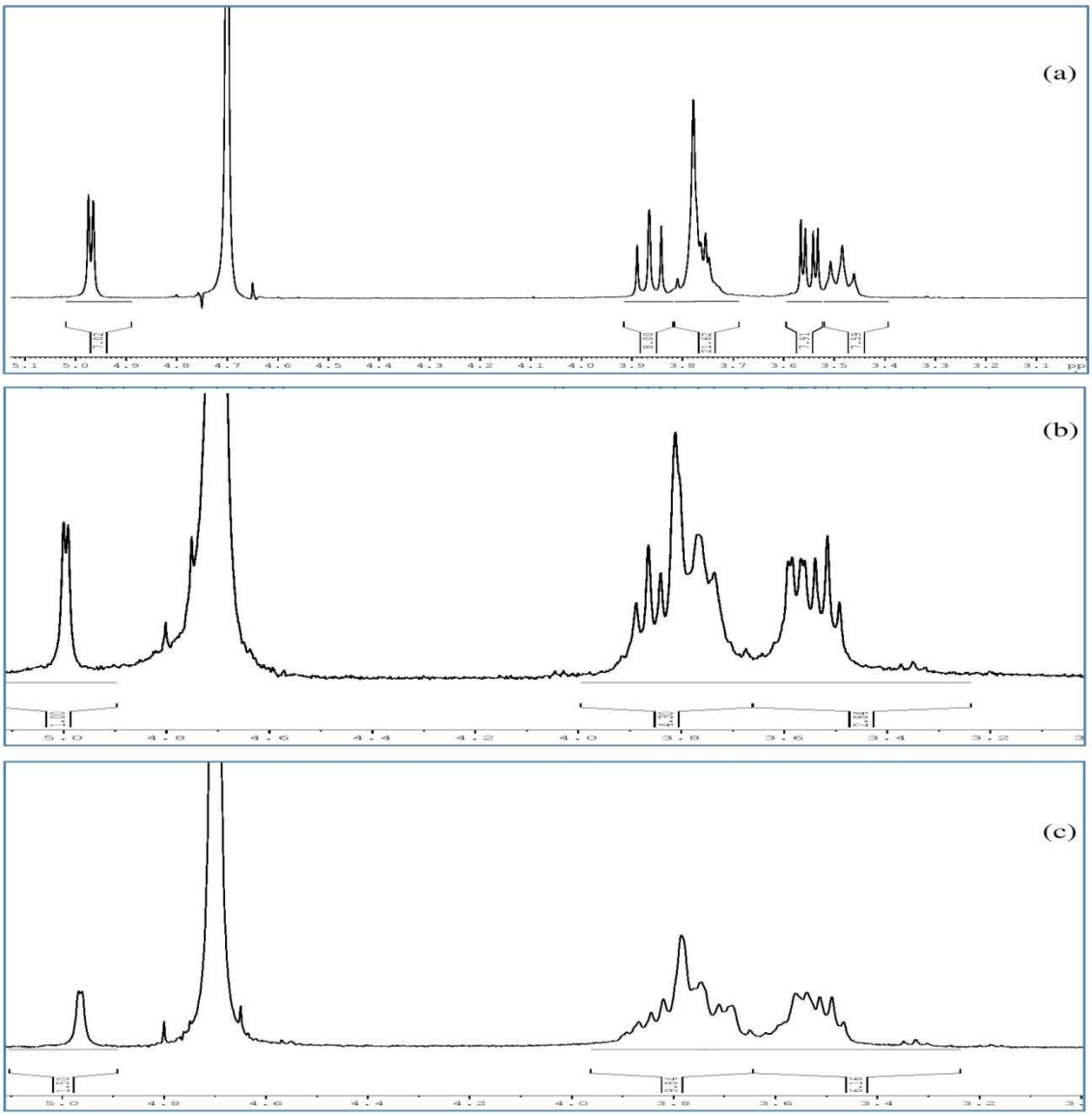

Figure 4: ^{1}H-NMR spectra of (a) β-cyclodextrin (b) co-precipitated complex (c) extended co-precipitation

The signals corresponding to β-cyclodextrin protons were assigned according to Botella et al. [7] and Polyakov et al. [11] and chemical shifts are shown in Table 2.

Table 2. Proton signals for β-cyclodextrin, co-precipitated complex and extended co-precipitated complexes and Chemical shifts for both complexes obtained by ^{1}H NMR in D_2O

Proton Signals

	H1	**H2**	**H3**	**H4**	**H5**	**H6**
β-cyclodextrin	4.9753 4.9658	3.5693 3.5578 3.5439 3.5336	3.8916 3.8662 3.8421	3.5060 3.4864 3.4634	3.7580 3.7480	3.7788 3.7640
Co-precipitated inclusion complex	5.0004 4.9919	3.5928 3.5853 3.5682 3.5609	3.8881 3.8647 3.8408	3.5403 3.5166 3.4934	3.7669 3.7359	3.8121

Extended co-precipitated inclusion complex	4.9678 4.9612	3.5616 3.5385	3.8700 3.8446 3.8208	3.5139 3.4900 3.4650	3.7112 3.6860	3.7851 3.7456

Chemical Shifts in proton signals

	H1	H2	H3	H4	H5	H6
Co-precipitated inclusion complex	-0.0251 -0.0261	-0.0235 -0.0275 -0.0243 -0.0273	+0.0035 +0.0015 +0.0013	-0.0343 -0.0302 -0.0300	-0.0089 +0.0121	- -
Extended co-precipitated inclusion complex	+0.0075 +0.0046	- -	+0.0216 +0.0216 +0.0213	-0.0079 -0.0036 -0.0016	+0.0468 +0.0620	-0.0063 +0.0184

"-" indicates downfield shifts. "+" indicates upfield shifts

The incorporation of the guest molecule into the cyclodextrin cavity causes changes in the chemical environment and significant chemical shifts in protons as shown in Table 1. In co-precipitation complex the doublet due to H6 merged into singlet suggesting that there is a change in the chemical environment upon complex formation. Also, upfield shift in the inner protons suggests the incorporation of the β-carotene in the β-cyclodextrin cavity. In extended co-precipitation complex the quartet due to H2 merges into a doublet suggesting chemical changes in the neighbouring protons upon guest entry. Also, significant upfield shift is observed in inside protons i.e. H3 and H5 suggesting the incorporation of guest. Based on this observation β-carotene docks into the cavity of the host in two possible conformations, one from the wide opened mouth and the other from the narrow base, as illustrated in Fig.5(a). Also, TEM of the inclusion complex, clearly shows β-carotene docked into the β-cyclodextrin cavity as shown in Fig 5(b).

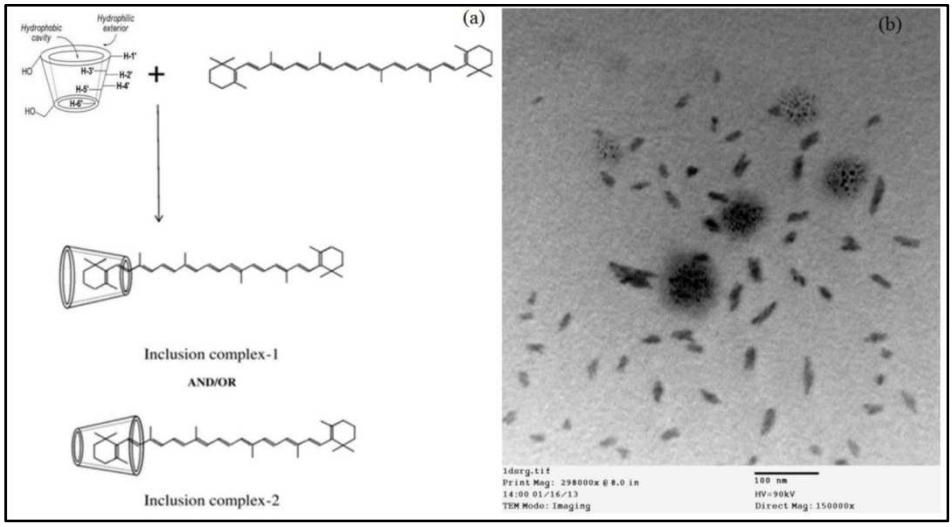

Figure 5. (a) Inclusion complex orientations; (b) TEM of β-carotene – β-cyclodextrin inclusion complex.

The magnitude of chemical shifts in H1 protons being higher than that of H6 protons suggests a higher possibility of docking from the wide-opened mouth.

From H^1-NMR it is clear that the extended co-precipitation method is better than co-precipitation because of substantial upfield shifts observed in the same which is further suggestive of a better inclusion complex formation.

3.5. Quantification of β-carotene in inclusion complexes The concentration of β-carotene in the inclusion complex formed by the two methods was quantified and it was observed that

inclusion complex prepared by extended co-precipitation loaded 1.2 µg β-carotene/ mg of complex, which was twice that found in the complex prepared by co-precipitation which loaded 0.6 µg of β-carotene/ mg of complex.

4. CONCLUSION

In conclusion, the work has reported the preparation and characterization of inclusion complex of β-carotene in β-cyclodextrin by extended co-precipitation. The complex which was characterized by UV-Vis spectroscopy, FTIR and ^{1}H-NMR spectroscopy and TEM revealed absolute docking of the guest molecule into the cavity of the host. Significant molecular interactions have suggested a reasonable degree of inclusion. The study proposes the docking from the open mouthed wide end of β-cyclodextrin. The work envisages the application of such inclusion complexes in the food industry and in the realm of bioavailability further *in vivo* studies are suggested.

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