Water Soluble Complexes of

Curcumin and Hydroxypropyl Cyclodextrins: Preparation and Characterization

A THESIS SUBMITTED

BY

S.V. Berwin Singh

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FOR THE DEGREE OF

MASTER OF PHILOSOPHY

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DECLARATION

I, **Berwin Singh**, hereby declare that I had personally carried out the work depicted in the thesis entitled "**Water Soluble Complexes of Curcumin and Hydroxypropyl Cyclodextrins: Preparation and Characterization**" under the direct supervision of **Dr. Roy Joseph, Scientist F, Polymer Processing Laboratory,** Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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The Thesis

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Submitted

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S.V. Berwin Singh

List of Abbreviation

S.No	Expanded forms	Abbreviated form
1.	Multiple Drug Resistance	MDR
2.	High performance Liquid Chromatography	HPLC
3.	Mille litre	mL
4.	Hours	h
5.	Mille gram per mille litre	Mg/mL
6.	Minutes	min
7.	Electron Spray Ionization	ESI
8.	Multiple Reaction Monitoring	MRM
9.	Bio Curcumax	BCx
10.	2-Hydroxypropyl alpha cyclodextrin	HPαCD
11.	2-Hydroxypropyl beta cyclodextrin	HPßCD
12.	2-Hydroxypropyl gamma cyclodextrin	HPγCD
13.	Nuclear factor kappa B	NF-κB
14.	Curcumin	CUR
15.	Demethoxy Curcumin	DMC
16.	Bis Demethoxy Curcumin	BDMC
17.	Vanillin	VAN
18.	Vanillic acid	VAD
19.	Ferulic acid	FAD
20.	Ultraviolet-visible spectrophotometry	UV-Vis
21.	Liquid chromatography–mass spectrometry	LC-MS/MS
22.	Fourier transform infrared spectroscopy	FTIR
23.	Fourier transform Raman spectroscopy	FT Raman
24.	Differential scanning calorimetry	DSC
25.	X-ray Diffraction	XRD
26.	scanning electron microscope	SEM
27.	Two Dimension	2D

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

Curcuma longa contains various Bio active principles which has been know centuries before traditional Indian and Chinese medicine used Turmeric in their formulations. The main biop active principle present in the turmeric is curcumin and curcuminoids. Curcuminoids contains various Bio active principles. Hundred percent thereuputic efficacy of curcumin and curcuminoid could not be utilized due to the poor aqueous solubility.

Cyclodextrin a cyclic oligosaccharides which has an ability to encapsulate various drug molecule were used to encapsulate these curcuminoids. There are various method of synthesizing complexes which are primitive yet pH shift method was found to be less described and more reliable method of complexation. So therefore the goal of the study was to synthesis cyclodextrin complex formulation using pH shift method, characterize those complexes and the check the stability of the complexes.

The dissertation is divided into four main chapters (1) Introduction (2) Materials and Methods (3) Results and Discussion (4) Summary and Conclusions.

Chapter 1 includes a brief outline of the study, review of literature, gap analysis and objective of the study for the dissertation. The reviewed literature proposes that compared to parent cyclodexrin hydroxypropyl derivates are highly soluble. Compared to pH sift method, tituration method, solvent evaporation method, kneading method.etc were not able to form complexes effectively. The problem with pH shift method is that the method has not been adapted to make a powdered formulation. Stability of the complexes was studied using UV-Vis Spectrophotometer individual curcuminoids could not be differentiated.

Therefore the study was designed based on the hypothesis that the among hydroxylpropyl alpha, hydroxypropyl beta and hydroxylpropyl gamma

could stabilizes all three curcuminoids. In order to test this hypothesis the specific objective were set.

The specific objectives adopted to test the hypothesis are:

I Optimization of complexation procedures of curcumin with cyclodextrins using the technique “pH shift method”.

II Synthesis of inclusion complexes of curcumin with various cyclodextrins (2-hydroxypropyl-α-cyclodextrin, 2-hydroxypropyl-β-clodextrin and 2-hydroxypropyl-γ-cyclodextrin).

III Characterization of these complexes by FTIR, FT Raman, UV-Vis Spectroscopy, Differential Scanning Calorimetry, X-Ray Diffraction Spectroscopy and Scanning Electron Microscopy (SEM) and Hemolysis.

IV Study solubility profile of the complexes using UV-Vis.

V Study the stability profile of the complexes using LCMS/MS.

Chapter 2 discusses briefly about the materials and methods which were involved in the study information regarding the raw materials, Chemicals used their purity, Instruments used for characterization their make, model number, manufacturer’s data etc. were provided. Standard solution preparation, sample preparations, LC/MS conditions and Synthesis of the complexes, molar ratio in which according to which the raw material were taken have been briefed. Special procedures like the preparation of the samples for the haemocompatablity evaluation which has been quiet modified so it has been described and discussed in this chapter.

Chapter 3 the results and discussion part in the result and discussion part the overlay characterization of the raw materials and the complexes and the scientific substantiation for the formation of complexes have been provided. Some highlighted information is as followed. In case of FTIR data of all three

cyclodextrin there are some characteristic disappearance of aromatic C=C stretching (1600 cm^{-1}) confirms that there is complexation formation occurring but there is no evidence of bond formation. In case of FT Raman in complexes presence of a characteristic peak which is seen eminently at the range of 1633 cm^{-1}-1636 cm^{-1} were observed only in complexes. Solubility of the complexes with different solvents was discussed, comparative stability of each complexes and their percentage of the individual curcuminoids were finally discussed along with the existing literature.

Chapter 4 is Summary and Conclusions the final part which discuss about whether the hypothesis has been proved along with conclusion and future directions derived from the study.

1. Introduction

1.1 Background

Usage of medicinal plants is not a new trend to humanity. Across the centuries many new types of medications, medical procedures, and medical formulations came into existence, which were charming at those times. Widespread use of synthetic drugs made us to forget our primitive medicines. Prolonged use of synthetic drugs gives more adverse effects than the beneficiary effects. The clinicians are now facing a dreadful condition called Multiple Drug Resistance (MDR) which would soon become the problem of the decade if remained unchecked. MDR arises due to the improper over usage of drugs. On the other hand, there are people who cannot afford 1$ for the medication due to their deprived economy. For these two strangulating global problems a common answer proposed is naturoceuticals.

Use of plants for healing purposes predates human history and forms the origin of much modern medicine. If we look back our ancestor's path they used extracts from plants in order to treat even complicated ailments, many usual drugs were derived from plant sources. Ayurvedha and Siddha, which dates back to antediluvian period, were known to use things which are obtained from natural sources, i.e., leaf, bark of trees, roots of plants, etc. Ayurvedha practices started over 5000 years ago. Examples: Neem (***Azadirachta indica***) leaves which were antibacterial and antiviral in nature are used in medicinal preparations, Tulsi (***Ocimum tenuiflorum***) leaves are given to young children in order to improve the function of immune system, Adhatoda ***(Justicia adhatoda)*** contains Vasicine, a bronco dilator used to treat respiratory ailments, ashwagandha ***(Withania somnifera)*** was used as an antioxidant and also used in medicinal preparation treating cough. Hippocrates said that "Let food be thy medicine and medicine be thy food."

Turmeric is one among the several plants which have been used as a food additive, coloring agent as well as a medicinal plant. There are various bioactive principles in turmeric. Since it is versatile in order to alter various physiological and metabolic pathways in biological system it is used in medicine. Polyphenolic compounds present in turmeric are Curcumin (diferuloylmethane), demethoxycurcumin and bisdemethoxycurcumin and collectively called curcuminoids. Curcuminoids are responsible for the antibacterial, antiseptic, antiapoptotic, antiinflammatory, analgesic, antioxidant properties of turmeric. Among the curcuminoids, curcumin is the major component and it is the more potent therapeutic bioactive principle.

Curcumin is photosensitive, pH sensitive and has reduced aqueous solubility. Poor aqueous solubility results in poor reabsorption and poor bioavailability in biological system (poor oral absorption, unstable in gastrointestinal tract). To increase the aqueous solubility of curcumin various techniques were used which in turn improve the bioavailability. The technique which is widely employed to increase the solubility and bioavailability of a drug is to encapsulate it in: (a) solid dispersions, (b) micro emulsions (water dispersed in oil), (c) liposome's (synthetically synthesized lipid bilayer), (d) cyclodextrins, (e) polymer/magnetic nanoparticles (particles in the range of less than 100nm which is used to carry drug particles), (f) microspheres (drug encapsulated by a uniform layer of resistive coating material), etc.

Cyclodextrin is formed during bacterial digestion of cellulose and is used for the encapsulation of various lipophilic drugs with less aqueous solubility. Parent cyclodextrins have limited solubility, so modified cyclodextrins have been used for pharmacological purposes. In this thesis we used α-cyclodextrin, β-cyclodextrin, 2-hydroxypropyl-α-cyclodextrin, 2-hydroxypropyl-β-clodextrin and 2-hydroxypropyl-γ-cyclodextrin for complexing curcuminoids.

Hydroxypropyl derivative were known for their higher water solubility so that they are widely used in pharmaceutical preparations.

There are several techniques reported for preparing curcumin-cyclodextrin inclusion complexes. Some of them are: Physical mixture, freeze drying, solvent evaporation technique and recently pH shift method. Complexes reported in the literature which are made by pH shift method have not been characterized in detail. In this thesis details of preparation of curcumin-cyclodextrin complexes by pH shift method and characterization of these complexes are presented.

Curcumin-cyclodextrin complexes are considered as host-guest complexes which were characterized in order to know its supramolecular chemistry. FTIR and FT Raman were used in order to characterize the nature of curcumin and cyclodextrin in the complex. X-ray diffraction was done to check crystalline/amorphous nature of the complex. DSC is carried out in order to know the melting and crystallization behavior of the guest (curcumin) and host (cyclodextrin).

UV-Vis spectroscopy and High performance Liquid Chromatography (HPLC) technique were used to study the amount of curcumin content in the complex and also to analyze the degradation products formed *in vitro.* Liquid chromatography–mass spectrometry (LCMS-MS) was used to detect the different curcuminoids present such as diferuloylmethane, demethoxycurcumin, and bisdemethoxycurcumin in the complex. Stability of curcumin and complexes was studied in PBS buffer solution. Hemocompatibility of the complexes was also carried out.

1.2 Review of Literature

1.2.1 Turmeric

Turmeric is the rhizome part of the plant *Curcuma longa* (Figure 1.1) which is yellow in color due to the presence of curcumin. A number of biologically active compounds are present in the turmeric, namely, curcumin, demethoxycurcumin, bisdemethoxycurcumin, eugenol, tetrahydrocurcumin, triethylcurcumin, turmerin, etc. Turmeric has been used by the people of China and Indian subcontinents [Aggarwal *et al*, 2007]. It has the ability to cure various ailments. Knowing the importance of curcumin our ancestors have given curcumin a great importance and made it as a part of the traditional practices to offer turmeric along with other things to gods and goddesses.

Figure1.1 Turmeric (*Curcuma longa*)

1.2.3 Scientific Classification

Scientific classification of turmeric (*Curcuma longa*) is as followed:

Kingdom: Plantae

Order: Zingiberales

Family: Zingiberaceae

Genus: Curcuma

Species:C.longa

1.2.4 Bio Active Principles of Turmeric

Constituents in turmeric are mentioned in Figure 1.2 Phytochemicals present in turmeric are turmerin, turmerones, atlantones, turmeronols, curcumol, eugenol, curcumenol, curcuminoids, tetrahydrocurcumin, triethylcurcumin and zingiberene. Phenolic compounds isolated from turmeric are known as Curcuminoids, which include Curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin whose chemical structures

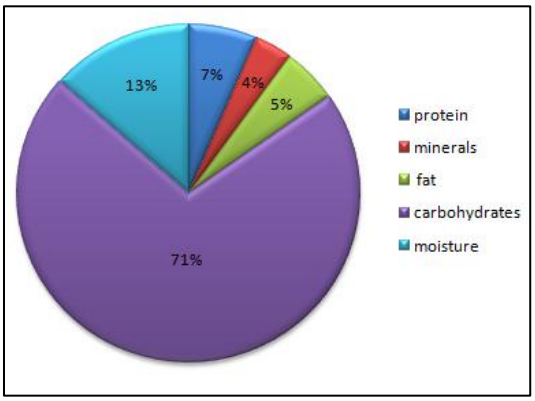

Figure 1.2: Constituents in turmeric

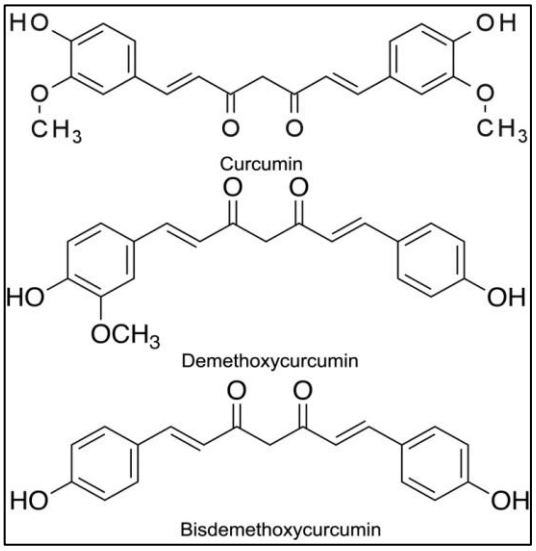

Figure 1.3: Chemical structure of Curcuminoids

are in given in Figure 1.3. In general turmeric powder preparations contain approximately 77% Curcumin, 18% demethoxycurcumin and 5% bisdemethoxycurcumin [Hoehle *et al*, 2006]. Curcumin is a low molecular weight (368.37) polyphenolic compound responsible for the yellow colour of curcumin [www.chemspider.com, curcumin Chem spider ID: 839564]. From turmeric curcumin was first isolated in the year 1815 [Vogel and Pelletier, 1815]. Despite the fact that turmeric was used in Indian/Chinese food and medicine for centuries its application and its bioactive principles were unknown to the scientific comunity, so not much work was carried out at that time. By the year 1910 nearly after one century Milobedzka and Lampe elucidated chemical structure of curcumin was [Milobedzka, 1910].

The commonly accepted chemical structural form of curcumin consists of two conjugated backbones each with a ketone and a –CH2 group separating the backbones and a terminal meta-methoxy-para-hydroxy phenyl rings on each side .

Curcumin possess a hyrophobic aliphatic bridge, a highly polar enolic and a phenolic group. The overall large dipole moment of 10.77 D confirms the polar ends and the middle group of curcumin in the enolic form. Hydrophobicity of curcumin is log P 2.5 [Fujisawa and Toshiko, 2005]. At pH between 1 and 7 Curcumin appears yellow in colour, a neutral stable state with low aqueous solubility; above pH 7 curcumin will become redish pink with slight increase in solublity [Aggarwal *et al*, 2007]. The degradation products under alkaline conditions have been identified as vanillin,vanillic acid and ferulic acid [Wang et al, 2004]. By the year 1949, a paper was published in Nature by Schraufstatter and colleague stating the anti-bacterial properties of curcumin [Schraufstatter and Bernt, 1949]. After this discovery attention of several researchers were turned to this compound and many were exploiting its bioactivity *in vivo* [Rao *et.al*.1970 , Srimal and Dhawan, 1973]. As of June

2013, in the National Institutes of Health PubMed database just a query “curcumin” gave more than 5000 articles yearly. This goes on increasing as people working in turmeric increased in the past decades [ncbi.nlm.nih.gov].

1.2.5 Curcumin In Biomedical application

Curcumin is an antioxidant [Ayed, 2006.], anti-ischemic [Pradeep *et al*, 2008], antibacterial [Schraufstatter and Bernt, 1949] antitumor and anti-inflammatory [Yadav *et al,* 2010]. It also helps in skin regeneration [Thangapazham *et al*, 2013]. For decades it has been used along with food by Indians in order to get rid of ailments like cancer, diabetic nephropathy, skin problems, inflammatory conditions, neurological disorders, etc.

Antioxidant activity of curcumin is known from its ability to protect the Red blood cells from the oxidation. In vitro experiments showed that there is significantly reduction in Reactive Oxygen Species (ROS) [Barzegar A and Moosavi-Movahedi].

Curcumin improves calcium transport in cardiac sarcoplasmic reticulum. This causes in replacement of defective calcium haomeostasis in the cardiac muscles [Nadkarni, 1976].

Curcumin has shown promise in cardiac conditions too. A study revealed that low doses of curcumin decreases low-density lipoprotein and total cholesterol levels in subjectes [Alwi, 2008].

Curcumin is a good anti-inflamatory agent. It has a good command over Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Curcumin reported to supress NF-κB by down regulation of cyclooxygenase-2 and nitrous oxide synthetase [Singh and Aggarwal, 1995].

Pharmacological studies in animal models and humans suggest that curcumin is safer. Safety evaluations carried out by giving 300-mg/kg body weight in small animal models (Wistar rats, Guinea pigs) and large animal

models (Primate) of both sexes observed no adverse reaction [Aggarwal *et al*, 2003]. To date, approximately 50 clinical trials using human subjects have been completed. Although still in the initial phases, most of these trials have suggested that curcumin is safe to humans [Gupta *et al,* 2012]. According to the clinicila trials.gov there are now 32 different groups evaluating curcumin

1.2.6 Bioavailability of curcumin - a big concern

Bioavailablity is defined as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. Though turmeric has more bioactive compounds the constraint in using turmeric is because of:

I. Degradation (photolytic/enzymatic)
II. Inability to reach circulation and
III. Low oral bioavailability and poor gastrointestinal reabsorption

In order to attain the desired dosage of curcumin in the blood it requires intake of more amount of drug. It is a known fact that bioavailability determine thereputic index of a drug. Lower the bioavailability lower the thereputic index which in turn decreases the thereapeutic activity.

1.2.7 Methods to enhance solubility of Curcumin

There are a number of techniques that are known to increase the solubility of curcumin [Challa *et al.* 2005]. These include nanoparticles [Thangapalam *et al,* 2008], kneading method [Fernandes CM and Veiga FJB, 2002], pH shift method [Yadav et al, 2009 and Suresh et al, 2012] and spray drying [Moyano et al, 1997].

In kneading method the guest molecule is made in the form of a paste by using a morter and pestleand then to that the drug which has to be complexed is added [Fernandes CM and Veiga FJB, 2002]

Hydrophilic and hydrophobic molecules can be accomdate in Liposomes. From the work carried out by in human prostate cancer cell lines it was oberved that antiproliferative activity of liposomal curcumin formulation had ten fold high than free curcumin[Thangapalam *et al,* 2008].

Curcumin loaded solid lipid nanoparticles were incorparatend in cream formulation it was found that the nano particles increased the efficiency of curcumin released from the cream [Anand *et al*, 2007].

Most of the literature describes synthesis of complexes by physical mixture technique (Chiou and Riegelman, 1971 and Joshi *et al*, 2010) but molecular characterization data of Mohan *et al*. [Mohan *et al,* 2012], suggest that physical mixing is not sufficient enough to produce an interaction between the guest and host molecule which was clear from the FTIR which showed no peak at the 1620cm^{-1} region but in case of freeze dried complex a characteristic peak was observed at 1620 cm-1 which is due to interference of OH in cyclodextrin.

In spray drying the complex is synthesized by using solvent suspension technique and then using a spray dryer the sample is being dried [Moyano, 1997]

Previous literature [Yadav et al, 2009 and Suresh et al, 2012] which described about the pH shift lacks characterization of curcumin-cyclodextrin complex, molar ratios used were disclosed, we planned to synthesis curcumin-cyclodextrin by pH shift method in molar ratio 2:1 as since the effectiveness of complexation is good [Dandawate *et al*, 2012] in 1:2 ratio (1 guest molecule: 2 host molecule guest-curcumin, host-cyclodextrin).

Due to poor bioavailability, oral administration of curcumin leads to very poor concentration of curcumin in plasma of rats [Ravindranath and Chandrasekhar, 1980]. Previous study conducted in rats showed that after [Shoba *et al*, 1998] administering 1g/kg of curcumin analysis of portal blood

samples collected at intervals of 15 to 24 hrs showed that only traces of curcumin found in blood plasma. A study on the effect of piperine on bioavailability of curcumin conducted in humans and suggested that bioavailability of curcumin in humans is higher than that of rats. This difference may be attributed to the species variation [Wang *et al,* 2011].

1.2.8 Analysis of curcuminoids by LCMS/MS

Most reports on solubility and stability of curcuminoids were done using UV-Vis spectroscopy [Yadav, 2009]. The problem with this technique is that it is quiet unspecific and it could not estimate the amount of individual curcuminoids (i.e., Curcumin, demethoxycurcumin, bisdemethoxycurcumin) present in the sample. Liquid chromatography Mass spectrometry/Mass spectrometry (LCMS/MS) is a valuable technique to characterize individual components in a mixture especially when the retention time of the components is the same [Anchang, 2006].

1.2.9 Cyclodextrins

Cyclodextrins are non-reducing cyclic glucose oligosaccharides resulting from the cyclomaltodextrin glucanotransferase catalyzed degradation of starch. In a nut shell Cyclodextrins formed during bacterial digestion of cellulose [Szejtli, 1982]. There are three parent cyclodextrins: 6 D-glucopyranonsyl residues (α-cyclodextrin), 7 D-glucopyranonsyl residues (β-cyclodextrin) and 8 D-glucopyranonsyl residues (γ-cyclodextrin). These 6, 7 and 8 D-glucopyranonsyl residues were linked by (α-1,4) glycosidic bonds (Figure 1.4). α-D-glucopyranose units contain a fairly lipophilic inner cavity and a hydrophilic outer surface. The polarity of the cavity has been estimated to be similar to that of an aqueous ethanolic solution. All three Cyclodextrins are the same in their bond lengths and orientations apart from number of glucose residues. Its molecular structural arrangement appears to be like a

truncated cone or a bottomless tub-shaped. Around the external rim the molecule is stiffened by hydrogen bonding (3-OH and 2-OH groups). The

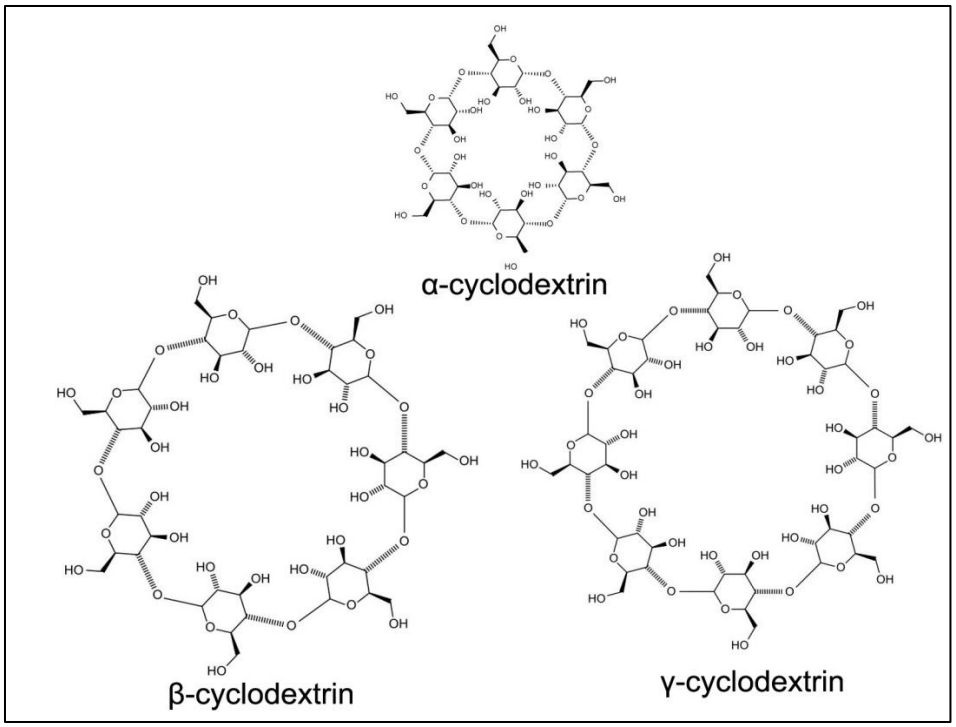

Figure 1.4: α-cyclodextrin β-cyclodextrin and γ-cyclodextrin

hydrogen bond strengths is higher for γ-cyclodextrin moderate for β-cyclodextrin. Compared to γ-cyclodextrin and β-cyclodextrin α-cyclodextrin is very less hydrogen bond strength. As far as solubility in water is concerned β-cyclodextrin has the least solubility. The reason for this poor aqueous solubility may be due to interaction of lipophiles with β-cyclodextrin. Due to relatively strong intermolecular hydrogen bonding in the crystal state aqueous solubility of the natural cyclodextrins is much lower than that of comparable acyclic saccharides. Structural parameters of cyclodextrins are described in Table 1.1 [Loftsson, 1995].

Apart from the parent Cyclodextrins there are a number of modified cyclodextrins which are water soluble [Challa *et al.* 2005]. Examples are: 2-hydroxypropyl α-cyclodextrin, 2-hydroxypropyl β-cyclodextrin, 2-hydroxypropyl

γ-cyclodextrin, glycosyl β-cyclodextrin, methylated derivatives of β-cyclodextrin, sulfobutylated β-cyclodextrin, etc.

Table 1.1: Structural parameters of parent cyclodextrins

Cyclo-dextrin	Cavity Diameter (nm)	Cavity Height (nm)	Cavity volume (ml mol-1)	Glucose Residue	Solubility (g/100ml)
α	0.47-0.53	0.79	174	6	14.5
β	0.6-0.66	0.79	262	7	1.85
γ	0.75-0.83	0.79	472	8	23.2

Cyclodextrins offer a number of advantages when they are used as complexing agents. Some of them are listed below [József Szejtli *et al*, 2004]:

- Improvement in the aqueous solubility of hydrophobic substances.
- Stabilization of light sensitive substances.
- Increases shelf life.
- Catalytic activity of cyclodextrin with guest molecules.

Pharmaceutical community uses these characteristics of cyclodextrin put them into use in drug formulation. Some of the commercially available cyclodextrins-curcumin drugs are mentioned in the Table 1.2.

Table 1.2: Various Drugs and its complexation with parent and Hydroxypropyl cyclodextrins

S.No	Cyclodextrin	Drug	Ref.
1	α	Curcumin	**Nagaraju *et al*,** 2013.
		Dehydroepiandrosterone	**Corvi *et al*,** 2003.
2	β	Erythro Mycin	**Song *et al*,** 2011.
		Glibenclamide	**Aiman and Nadia,** 2009.
3	γ	Doxorubicin	**Monnaert *et al,*** 2004.
		Prednisolone	**Yano *et al*,** 2001.
4	2-Hydroxy propyl α	Fexofenadine	**Ali *et al,*** 2012.
		Ferulic acid	**Monti *et al,*** 2011.
5	2-Hydroxy propyl β	Spironolactone	**Tötterman *et al***, 1997.
		Curcumin Analogue	**Dandawate *et al.*** 2012.
6	2-Hydroxy propyl γ	Doxorubicin	**Monnaert *et al.***2004.
		Curcumin	**Yadav *et al.*** 2010.

1.2.10 Curcumin-cyclodextrin complexes

Cyclodextrin is the host molecule curcumin the drug molecule is considered as the host molecule. In drug complexation the cyclodextrin is solubilised in water

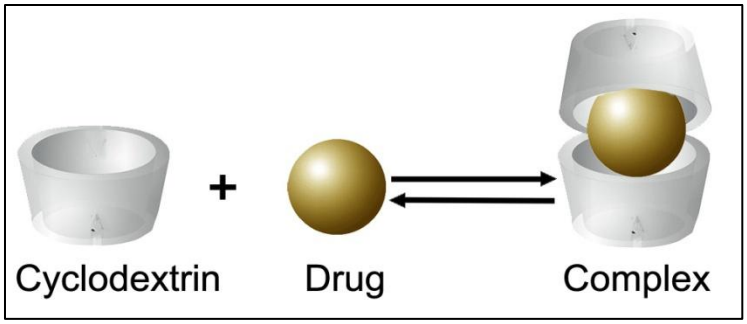

Figure 1.5: Complex formation (1:2 drug:Cyclodextrin)

curcumin which insoluble in water is dissolved in appropriate solvent and mixed with the cyclodextrin which lead to the formation of solid inclusion complexes [Kayaci and Uyar, 2011]. Binding of the molecule with the cyclodextrin has not known clearly. Solid inclusion complex formation is a dynamic process during complexation the lipophilic cavity of cyclodextrin molecule acts as a microenvironment to accommodate the incoming non polar guest molecule. In inclusion complex there is no formation or breakage of a covalent bond. [Stella Valentino, 1999]

Studies on the complexation of curcumin with various hydroxypropyl derivatives of cyclodextrin such as 2-hydroxypropyl-α-cyclodextrin, 2-hydroxypropyl-β-clodextrin, and 2-hydroxypropyl-γ-cyclodextrin have been previously reported [Tobar et al, 2012 and Dandawate PR *et al*, 2012].

A study on the comparative solubility analysis of γ-cyclodextrin (γ-CD), methyl β-cyclodextrin (MβCD), Hydroxypropyl β-cyclodextrin (HPβCD) and β-cyclodextrin (βCD) complexed with curcumin by kneading method showed that solubility of curcumin-γCD complex is higher than curcumin-βCD complex. Least solubility was observed in case of HPβCD whereas MβCD is neither a good complexing agent [Yadav, 2009].

Yadav *et al* compared the effect of the complexed with uncomplexed curcumin and obsserved that complexation increases reabsorption of the curcumin but the lacunae in the study was it did not explain the method of complexation (pH shift method) curcumin content is not being mentioned [Yadav, 2010]. Stability profile of the complexes which has been previously reported uses the spectrophotometric method [Gonçalves et al, 2012] Using spectophotometry total curcumin content can be analyzed but it is impossible to analyze the individual curcuminoids so we planned to carry out an LC-MS/MS analysis.

1.3 Hypothesis

Based on the review of literature made the study was designed to experiment the hypothesis that hydroxylpropyl gamma could stablises all three curcuminoids than hydroxylpropyl alpha and hydroxylpropyl beta. In order to test this hypothesis the specific objective were set.

1.4 Objective of the work

The objectives of this work are listed below:

I Optimization of complexation procedures of curcumin with cyclodextrins using the technique “pH shift method”.

II Preparation of inclusion complexes of curcumin with various cyclodextrins (2-hydroxypropyl-α-cyclodextrin, 2-hydroxypropyl-β-clodextrin and 2-hydroxypropyl-γ-cyclodextrin).

III Characterization of these complexes by FTIR, FT Raman, UV-Vis Spectroscopy, Differential Scanning Calorimetry, X-Ray Diffraction Spectroscopy and Scanning Electron Microscopy (SEM) and hemolysis.

IV Study Solublity profile of the complexes using UV-Vis.

V Study the stability profile of the complexes using LCMS/MS.

2. Materials and Methods

2.1 Materials Used

The raw materials, solvents and standards used for this work and their sources and purity are listed below in table 2.1.

Table 2.1 Source and purity of raw materials used

Materials	Abbreviation used	Source	Purity
Curcumin (Curcuminoid with trade name Biocurcumax®)	BCx	M/s. Arjuna Naturals Extracts Ltd, India	95%
2-Hydroxypropyl α Cyclodextrin	HPαCD	M/s. Aldrich Chemistry Ltd, Germany	Not Available
2-Hydroxypropyl ß Cyclodextrin	HPßCD	M/s. Aldrich Chemistry Ltd, Germany	Not Available
2-Hydroxypropyl γ Cyclodextrin	HPγCD	M/s. Aldrich Chemistry Ltd, Germany	Not Available
Vanillin	VAN	M/s. Sigma Aldrich, USA	99%
Vanillic acid	VAD	M/s. Fluka, USA	≥97.0%
Ferulic acid	FAD	M/s. Aldrich, USA	99%
Ethanol		M/s. SD fine Chemicals, Mumbai	99.9% absolute
Methanol		M/s. Merck Specialties Pvt. Ltd, Mumbai	HPLC grade

2.2 Methods

The process equipment used for the work and the analytical instruments used for the characterization of the materials are given in tables 2.2 below along with their sources / suppliers.

Table2.2: Process equipments and their suppliers

Instruments	**Supplier/Manufacturer**	**Model No**
Electronic weighing balance	M/s. Sartorius	LA230 S
Shaker bath	M/s. Julabo, Germany	SW 22
Vortex mixer	M/s. Labtop, India	VM 301
Ultrasonicator	M/s. COscar Ultra Sonicator, India	Microclean 103
Lyophilizer	M/s. Bioasset,India	SLN 0202
Centrifuge	M/s. Heraeus Biofuge, Germany	-

Table 2.3 Analytical Instruments used and their sources

Instruments	**Model Name and Manufacturer**	**Model No.**
UV/Visible spectrophoto-meter	M/s. Shimadzu, Japan.	UV-1800
FT IR spectroscopy	M/s. Jasco, Madison USA.	6300
FT RAMAN spectro-scopy	M/s. Bruker, Switzerland.	RFS 100/S
Scanning Electron Microscope	M/s. HITACHI, Japan.	S-2400
High Performance Liquid Chromatography	M/s. Waters Co, Milford, MA, USA.	Alliance 2695
Mass Spectrometry	M/s. Micromass UK Ltd, Manchester, England.	Quattro micro
pH meter (Digital)	Cyberscan, Japan (Eutech Instruments, Singapore)	5.1
Differential Scanning Calorimeter	M/s. TA Instruments Inc., USA	SDT 2920
X-Ray Diffraction Spectroscopy	M/s. Siemens diffracto-meter, USA	D-5005
Scanning Electron Microscopy	M/s. HITACHI, Japan	S-2400
Automated hematology analyzer	M/s. Sysmex	K 4500
Software Used	Orgin Pro	Version 8

2.3 Characterization of the Raw Materials

2.3.1 FTIR Spectroscopy

FTIR-ATR spectra of BCx and cyclodextrins were recorded in an Attenuated total reflection infrared spectrophotometer (Jasco 6300) in the scan range of 399–4000$cm^{-1}$with a 4cm^{-1} resolution and 64 scans per spectrum.

2.3.2 Raman Spectroscopy

Raman spectra of BCx and cyclodextrins were recorded at 22^{o}C using a Bruker RFS100/S Raman spectrometer. A scan range of 500–3500cm^{-1} with a resolution of 2cm^{-1} was used and the spectra were the averages of 32 scans. The spectra were generated using a laser power of 150mW and source wavelength of 1064nm using Nd:YAG (Neodymium-doped yttrium aluminum garnet) laser source. The detector used was standard germanium.

2.3.3 X-Ray Diffraction Spectroscopy

Using the X-ray Diffractometer (Siemens diffractometer D-5005) with Cu radiation source patterns of the BCx and cyclodextrins were obtained. The measurements were done at 40kV voltage and 30mA current and scanned at a rate of 2° min^{-1} with scanning angle10°$\leq 2\theta \geq$40°

2.3.4 Differential scanning calorimetry (DSC)

As per ASTM E 537-07 standards DSC measurements were carried out in a DSC Q100 V9.6 Build 290 differential scanning calorimeter (TA Instruments Inc., New Castle, DE). Curcumin and cyclodextrins were scanned in the temperature range 25–210◦C and the heating rate employed was 10◦C/min. The measurements were carried out under dry N_2 at a flow rate of 50 ±5 mL/min.

2.3.5 Scanning Electron Microscopy (SEM)

Surface morphology of the BCx and cyclodextrins was observed in scanning electron microscopy (HITACHI model, S-2400, Japan). Specimens were mounted on

aluminum stubs with the help of a double side adhesive tape and coated with an ultra thin (300 Å) layer of gold in a coating apparatus(Hitachi E101). After coating the stub is mounted and observed in various magnifications 500X, 3000X and 5000X.

2.3.6 Drug Content determination using UV–Vis spectroscopy:

Curcumin content in the complex was determined by using a UV–Vis spectrophotometer (model UV-1800, Shimadzu, Japan). For this 1mg complex was dissolved in 1 mL of ethanol and the absorbance of the solution was recorded at 427 nm. The curcumin content was estimated from a standard X–Y plot of BCx (dissolved in ethanol) vs. concentration. The path length of the quartz cell was 10 mm. For each measurement the baseline was established by placing blank ethanol in the reference compartment. All measurements were carried out at 25 ± 1 ◦C.

2.3.7 In-Vitro Hemocompatibility:

As per ISO Document 10993-4, (Biological evaluation of medical devices. Part 4. International Organization of Standardisation, Geneva 2002) percentage hemolysis test for the complexes was done.

- Blood from human volunteer was collected into the anticoagulants.
- The test materials were placed in polystyrene culture plates and agitated with phosphate buffered saline before they were exposed to blood.
- Samples (10mg each) were suspended in 1mL of PBS and 100µl of the samples were placed in polystyrene plates and 2mL of blood was added 1mL of blood was taken immediately for initial analysis and remaining 1mL blood was incubated with the sample for 30 min under agitation at 75 ± 5 rpm using an Environ shaker thermo stated at 35 ± 2°C.
- Four empty polystyrene culture dishes were exposed with blood as reference.

2.3.7.1 Calculating Percentage of Hemolysis:

The total hemoglobin in the initial samples was measured using automatic hematology analyzer (Sysmex-K 4500). The platelet-poor plasma from blood exposed for 30 min was prepared. The free hemoglobin liberated in the plasma after exposure was measured ineach sample using diode array spectrophotometer and

the percentage hemolysis was calculated using the formula.

% Hemolysis = Free Hb/total Hb *100

2.4 Preparation of Curcumin-CD complex by pH shift method

Complexes of Cyclodextrin and curcumin were prepared by adopting the following procedure:

I BCx and cyclodextrin were taken in molar ratio 1:2 (Table 2.4).

II Cyclodextrin was dissolved in 4mL water, mixed well by vortexing for 2 min and the pH was adjusted to 11.5 by using 4N NaOH. Whenever the pH exceeded 11.5, it was brought down by adding 1N HCl. Care was taken to maintain the final volume of the solution to 8 mL.

III Suspended BCx in 1mL Ethanol and vortexed for 30 sec, Added 4N NaOH drop-wise till the pH reached 12. Care was taken to maintain the final volume of the solution to 2mL. Since BCx is light sensitive, its solution was prepared in amber colored beakers and in the dark conditions.

IV Soon after adjusting the pH of BCx to 12, cyclodextrin was added to the beaker containing BCx and then mixed thoroughly. After mixing it thoroughly the pH was brought down to 7 by adding 1N HCl drop-wise.

V After attaining neutral pH, the solution was filtered using 0.45µm membrane filter the filtrate was freeze-dried in lyophilizer.

Table 2.4: Weight of BCx and cyclodextrins taken for preparing complexes having mole ratio 1:2.

S.No	Name of the compound	Molecular Weight	Quantity taken
1	HPαCD	1180 (average)	2 g
	BCx	368.38	0.315 g
2	HPßCD	1460 (average)	2 g
	BCx	368.38	0.253 g
3	HPγCD	1580 (average)	2 g
	BCx	368.38	0.240 g

2.5 Characterizations of the Complexes

Characterization of complexes was carried out by UV-Vis Spectroscopy, FT Raman Spectroscopy, FT-IR Spectroscopy, DSC and TGA as previously described in section 2.3.

2.6.1 Preparation of standard curve for BCx in Ethanol

- The standard solution was prepared according to the method used by Wang et al. (1997). Accurately weighed 10 mg of BCx was taken in a 10 mL standard flask and dissolved in 5 mL of ethanol and made up to the mark with the same solvent to get a stock solution of 1000 µg/mL- Stock solution-I.
- From the stock solution-I, 5 mL was taken and made up to 50 mL with ethanol in a standard flask to get a stock solution of 100µg/mL- Stock solution-II.
- From the stock solution-II, 5 mL was taken and made up to 50 mL with ethanol in a standard flask to get working stock solution of 10 µg/mL

- Aliquots of 2, 4, 6 and 8 µL working stock solution were taken in 10 mL standard flasks and made up to the volume with ethanol and 10 µL working stock solution taken as such to get final concentrations 2,4,6,8 and 10 µg/mL.
- The samples were then analyzed spectrophotometrically by using a double beam UV spectrophotometer at 427 nm using ethanol as blank.

2.6.2 Standard curve for BCx in PBS:

- The standard solution was prepared according to the method used by Wang et al. (1997). Accurately weighed 10 mg of BCx was taken in a 10 mL standard flask and dissolved in 5 mL of ethanol PBS mixture (ratio 1:1) and made up to the mark with the same solvent to get a stock solution of 1000 µg/mL-Stock solution-I.
- From the stock solution-I, 5 mL was taken and made up to 50 mL with ethanol PBS mixture (ratio 1:1) in a standard flask to get a stock solution of 100µg/mL-Stock solution-II.
- From the stock solution-II, 5 mL was taken and made up to 50 mL with PBS in a standard flask to get working stock solution of 10 µg/mL
- Aliquots of 2, 4, 6 and 8 µL working stock solution were taken in 10 mL standard flasks and made up to the volume with PBS and 10 µL working stock solution taken as such to get final concentrations 2,4,6,8 and 10 µg/mL.
- The samples were then analyzed spectrophotometrically by using a double beam UV spectrophotometer at 427 nm using PBS as blank.

2.6.3 Standard curve for BCx in Distilled water

- The standard solution was prepared according to the method used by Wang et al. (1997). Accurately weighed 10 mg of BCx was taken in a 10 mL standard flask and dissolved in 5 mL of ethanol distill water mixture (ratio 1:1) and made up to the mark with the same solvent to get a stock solution of 1000 µg/mL-Stock solution-I.

- From the stock solution-I, 5 mL was taken and made up to 50 mL with ethanol distill mixture (ratio 1:1) in a standard flask to get a stock solution of 100µg/mL- Stock solution-II.
- From the stock solution-II, 5 mL was taken and made up to 50 mL with distill water in a standard flask to get working stock solution of 10 µg/mL
- Aliquots of 2, 4, 6 and 8 µL working stock solution were taken in 10 mL standard flasks and made up to the volume with PBS and 10 µL working stock solution taken as such to get final concentrations 2,4,6,8 and 10 µg/mL.
- The samples were then analyzed spectrophotometrically by using a double beam UV spectrophotometer at 427 nm using distilled water as blank.

2.6.4 Sample preparation for solubility study

Weighed 1mg each of BCx -cyclodextrin complexes and dissolved in distilled water. Since the curcumin content in the complex was under 10%, the amount of BCx weighed was such that the complex and BCx had equivalent quantity when added into distilled water. In the case of HPγCD-BCx, 1 mg of complex and 0.07mg BCx were taken for UV measurements. The samples were vortex mixed and sonicated for 5min to dissolve completely. The sample was kept in a shaker bath at 37°C and at 70rpm. Later it was centrifuged at 2500 RPM for 5 min and the supernatant was analyzed in UV-Vis Spectroscopy. It was found that complexes were fully soluble whereas major quantity of BCx remained as precipitate. The absorption maxima of each sample were measured and plotted against standard curve. The samples were analyzed in triplicate and final concentration of each samples were expressed as mean.

2.7 Stability Studies by LC-MS/MS

2.7.1 Preparation of standard solutions

Stock solution of BCx was prepared by weighing accurately 2.7 mg of BCx into 1.5 mL amber colored centrifuge tube containing 1 mL of methanol and vortex mixed to dissolve it completely. The working standard solutions were prepared by serial dilution using the solution, phosphate buffer of pH 7.4: methanol (1:9 v/v), as diluent. The

concentration of individual curcuminoids was calculated according to the composition in which they present in BCx (Curcumin (CUR) - 70.43%, Demethoxycurcumin (DMC) - 25.67% and Bisdemethoxycurcumin (BDMC) - 3.90%). The final concentration of calibration standards were 0.12, 0.26, 0.59, 1.18, 2.35, 4.71 and 9.41 µg/mL for CUR, 0.04, 0.10, 0.21, 0.43, 0.86, 1.72 and 3.43 for DMC and 0.007, 0.015, 0.033, 0.065, 0.130, 0.261 and 0.521 for BDMC. The calibration graph was obtained by plotting peak area of the analyte (Y) against concentrations (X, µg/mL) of each analyte and the data obtained were calculated by linear regression analysis.

2.7.2 Sample preparation for LC-MS/MS studies

In order to carry out stability studies in LCMS, 1mg/ml of complex was dissolved in PBS (pH 7.4) buffer and the sample was vortexed to get a thorough dissolution and sonicated for five minutes. The solution was then kept in a shaker bath set at 37°C and at 70rpm. In previous studies (Patro *et al*, 2012 and Yadhav *et al*, 2010), the complex and curcuminoids were taken at the concentration of 1mg/mL but in this study, the concentration of the control (BCx) was taken based on the curcumin content in each respective sample as described in Table 2.5. Aliquot of 100 µL samples were taken at periodic intervals (0, 2, 4, 6, 8 and 24 h) and 900 µL of methanol was added, vortex mixed and centrifuged at 2500 rpm for 5 minutes. The supernatant was analyzed by LC-MS/MS. The absorption maxima of each sample were measured and plotted against standard curve. The samples were analyzed in triplicate and the mean value is reported.

Table 2.5: Concentration of BCx taken for LCMS Study

Name of the Complex	Curcuminoid content in 1 mg of complex	Amount of BCx added to 10 mL PBS solution
BCx-HPαCD	0.0203 mg	0.203 mg
BCx-HPßCD	0.0300 mg	0.300 mg
BCx-HPγCD	0.0749 mg	0.749 mg

2.7.3 LC-MS/MS Conditions

High-performance liquid chromatography separations were performed on a Waters Alliance (Waters Co., Milford, MA, USA) system. The column utilized for separation was a 50mm x 2.1mm, Symmetry C18 column (Waters, Milford, MA, USA) with a particle size of 3.5 µm. Gradient elution was carried out using 0.1% acetic acid in water as component A and 0.1% acetic acid in acetonitrile as component B. All separations were carried out at ambient temperature with a flow-rate of 0.2 mL/min and a total run time of 10 min. The injection volume was 20 µL for all the samples.

Mass spectrometric analyses were performed with a Micromass Quattro micro triple quadrupole mass spectrometer (Micromass UK Limited, Manchester, England) equipped with an electrospray ion source (ESI) operating in negative mode. Data were acquired with MassLynx4.1 software. The following instrumental parameters were used for LC/MS/MS detection and quantification of the analytes in MRM (Multiple Reaction Monitoring) mode: dwell time 0.05 s, inter channel delay 0.01s, inter scan delay 0.01 s, capillary voltage 3.50 kV, source temperature 120°C, desolvation temperature 350°C, desolvation gas flow 700 L/h, ion energy 0.5 V, multiplier voltage 650 V, entrance lens voltage 20 V, exit lens voltage 20 V, collision gas argon pressure 2.50 $x10^{-3}$Torr. The analyte specific parameters were optimized by continuously infusing standard solutions dissolved in water (1 $\mu g/mL^{-1}$) at a rate of 10$\mu Lmin^{-1}$.

The chromatography and the retention time of the individual curcuminoids and degradation products was optimized by injecting known quantity of BCx and anticipated degradation products of curcuminoids (VAN, VAD, FAD) mixed together in HPLC grade methanol. The injection volume for sample and standards were 20 µL.

3. Results and Discussion

3.1 Characterization of raw materials

3.1.1 Curcumin

FT IR and FT Raman spectra of curcumin (BCx) are shown in Fig 3.1 and 3.2.

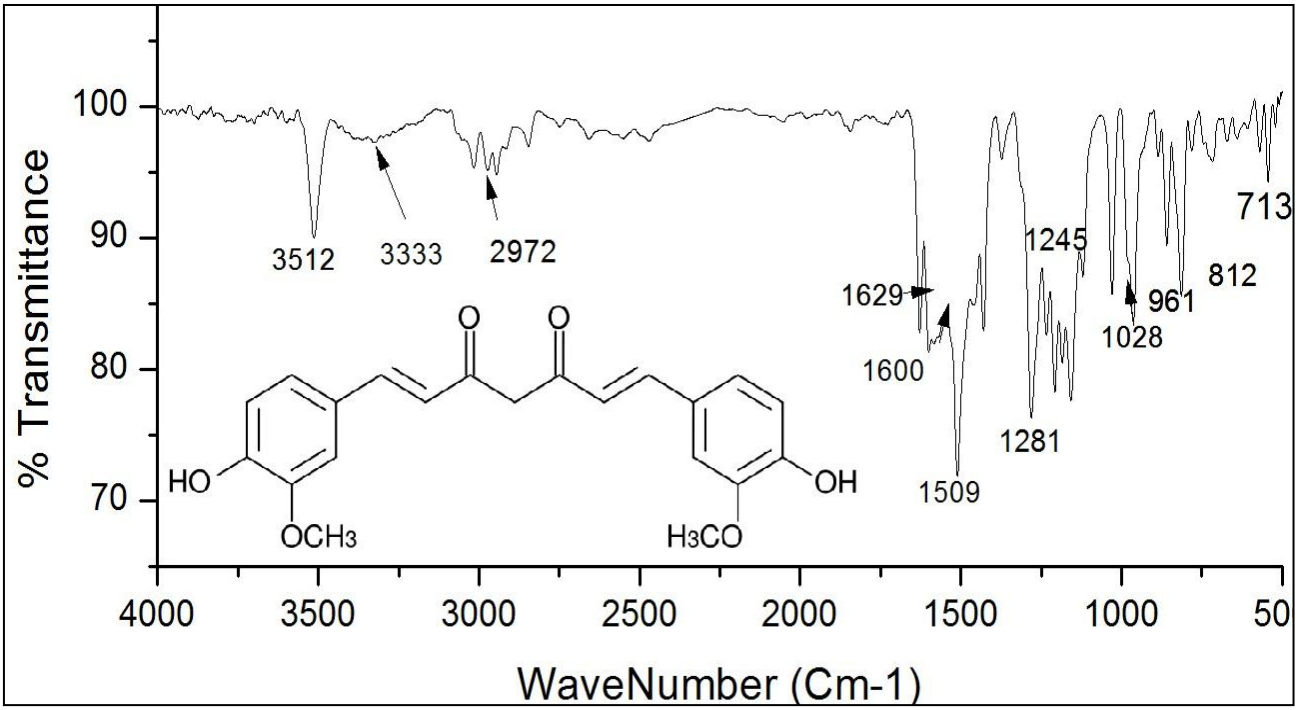

Figure 3.1 FT IR spectra of BCx

A sharp peak at 3512 cm^{-1} and a broad peak at 3333 cm^{-1} indicate the presence of OH. Peak at region 1629 cm^{-1} consist of two distinct characteristics, i.e., v(C=C) and v(C=O). Peak at 1601 cm^{-1} attributed to $v(C=C_{ring})$ aromatic ring stretching vibration. The peak at 1508 cm^{-1} corresponds to v(C=O) in plane bending aromatic stretching vibration, peak at 1272 cm^{-1} corresponds to enolic C-O, peak at 1028 cm^{-1} corresponds to C-O-C. Benzoate trans-CH vibration is indicated by the peak at 961 cm^{-1}. Cis CH vibration of aromatic ring is seen at 731 cm^{-1}.

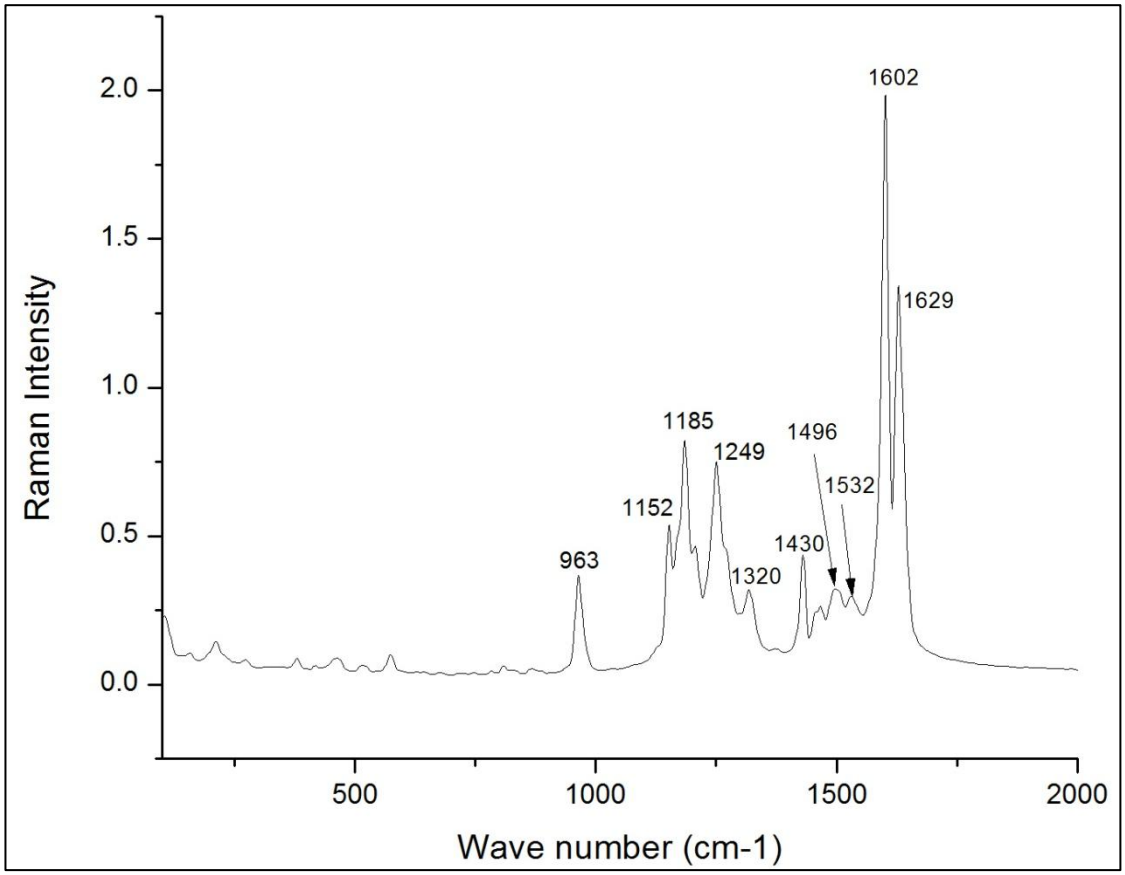

Figure 3.2 FT Raman spectra of BCx

In the Raman spectrum, peak at 1430cm^{-1} corresponds to O-CH_3 deformation vibration, CH_2 scissoring vibration due to alkane CH_2, symmetric COO stretching of acetate groups and CH deformation. The peak at 1532 cm-^{1} corresponds to aromatic C=C stretching vibration. The peak at 1602cm^{-1} corresponds to C=C stretching and 1629cm^{-1} corresponds to C=O stretching.

3.1.2 FTIR spectra of Cyclodextrins

FT IR spectra of HPαCD HPßCD and HPγCD are shown in Figure 3.3

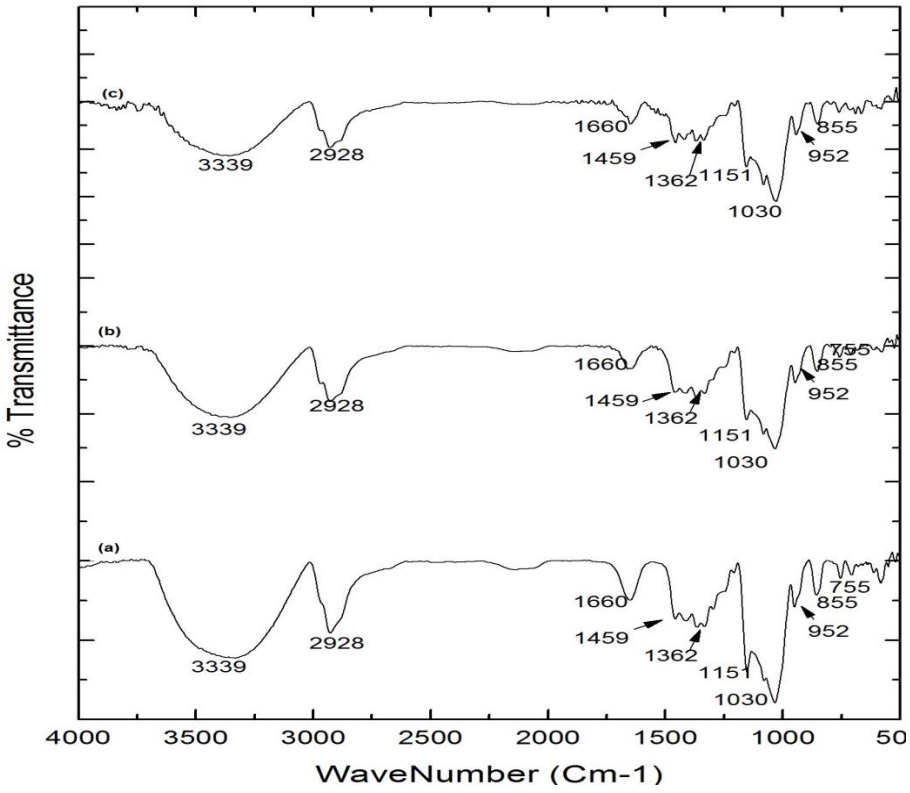

Figure 3.3 FT IR spectra of (a) HPαCD, (b) HPßCD and (c) HPγCD

IR spectra of all three cyclodextrins were almost similar yet there were slight variation observe in intensities. Vibrations observed at region 3600-3100 cm^{-1} corresponds to OH stretching which is present in all three cyclodextrins. Peak observed at region 2928cm^{-1} were due to symmetric/asymmetric C-H stretching. Deformation band of water H-O-H in cyclodextrin were observed at 1660 cm^{-1}. Strong peak at the region 1151cm^{-1} corresponds to C-H overtone vibrations.

3.2 Characterization of curcumin-cyclodextrin complexes

3.2.1 FT IR spectroscopy

FT IR spectrum of HPαCD-BCx complex is shown in figure 3.4. For comparison spectra of BCx, HPαCD are also shown. Similarly the spectra of other complexes are shown in Figures 3.5(HPßCD) and 3.6(HPγCD).

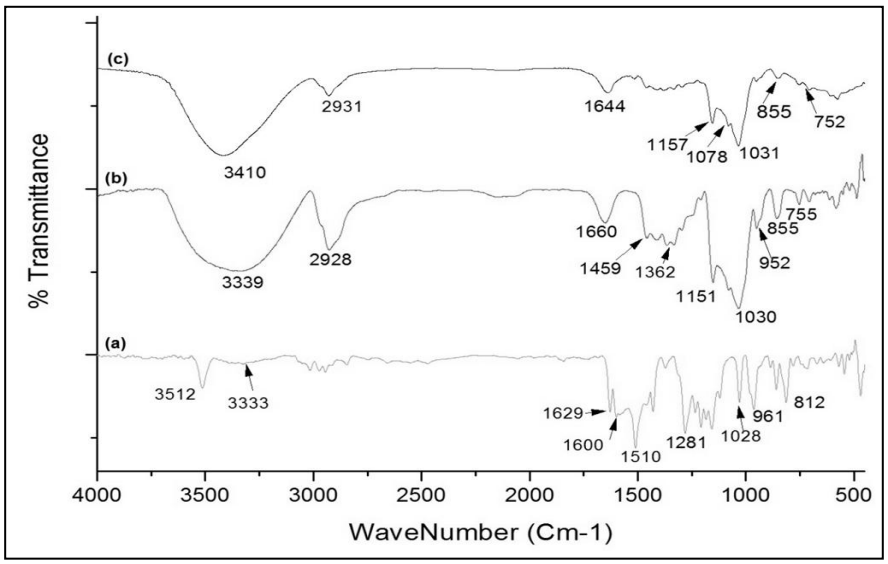

Figure 3.4 FT-IR spectra of (a) BCx, (b) HPαCD and (c) HPαCD-BCx.

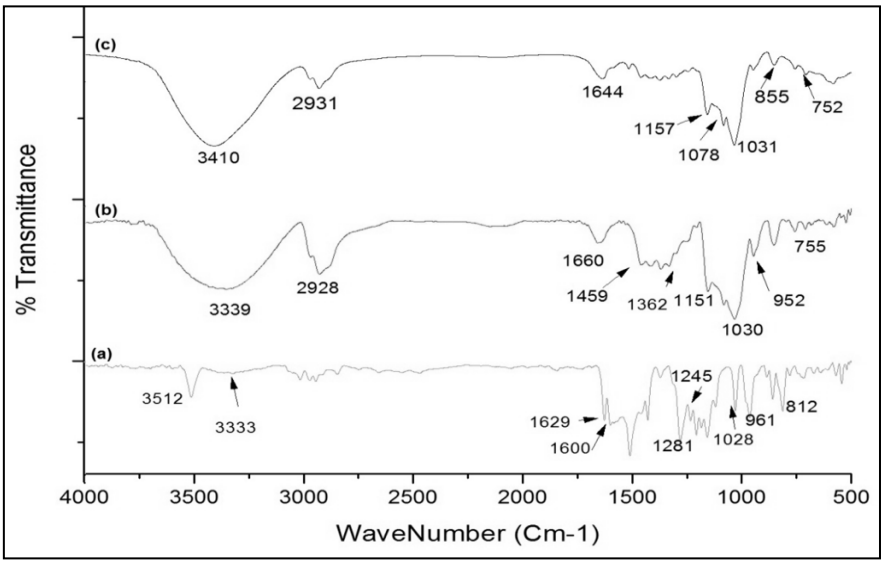

Figure 3.5 FT-IR spectra of: (a) BCx, (b) HPßCD and (c) HPßCD-BCx.

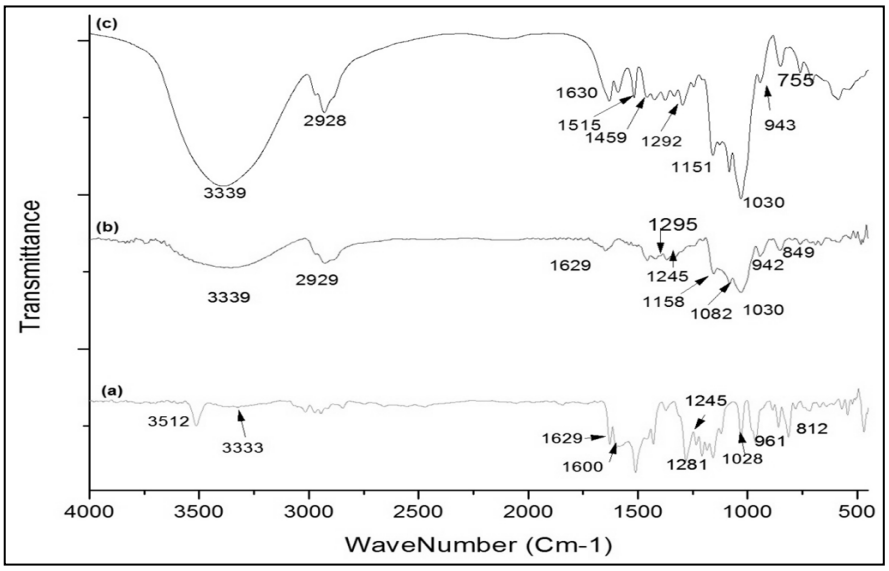

Figure 3.6 FT-IR spectra of: (a) BCx, (b) HPγCD and (c) HPγCD-BCx.

The complexes HPαCD-BCx , HPßCD-BCx and HPγCD-BCx follow a similar kind of peak arrangement as shown in Table 3.1. The peak for O-H stretching in the complex is shifted to higher wave number and also sharpening of the peak is observed at the region 3200-3500cm^{-1}. This is an evidence of increased inter molecular H bonding in the complex. Shift of peak in the range of 1000-4000cm^{-1} supports the formation of complex. The complex formation may be formed due to the interaction occurring between C=C, C=O, -OH, aromatic ring, etc. present in curcumin and cyclodextrin which is supported by shift in the C=O stretching of the complex. Peak corresponds to enolic COH bending (at 1362 cm^{-1}) in cyclodextrin disappears in complex which signifies involvement of enolic groups in some interaction between BCx and cyclodextrins. Strong peak at 1281 cm-1 of curcumin disappears in complex which denotes formation of complexes. Disappearance of the peak of C=C stretching at 1600 cm-^{1} indicates host-guest complex formation. Strong peak at 1510 cm-^{1} corresponds to CC=O in plane deformation disappears in the complex. In all three complexes there is no bond formation involved between curcumin and complex which were similar to the observation by Dandawate *et al* (2012). In HPγCD-BCx sharpening of OH stretching, disappearance of aromatic C=C stretching (1600 cm^{-1}), increase in intensity of 1030 cm^{1} (due to C-O-C stretching) was observed.

Table3.1 IR peak assignments of curcumin, cyclodextrins (HPαCD HPßCD and HPγCD) and complexes (BCx-HPαCD BCx-HPßCD and BCx-HPγCD)

BCx [cm^{-1}]	HPαCD [cm^{-1}]	HPßCD [cm^{-1}]	HPγCD [cm^{-1}]	HPαCD BCx [cm^{-1}]	HPßCD-BCx [cm^{-1}]	HPγCD-BCx [cm^{-1}]	Peak assignment
3512							OH stretching of phenol group, intra-molecular H bond
	3339	3339	3339	3401	3401	3401	OH Stretching
3333							CH stretching, intermolecular H bond stretching
	2928	2928	2928	2931	2931	2931	Asymmetric CH stretching of CH_2
1629							C=O, C_{ring}-C=C stretching
				1644	1644	1644	HOH of water of crystallization, C=C stretching
1600							Aromatic C=C Stretching
1510	1530	1530	1530			1515	C=O Stretching, CCC, CC=O in plane bending due to CH_2
	1459	1459	1459		1459	1459	In plane bending of CH_3
1372	1362	1362	1362				In plane bending of CH, enolic COH, skeletal CCC
1281						1292	CH in plane bending of C=CH, aromatic C-O stretching
	1155	1155	1155	1157	1157	1151	CH overtone stretching, C-O -C stretching
1150							In plane bending of aromatic CCH, Skeletal CCH
	1082	1082	1082	1078	1078	1078	C-O,C-C stretching,in plane bending of OCH
	1030	1030	1030	1031	1031	1030	C-O,C-C,CCO,C-O-C stretching of glucose units
1028							C-O-C stretching,out of planebending of CH_3 in plane bending of aromatic CCH
961	944	944	944			943	O stretching, in plane bending of CCH
854	855	855	855	855	855	855	CH out of plane bending of aromatic CCH and skeletal CCH
	753	753	753	752	752	755	Skeletal C-C stretching, CH out of plane bending
713	711	711	711				C stretching

3.4 FT Raman spectroscopy

FT Raman spectra of BCx, HPαCD, HPßCD, HPγCD, HPαCD-BCx, HPßCD-BCx and HPγCD-BCx are shown in Figure 3.7. Two prominent peaks were observed for BCx in the region 1600 cm^{-1} and 1629 cm^{-1} and a small peak at 1430cm^{-1}. In all three cyclodextrins (HPαCD, HPßCD and HPγCD) there were no peaks in the range 1500-1700 cm^{-1}. The only major peak observed was that at 1461 cm^{-1}. It is observed that the peaks in the region 1600cm^{-1} corresponds to aromatic ring vibration v(C-C_{ring}). In all complexes (BCx-HPαCD, BCx-HPßCD and BCx-HPγCD) reduction in the intensity of the v(C-C_{ring}) peak at 1600 cm^{-1} was observed and it may possibly due to the restriction imparted by cyclodextrin cage and the shielding effect on the benzene ring by the cyclodextrin cavity (Mohan *et al.*, 2012).

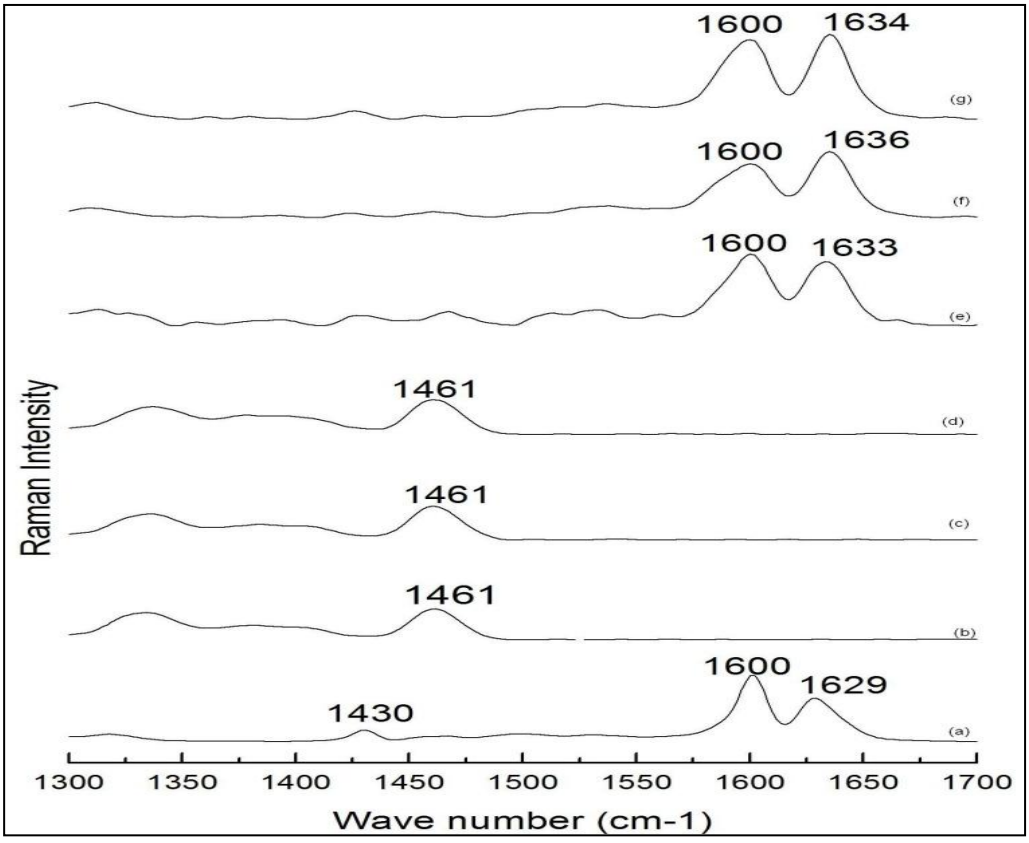

Figure 3.7: FT Raman spectra of: (a) BCx (b) HPαCD (c) HPßCD (d) HPγCD (e) BCx-HPαCD,(f) BCx-HPßCD and (g) BCx-HPγCD

3.5 Differential Scanning Calorimetry

In order to evaluate the thermal properties of BCx, cyclodextrins (HPαCD, HPßCD and HPγCD) and curcumin-cyclodextrin complexes (HPαCDBCx, HPßCDBCx and HPγCDBCx) DSC was carried out. The complex formation with a guest molecule may result in the complete disappearance of endothermic peak or shifting of peak to the other temperatures indicating changes in crystal lattice, melting, boiling or sublimation points. Apart from thermal properties encapsulation of a drug molecule with a host molecule can also be quantitatively evaluated (Yadav *et al*, 2009). Thermograms of BCx, HPαCD, HPßCD, HPγCD, HPαCD-BCx, HPßCD-BCx and HPγCD-BCx are overlaid and are shown in

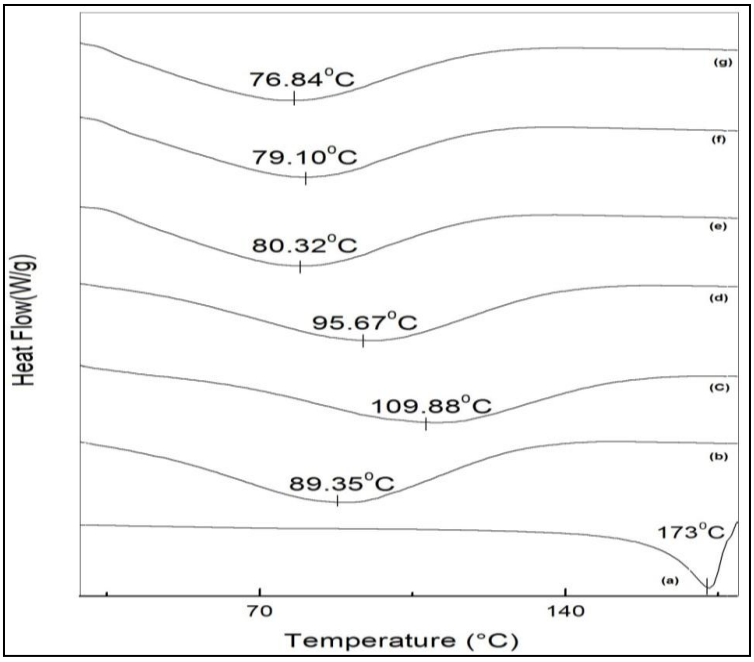

Figure 3.8. DSC analysis of: (a) BCx, (b) HPαCD, (c) HPßCD, (d) HPßCD (e) HPαCD-BCx, (f) HPßCD-BCx and (g) HPγCD-BCx

Figure 3.8. BCx and HPßCD displayed the higher endothermic peaks at 173°C and 109.88°C whereas HPαCD and HPγCD showed lower endothermic peak in between appears at 85°C and 95°C. Due to the complexation of Curcumin with HPαCD (HPαCD-BCx) the occurrence of endothermic peak shifted to a lower value at around 80.32°C and in case of HPßCD-BCx and HPγCD-BCx the

endothermic peak shifted to even lower values of 79.1°C and 76.8°C. While comparing the BCx, HPαCD, HPßCD and HPγCD with that of HPαCD-BCx HPßCD-BCx and HPγCD-BCx it is so evident that the change in endothermic peak in complexes is due to the complex formation (Patro, 2013).

3.6 X-ray diffraction

XRD spectra of BCx, HPαCD, HPßCD, HPγCD, HPαCD-BCx, HPßCD-BCx and HPγCD-BCx are overlaid and are shown in Figure 3.9. Results reveal that the BCx exists in a crystalline form whereas HPαCD, HPßCD and HPγCD are amorphous in nature. In the case complexes, HPαCD-BCx, HPßCD-BCx and HPγCD-BCx, their spectra show that the crystalline nature of BCx appeared lost due to complexation (Dandawate *et al*. 2012).

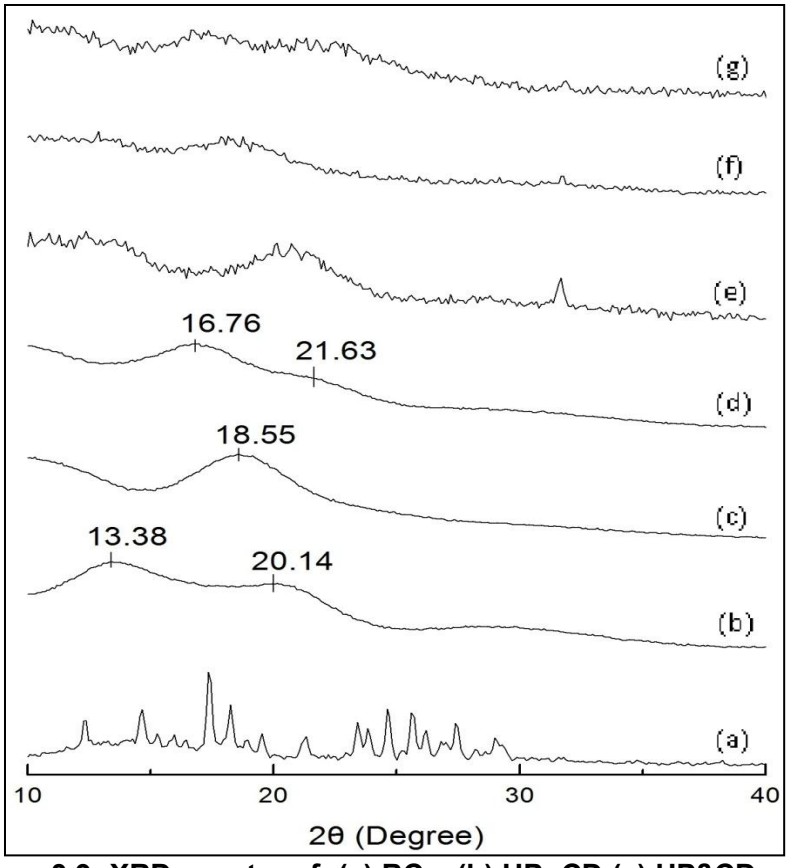

Figure 3.9: XRD spectra of: (a) BCx, (b) HPαCD,(c) HPßCD, (d) HPγCD,(e) HPαCDBCx, (f) HPßCDBCx and (g) HPγCDBCx

3.7 Scanning Electron Microscopy

To check bulk morphology of BCx, HPγCD and HPγCDBCx SEM studies was carried out and morphological features are shown in Figure 3.10. It has been observed that turmeric powder showed crystalline morphology, whereas cyclodextrin exhibits crystalline Golf ball like structure. In Biocurcumax-cyclodextrin complexes it was found that crystalline flakes surrounded distorted spherical-crystals, however uneven aggregations were seen in case of complexes. Due to inclusion of turmeric into cyclodextrin surface morphology of complex has been altered.

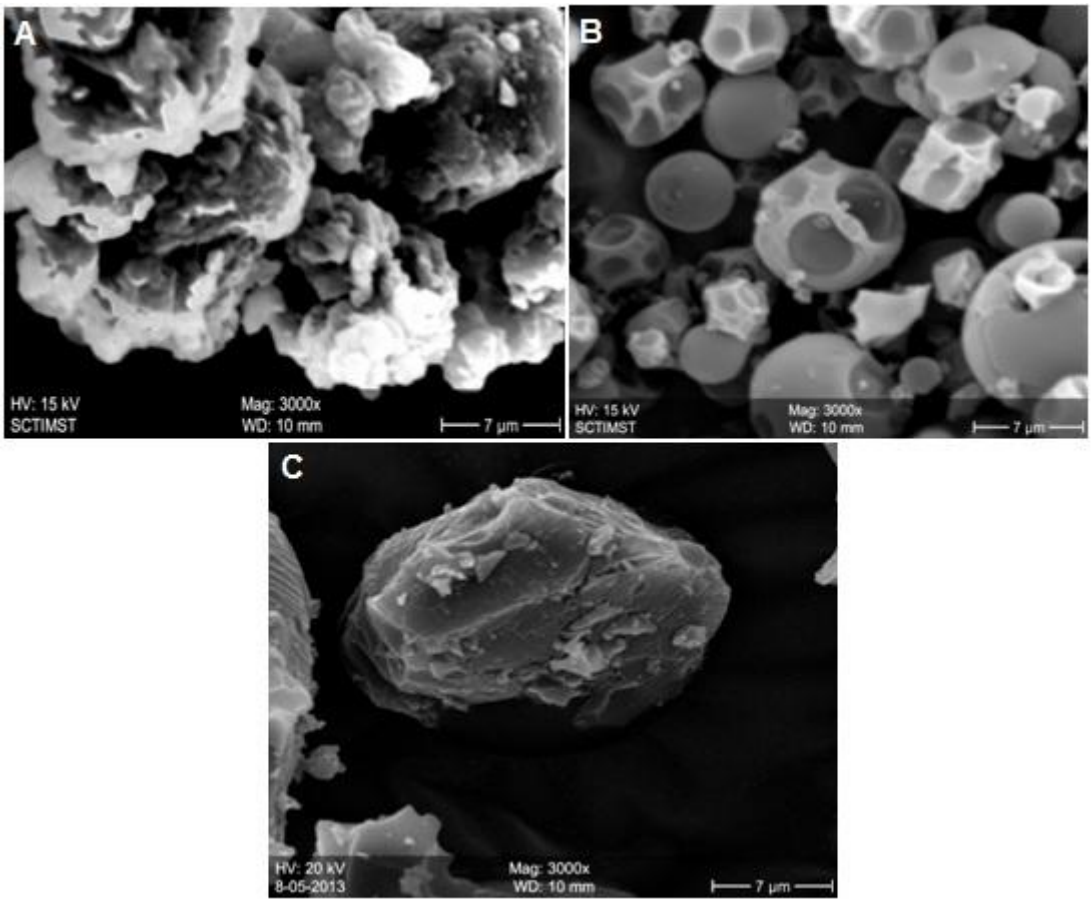

Figure 3.10: SEM Micrograph of (A) BCx, (B) HPγCD and (C) HPγCDBCx (magnification 3000X)

3.8 Haemocompatibility

Hemolytic potential of HPαCD, HPßCD, HPγCD, BCx-HPαCD, BCx-HPßCD and BCx-HPγCD was evaluated and the results are as shown in Figure 3.11. This test was done in order to give an idea of the hemolytic properties of the complex which were intended to be used for intravenous administration.

Acceptable value for hemolysis is less than 1%. All three Complexes recorded hemolysis values below 1% (Table 3.2). Hence, it can be concluded that the complexes are non-hemolytic and can be used for intravenous administration. If we compare the hemolysis of cyclodextrins HPαCD is higher where as HPßCD and HPγCD were less than 0.06. In case of complex it was observed that BCx-HPßCD and BCx-HPγCD has the highest percentage hemolysis whereas in case of BCx-HPαCD, the hemolysis was only 0.04%.

Table3.2: Percentage of Hemolysis of HPαCD, (c) HPßCD, (d) HPγCD, (e) HPαCDBCx, (f) HPßCDBCx and (g) HPγCDBCx

Cyclodextrin	Percentage of hemolysis
HPαCD	0.10
HPßCD	0.03
HPγCD	0.06
HPαCD-BCx	0.04
HPßCD-BCx	0.10
HPγCD-BCx	0.10

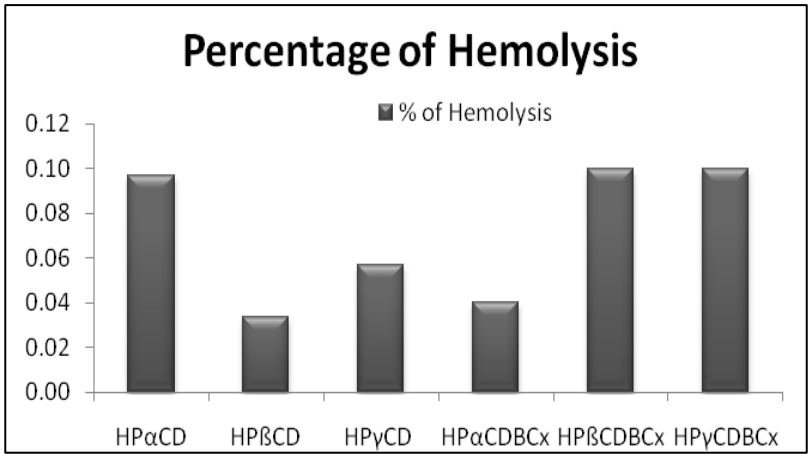

Figure 3.11: %Hemolysis of: HPαCD, (c) HPßCD, (d) HPγCD, (e) HPαCDBCx, (f) HPßCDBCx and (g) HPγCDBCx

3.9 Estimation of curcumin content in the complex using ethanol, PBS and distilled water as solvents

In order to estimate curcumin content in the complex, samples (1mg each) were dissolved ethanol, PBS of pH 7.4 and distilled water of pH 5.5. Standard curves produced for the estimation of curcumin content is shown in figure 3.12. slope value and regression coeficient of the respective are represented in table 3.3 Estimated curcuminoid in the complexes were listed in table 3.4.

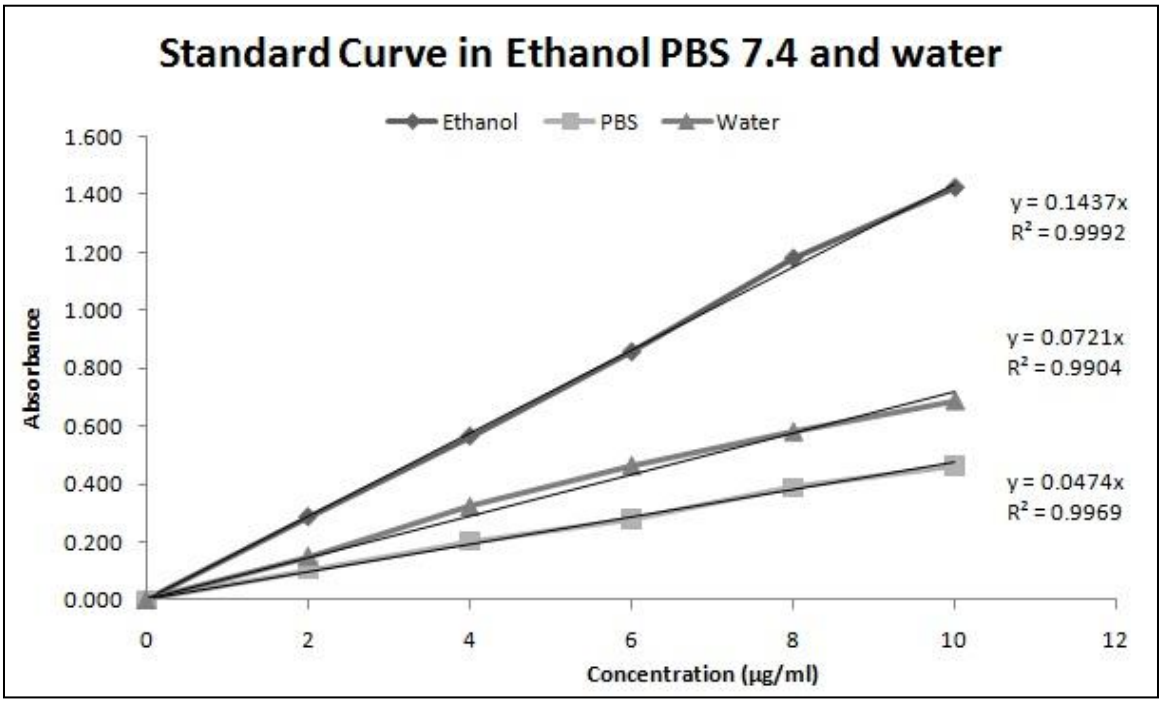

Figure 3.12: Standard Curves of BCx in Ethanol, PBS pH 7.4 and Distilled Water

Table 3.3: Regression equation and standard slope values of Ethanol, PBS pH 7.4 and Distilled Water of pH 5.5

Solvent	Ethanol	PBS pH 7.4	Distilled Water
Slope	0.1437	0.0474	0.0721
Regression Coefficient	0.9992	0.9969	0.9904

Results showed that curcumin absorbs strongly when dissolved in ethanol but moderately absorbed in PBS medium. In the case of complexes, it was found that the amount of curcumin got into host-guest complex is highest in the case of HPγCD and curcumin content in the complexes are in the following order HPγCD-BCx > HPßCD-BCx > HPαCD-BCx.

Table 3.4. Curcuminoid content in the complexes

Sample details	**Curcuminoid content (%) in the complex, estimated using the solvent:**		
	Ethanol	PBS	Distiled water
HPαCD-BCx	2	1.8	1.7
HPßCD-BCx	3	2.8	3.1
HPγCD-BCx	7.5	7.1	7.2

3.10 Solubility in Water

Complex formation substantially enhanced the solubility of the curcumin. UV/Vis spectra of aqueous solutions of BCx and HPγCD-BCx are shown in Figure 3.13. In the case of BCx, its solubility is so poor that no absorption

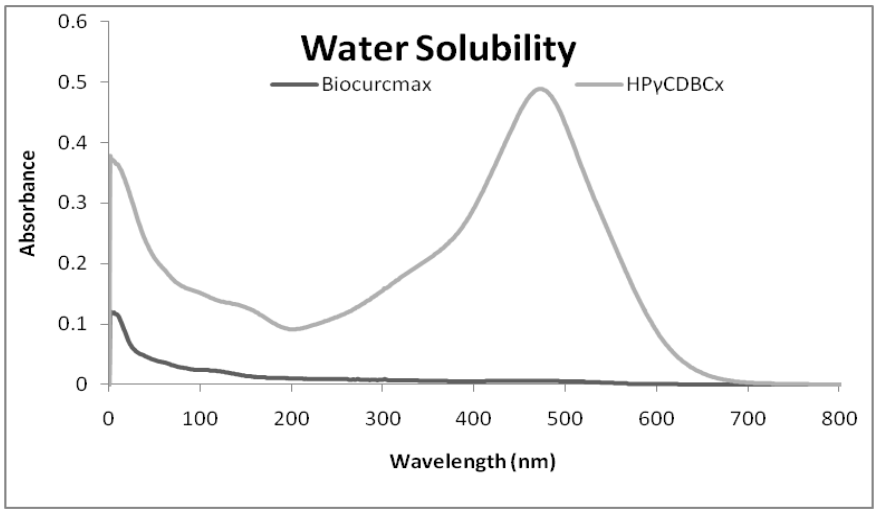

Figure 3.13: UV/Vis absorption Spectra of BCx and HPγCD-BCx

maxima were recorded at 427nm whereas in the case of complex, curcumin strongly absorbs at this wavelength due to its high solubility in water.

3.11 Stability of curcuminoids and complexes in PBS of pH 7.4

3.11.1 Method optimization for Chromatography and Mass spectrometry

Stability studies were assessed by analyzing 24 h samples of pure biocurcumax and biocurcumax-cyclodextrin complexes in pH 7.4 phosphate buffer using LC-MS/MS. The samples were estimated for percentage loss of individual curcuminoids along with possible formation of degradation products. The optimized analyte specific parameters for the Mass spectroscopy are shown in Table 3.5 and the mass spectra for curcuminoids and degradation products are shown in Figure3.15 respectively. The chromatographic trials were taken for curcuminoids and degradation products using different mobile phase compositions in isocratic and gradient elution mode. The degradation products VAN, VAD and FAD are more polar, elutes faster in isocratic conditions and curcuminoids gets retained. To achieve better retention for degradation products and curcuminoids gradient elution was optimized using 0.1% acetic acid in water as component A and 0.1% acetic acid in acetonitrile as component B. The gradient program is presented in Table 3.6. All the samples were analyzed in MRM (Multiple Reaction Monitoring) mode. The retention time for CUR, DMC, BDMC, VAN, VAD and FAD was 7.37, 7.20, 6.99, 1.27, 1.21 and 1.42 respectively. The overlay of chromatograms of curcuminoids and degradation products are given in Figure 3.14.

Table 3.5: Mass transitions and parameters for individual analytes

Analyte	Precursor ion (m/z)	Fragment ion (m/z)	Cone (V)	Collision energy (eV)
CUR	367	217	20	12
DMC	337	216.9	20	12
BDMC	307.2	187	20	10
VAN	150.9	135.8	20	12
VAD	166.9	122.8	20	12
FAD	192.9	133.8	20	15

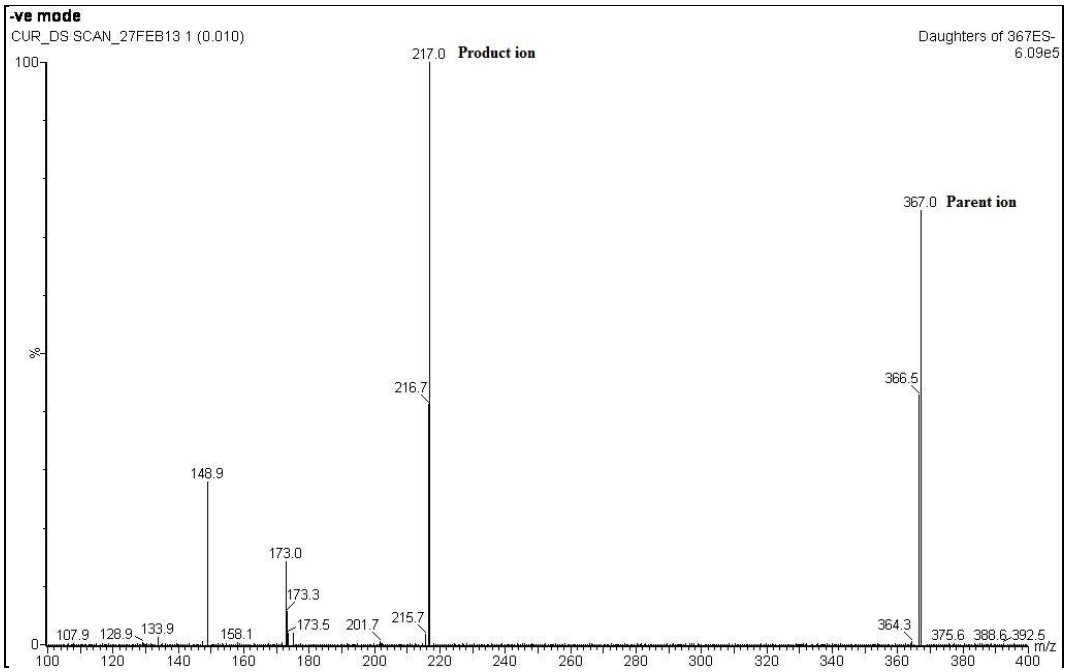

Figure3.14a : Mass spectra of CUR

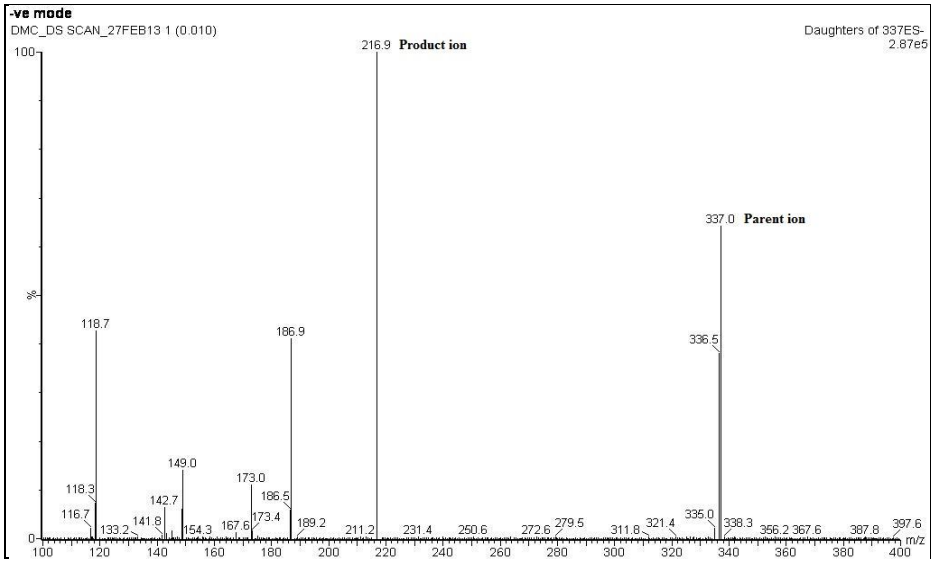

Figure 3.14b: Mass spectra of DMC

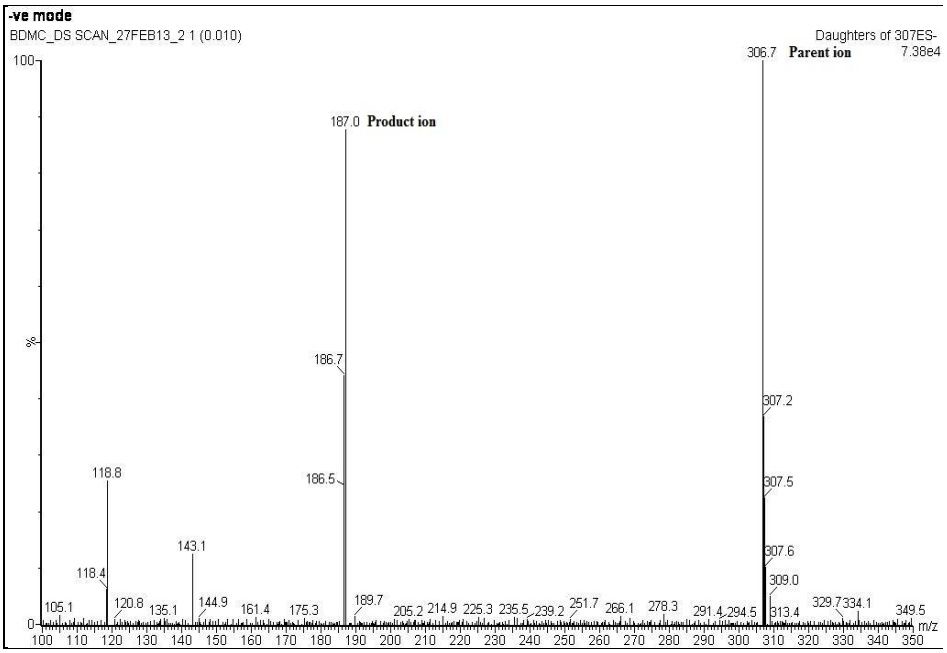

Figure 3.14c : Mass spectra of BDMC

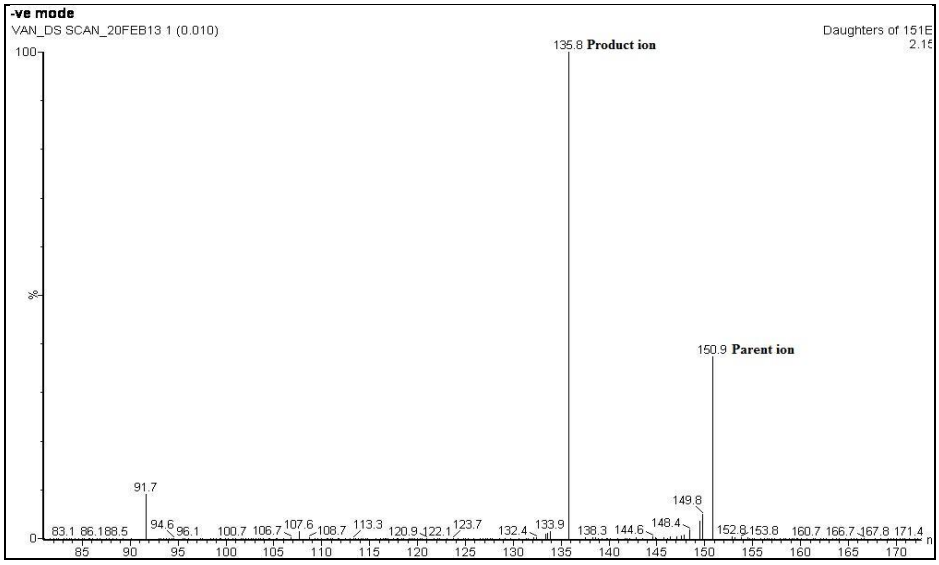

Figure 3.14d : Mass spectra of VAN

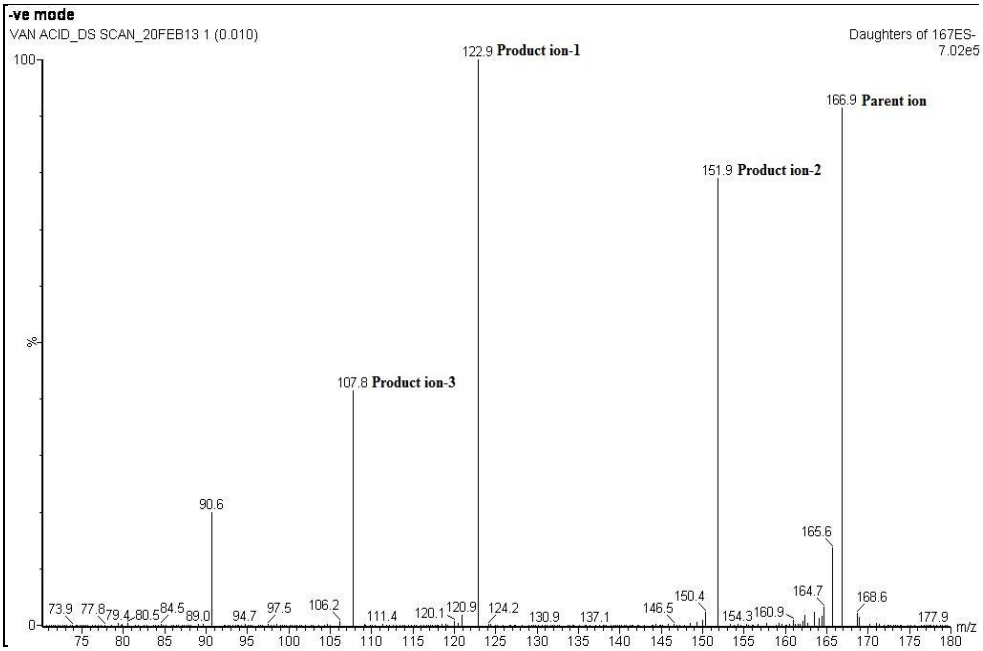

Figure 3.14e : Mass spectra of VAD

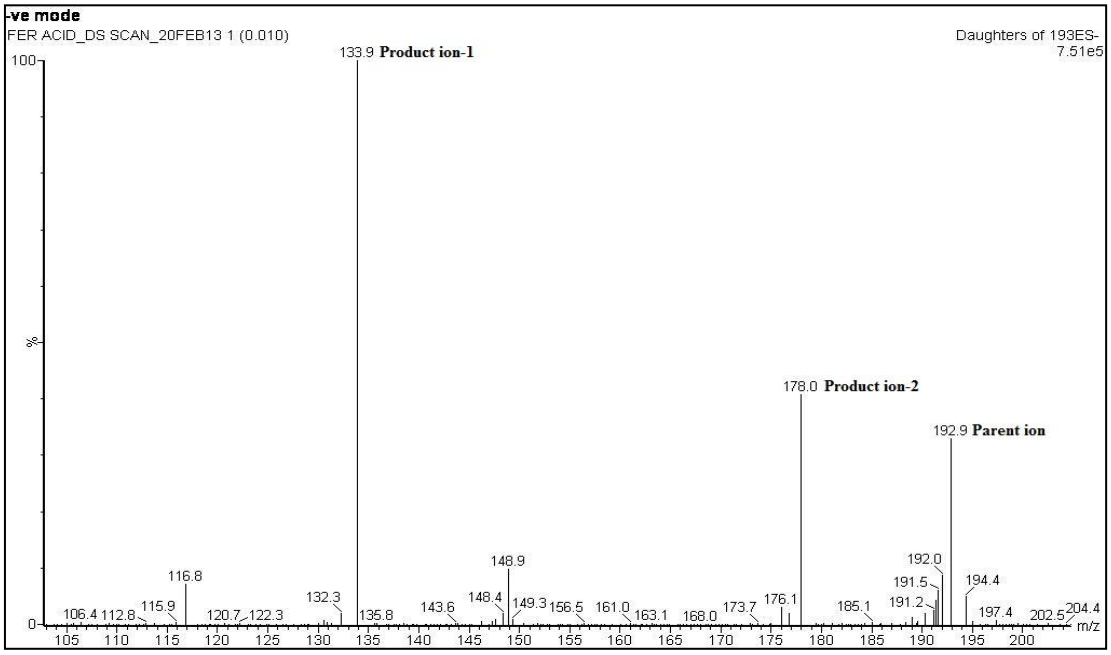

Figure3.14 : Mass spectra of FAD

Table3.6: Gradient program

Time (min)	A (%)	B (%)
0	90	10
2	90	10
3	10	90
6	10	90
9	90	10
10	90	10

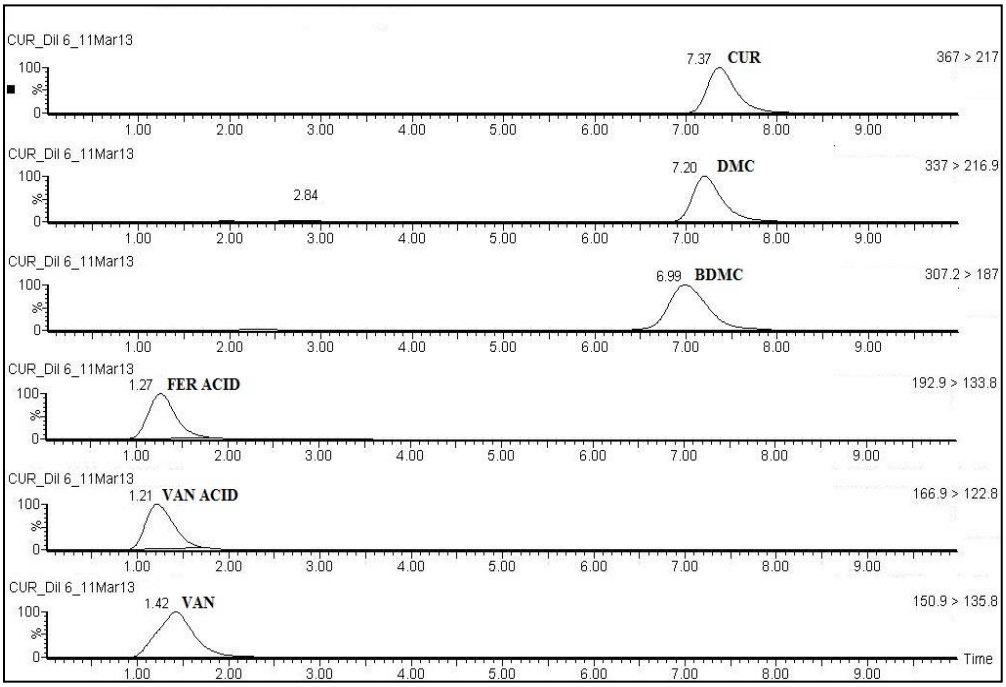

Figure 3.15: Overlay Chromatogram of curcuminoids and degradation products

3.12 Quantification of curcuminoids using LC-MS/MS:

Calibration curve Calibration curve for curcuminoids was achieved for a set of seven non-zero calibration standards. The calibration standards for CUR was 0.12, 0.26, 0.59, 1.18, 2.35, 4.71 and 9.41 µg/mL and the regression equation obtained was y=60029x+24132. The calibration standards for DMC was 0.04, 0.10, 0.21, 0.43, 0.86, 1.72 and 3.43 µg/mL and the regression equation obtained was y=28379x+3334.3. The calibration standards for BDMC was 0.007, 0.015, 0.033, 0.065, 0.130, 0.261 and 0.521 µg/mL and the regression equation obtained was y=7440.4x+318.96. The method was linear in the concentration range used with good r^2 values 0.9932, 0.9982 and 0.9909 for CUR, DMC and BDMC respectively. The calibration graphs are shown in (Figure 3.16)

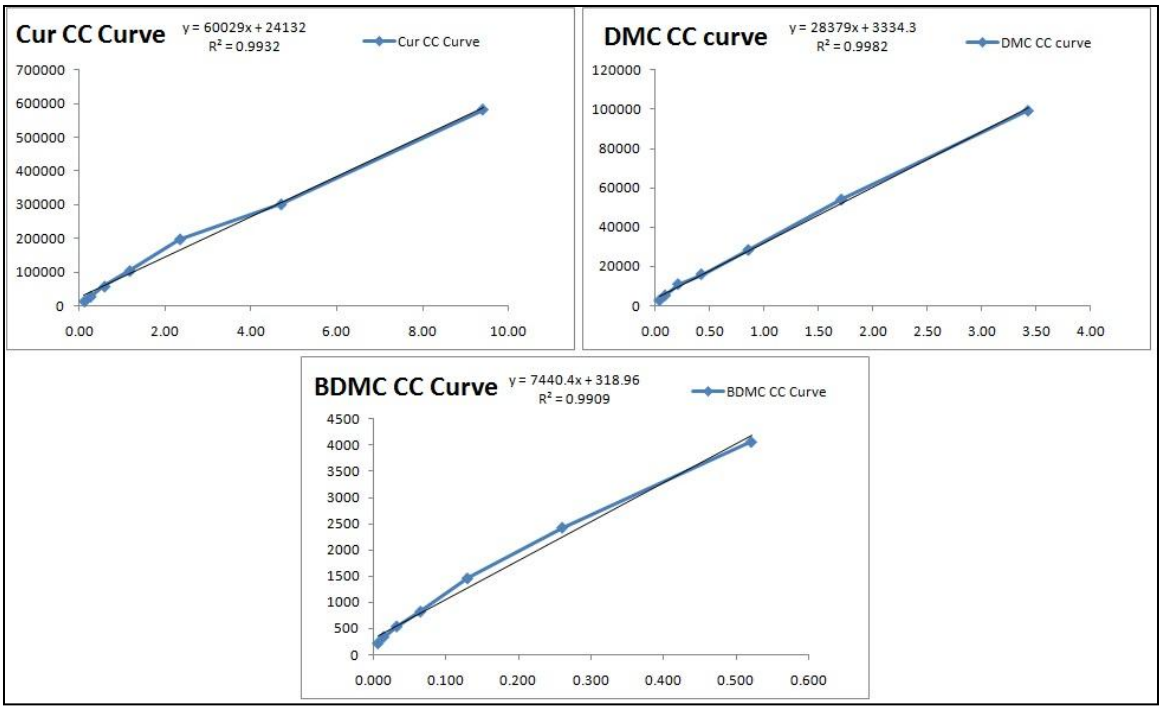

Figure 3.16 LC-MS/MS calibration graphs

3.12.1 Stability profile of individual complexes observed in LC-MS/MS:

The BCx as such in pH 7.4 buffer degrades rapidly and the % content goes down from 0.08 to 0.05% for CUR, 0.06 to 0.03% for DMC and degrades completely from 0.01% for BDMC.

The HPαCDBCx complex in pH 7.4 buffer showed better stability for DMC and BDMC, whereas the content of CUR reduced from 0.07% to 0.02% in 8h and degrades completely at 24h.

In case of HPßCDBCx , BDMC content remains unaffected, whereas the content of CUR and DMC gets reduced to almost half in 2h.

About 80% of the content was found after 24 h in HPγCD-BCx which provided better protective effect for all the curcuminoids. The % content during the time intervals from 0 h to 24 h is shown in Table 3.7 and figure 3.17

Table 3.7: Stability profile of complexes in PBS 7.4 pH observed at various time intervals.

Sample		Curcumin content (µg/mL)			Curcumin content (%)		
	Time (h)	CUR	DMC	BDMC	CUR	DMC	BDMC
BCx	0	0.80	0.63	0.07	0.08	0.06	0.01
	2	0.47	0.28	0.02	0.05	0.03	0.00
	4	0.00	0.00	0.00	0.00	0.00	0.00
	6	0.00	0.00	0.00	0.00	0.00	0.00
	8	0.00	0.00	0.00	0.00	0.00	0.00
	24	0.00	0.00	0.00	0.00	0.00	0.00
HPαCDBCx	0	0.7	0.6	0.1	0.07	0.06	0.01
	2	0.3	0.5	0.3	0.03	0.05	0.03
	4	0.3	0.4	0.1	0.03	0.04	0.01
	6	0.3	0.5	0.2	0.03	0.05	0.02
	8	0.2	0.5	0.3	0.02	0.05	0.03
	24	0.0	0.4	0.4	0.00	0.04	0.04
HPßCDBCx	0	1.0	0.9	0.5	0.10	0.09	0.05
	2	0.5	0.6	0.2	0.05	0.06	0.02
	4	0.4	0.6	0.3	0.04	0.06	0.03
	6	0.4	0.6	0.6	0.04	0.06	0.06
	8	0.3	0.6	0.3	0.03	0.06	0.03
	24	0.1	0.5	0.3	0.01	0.05	0.03
HPγCDBCx	0	4.5	3.9	0.4	0.45	0.39	0.04
	2	5.3	3.9	0.5	0.53	0.39	0.05
	4	4.9	3.7	0.5	0.49	0.37	0.05
	6	4.9	3.8	0.6	0.49	0.38	0.06
	8	4.8	3.8	0.5	0.48	0.38	0.05
	24	4.0	3.1	0.5	0.40	0.31	0.05

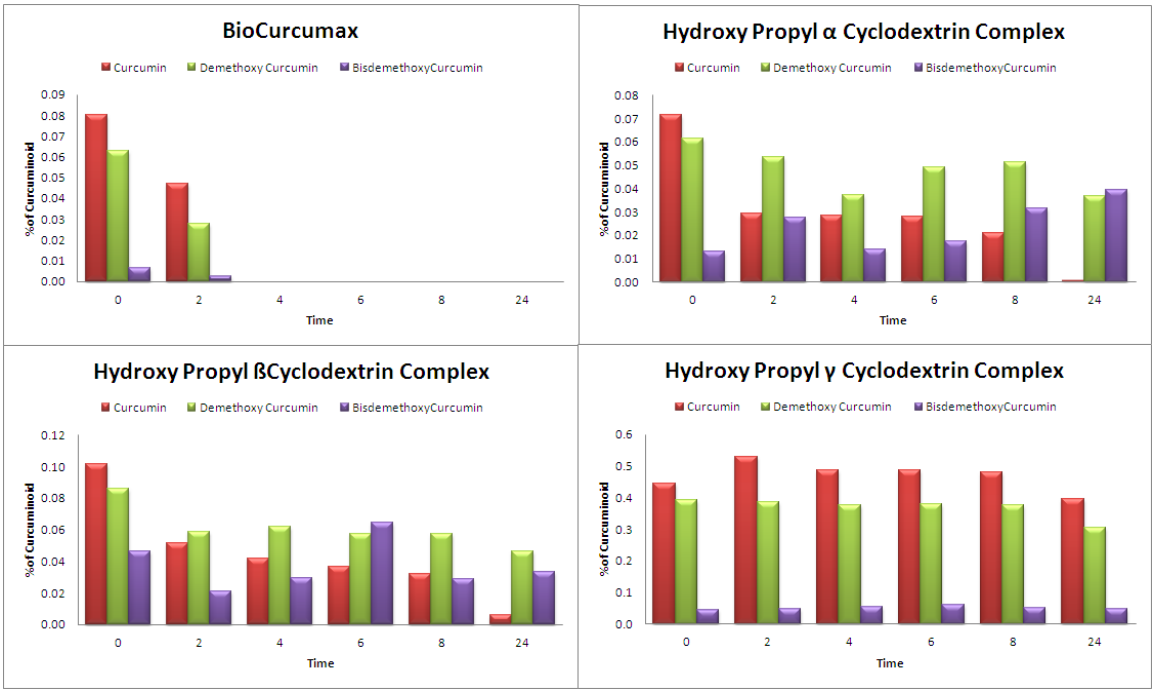

Figure 3.17: Curcuminod content in percentage expressed in bar diagram.

Although the percentage loss in the curcuminoids was observed, the formation of the expected degradation products vanillin, vanillic acid and ferulic acid was not seen in the chromatography. The results were similar to the observations by Odaine N. Gordon and Claus Schneider (Gordon ON and Schneider C, 2012), where the major degradation product observed was a bicyclopentadione derivative and the formation of VAN, VAD and FAD was negligible.

Percentage of individual curcuminoids after 24 h was calculated and tabulated (table 3.8) and represented HPγCDBCx in the form of chart. It was observed that the percentage of CUR is higher in HPγCDBCx in HPßCDBCx, and HPαCDBCx 6%, 1% respectively HPαCDBCx, HPßCDBCx and HPγCDBCx conserves DMC more than 50% even after 24 hrs but in case of BCx DMC is 0%. Percentage of BDMC after 24 h in all three complexes were more than 70% but in BCx only zero percent of BDMC was observed.

Table 3.8: Curcuminoids remained in PBS buffer after 24h

Compound	CUR remained after 24h /%	DMC remained after 24 h /%	BDMC remained after 24 h /%
BCx	0	0	0
HPαCD-BCx	1	59	100
HPßCD-BCx	6	53	72
HPγCD-BCx	89	78	100

To monitor pattern changes that occur at pH 7.4 due to degradation, mass spectra of samples were recorded. Samples of BCx and complexes dissolved in PBS for 8 h were injected directly into mass spectrometer. The spectral pattern shows the presence of peaks of curcuminoids with increased intensity in biocurcumax gamma cyclodexrtin complex in comparison to other complexes and pure biocurcumax. Among degradation products, only vanillin was observed to certain extent. The peak at m/z 150.6 corresponding to vanillin was found with high intensities in alpha and beta complexes in comparison to gamma indicating the protective effect of gamma complex (Figure 3.18). In case of pure biocurcumax, the peaks curcuminoids were negligible and none of the peaks corresponding to degradation products were found. The observation indicates that the degradation products formed may not be stable and undergo further degradation and this may be the reason for absence of degradation peaks in chromatography.

While comparing the different cyclodextrin complexes it was observed that Hydroxypropyl gamma cyclodextrin has an ability to conserve the drug within its cavity such that it is able to maintain the curcuminoids until 24 h but rest of the cyclodextrins i.e hydroxypropyl alpha was able to conserve bis demethoxy and beta cyclodextrin was not able to conserve. As per the complexation scheme described by Mohan *et al.,* and Tobara *et al.,* (Mohan *et al.,* 2012 and Tobar *et*

al., 2012) the reason of this distinctive stability is possibly due to the variation in the inner diameter of the cyclodextrins ring.

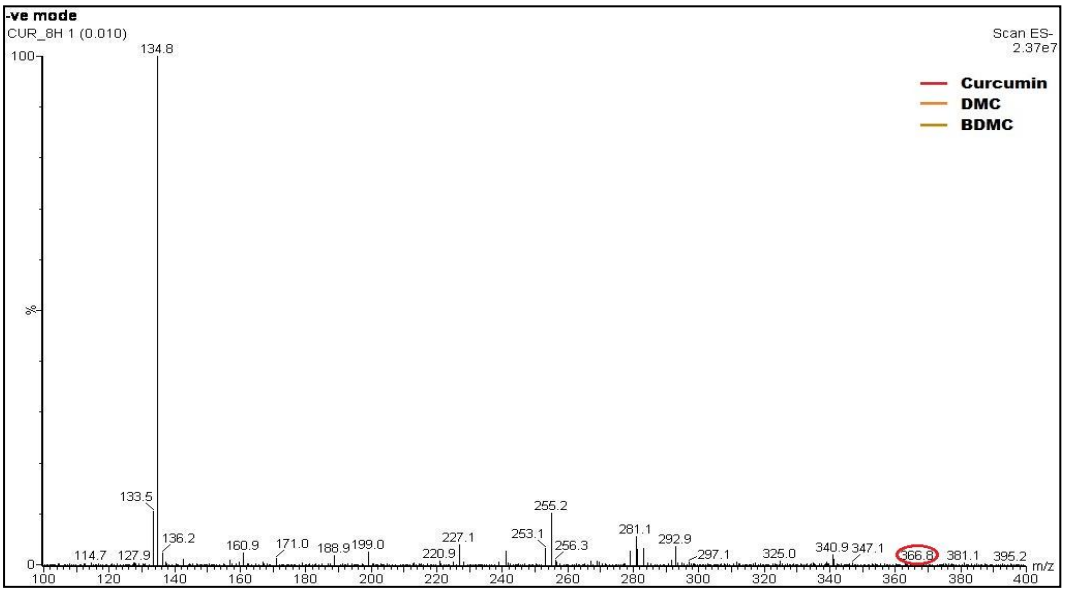

Figure 3.18. a.of HPαCDBCx 8Hrs in PBS pH 7.4

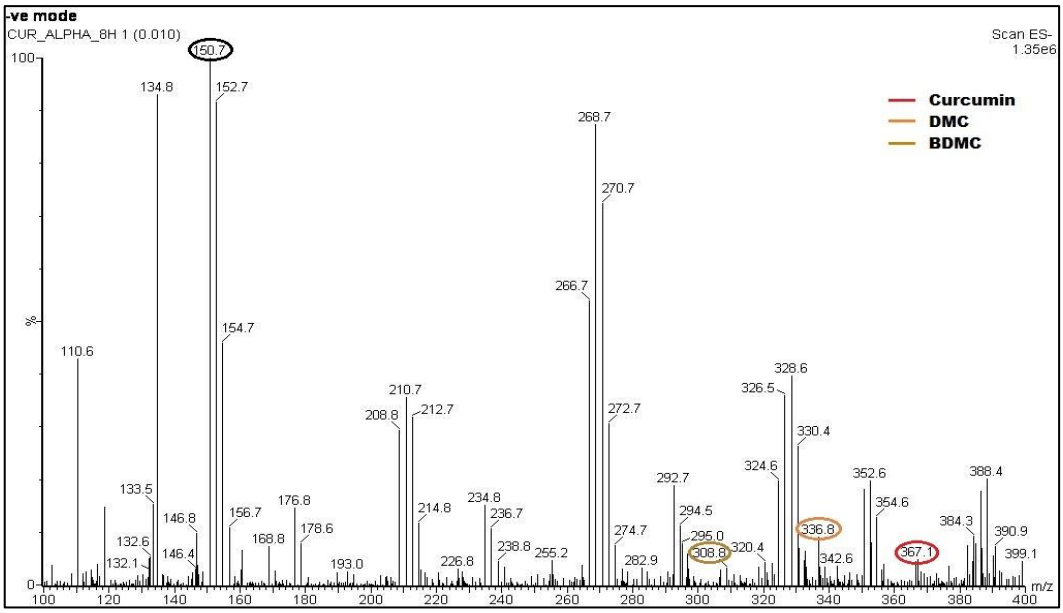

Figure 3.18. b. Mass spectra of BCx 8Hrs in PBS pH 7.4

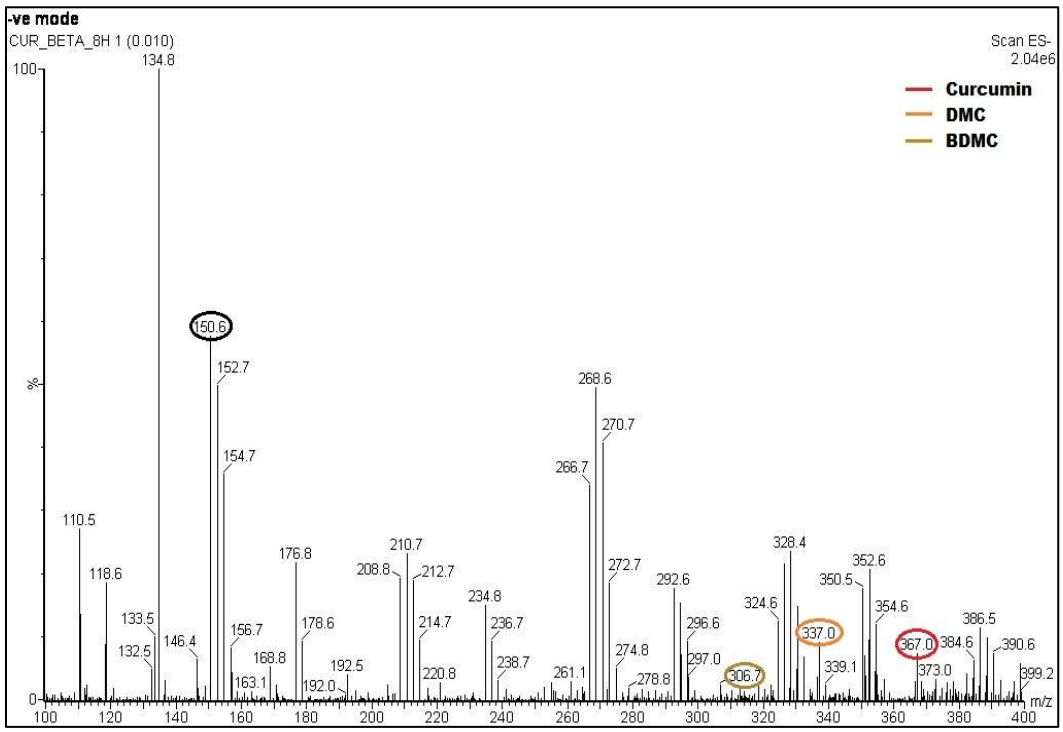

Figure 3.18. c. Mass spectra of HPßCDBCx 8Hrs in PBS pH 7.4

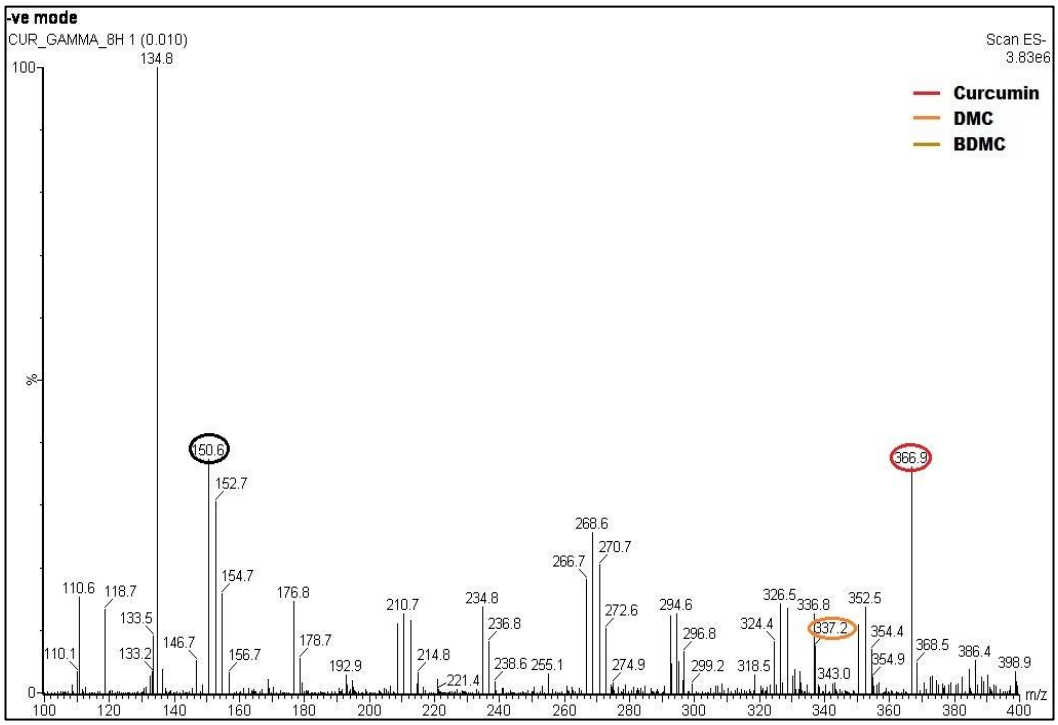

Figure 3.18.d. Mass spectra of HPγCDBCx 8Hrs in PBS pH 7.4

4. Summary and Conclusion

4.1 Conclusion:

Cyclodextrin inclusion complexes are used widely in pharmaceutical application due to good bio activity and cytocompatibility. In the present study curcuminoid-cyclodextrin inclusion complexes were developed using pH shift method and certain modifications and optimizations have been carried out with the future prespective of being used in *in vitro* and *in vivo* models.

Different formulations were prepared in combination with various Hydroxypropyl derivatives of cyclodextrins (2-Hydroxypropyl-α-cyclodextrin, 2-Hydroxypropyl-β-cyclodextrin and 2-Hydroxypropyl-γ-cyclodextrin). The inclusion complexes were analyzed for its solubility in water and the results confirmed that the complexes showed better solubility than the uncomplexed Bio Curcumax. Complexation efficiency and curcuminoids content in the samples were in the order of 2-Hydroxypropyl-γ-cyclodextrin>2-Hydroxypropyl-β-cyclodextrin>2-Hydroxypropyl-α-cyclodextrin. Using LC-MS/MS studies individual curcuminods were analyzed in case of all complexes, which showed stability up to 24h. On comparison of stability of all 3 complexes obtained 2-Hydroxypropyl-γ-cyclodextrin was found to be more promising candidate for *in vivo* and *in vitro* applications. Complex was found to be the most efficient one in stabilizing all three curcuminoids effectively.

The 2D surface morphology evaluation of the freeze dried complex using SEM showed changes in the amorphous nature of the Bio Curcumax Biological Characterization of the samples was carried out by haemocompatibility studies according to ASTM standards and the results displayed mild haemolysis within the accepted range.

From the results obtained it can be concluded that inclusion complex posses good solubility and stability leading to its increased bioavailability in the biological system.

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