# PREPARATION AND CHARACTERIZATION OF SILK FIBROIN BASED MATERIALS LOADED WITH NATURAL COMPOUNDS

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**by**
**Merve ŞAMLI**

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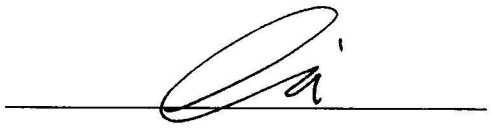
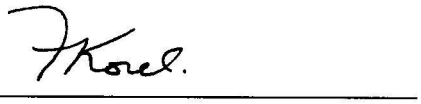
We approve the thesis of **Merve ŞAMLI**



**Assoc. Prof. Oğuz BAYRAKTAR**
Supervisor

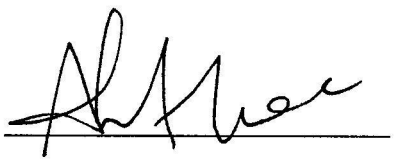


**Assoc. Prof. Figen KOREL**
Co-supervisor

**Assist. Prof. Ayşegül BATIGÜN**
Committee Member

**Assist. Prof. Çağatay CEYLAN**
Committee Member

**12 July 2010**



**Assoc. Prof. Ahmet KOÇ**
Head of the Department of
Biotechnology and Bioengineering

**Assoc. Prof. Talat YALÇIN**
Dean of the Graduate School
of Engineering and Sciences

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# ABSTRACT


## PREPARATION AND CHARACTERIZATION OF SILK FIBROIN BASED MATERIALS LOADED WITH NATURAL COMPOUNDS

In this study, it was aimed to increase solubility of flavonoids via inclusion complexation. Silk was also selected as carrier material. Firstly we have prepared cyclodextrin inclusion complexes with rutin via co-precipitation method. Stability constant of Beta-cyclodextrin complex was calculated as 262 $M^{-1}$. Aqueous solubility of rutin was increased with inclusion complex of Beta-Cyclodextrin. Effect of temperature on aqueous solubility of free rutin, and its complex were studied. Also solubility energies are calculated. Characterization of cyclodextrin complexes were conducted with the help of UV-visible spectroscopy, X-Ray Diffractometry, Differential Scanning Calorimetry, Thermal Gravimetric Analysis, Fourier Transform Infrared spectroscopy and Scanning Electron Microscobe techniques. Characterization results supported formation of inclusion complexes which were compatible with the previous literature studies. Before release tests, dissolution profile of rutin, physical mixture and inclusion complex was observed at 37°C and in PBS; results show that addition of cyclodextrin has an increasing effect on solubility rate and amount. Then silk fibroin based films were prepared and used as carriers for natural compounds. After loading flavonoids and complexes into silk fibroin based films, release tests were done at 37 °C in neutral pH conditions for 24 hours. Most of the Rutin – *independent from the form, whether free or complexed*- released from Silk Fibroin films within the first 5 hours (burst release occurs) and the rest of it released slowly within 24 hours. Electron microscobe analyses showed that films have a homogenous and dense morphology. Consequently, silk fibroin is useful to load natural compounds into silk fibroin films in order to modify their release period within physiological conditions.

# ÖZET

## DOĞAL BİLEŞİKLERLE YÜKLÜ İPEK FİBROİN ESASLI MALZEMELERİN HAZIRLANMASI VE KARAKTERİZASYONU

Bu çalışmada, inklüzyon kompleksleşmesi yoluyla rutinin çözünürlüğünün artırılması hedeflenmiştir. İpek fibroin lifi taşıyıcı malzeme olarak seçilmiştir. İlk olarak rutin-siklodekstrin inklüzyon kompleksleri birlikte çöktürme yöntemi ile hazırlanmıştır. Elde edilen kompleks bileşiğin kararlılık sabiti 262 $M^{-1}$ olarak hesaplanmıştır. Beta siklodekstrin ile kompleksleşmesi yolu ile rutinin sudaki çözünürlüğü artırılmıştır.Ayrıca sıcaklığın rutinin (serbest ya da kompleksleşmiş formunun) çözünürlüğü üzerindeki etkisi incelenmiş ve bileşiklerin çözünürlük enerjileri hesaplanmıştır. Siklodekstrin komplekslerinin karakterizasyonu, UV-görünür spektroskopisi, X-Ray kristallografisi, diferansiyel taramalı kalorimetri, fourier transform infrared spektroskopisi, taramalı elektron mikroskobu yardımıyla gerçekleştirilmiştir. Karakterizasyon deneylerinin sonuçları,*önceki literatür çalışmaları ile uyumlu bir biçimde*, kompleks oluşumunu doğrular niteliktedir. Salım deneyleri öncesinde serbest rutin,fiziksel karışımı ve inklüzyon kompleksinin fizyolojik koşullarda çözünme profili incelenmiştir, sonuçlar siklodekstrinin çözünürlük hızı ve miktarını artırdığını göstermektedir. İpek bazlı filmler hazırlanarak doğal bileşiklerin taşınımı sağlanmıştır. Flavonoidlerin ve komplekslerinin ipek filmlerine yüklenmesi sonrasında, 37 °C'de nötral pH koşullarında 24 saat boyunca salımı gözlenmiştir. Rutinin – *bulunduğu formdan bağımsız olarak, serbest ya da kompleksleşmiş* – ipek filmlerinin içinden ilk beş saat içinde büyük bir kısmının salındığı (ani salım) ve geri kalanın 24 saat içinde yavaşça salındığı gözlemlenmiştir. Elektron mikroskobu analizleri filmlerin yoğun ve homojen bir morfolojide olduğunu göstermiştir. Sonuç olarak, ipek fibroin filmleri içine doğal bileşikleri yüklemek ve sonrasında fizyolojik koşullarda salımını modifiye edebilmek için uygun bir malzemedir.

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# CHAPTER 1

# INTRODUCTION

Flavonoids are one of the most important naturally occurring antioxidant sources. Beside the beneficial effects of flavonoids on cardiovascular health and cancer preventing ability or suppressing cancer cell growth, their low aqueous solubility is a challange to make these compounds bioavailable. Their low aqueous solubility make (or cause) these substances to have lower *in vivo* antioxidant and/or anticarcinogenic activities compared with their *in-vitro* activities. Also, these beneficial compounds are very sensitive to temperature, light, oxygen; they can easily degrade via these factors.

At this point utilization of cyclodextrins (CDs) would provide a practical solution to these problems.Cyclodextrin (CD) is a torus shaped molecule having hydrophobic cavity. This structure provides CDs to form complex compounds with the molecules having ability to locate the cavity. Cyclodextrin complexes also known as inclusion complexes (IC) have found many applications for many years. Inclusion complexes have been used in order to control the activity and / or dose of flavonoids in the body.

Silk fibroin was also selected as carrier material since it is biologically active and biocompatible. Usage of silk fibroin as fibroin based carrier material helps to modify the release behaviour of the active natural compound. In this study, Inclusion complexes between CD and Rutin were prepared to increase its aqueous solubility. Characterization of the resulting complex compounds, were conducted with the help of UV-vis spectroscopy, X-ray crystallography, differential scanning calorimetry, thermal gravimetric analysis and Fourier transform infrared spectroscopy. The change of solubility energies and aqueous solubilities with respect to temperature were determined for both free Rutin and inclusion complex of rutin and form of the drug (free or complex) are calculated. Before release tests, dissolution profile of rutin (Rt), physical mixture (PM) and inclusion complex (IC) were also evaluated. Then silk fibroin based films were prepared and used as water insoluble carrier material for IC and Rt. After loading rutin and inclusion complexes into silk fibroin based films, release tests were performed under acidic and neutral pH conditions.

# CHAPTER 2

# THEORY

## 2.1. What is Inclusion Complexation

A *complex* in which one component (the *host*) forms a cavity or, in the case of a crystal, a crystal lattice containing spaces in the shape of long tunnels or channels in which molecular entities of a second chemical species (the *guest*) are located. There is no covalent bonding between guest and host, the attraction being generally due to *van der Waals forces*. If the spaces in the host lattice are enclosed on all sides so that the guest species is 'trapped' as in a cage, such compounds are known as *clathrates* or 'cage' compounds (IUPAC 2006). Inclusion complexes are entities comprising two or more molecules. Complex formation is a dimensional fit between host cavity and guest molecule. One of the molecule, the ''host'', includes, totally or partly, the ''guest'' molecules by physical forces. Cyclodextrins are considered typical host molecules. Because of their ability to form solid inclusion complexes (host–guest complexes) with a very wide range of solid, liquid and gaseous compounds by a phenomenon of molecular complexation. The lipophilic cavity of cyclodextrin molecules provides a micro environment into which an appropriately sized non-polar moiety can enter to form inclusion complex (Loftsson and Brewster 1996). No covalent bonds are broken or formed during formation of the inclusion complex (Schneiderman and Stalcup 2000). The main driving force of complex formation is the release of enthalpy-rich water molecules from the cavity. Water molecules are displaced by more hydrophobic guest molecules present in the solution to attain an apolar–apolar association and decrease of cyclodextrin ring strain resulting in a more stable lower energy state (Szetjli 1998). The binding of guest molecules within the host cyclodextrin is not fixed or permanent but rather is a dynamic equilibrium. Binding strength depends on how well the 'host–guest' complex fits together and on specific local interactions between surface atoms. Complexes can be formed either in solution or in the crystalline state and water is typically the solvent of choice. Inclusion complexation can be accomplished in co-solvent system, also in the presence of any nonaqueous solvent. Cyclodextrin

architecture confers upon these molecules a wide range of chemical properties markedly different from those exhibited by non-cyclic carbohydrates in the same molecular weight range (Singh, et al. 2002).

Complex formation in solution is a dynamic equilibrium process which can be illustrated by the equation (2.1.) where CD is the cyclodextrin, G is the guest molecule, and CD-G is the inclusion complex (Astray, et al. 2009). The stability of the inclusion complex can be described in terms of recombination constant ($k_R$) or a dissociation constant ($k_D$) in equation 2.1. :

$$CD + G \underset{k_D}{\overset{k_R}{\rightleftharpoons}} CD\text{-}G \quad K = k_R / k_D \qquad (2.1)$$

Ionization decreases the rate of complex formation and decomposition. This recombination-dissociation equilibrium is one of the most important characteristics of this association (Astray, et al. 2009).

*Energy and Inclusion Mechanisms*

The inclusion of a guest in a CD cavity consists basically of a substitution of the included water molecules by the less polar guest. The process is energetically favoured by the interactions of the guest molecule with the solvated hydrophobic cavity of the host. In this process entropy and enthalpy changes have an important role (Astray, et al. 2009). In spite of the fact that the ‘‘driving force’’ of complexation is not yet completely understood, it seems that it is the result of various effects:

a. Substitution of the energetically unfavoured polar–apolar interactions (between the included water and the CD cavity on the one hand, and between water and the guest on the other) by the more favoured apolar–apolar interaction (between the guest and the cavity), and the polar–polar interaction (between bulk water and the released cavity-water molecules).

b. CD-ring strain release on complexation.

c. van der Waals interactions and hydrogen bonds between host and guest.

CDs are hydrophobic molecules, since their solubility improves when a small amount of ethanol is added to water. Water molecules in the CD cavity cannot satiate their hydrogen bonding capacity as occurs with those in the bulk of the solvent. These water molecules have enhanced energy or enthalpy. The decrease in the energy of the system is caused by the reduction of the solvent-guest molecule and solvent-cavity interactions (Astray, et al. 2009). The energy of covalent chemical bonding is 400 kJ/mol. The energy of hydrogen bond is about 40 kJ/mol, and the Van der Waals forces represent only about 4 kJ/mol of bond energy. In the case of inclusion complexes, the species may achieve a stability which is proportional to covalent bonding due to the spatial arrangement produced. Van der Waals forces, hydrophobic interactions and hydrogen bonds hold the CD and its guest together (Astray, et al. 2009). The energy of Van der Waals forces is proportional to molecular polarizability and molecular refraction. For structurally analogous compounds, a linear correlation exists between the molecular refractions (refraction index nD) and the dissociation constants of their CD complexes (Gelb, et al. 1981). The other dominating stabilizing force is the hydrophobic or solvophobic. Hydration of the CD complex is energetically favoured when compared with the separate hydration of the components. The role of the hydrogen bonding is not universal because stable compounds are formed for example with benzene which cannot form hydrogen bonds.

The stability of the complex grows with the increase in the electron-donor character of the substituent of the included molecule. The nature of the guest molecule must be taken into account, (Takagi and Maeda 1984), although the thermodynamic parameters do not show variations (van Etten, et al. 1967). Inclusion hinders moderately the free rotation of the included molecule around its symmetry axis. This fact is the origin of the unusual entropy associated with the CD-guest interaction (Cooper and MacNicol 1978). The thermodynamic parameters, enthalpy ($\Delta H$) and entropy ($\Delta S$) can be obtained from the temperature dependence of the dissociation constant (Cramer, et al. 1967). The $\Delta H$ and $\Delta S$ values can be calculated from spectrophotometric data (Dodziuk 2006), but the most reliable data are obtained by calorimetric determinations (Barone, et al. 1985).The thermodynamic data from the literature suggest that CDs are not very discriminating host molecules.Effect of cyclodextrin structure on the inclusion looking for the most appropriate host, CDs with various cavity diameters and chemically modified CDs must be discriminated. The $\alpha$–CD forms insoluble complexes

with fatty acids and they have been used successfully in clinical diagnostic (Szejtli 1988). The unsaturated fatty acids form more stable β-CD complexes; however, this can cause an imbalance of fatty acids in the brain (verified experimentally in rats) (Sun and Gilboe 1994). Fortunately, the orally and intravenously administered CDs are unable to pass the blood-brain barrier. Using the higher soluble CDs derivatives, high solubilising effects can be attained. In this way, the substitution of hydroxyl groups of the CD structure by other groups can be carried out. Thus, their hydrophilicity/hydrophobicity can be modified, or the axial length of the cavity can be increased. Consequently, a CD derivative, which is highly soluble, can form a stable complex with the given guest (e.g. drug molecule), it is stable during storage, but will be rapidly decomposed in the biological media (Szejtli 1995).

*Effect of Guest Properties*

The geometric compatibility between host cavity and guest, the structure, charge and polarity of the guest, the effect of the reaction medium (solvent) and temperature are important for inclusion complex formation. It is important that the geometrical dimensions of the guest molecules are rather close to those of substituted benzene ring or its condensed homologues. The host molecule β-CD provides the most versatile cavity for molecular entrapment among the available parent CDs. For instance, β-CD forms stable inclusion complexes with mono- and sesquiterpenes (Szente and Szejtli, 1988). The charge and polarity of the guest play also an important role in the CD-substrate host–guest interaction; however, it is less decisive than that of the geometric fitting. Molecules can be complexed by CDs when they are less hydrophilic or less polar than water, and there is a positive correlation between stability of CD complexes and the hydrophilic character of molecules or certain parts of the guest molecules. In the case of the charge, the complexation of neutral molecules is easier than the ionized counterpart (Bergeron, et al. 1977; Bergeron, et al. 1978; Matsui, et al. 1985). The role of the medium in which complex formation takes place plays an important role, since its presence influences the inclusion equilibrium process in both directions. The CD complex formation does not require any extra solvents, but the presence of at least a minimum amount of water is necessary for the inclusion processes (Kamihira, et al. 1990). Some of the applied co-solvents in ‘‘wet’’ complexation technologies are known to form ternary complexes with the guest and CD (Redenti, et al. 1993). Ethanol is a

rather frequently applied co-solvent in the preparation of CD-drug and CD-flavour complexes. The ethanol content is around 0.01–0.5%. The stability and aqueous solubility of the complexes formed can be modified by applying inorganic salts (Buva´ ri and Barcza 1979). CD can also modify the stability of colloidal aggregates, showing inclusion phenomena with surfactants and, hence, inducing changes in the aggregation processes (Cabaleiro-Lago, et al. 2006b).

*Properties of Cyclodextrin-Included Guests in Solution*

When a more or less non-polar poorly water-soluble substance reacts with an aqueous CD solution, the following consequences can occur (Liu, et al. 1999):

1. The concentration of the guest in the dissolved phase significantly increases, and when solid complexes are simultaneously formed, the concentration of dissolved CD decreases (except with ionized guests, or hydrogen bond establishing).
2. The spectral properties of the guest are modified.
3. The reactivity of the included molecule is modified. In most cases their reactivity decreases.
4. The formerly hydrophobic guest in complexed form becomes hydrophilic.

## 2.1.1. Forces Contributing to Inclusion Mechanism

The driving forces leading to the inclusion complexation of cyclodextrins should include the electrostatic interaction, van der Waals interaction, hydrophobic interaction, hydrogen bonding, and charge–transfer interaction. However, due to enthalpy-entropy compensation, release of conformational strain and exclusion of cavity-bound high-energy water are not energetically contributive to the complex formation, and the enthalpy and entropy changes of the complexation are not good criteria to be used in judging whether a particular driving force is present or important. Nevertheless, the multivariate quantitative structure-activity relationship analyses not only are useful in predicting the binding constants of the inclusion complexation, but also can illustrate which driving forces are important in particular complexation systems. Usually, it is found that van der Waals interaction and hydrophobic interaction constitute the major driving forces for cyclodextrin complexation, whereas electrostatic interaction and

hydrogen bonding can significantly affect the conformation of a particular inclusion complex (Liu and Guo 2002).

### 2.1.1.1. Electrostatic Interaction

The electrostatic interaction energy is the energy of interaction between the undistorted charge distributions of the two molecules interacting with each other. It includes all electrostatic forces between permanent charges, dipoles and higher multipoles present in the system. Usually, three types of electrostatic interactions are the most important, i.e., ion–ion interaction, ion–dipole interaction, and dipole–dipole interaction. Apparently, as CDs are neutral molecules, the ion–ion interaction does not occur in CD complexation, unless the CD is appropriately substituted. On the other hand, the ion–dipole interaction is expected to take place in CD complexation for the apparent reason that CDs are polar molecules (Matsui and Okimoto 1978). Unfortunately, the occurrence of this interaction is difficult to show (Suzuki, et al. 1993). In fact, any strong ion-dipole interaction is not necessarily favorable for the CD complexation in aqueous solution because under this condition the interaction between the substrate and water will also be strong (Bernad, et al. 1999). Thus, the dipole–dipole interaction was concluded to be important in CD complexation. The importance of the dipole–dipole interaction in CD complexation can also be shown with the free energy relationship analyses.

### 2.1.1.2. Van der Waals Interaction

Many authors have claimed the involvement of van der Waals interaction in CD complexation, but the arguments of some of them are in fact weak. For example, as it is generally believed that the hydrophobic interaction between two nonpolar molecules is with a positive enthalpy, the observation of a negative enthalpy change in CD complexation is often considered to indicate the dominance of van der Waals interaction instead of the hydrophobic interaction (Barone, et al. 1986). The involvement of van der Waals interaction in CD complexation can also be shown by the structures of the complexes. In fact, numerous studies have revealed that bulky guest molecules are in

close van der Waals contact with the CD cavities (McMullan 1973). Interestingly, sometimes van der Waals interaction might be so strong that the hydrophobic but bulky side of the guest molecule can enter the CD cavity (Suzuki, et al. 1992). Another method to show the involvement of van der Waals interaction in CD complexation is molecular modeling (Schneider 1993), which is usually performed with molecular mechanic and molecular dynamic calculations.

### 2.1.1.3. Hydrophobic Interaction

Traditionally, hydrophobicity was considered to be the result of the enhanced structure of the water molecules in the near vicinity of the non-polar solute, which would bring about a usually large entropy loss during the hydration (Frank and Evans 1945). Sometimes, this explanation was even overemphasized, resulting in the postulation of the iceberg- or clathrate-like structures of the hydrophobic hydration shell (Glew 1962). According to the model, the destructive overlap of the hydrophobic hydration shell, which is entropically favorable due to the release of the structured hydration water, constituted the driving force for the aggregation of nonpolar solutes in aqueous solution (Némethy and Scheraga 1962). This driving force is usually named the hydrophobic interaction. As the interaction is attractive in nature and it tends to restrict the conformation freedom of the complex, it is possible that the total enthalpy and entropy of the complexation are both negative in spite of the presence of the hydrophobic interaction (Uekama, et al. 1978). In CD chemistry, the most compelling evidence in favor of the presence of the hydrophobic interaction is the repeated observation that in the CD complexes the most nonpolar portions of the guest molecules are usually enclosed in CD cavities (Breslow and Campbell 1971). The involvement of the hydrophobic interaction in CD complexation can also be shown by the correlation analyses, as in general increasing the hydrophobicity of the substituent of the guest molecule enhances the complexation (Matsuura, et al. 1977).

### 2.1.1.4. Hydrogen Bonding

The hydrogen bond is typically an interaction involving an electronegative donor *X*, a hydrogen H, radical group R and a electronegative acceptor *Y* :

$$R - X - H \cdots Y - R.$$

Though many authors claim that the fundamental nature of the hydrogen bond remains somewhat obscure, great emphasis has been placed on interpreting the bond on a purely electrostatic basis. In CD chemistry, the important role of hydrogen bonding in the complexation has been well established for the complexes in the solid state (Saenger and Steiner 1998). A number of crystal structures of CD complexes have clearly shown the well-defined hydrogen bonds between the substrates and the hydroxyls of CDs (Harata, et al. 1984). Computational studies also showed the energetic advantage of adopting a hydrogen-bonded conformation in the complexation (Tong, et al. 1992). Usually, the host–guest hydrogen bonding is restricted to the primary O(6)—H groups of CDs because they are flexible and can rotate about the C(5)—C(6) bond in contrast to the secondary O(2) and O(3) atoms which are rigid due to the preferred $^4C_1$ form of the glucose units (Nakagawa, et al. 2000). Several spectroscopic studies later also suggested the occurrence of hydrogen bonding in CD complexation in aqueous solution (Leclercq, et al. 1998).

### 2.1.1.5. Exclusion of Cavity-Bound High-Energy Water

As the CD cavities are nonpolar, it is not unexpected that the water molecules included in CD cavities should lack the complement of stabilizing hydrogen bonds that would be available to them in the bulk aqueous solution (Chacko and Saenger 1981). Thus, the water molecules in CD cavities are at higher level of energy than those in bulk solution, whose release upon the CD complexation with the guest molecules was postulated as a driving force leading to the complex formation (Van Etten, et al. 1967). However, some authors disagreed with the above postulation (Connors 1996). In fact, though the cavity-bound water molecules are at a higher energy, or in other words, they are “enthalpy rich”, they should have more conformational freedom than the water molecules in the bulk solution because of the lack of hydrogen bonding. Thus, although the exclusion of the cavity-bound water is accompanied with a negative enthalpy

change, the free energy change of the process is not necessarily negative. As shown below, the reorganization of solvent molecules is actually a process of enthalpy-entropy compensation without any free energy contribution. As a result, the exclusion of cavity-bound water is not a driving force of the complexation (Liu and Guo 2002).

### 2.1.1.6. Charge–Transfer Interaction

Charge–transfer interaction is in fact a type of van der Waals Interaction (Schwartz 1990). In CD chemistry, in addition to the charge–transfer interaction between the substitution groups of CDs and the guest compounds, charge–transfer interaction directly between the CD skeleton and the substrate has also been observed (Rademacher and Czarnik 1993). Though sometimes an explanation based on dipole–induced dipole interaction was proposed, the distortion of the electrons within the substrate molecule itself is not sufficient to account for the above behaviors. That is why charge–transfer interaction should be paid attention to, but in fact the interaction is nothing new but one type of van der Waals interaction (Liu and Guo 2002).

### 2.1.1.7. Relations between Different Driving Forces

*Enthalpy-Entropy Compensation*

Enthalpy-entropy compensation is the phenomenon in which the change in enthalpy is offset by a corresponding change in entropy resulting in a smaller net free energy change (Leffler 1955). Nevertheless, it is generally believed that enthalpy-entropy compensation plays an important role in the reactions in solution. In CD chemistry, the occurrence of enthalpy-entropy compensation was observed early and has been well documented (Lewis and Hansen 1973). In particular, it has been suggested that the slope and intercept of the enthalpy-entropy $(\Delta H - T\Delta S)$ plot could be quantitative measures of the conformational change and extent of desolvation upon complexation (Inoue, et al. 1993). However, there remains some controversy concerning the enthalpy-entropy compensation in CD complexation. First, it remains unknown whether or not the observed compensation is a fact or an artifact. As known, the correlation between the enthalpies and entropies obtained from the van't Hoff plots

could be an artifact because the experimental errors of the two quantities tend to be dependent on each other (Linert and Jameson 1989). Nevertheless, most of the enthalpy and entropy data of CD complexation were obtained from calorimetric measurements, and it has been shown recently on the basis of computer simulations that the compensation between them should be a real one (Liu, et al. 2001).

*The Detailed Thermodynamic Steps in CD Complexation*

As anticipated, the detailed mechanism of CD inclusion complexation is quite complicated. However, it is still possible to break the binding process into several steps, which is helpful in illustrating the driving forces of the complexation. Here presented a comprehensive model of the inclusion process of CDs, in which the binding was broken into the following steps (Tabushi and Mizutani 1987).

(1) Release of two water molecules from the CD cavity into the gas phase.
The step accompanies losses of van der Waals interaction and hydrogen bonding between the two water molecules, gains of motional freedom of the two water molecules, and a change in conformation energy of the host.

(2) Transformation of the extruded gaseous water molecules into a liquid phase, which is apparently accompanied with a negative enthalpy and entropy change.

(3) Transfer of a nonpolar guest molecule from water to an ideal gaseous state leaving a structured cavity behind, which collapses with redistribution of the water molecules.

(4) Binding of the guest molecule by the host, accompanied by the turning on of the host–guest intermolecular interaction and a change in the conformation energy of the host.

On the basis of the model, calculated binding energy results are modestly close to the experimental values. The results from the above calculation are interesting. Firstly, it was found that van der Waals interaction between CD and the guest is very important because it provides a large negative enthalpy. This enthalpy is to some extent compensated by the freezing of the motional freedom of the guest molecule in the

complex formation, but not completely. Therefore, van der Waals interaction is a driving force of the complexation. However, it was shown that the release of the cavity-bound water molecules is also accompanied by a large negative enthalpy. Thus, the experimental negative enthalpy cannot be used to demonstrate the dominance of van der Waals interaction in CD complexation. Secondly, it was found that the conformation energy of the host molecule increases from the hydrate to an inclusion complex. Apparently, instead of relief of the conformational strain, the inclusion complexation leads to a more strained conformation of CD with higher conformation energy. Presumably, by changing the conformation of the host, the complex can optimize the interactions between the host and guest and lower its energy. Nevertheless, it is clear that the relief of conformation strain is not a driving force in CD complexation (Liu and Guo 2002).

### 2.1.2. Factors Affecting Inclusion Complexation

*Solution Dynamics*

In crystal form only at surfaces complex molecules are suitable for complexation. But in solution form more cyclodextrin molecule becomes suitable for complexation. Guest molecules' solubility is also a determinant factor.

*Usage of Solvents*

Presence of more cyclodextrin molecules dissolved in solvent, the more molecules are suitable for complexation. The guest (in this situation) can be interchangeable with the solvent found in the cavity. Solvents that are making complexation with CD's should not be used for dissolving drug (Del Valle 2004).

*Temperature Effect*

Temperature increases the solubility while decreasing the stability. The heat stability of complexes varies due to guest molecules' characteristics (Del Valle 2004).

*Effect of Water*

Amount of water affects the reaction rate (Akçakoca and Atav 2006).

## 2.1.3. Methods for Preparation of Inclusion Complexes

There so many techniques for preparation cyclodextrin complexes. Below some of the methods are explained briefly. These methods based on some simple chemical rules and methods can be altered "reasonably" a little due to the guest molecules' physicochemical properties (Hirayama and Uekama 1987).

*In Solution*

Usually this method is applied to water soluble active components (drugs). Drug is added into hot CD solution and mixed overnight until a precipitate occurs (Akçakoca and Atav 2006).

*Co-Precipitation*

If guest molecule is not water soluble, this method can be suitable. Appropriate amount of drug is dissolved in hot organic solvent. This solution is added slowly into aqueous CD solution. After a while occurrence of complex can be observed from the precipitate. Then organic solvent is evaporated under vacuum and product is dried and sieved (Akçakoca and Atav 2006).

*Neutralization Method*

This method generally used for ionizable drugs. Basic drugs are dissolved in acidic solution and acidic drugs dissolved in basic solution, completely. After complete dissolution, CD is added slowly into the solution. CD ionizes and makes a clear solution with drug. The pH of this solution is adjusted in order to precipitate complexed drug; and then via centrifugation and filtration solid complex is separated (Loftsson and Brewster 1996).

*Lyophilization Technique*

Drug in the organic solvent and CD in water are mixed and then freezed. The mixture is sublimed under vacuum (Del Valle 2004).

*Dry Mixing*

Mostly applied to the guests in liquid form (oils etc.). Mixing is applied at room temperature (Del Valle 2004).

## 2.1.4. Determination of Complexed Drug's Solubility

One of the most important applications of cyclodextrins in pharmaceutical fields is to enhance aqueous solubility of drugs through inclusion complexation.The solubilization ability of cyclodextrins can be quantitatively evaluated by the phase solubility method developed by Higuchi and Connors. The phase solubility diagrams (Akçakoca and Atav 2006) i.e., plots of solubility of guest as a function of cyclodextrin concentration, are generally classified as either type A (a soluble complex is formed) or type B (a complex with definite solubility is formed), as shown in Figure 2.1.

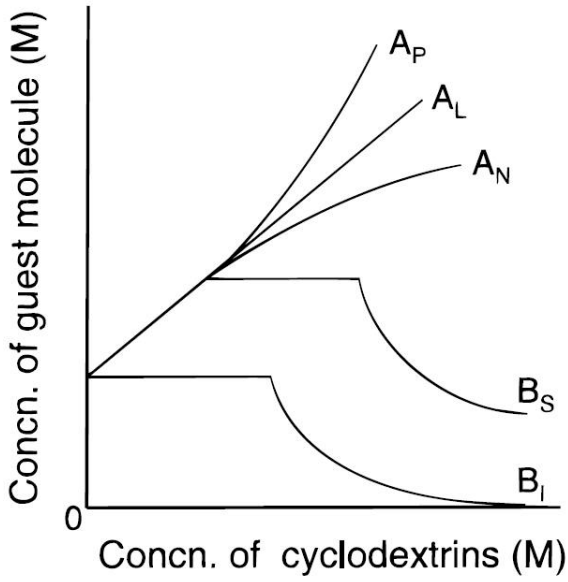


Figure 2.1. Type of Phase Solubility Diagram.
(Source: Uekama 1998)

The type A can be further classified in subtypes $A_L$, $A_P$, and $A_N$, where the guest solubility of the first type increases linearly with cyclodextrin concentration while those of the second and third types deviate positively and negatively, respectively, from the straight line. The complex formation with a 1:1 stoichiometry gives the $A_L$ type diagram, whereas the higher order complex formation in which more than one

cyclodextrin molecules are involved in the complexation gives the $A_P$ type. The interaction mechanism for the $A_N$-type is complicated, because of a significant contribution of solute-solvent interaction to the complexation. In the case of the $B_S$ type, the initial ascending portion of the solubility change is followed by a plateau region and then a decrease in the solubility at higher cyclodextrin concentrations, accompanying a microcrystalline precipitation of the complex. The $B_I$ -type diagram is indicative of the formation of insoluble complexes in water. The stability constant and stoichiometry of complexes are determined by analyzing quantitatively the phase solubility diagram. The solid cyclodextrin complexes can be prepared by referring the B-type solubility diagram (Uekama 1998). The apparent stability constant ($K_c$) –that shows the affinity of the guest molecule to the host molecule-will be calculated from the phase solubility diagrams.

## 2.2. Drug Release

Drug delivery refers to the delivery of a pharmaceutical compound to humans or animals. Since the drug is toxic if the concentration is higher than the toxic level or has no therapeutic effect if the concentration is less than the minimum therapeutic level, it is important to release the drug at a 'controlled' rate (Xu 2009). When you put the drug into a polar or apolar solution; the amount of substances that drug leaving into solution gives its release profile. It is an indicator used in drug delivery or food industry for determination of the level of the effectiveness and quantities of bioactive compounds. The results of this test give us the level of effect of the natural compound to human body (Uekama 1998). The goal of many of the original controlled-release systems was to achieve a delivery profile that would yield a high blood level of the drug over a long period of time. With traditional tablets or injections, the drug level in the blood follows the profile shown in Figure 2.2. (a), in which the level rises after each administration of the drug and then decreases until the next administration. The key point with traditional drug administration is that the blood level of the agent should remain between a maximum value, which may represent a toxic level, and a minimum value, below which the drug is no longer effective. In controlled drug delivery systems designed for long-term administration, the drug level in the blood follows the profile shown in Figure 2.2. (b), remaining constant, between the desired maximum and minimum, for an extended

period of time. Depending on the formulation and the application, this time may be anywhere from 24 hours to 1 month to 5 years.

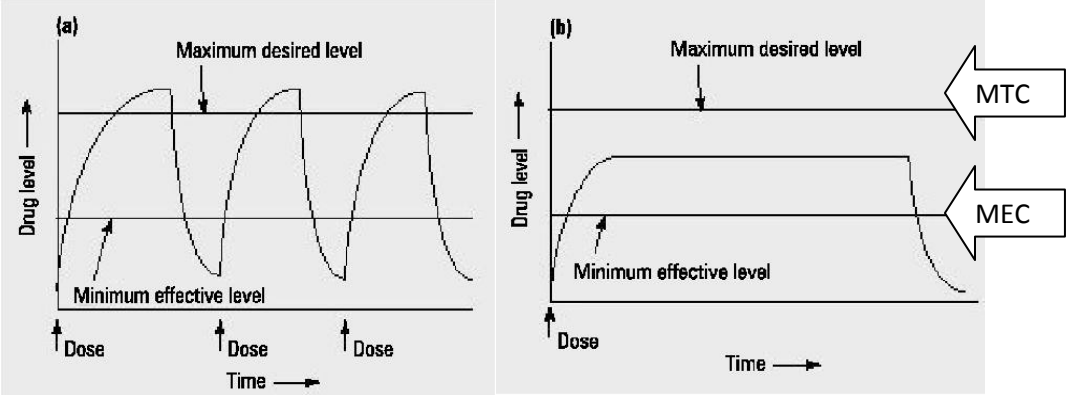


Figure 2.2. Drug levels in the blood with (a) traditional drug dosing and (b) controlled-delivery dosing. MTC: minimum toxic concentration; MEC: minimum concentration. (Source: Peppas 1997)

## 2.2.1. Typical Drug Release Profiles Following Oral Administration

*Immediate Release*

Immediate release formulation of analgesics, antipyretics, coronary vasodilators, etc., is particularly useful in emergency situations. Since the dissolution rate of the poorly water-soluble drugs is mainly responsible for both the rate and extent of oral bioavailability of the drugs, various hydrophilic materials are used to attain the immediate release formulation.

*Delayed Release*

*Delayed release* is a mechanism whereby the release of an active substance is delayed from a finite "lag time" up to a point when/where its release is favored and is no longer hindered (Lakkis 2007). An enteric preparation can be classified as time controlled release, since the drug is preferentially released in the intestinal tract (Uekama 1998).

*Prolonged Release*

Most of the slow-release preparations have been aimed at achieving the zero-order or pH-independent release of drugs to provide a constant blood level for a long period of time. This kind of formulation has many advantages such as reducing the

frequency of dosing, prolonging the drug efficacy, and avoiding the toxicity associated with the administration of a simple plain tablet (Uekama 1998).

*Modified Release*

*Modified release* is a mechanism designed to maintain constant concentration of an active at its target site. Examples of this release pattern include encapsulating flavors and sweeteners for chewing gum applications so that their rate of release is reduced to maintain a desired flavor effect throughout the time of chewing (Lakkis 2007).

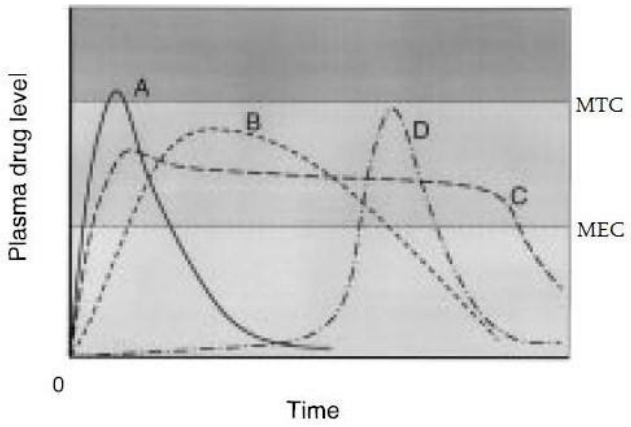


Figure 2. 3. Typical drug release profiles following oral administration.
(Source: Uekama 1998)

## 2.2.2. Overview of Release Mechanisms

Despite the far-reaching applications of complexation and controlled-release technologies in many industries, predicting the release of encapsulated actives, especially in biological systems (foods included), remains a challenge. In the human gastrointestinal tract (GIT), for example, the release of complexes is a function of the physiological conditions, presence of food as well as the physicochemical properties of the ingested dosage. One of the essential requirements for predicting release mechanisms of complexed dosages is by identifying parameters involved in mass transport and diffusion of the actives from a region of high concentration (dosage) to a region of low concentration in the surrounding environment (Lakkis 2007). Kinetically, two main release patterns are identified, zero-order and first-order (Figure 2.4.).

Zero-order release equation $-dA/dt = k$ (2.2)

First-order release equation $-dA/dt = k[C]$ (2.3)

where $-dA/dt$ is the change in active concentration over time, k is the rate constant, and [C] is the active's concentration. In designing microcapsules with controlled-release systems, it is critical to identify desirable release profile so that adequate materials and technology can be chosen.

*Burst release* is simply described by a high initial delivery of an entrapped active, before the release reaches a stable profile, thus reducing the system's effective lifetime and complicating the release control. Although burst release may be preferred for flavor high impact applications, in drugs this mechanism may lead to high toxicity levels and in effective administration of the active (Huang and Brazel 2001).

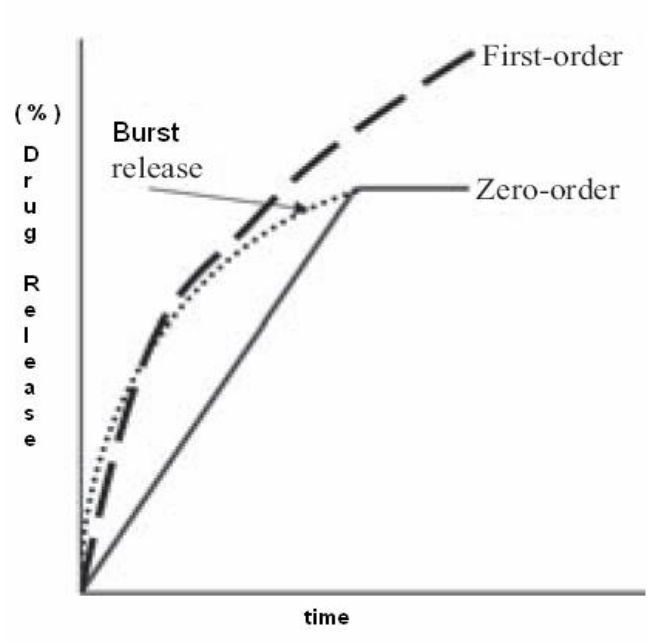


Figure 2. 4. Release rates (zero-order, first-order and burst) of microencapsulated systems. (Source: Lakkis 2007 )

When placed in a release medium, the active can quickly diffuse out of the membrane surface causing a burst effect. Low-molecular-weight actives frequently undergo burst release, a result of high osmotic pressure and increased concentration gradient.

### 2.2.3. Modes of Release

When a compound is encapsulated, it should be released from that encapsulant in order to show its activity at the desired time and place. The most common modes of release are detailed below:

*Thermal release*—whereby the encapsulant melts at a certain temperature, usually during cooking of the product, releasing the ingredient. By altering the type of coating and its thickness, it is possible to ensure release of an ingredient within a few degrees of the required temperature (Lakkis 2007).

*Physical release*—requiring the physical breaking of the microcapsules; usually this mode of release is designed for ingredients that need to be released during chewing. Factors which can be altered to prolong or otherwise the release profile include the size of the capsules and the strength and flexibility of the coating (Lakkis 2007).

*Dissolution*—most food products contain at least a small amount of water, which can be used to ensure the release of an ingredient enclosed in a water-soluble coating. The chemistry of the coating can be designed so that this only occurs at a particular pH, temperature, or salt concentration (Lakkis 2007).

### 2.2.4. Controlled Drug Release for CD Complexes

When the complex molecule is put into water, release of complexed guest occurred at two stages ;

1. Dissolution of the complex
2. Exchange of complexed guest with the water molecules

$$\text{I.C.} \leftrightarrows \text{Drug} + \text{CD} \tag{2.4}$$

$$\text{Drug} \leftrightarrows \text{Dissolved I.C.} + \text{Undissolved I.C.} \tag{2.5}$$

There is an equlibrium between free and complexed CD, guest and dissolved-undissolved complex.

# CHAPTER 3

# LITERATURE REVIEW

## 3.1. Phenolic Compounds

Flavonol glycosides and their aglycones are generally termed flavonoids. Many different flavanoids occur in nature these are distributed throughout the higher plants. Rutin is an important member of this family. Rutin is a naturally occurring phenolic flavonol glycoside, which is named as vitamin P and was thought to be an activating factor for vitamin C in the nutrition literature (Song and Wang 2001). Rutin is [2-3, 4-dihydroxyphenyl - 3, 5, 7-trihydroxy-4-oxo-chromen-3yl rutinoside], an O-glycoside composed of aglycon of quercetin and rutinose. Rutin has found in many of the plants. Rutin is found mostly in the leaves (olive leaf) and rarely in some vegetables (spinach). For example, it was reported that the upper youngest asparagus shoot tissues contained the highest amount of rutin in the shoot and it was 0.03-0.06% of tissue fresh weight (Wang, et al. 2003). On average buckwheat leaves, stems, and flowers were reported to contain, about 300, 1,000, and 46,000 ppm of rutin respectively. *Hypericum perforatum* L (Hypericaceae) is a one of the plants carries rutin in its parts between 0.095–0.2 % (Kreft, et al. 1999). Rutin (quercetin-3-O-rutinose) is one of the most bioactive flavonoids and the second abundant component found in olive leaves (Bayçın, et al. 2007; Altiok, et al. 2008). It is 3', 4', 5, 7- tetrahydroxyflavone-3β-D-rutinoside and the corresponding chemical structure is given in Figure 3.1.

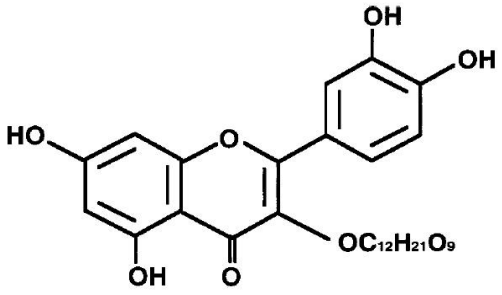


Figure 3. 1. The chemical structure of rutin molecule.
(Source : Miyake, et al. 2000)

### 3.1.1. Physicochemical Properties of Rutin

The solubility data of rutin (MW: 664.58 Da) in 8 different solvents and at 5 different temperatures are given below, that proving the low aqueous solubility of rutin.

Table 3. 1. Solubility data, x of rutin in eight different solvents at T= 283.15, 298.15, 313.15, 323.15 and 333.15. [a] Expanded uncertainty (±) were calculated using Standard deviation S.D. x coverage factor k, k = 2. (Source: Zi, et al. 2007)

| **Solvents** | $x^a$ ($\times 10^5$) | | | | |
|---|---|---|---|---|---|
| Temperature | **283.15 K** | **298.15 K** | **313.15 K** | **323.15 K** | **333.15 K** |
| Water (pH 6.4) | 0.53 ± 0.02 | 0.66 ± 0.03 | 1.02 ± 0.05 | 1.42 ± 0.04 | 1.96 ± 0.06 |
| Methanol | 228.9 ± 1.5 | 303.4 ± 0.7 | 398.9 ± 2.6 | 466.0 ± 2.0 | 523.8 ± 3.1 |
| Ethanol | 372.1 ± 3.4 | 436.5 ± 2.1 | 591.8 ± 2.4 | 653.2 ± 1.3 | 754.3 ± 2.1 |
| 1-Propanol | 149.8 ± 0.4 | 179.2 ± 1.5 | 234.7 ± 0.4 | 259.5 ± 2.5 | 297.0 ± 6.7 |
| 2-Propanol | 130.3 ± 2.5 | 157.0 ± 1.9 | 207.7 ± 2.8 | 236.1 ± 0.8 | 266.9 ± 3.2 |
| 1-Butanol | 30.5 ± 0.5 | 34.6 ± 0.2 | 39.69 ± 1.2 | 44.6 ± 1.1 | 50.8 ± 0.6 |
| Acetone | 16.9 ± 1.7 | 27.1 ± 0.9 | 54.1 ± 1.1 | 68.5 ± 0.5 | |
| Ethyl acetate | 114.4 ± 3.7 | 108.5 ± 2.1 | 100.3 ± 1.3 | 95.3 ± 0.6 | 89.4 ± 0.8 |

Also, solubility of rutin at room conditions was investigated as 45 mg/L in distilled water, 26 mg/L in simulated gastric fluid (pH = 1.2) and 128 mg/L in simulated intestinal fluid (pH = 7.5) (Lauro, et al. 2002).

The absorption of rutin *in vivo* was also investigated but there are conflicting statements in the literature. It was reported that dietary rutin was recovered in a substantial concentration in rat plasma as two conjugated metabolites. Rutin could be hydrolyzed by the intestinal microflora with R-rhamnosidase and β-glucosidase to isoquercitrin (quercetin3-glucoside) and quercetin. On the other hand, rutin is absorbed into the bloodstream in the upper part of the small intestine. Excessive amounts were excreted in the urine. These two conflicting statements demonstrate the sorption of rutin is certain *in vivo* but its absorption mechanism has not been enlightened yet (Song and Wang 2001). Also in Table 3.2. the change in % mass at 4 different temperature intervals that obtained from TGA analysis of Rt can be observed .

Table 3. 2. (%) Mass losses and Decomposition stages of Rutin. (Source: Costa, et al. 2002)

| **Nitrogen Atmosphere** | **First** | **Second** | **Third** | **Fourth** |
|---|---|---|---|---|
| **Temperature interval (°C)** | 25–139 | 139–304 | 304–511 | 511–892 |
| **Mass losses (%)** | 5 | 15.8 | 15.5 | 13.3 |

### 3.1.2. Health Benefits

Rutin is a physiologically active material on the blood vessels as it increases the capillary fragility and act as an antioxidant by scavenging free oxygen radical. Therefore, the pharmaceutical preparations containing this material have been produced. Rutin has several biological effects:

(a) an antioxidant effect (an inhibitory effect of the oxygen radical formation and a scavenging effect for active oxygen and nitric oxide radicals), and

(b) inhibitory effects on enzymes such as xanthine oxidase, phospholipase A2 group II, and hyaluronidase. The latter leads to a protective effect against the cytotoxicity of oxidized low-density lipoproteins (LDL) to vascular endothelial cells and gastric lesions induced by ethanol, and a colonic neoplasis and lung metastasis (Timberlake, et al. 1988; Havsteen 1989 ; Pathak, et al.1991; van Acker, et al. 1995).

(c) In clinical use, rutin has been used to treat disease states characterized by capillary bleeding (antihemorrhagical activity) associated with increased capillary fragility ( i.e., purpura and hypertension) strengthens the capillaries of blood vessels and the regulates the capillary permeability (Ljungman , et al. 1996; Hanasaki , et al. 1994; Yoshino and Murakami 1998).

Rutin has a broad range of physiological activities such as anti-inflammatory, antitumoral, anticataract, antihepatotoxic, antivaricose antioxidative and antibacterial properties (Sheu, et al. 2004). There exist many studies on the health effects and mechanisms of rutin. For instance, investigated the mechanism of rutin in inhibition of platelet aggregation and found that rutin may be an effective agent in treating thromboembolicrelated disorders. It can be used as antiplatelet agents such as aspirin and ticlopidine in intravascular thrombosis (Sheu, et al. 2004). These properties are potentially beneficial in preventing diseases and protecting the stability of the genome.

## 3.2. Cyclodextrins

### 3.2.1. Evolution of Cyclodextrins and Their Properties

#### 3.2.1.1. Introduction to Cyclodextrins

Cyclodextrins (CDs) are cyclic molecules composed of glucopyranose ring units to form truncated cone type, doughnut structures. The most common are the α, β, and γ-cyclodextrins which are composed of six, seven, and eight sugar units respectively (Frömming, et al. 1994). The exterior of the CDs are hydrophilic while the interior is hydrophobic, and the different CDs possess different cavity sizes according to the number of glucopyranose rings present (Figures 3.2. and 3.3.).

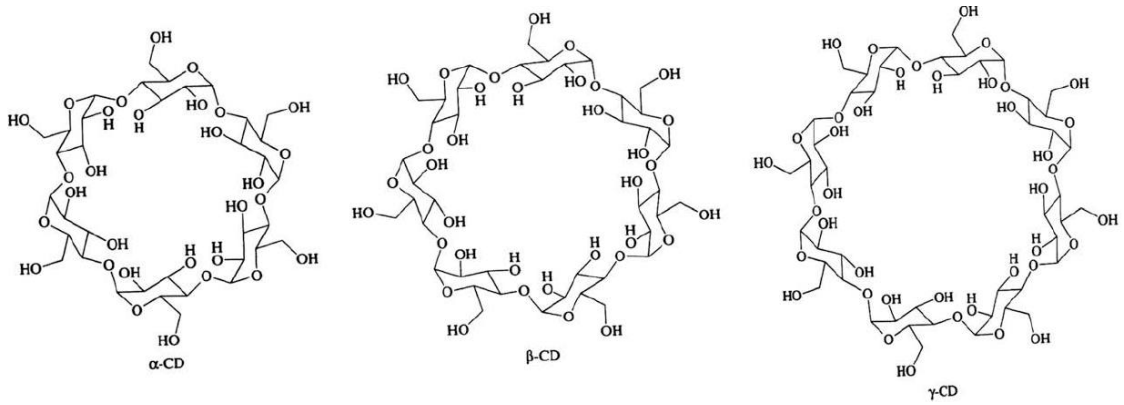


Figure 3. 2. Types of natural cyclodextrins.
(Source : Astray, et al. 2009)

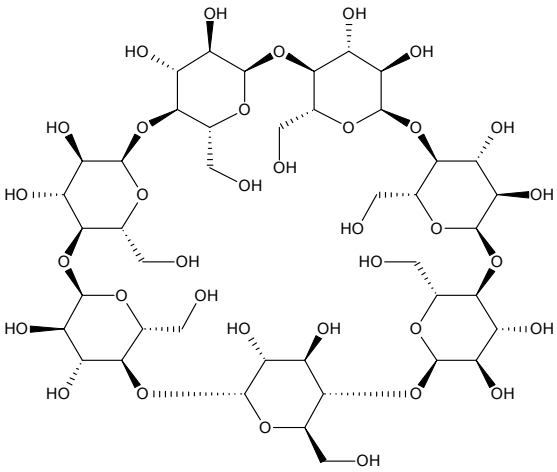

Figure 3. 3. Beta-cyclodextrin molecule.

Various cyclodextrins can be considered as empty capsules of molecular size (Frömming, et al. 1994).

### 3.2.1.2. Production of Cyclodextrins

Cyclodextrins are produced from starch by means of enzymatic conversion (intramolecular transglycosylation reaction). Some microorganisms (i.e *Bacillus* species) produce cyclodextrin glycosyltransferase (CGtase) enzyme which degrade starch into cyclodextrins (Taneri, et al. 2003).

Production of cyclodextrins are carried out through four stages;

- Production of microorganisms that have ability to produce CGtase enzyme
- Separation of enzyme from the medium, determination of the concentration and finally purification of the enzyme
- Enzymatic conversion of hydrolyzed starch into cyclic and non-cyclic dextrin mixture
- Seperation, purification and crystallization of cyclodextrins from mixture

Formation of different cyclodextrins and the ratio between their amounts are related to the incubation period of enzyme within the starch (Bekers, et al. 1991).

### 3.2.1.3. Parent CDs and CD Derivatives Formation

Cyclodextrins ar e of three types: α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, referred to as first generation or parent cyclodextrins. α-Cyclodextrin, β-cyclodextrin and γ-cyclodextrins are composed of six, seven and eight α-(1,4) linked glycosyl units, respectively. β-Cyclodextrin is the most accessible, the lowest-priced and generally the most useful. Each cyclodextrin is a torus (doughnut-shaped) molecule (Singh, et al. 2002). The internal cavity of the doughnut is hydrophobic, whereas the external surface is hydrophilic. These act as a host for entrapping either wholly or partially other chemicals without the formation of covalent bonds. Chemical modifications of CDs can alter their physical properties, see Figure 3.4.

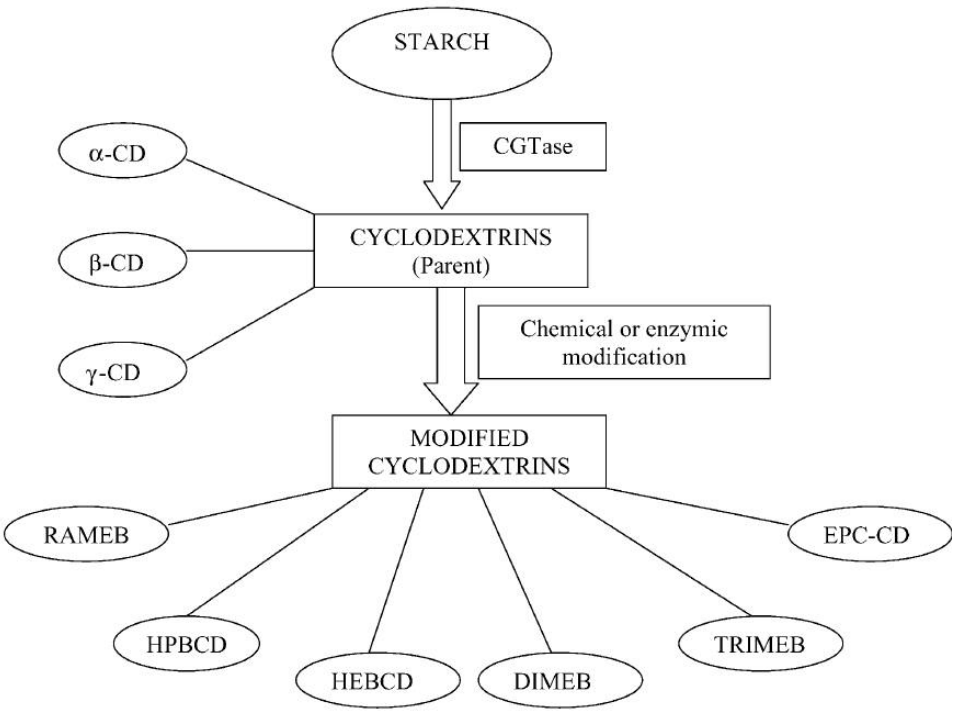


Figure 3. 4. Parent CDs and CD derivatives formation scheme{RAMEB = randomly methylated beta cyclodextrin; HPBCD = hydroxypropyl beta cyclodextrin; HEBCD = hydroxyethyl beta cyclodextrin; DIMEB = Heptakis (2,6-dimthyl)-beta cyclodextrin; TRIMEB = Heptakis (2,3,6-trimthyl)-beta cyclodextrin; EPC-CD = Cyclodextrin crosslinked with epichlorhydrin}. (Source: Singh, et al. 2002)

These modifications can improve solubility, stability against light or oxygen and help control the chemical activity of guest molecules (Singh, et al. 2002).

### 3.2.1.4. Chronological Evolution of Cyclodextrins

CDs were first noted in an 1891 publication as simply a crystalline substance obtained from digestion of starch. Villiers referred to the substance as cellulosine. Twelve years later, Schardinger observed the same substance and was able to isolate and examine it. Schardinger was the first to extensively examine CDs in the late 1800s and for this reason CDs are often referred to as Schardinger dextrins (Madison 2001). Freudenberg and co-workers in the 1930s established the true structure of CDs and suggested that larger cyclodextrins could exist, with assistance from the observations made by Karrer and Miekeley. They showed that cyclodextrins were cyclic oligosaccharides formed by glucose units and somewhat later Cramer and co-workers described their ability to form inclusion complexes. By the early 1950s the basic physicochemical characteristics of cyclodextrins had been discovered, including their

ability to solubilize and stabilize drugs. The ability of CDs to complex with a variety of drug molecules, and noted the stabilization, volatility reduction, and solubility changes that occurred as a result of complexation was examined by Cramer and coworkers. The first cyclodextrin-related patent was issued in 1953 to Freudenberg, Cramer and Plieninger, that encompassed the potential drug related applications foreseen as a result of their studies (Vyas, et al. 2008). However, pure cyclodextrins that were suitable for pharmaceutical applications did not come available until about 25 years later and at the same time the first cyclodextrin containing pharmaceutical product was marketed in Japan. Later cyclodextrin-containing products appeared on the European market and in 1997 also in the US. New cyclodextrin-based technologies are constantly being developed and thus, 100 years after their discovery cyclodextrins are still regarded as novel excipients of unexplored potential. More than 30 different pharmaceutical products containing cyclodextrins are now in the market worldwide (Vyas, et al. 2008). Among the different compounds that can act as host molecules (i.e. cyclodextrins, calixarenes, cucurbiturils, porphyrins, crown ethers, zeolites, cyclotriveratrylenes, cryptophanes and carcerands) CDs are preferred for a number of different reasons. The first advantage of CD is its relatively nonreactive nature. CD is stable in alkaline solution, has fairly good resistance to UV and IR light, is thermally stable at up to 270 °C, and acid hydrolysis only results in nontoxic glucose products. CDs are also nonreducing and periodate oxidation does not produce formic acid or formaldehyde, which is a concern for food and drug applications. CDs are also readily available, thoroughly studied, natural products that have been found to form stoichiometric complexes with a wide range of molecules (Huang and Tonelli 1998).

### 3.2.1.5. Toxicity of Natural Cyclodextrins

If CDs or their inclusion complexes are orally administered, the absorption of the free CD deserves attention, because the inclusion complexes dissociate under physiological conditions (Bekers, et al. 1991). Orally administered CDs have shown to be harmless, probably because insignificant amounts of CD are absorbed from the intestinal tract (Jones, et al. 1984). The metabolism of β-CD occurs by the action of amylases of the bacterial flora of the colon where they are converted to glucose**;** however hydrolysis by α-amylases occurs only at a slow rate. Only after parenteral administration of high doses of CD severe signs of toxicity are observed. This toxicity is

characterized by nephrosis. Main properties of β-cyclodextrins are: less irritating than α-CD after injection; binds cholesterol; very small amounts (1-2 %) absorbed in the upper intestinal tract after oral administration; no metabolism in the upper intestinal tract; metabolized by the bacteria in caecum and colon; currently the most common cyclodextrin in pharmaceutical formulations and thus, probably the best studied cyclodextrin in humans (Del Valle, et al. 2004).

## 3.2.1.6. Physicochemical Properties of Cyclodextrins

- β-CDs have water solubility of 1.8% (w/w) at ambient temperature.
- β-CDs have equilibrium moisture content of 14.5%. At this moisture level, the CDs remain a pourable powder which is dry to the touch (Bekers, et al. 1991).
- General physical properties of α-CD, β-CD, γ-CD, DM-β-CD, HP-β-CD are summarized in Table 3.3.

Table 3. 3. Physical properties of the CDs and some derivatives
(Source: Bekers 1991)

| | α | β | γ | DM-β[1)] | HP-β[2)] |
|---|---|---|---|---|---|
| * Number of glucose residues: | 6 | 7 | 8 | 7 | 7 |
| * Cavity dimensions (Å) | | | | | |
| - Cavity diameter: | 5 | 6 | 8 | 6 | 6 |
| - Height of torus: | 7.9 | 7.9 | 7.9 | 10.0 | |
| - Diameter of periphery: | 14.6 | 15.4 | 17.5 | | |
| * Molecular weight: | 973 | 1135 | 1297 | 1331 | ±1300 |
| * Aqueous solubility [3)]: | 14.5 | 1.85 | 23.2 | 57 | >50 |
| * Melting point (°C): | 275 | 280 | 275 | 295-300 | |
| * pKa [4)]: | 12.3 | 12.2 | 12.1 | | |
| * Half-life of ring opening [5)] (hr): | 6.2 | 5.4 | 3.0 | 8.5 | |
| * Enzymatic hydrolysis [6)]: | negli-gible | slow | rapid | | |

[1)] heptakis-2,6-di-O-methyl-β–CD; [2)] 2-hydroxypropyl-β-CD; [3)] in grams per 100 ml water at ambient temperature; [4)] pKa: by potentiometry at 25 °C; [5)] Half-life of ring opening: in 1 N HC1 at 60 °C; [6)] by Aspergillus oryzae α-amylase.

### 3.2.1.7. Research Interests Involving Cyclodextrins

The largest area of interest involving cyclodextrins for both industrial and academic scientists' lies in the research of cyclodextrin based inclusion complexes (Hedges 1998). Though the general structure of cyclodextrin was well established in 1950 (Hedges 1998), there are still substantial research efforts focusing on the study of the structural aspects of cyclodextrin and its derivatives. There has been a great deal of effort involved in the study of how substitution of the hydroxyl groups of cyclodextrin alters fundamental parameters. Modification of cyclodextrin has been incorporated into a number of research projects (Nishiki, et al. 1999; Renard, et al. 1997; Tian, et al. 2000). Derivatization of cyclodextrin has been an important factor in the success of many projects and has been the topic of a recent review (Khan, et al. 1998). Modification of cyclodextrin has been shown to greatly alter many parameters including complexation stability and overall solubility. The majority of cyclodextrin complexation studies are done in aqueous environments because in a non-aqueous solution the solvent may actively compete with the desired guest molecule to form an inclusion complex with cyclodextrin. Cyclodextrin, cyclodextrin derivatives, and cyclodextrin based complexes have been studied by many techniques including NMR and 2D NMR analysis (Schneider, et al. 1998), calorimetry and TGA (Hedges 1998), X-ray methods (Hirotsu, et al. 1982), electric field pulse techniques, volumetric techniques, electron microscopy (Hodi, et al. 1985), circular dichroism (Meyer, et al. 2000), and electron paramagnetic resonance (EPR) to name a few. Inclusion complex formations involving cyclodextrins have been utilized in catalysis of reactions. They have been incorporated into biomimetic reactions where cyclodextrin acts as an enzymatic model. Once a guest is accommodated within the host cavity several of its parameters, including solubility, are altered. This alteration in solubility allows for the guest compound to be transferred between phases where it is then available to react. The observation of increased availability of a guest also has been utilized in drug delivery systems. Cyclodextrin's potential in drug carrier systems is another area of great interest from an industrial and academic viewpoint. Factors such as skin permeation, increased photostability, and water solubility are but a few of the areas being investigated.

### 3.2.2. Applications of CD Complexes

Inclusion in cyclodextrins exerts a profound effect on the physicochemical properties of guest molecules as they are temporarily locked or caged within the host cavity giving rise to beneficial modifications of guest molecules, which are not achievable otherwise (Singh, et al. 2002). Negligible toxicity allows for its application in everyday products such as shampoo and toothpaste. CDs have even been found to be safe enough to be used in food and drug systems. This section will include a broad but brief introduction into the different ways cyclodextrins and their ability to accommodate guest molecules are being used today in industry. Cyclodextrins and their derivatives have found a place in a number of consumer products and in many industrial processes (Madison 2001).

#### 3.2.2.1. CD Applications in Foods

*Stabilization by Powdering*

Flavor components such as apple, citrus fruits, and plums, and spices such as allspice, cinnamon, garlic, or ginger, and herbs such as peppermint and basil, included into CDs are available on the market and have a good reputation for the high stability they exhibit when they are heated during industrial food processing. For example, in the production of candies, cookies, and chewing gum using these flavors it is normally necessary to use a larger quantity of flavor than that which remains in the finished product. However, when CD inclusion complexes of these flavors are used for processing, a product can be obtained using far smaller amounts of flavor than would be required conventionally. Furthermore, CD inclusion complexes of these components are stable and last longer than liquid essences or the components themselves (Lindner, et al. 1981). Another more elementary aspect associated with the processing of CD complexes is the fact that they are solids. When a liquid component is complexed with CD it forms a solid complex, which is then much easier to handle from an industrial standpoint (Szejtli 1988).

*Reduction of Bitter/Astringent Tastes*

The catechins contained in tea leaves are called also ‘‘tannin’’ and are the source of the astringent taste specific to green tea. The strong bitter/astringent taste of catechins is a big problem in the marketing of teas as daily drinks. By adding β-CD to catechins, the bitter taste can be reduced allowing less-bitter tea drinks to be prepared. Currently, several tea drinks containing catechins at high concentrations are marketed as popular health commodities (Dodziuk 2006).

*Improvement of Taste and Other Properties*

When γ-CD is added to ginseng extract powder, the hygroscopic nature is reduced and the bitter taste specific to ginseng can be masked, resulting in improvement of the taste. In addition, the solubility and dispersibility in water can be improved (Dodziuk 2006).

*Anti-oxidation, Stabilization, and Improvement of Bioavailability*

Many vitamins, fatty acids, pigments, and various other physiologically active substances are unstable and easily destroyed by heat, light (UV radiation), or acids, or are susceptible to oxidation, and they are not efficiently absorbed into the body. By adding such physiologically active supplements, CD inclusion technology can be utilized to solve various problems such as unfavorable taste, disagreeable odor, instability, low absorption rate, and low bioavailability (Dodziuk 2006).

- Since vitamins are unstable and easily deteriorate, it is necessary to devise a formulation that will stabilize them. One effective method of achieving this is the use of various CD inclusion complexes. Even the liposoluble vitamin E (tocopherol), which is unstable under sunlight, UV light, or heat, can be stabilized with γ-CD. When a mixture of tocopherol and starch was kept at 45ºC, 60% of its activity was lost in 17 weeks. But under the same conditions, γ-CD-included tocopherol lost only 20% of its activity (Dodziuk 2006).
- When a compound is contained within the cavity of the CD, attack on that compound by other molecules is severely limited by the shielding of the bulky CD. The movement of the guest is also restricted (Szejtli 1988). It has been found that both thermal and oxidative stabilities have increased upon complexation with CD (Hedges 1998).

- CDs are used for the stabilization and solubilization of natural colorants. There is a strange common characteristic among the natural red colorants such as anthocyanin, lycopene, and astaxanthine, namely that of the native α-, β-, and γ-CDs it is γ-CD that is most effective in stabilizing all three natural red colorants (Dodziuk 2006).
- CDs have been found to alter the solubility properties of complexed guests, and have been utilized to solubilize hydrophobic compounds in aqueous media. Increased solubility has also been found to result in increased bioavailability in drug systems (Konno, et al. 1982).

*Solubilization of Insoluble Substances*

By forming CD inclusion complexes it is possible to solubilize, as stable solutions, some substances that are hardly soluble in water or that have been regarded as unsuitable for the preparation of drinks.

- In general, many flavonoids are solubilized, as stable solutions giving no precipitates, by adding both β- and γ-CDs in appropriate amounts tailored to each flavonoid instead of using either β- or γ-CDs alone.
- When isoflavone derivatives are converted into inclusion complexes with CDs the inclusion complexes of isoflavone derivatives are absorbed more efficiently into human bodies and exhibit more pronounced physiological effects than uncomplexed ones (Dodziuk 2006).

Compatibilization is another area where CDs have made their mark. They have been incorporated into pesticide formulations in order to improve their wettability. CDs also have been incorporated into packaging films for a number of different applications. When incorporated into some films, CDs have been shown to increase the biodegradation. CDs are also being used in films to control the release and uptake of a variety of compounds. They have been incorporated to produce permiselective films for food packaging, and some have found CD containing films to exhibit controlled release of a perfume for an extended period of time (Szejtli and Osu 1996).

### 3.2.2.2. CD Applications in Drug Delivery & Drug Release

*Some Characteristics of CDs as Drug Carriers*

The desirable attributes of drug carriers in drug delivery systems are the multifunctional properties such as controlled-release, targeting, and absorption enhancing abilities (Uekama 2004). From the safety aspect, bioadaptability is a necessity, and quality, cost-performance, etc. are additional requirements for drug carriers. CDs have such characteristics e.g. they are fairly bioadaptable and hardly absorbable from the gastrointestinal (GI) tract, they interact with specific components of biomembranes such as cholesterol and lipids, their macrocyclic ring survives in the stomach and small intestine, but they are biodegradable in the colon and large intestine, and more-functional CD derivatives are available to modify the physicochemical and inclusion properties of the host molecules. An important characteristic of CDs is the formation of inclusion complexes in both the solution and the solid states, in which each guest molecule is surrounded by the hydrophobic environment of the CD cavity. This can lead to the alteration of physicochemical properties of guest molecules such as solubility, chemical stability, dispersibility and so on, which can eventually have considerable pharmaceutical potential (Dodziuk 2006).

*Drug Solubility and Absorption/Bioavailability*

Inclusion complexation or solid dispersion with cyclodextrins can improve drug solubility or dissolution of poorly water-soluble drugs. In case of drugs with inadequate molecular characteristics for complexation cyclodextrin act as hydrophilic carriers, or as tablet dissolution enhancers for drugs with high dose (with which use of a drug/CD complex is difficult) e.g., paracetamol. Reduction of drug crystallinity on complexation or solid dispersion with CDs also contributes to the CD increased apparent drug solubility and dissolution rate. CDs can enhance drug dissolution even when there is no complexation. CDs can also act as release enhancers. CDs can enhance the oral bioavailability of drugs in different ways, and the enhancing mechanism of CDs on the oral bioavailability of drugs may be more complicated than we have so far believed (Dodziuk 2006). In case of hydrophobic drugs, CDs increase the permeability by increasing drug solubility, dissolution and thus making the drug available at the surface of the biological barrier, from where it partitions into the membrane without disrupting the lipid layers of the barrier. In such cases it is important to use just enough CD to

solubilize the drug in the aqueous vehicle since excess may decrease the drug availability (Dodziuk 2006).

*Control of Drug Release*

There are two types of control on drug release via oral delivery i.e., rate-controlled release and the time-controlled release. The hydrophobic CDs such as ethylated and acylated CDs with low aqueous solubility are known to work as prolonged-release carriers of water-soluble drugs. The combined use of CDs complex and CDs conjugate will be useful for designing various kinds of time-controlled type oral drug delivery preparations. The release of drug from the drug/CDs conjugate after oral administration shows a typical delayed-release behavior. Therefore, when the CDs conjugates are combined with other different release preparations, we can obtain more advanced and optimized drug release system, securing balanced oral bioavailability, and prominent therapeutic efficacy (Uekama 2004). For example, a repeated-release preparation may be designed by combining the CDs conjugate with a fast releasing fraction, while a combined preparation of the conjugate with a slow-releasing fraction may provide a prolonged release preparation (Kamada, et al. 2002). Since pharmaceutical preparations are usually composed of considerable amounts of pharmaceutical excipients and additives to maintain the efficacy and safety of the drug molecules, suitable combination of the CDs complex and the third component can markedly extend the actions of CDs for the design of advanced drug release formulations (Dodziuk 2006).

## 3.2.2.3. Previous Studies for Rutin-CD Complexes

Results of Rt-CD phase solubility studies at literature are summarized in Table 3.4. As we seen from the table highest stability constants were obtained via usage of BCD and its hydrophilic derivative HP-β-CD (Miyake, et al. 2000). The higher stability constant value means longer time and more harsh conditions needed for degredation of complex.

Table 3. 4. Summary of Rutin-CD Phase Solubility Studies at literature

| Type of CD | Stability Constant ($M^{-1}$) | Ref. |
|---|---|---|
| HP-β-CD | 341 | Sri, et al. 2007 |
| β-CD | 260 | |
| HP-γ-CD | 87 | Miyake, et al. 2000 |
| β-CD | 266 | |
| γ-CD | 112 | |
| HP-β-CD | 282 | |

The stability constant and stoichiometry of the inclusion complexes, depending on the guest/host concentration employed are useful indices for estimating the binding strength of the complex and changes in the physicochemical properties of the guest molecule in the complex. Moreover, attention should be directed towards various environmental factors, such as dilution, temperature, pH, and additives, in the design of practical formulations and routes of administration (Dodziuk 2006).

## 3.3. Silk Fibroin

Silks are generally defined as protein polymers that are spun into fibers by some lepidoptera larvae such as silkworm, spiders, scorpions, mites and flies. Silks differ widely in composition, structure and properties depending on the specific source (Altman, et al. 2003). Silk protein consists of two kinds of proteins, silk fibroin and silk sericin (glue-like protein). The ratio of silk fibroin to silk sericin is about 3:1. Both proteins consist of 18 amino acids; about 85% of them are Glycine, Alaline, Serine and Tyrosine (Becker, et al. 1997). Many studies on amino acids indicated that Glycine had an effect on reducing the cholesterol level in blood, Alaline could accelarate alcoholic metabolism and Tyrosine served the function of preventing senila dementia. Also it was shown that various degrees of peptide chains in size made of many kinds of amino acids had tangible results of adjusting the physiological action and the immunity system (Luo, et al. 1993). The primary structure of silk fibroin depicted as $(\text{-Gly-Ala-Gly-Ala-X-})_n$ where X=Ala or Ser. Most of the component amino acid residues are hydrophobic, while some sequences including hydrophilic residues were also isolated. Therefore silk fibroin is regarded as a block-type copolymer composed of both hydrophobic chains (crystalline region) and hydrophilic chains (amorphous region). Its average molecular weight is about 300,000 (Tsuruta, et al. 1993).

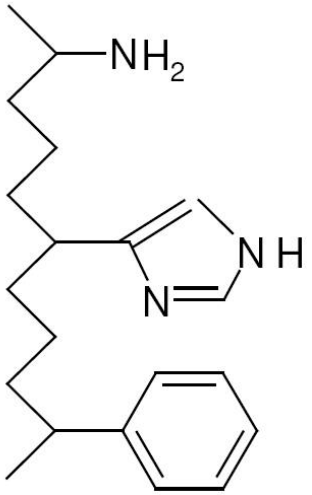


Figure 3. 5. Molecular structure of fibroin.
(Source: Özgarip 2004)

Macromolecules of fibrillar (fibrous) proteins like fibroin have porous ball conformations in aqueous solutions. These are formed by complicated constructs in that combine in concentrated solutions into complex super molecular associates. Destruction of the regular structure of the macromolecular fibroin chain during chemical and physical modifications, decrease the molecular weight, changes and decrease the strength of intermolecular interactions, and change the structure of concentrated solutions (Mukhamedzhanova, et al. 2001). Silk fibroin is a protein of a natural origin with beta structure. The structural layers in silk thread are bonded to each other only through Van-der-Waals interactions which makes the fibroin flexible. The polypeptide chains in silk fibroin are situated antiparallel (Mukhamedzhanova, et al. 2001). Along the plated beta sheet structure, there are also amorphous regions in the fibroin in which bulky residues of Tyr, Val, Arg. The amorphous region increases the extensibility while diminishing elasticity. The silk film cast from aqueous solution consists of alpha form and beta form (Mukhamedzhanova, et al. 2001). The raw silk thread produced by a silkworm is composed of fibroid in the core and is covered with sericine. If the sericine is removed by degumming, water-insoluble fibroin remains as a fine silk thread. This thread has a triangular cross-section and, therefore, exhibits a characteristic lustre, pleasant handling and an elegant drape (Onar 2004).

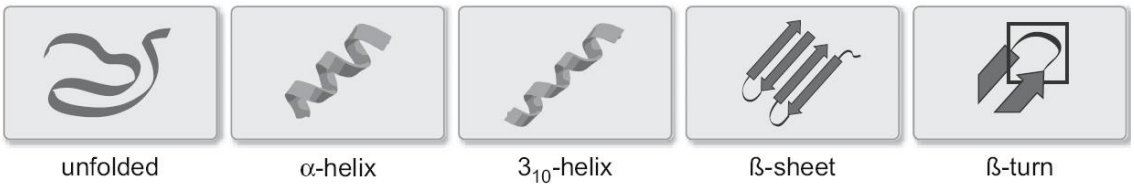


Figure 3. 6. Common secondary structural motifs in proteins.
(Source: Hardy, et al. 2008)

*Silkworm Silk*

The most extensively characterized silks are form of the domesticated silkworm, Bombyx mori which is the most abundant and is therefore obtained easily and cheaply (Altman, et. al. 2003). Of all natural silk-producing animals, mulberry silkworms (*Bombyx mori*) are of the most economic importance, because it is possible to rear them in captivity. After the eggs laid by adult moths hatch, the caterpillars are fed fresh mulberry leaves for a month until they are mature enough for metamorphosis into a moth, which requires the construction of a protective cocoon of silk (Adachi, et al. 2006; Zhao, et al. 2007; Zhao, et al. 2005). Once the cocoon is complete, silk farmers kill the caterpillar via exposure to hot water/steam, and the silk can be harvested. The crude silk needs both degumming (from the glue-like sericin) and processing before it can be dyed and sold (Freddi, et al. 2003; Mossotti, et al. 2006). The fact that there is a readily available source of silkworm silk has facilitated an understanding of its structure and function (Pérez-Rigueiro, et al. 2007 ; Marsh, et al. 1955). *B. mori* silk has a core–shell type structure, with its core composed of a complex of 3 proteinaceous components: a large protein, known as heavy chain fibroin (H-chain, ca. 350 kDa) that is linked to a second small protein, known as light chain fibroin (L-chain, ca. 25 kDa) via disulfide bonds; and a third small glycoprotein, known as the P25 protein (P25, ca. 30 kDa) is associated via non-covalent hydrophobic interactions (Inoue, et al. 2000; Tanaka, et al. 1999). The molar ratios of H-chain:L-chain:P25 are 6:6:1; the H-chain is hydrophobic and provides crystalline like features to the silk thread, whereas the L-chain is more hydrophilic and relatively elastic, and the P25 protein is believed to play a role in maintaining the integrity of the complex (Inoue, et al. 2000; Tanaka, et al. 1999). Before fiber formation, a solution of the three proteins is secreted from two glands within the silkworm, assembling into twin filaments that emerge from an exit tube in its head (known as the spinneret) and dry upon exposure to air. The resulting core contains anisotropic β-sheet-rich nanocrystals that are loosely aligned with the fiber axis and dispersed in an unstructured matrix (Marsh, et al. 1955). Another pair of glands secretes

glue-like sericins (a set of serine-rich glycoproteins) that coat the fibroin filaments and ensures the cohesion of the cocoon by sticking the twin filaments together (Tsukada, et al. 1979; Iizuka 1969 ). Finally the fiber is coated with a variety of other proteins postulated to protect the cocoon against microbes and predators.

### 3.3.1. Usage of Silk Fibroin in Drug Delivery

For a drug to have its optimal effect it is important that its release profile is both reliable and controlled, particularly important in cases where the drugs have undesirable side effects. Silk proteins may find application in drug delivery as drug carriers owing to their biocompatibility and their highly tunable morphologies. Silk was also found to be biologically active. More recent studies with well-defined silkworm silk fibers and films suggest that the core silk fibroin fibers exhibit comparable biocompatibility *in vitro* and *in vivo* with other commonly used biomaterials such as polylactic acid and collagen (Onar 2004). Foams prepared via freeze-drying aqueous solutions of *B.mori* fibroin and aspirin were demonstrated to be capable of controlled release of the aspirin trapped in the scaffold. Preliminary in vitro kinetic studies showed a burst release profile for the aspirin, with a significant quantity of aspirin released in the first 2 h, followed by an almost constant rate of release thereafter (Tsukada, et al. 1994). Alginate and PLGA microspheres containing model enzyme drugs were coated with a *B. mori* fibroin film. The as-cast films retarded the rate of release of the drugs when compared to uncoated controls in vitro, and could be further retarded by induction of β-sheet formation by treatment of the films with methanol (Wang, et al. 2007b). *B. mori* fibroin microspheres prepared using lipid vesicle templates have been used for the efficient encapsulation and controlled release of an active model protein drug (horseradish peroxidase) *in vitro* (Wang, et al. 2007a).It was demonstrated that microspheres prepared using spidroins (eADF-4) are capable of the encapsulation of poorly water soluble substances such as β-carotene, and that these microspheres were undigested in artificial gastric fluid and completely digested in artificial intestinal fluid at 37$^{o}$C. This sort of controlled release gives them potential use as delivery vehicles for hydrophobic compounds (such as drugs or food ingredients) that remain intact in the stomach and release the compounds in the small intestine (Liebmann, et al. 2008).

## 3.4. Objective of the Study

Cyclodextrin complexation is an efficient tool to enhance bioavailability. In this study, it was aimed to increase solubility of rutin via inclusion complexation. Cyclodextrins increase the dissolution efficiency of rutin in water while bioactivity is preserved. In oral formulations, bioavailability is directly related with aqueous solubility so good bioavailability can be maintained when cyclodextrins are used. Also in drug delivery, it is important to be able to modify release behavior, by encapsulating Rt into CD's cavity release behavior of Rt could be changed, as well. Silk fibroin was selected as carrier since it is a biocompatible material and also biologically active. The main target for loading the active compound into silk fibroin film is to provide a support protecting antioxidant stability and modifying release behaviour of the compound.

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1. Materials

Dialysis tubes (6,000-8,000 MW, prepared as described in the product manual) were obtained from Milipore Chem. Co. (St. Louis, MO, USA). Gallic acid was purchased from Merck. Methanol (99.7%) and ethanol absolute-chromasol (99.8%) were obtained from Riedel. Formic acid (98-100 %) supplied from Riedel-de Haen. Hydrochloric acid and sodium hydroxide were obtained from Riedel. PBS tablets purchased from OXOID (Dulbecco's PBS tablets). Rutin (98.5 + %; MW: 664.58) was obtained from Merck Co. (Darmstadt, Germany). β-Cyclodextrin (MW: 1135) obtained from Sigma Aldrich. Water was deionized with Sartorious Unit Device. Silk fibroin from KOZA BİRLİK (Bursa, Turkey) was used as biopolymeric carrier to determine the release behavior of rutin from prepared silk fibroin films.

## 4.2. Methods

The methods included in this study can be summarized in two parts;

- ✓ The first part is the preparation of inclusion complex (IC) of rutin (Rt) with β-cyclodextrin (BCD). Change in the solubility of rutin before and after complexation with BCD was determined with phase solubility experiments. In addition, identification and characterization of rutin inclusion complex were carried out by spectrophotometric and thermal techniques.

- ✓ The second part is the incorporation of rutin into silk fibroin films. In this part, the characterization of silk fibroin before and after incorporation of rutin inclusion complex and free rutin was performed. Release behaviour of rutin and rutin inclusion complex from silk fibroin films was investigated. The antioxidant capacity of rutin released from silk fibroin was also determined.

The experimental procedure followed during this study is schematically represented in Figure 4.1.

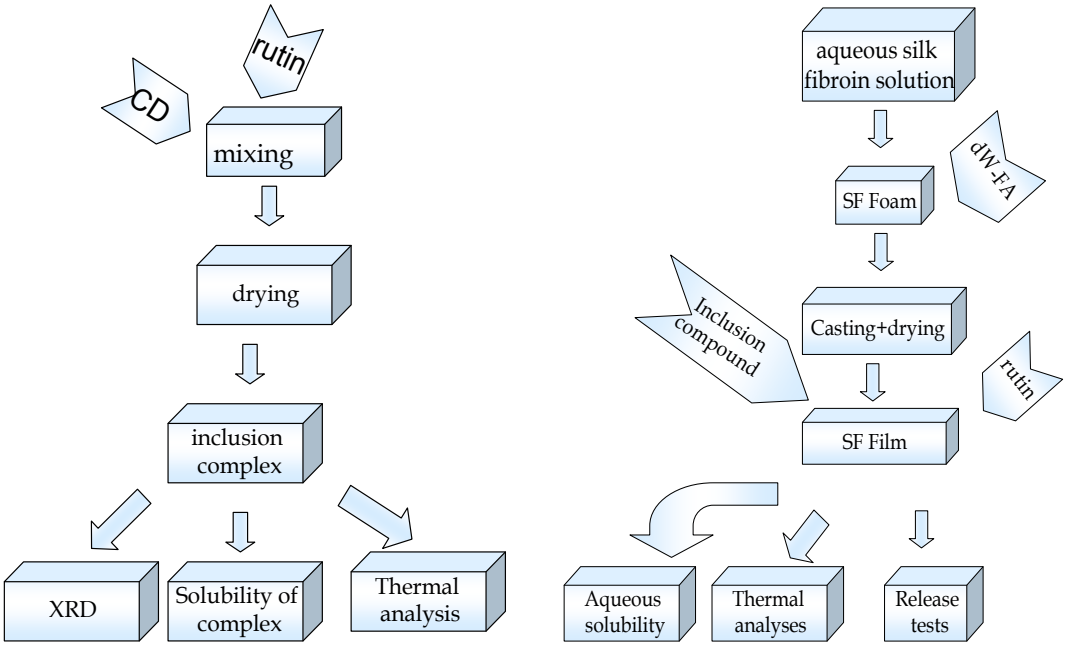


Figure 4. 1. Experimental Procedure.

## 4.3. Solubility Determination

### 4.3.1. Solubility of Rutin

Solubility of the rutin compound was determined by spectrophotometric method. Absorbance measurement was carried out at 255nm. In this method ;

- Aqueous solutions of rutin between 0 to 0.1 mM concentrations were prepared.
- The solution was stirred, till the rutin was mostly dissolved.
- After the filtration step, solutions were analyzed with respect to blank solution in UV-visible spectrophotometer (Thermo Multiscan spectrophotometer), in order to find rutin's saturation concentration in its aqueous solution.
- Calibration graphic of rutin is plotted, see Appendix A.

### 4.3.2. Solubility of Rt-BCD Inclusion Complex

Calculation of the solubility of the IC of Rt was done via spectrophotometric method, which mentioned above (also refer to section 4.4.1 for preparation of IC).

### 4.3.3. Dissolution Energy of Rutin

Free Rt, IC and PM of Rt that containing the same amount of rutin were suspended in 50 ml deionized water. These suspensions were placed in water bath at 25°C - 45°C - 65°C and shaked during 24 (or 6) hours. Samples are taken at 1st, 2nd, 3rd, 4th, 5th, 6th and at 24th hours and active constituent concentrations were determined spectrophotometrically. Then solubility energies were determined via Clasius-clapeyron equation (4.1.) that written below.

$$\log \frac{c_1}{c_2} = \frac{\delta Q_{sol}}{4.573} * \frac{T_1 - T_2}{T_1 * T_2} \qquad (4.1.)$$

$\delta Q_{sol}$ = dissolution energy (kJ/mol)

$T_1$ , $T_2$ = absolute temperatures (K)

$c_1$ and $c_2$ = solubilities (mM) at $T_1$-$T_2$ temperatures

## 4.4. Preparation of Materials

### 4.4.1. Inclusion Complexes of Rutin

The solid rutin complexes with BCD in a molar ratio of 1:1 were obtained suspending adequate amounts of both molecules in 40 ml deionized water. Stirring was carried out for 144 h, under controlled temperature (25± 0.01 °C) and then solutions were centrifuged in order to eliminate the uncomplexed rutin which had not reacted with BCD. Samples are freezed -18°C and solvent was sublimed under vacuum (- 45°C) yielding the solid complex as a pale yellow powder which were sieved to a fine particle powder. For pictures of IC and Rt see Appendix D1.

### 4.4.2. Silk Fibroin

#### 4.4.2.1. Silk Fibroin Solution

To handle the silk fibroin solution, raw silk was treated three times in boiling 0.5 wt % $Na_2CO_3$ solution (98 ºC) for 1.5 hour to remove the sericin (degumming). At each batch the silk was rinsed with distilled water. Degummed silk was dissolved in a ternary solvent system of $CaCl_2.2H_2O$ / $H_2O$ / EtOH solution (1:2:8 mole ratio) at 78ºC for 2 hours with shaking in water bath. The ratio of degummed silk to solvent was 1: 20 (weight/volume respectively). This solution was dialyzed for 3 days at 4 ºC in cellulose tubing with a molecular weight cutoff value of 6,000 against deionized water to remove $CaCl_2$ and ions. The ions check test was done by silver chloride. Silk fibroin solution was used after filtrating through Whatman No.1 filter paper. For pictures of materials see Appendix D2.

#### 4.4.2.2. Regenerated Silk Fibroin (RSF)

To obtain regenerated silk fibroin, filtrated silk fibroin solution was quickly put in a deep freezer (-80°C or -18°C) and then the solvent was sublimed under vacuum until foam like dry substance was supplied. For pictures of materials see Appendix D2.

#### 4.4.2.3. Silk Fibroin Film

2.5 % RSF is slowly dissolved in formic acid: water (1:1) binary solvent system. The mixture was stirred for 2 hours. Free Rt, PM of Rt and IC of Rt are individually added into film solution. In case of drug loading, the films were mixed for 2 more hours after drug addition. After all of the compounds are fully dissolved, film solutions poured into Polystyrene sterile petri dishes and casted under 25°C within working fume hood during 48 hours. The dried films were stored in a desiccator at 20°C until used to avoid contamination. The solubility of the films was controlled by immersing in deionized water and buffer solutions. See Appendix D3. for pictures of films.

## 4.5. Analysis of Inclusion Complexes via Phase Solubility Method

Excess amount of rutin was added to β-CD solutions prepared at different concentrations (0, 0.01,...., 0.30 M) with deionized water at pH 6.0. The suspensions were vortexed and sonicated for 1 h and kept on a horizontal rotary shaker (200 rpm) for 3 days. The suspension was filtered in order to obtain a clear solution. All samples were prepared in triplicate. Final clear solutions were diluted properly with deionized water (pH 6.0) to the calibration curve range, the concentrations of rutin in inclusion complex solutions were measured by UV-spectrophotometer (255nm). The apparent inclusion rate constant ($K_{1:1}$) can be calculated from the slope and intercept of phase solubility curve through use of equation 4.2. ;

$$K_{1:1} = \frac{slope}{S_o(1 - slope)} \qquad (4.2.)$$

where $S_0$ is the intrinsic solubility of drug.

## 4.6. Characterization

### 4.6.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were obtained using the standard KBr pellet technique (sample / KBr : 1 / 200 by weight); they were collected with Digilab FTS 3000 Mx FTIR spectrophotometer, at room temperature under vacuum in the wavenumber range from 4000 to 400 $cm^{-1}$ with a resolution of 2 $cm^{-1}$. The spectrometer was equipped with a DTGS-TEC detector. For all the spectra, the baseline correction and normalization (with respect to the most intense band) were carried out.

### 4.6.2. X-ray Diffractometer (XRD)

The changes in the crystalline state were monitored by X-ray diffractometer (Philips X'pert Pro) equipped with Ni filtered CuKα radiation source (λ=1.540560 Å) over 2θ range of 5° to 50° at a 10.15 s measurement time per step and a step size of 0.0334225°. The tube electric current was was operated at 40 mA and tube voltage was at 45 kV. Sample preparation for the X-ray analysis involved gentle grinding of the particles into a fine powder and packing of the powder into an aluminum sample holder with light compression to make it flat and tight.

Measurements with Powder X-ray diffractometry was applied to Rt, β-CD, PM and IC. X-ray diffractograms were used to determine whether crystallinity varies due to different forms of Rt (free, physically mixed or complexed).

### 4.6.3. Thermal Analysis

Thermal behaviour of free Rt , IC of Rt and PM of loaded SF film were determined by DSC ,TGA, and DtG analyses.

#### 4.6.3.1. Differential Scanning Calorimeter (DSC)

Thermal treatment was done for rutin, B-CD, their physical mixture and complex, respectively, in the 50 – 280°C temperature range at a 10°C / min heating rate, using aluminum pans under 50.0 ml/min nitrogen flow with DSC Q10 V9.4 Build 287 (TA Instruments, USA) instrument. Also SF films and Rt, PM, IC loaded SF films were also tested in the 20 – 520°C temperature range.

#### 4.6.3.2. Thermal Gravimetric Analysis (TGA) & Derivative Thermal Gravimetry (DtG)

The variation in thermal stability of Rutin due to its form (free, PM or IC) and Rt loaded SF films were also tested gravimetrically on a thermal gravimetric analyzer (TGA-51, Shimadzu) , using stainless-steel pans in the 40 – 800°C temperature range at

a heating rate of 10 °C/min, under 40 ml/min nitrogen flow. Its derivative was taken and analysed via the software in order to find degredation temperatures (Derivative Thermal Gravimetry).

## 4.7. Parameters Investigated

Significant parameters to be investigated was decided for *in vitro* drug release study in order to evaluate effect of some predetermined factors's on release behaviour of rutin from silk fibroin materials. Here are the predetermined parameters of release experiment;

1. Release temperature setted at 37°C
2. Form of Rutin ( free, complexed, and physically mixed with CD )
3. pH of the release medium (4.0 ; 7.3) [ isoelectric point of silk fibroin; physiologic pH]

## 4.8. Dissolution Profile and *in vitro* Rutin Release

Before release tests, dissolution profile of rutin (Rt), physical mixture (PM) and inclusion complex (IC) was also evaluated. Rutin in amounts that exceeded its aqueous solubility was carefully weighted into erlen mayer flasks, deionized water added and then slowly stirred at 37°C. Aliquots were taken from solution with 15 min time intervals and the same volume of blank medium with the same temperature as that of the tested medium was added immediately. Samples are centrifuged. The concentration of rutin from the supernatant was measured spectrophotometrically at 255 nm with Thermo Multiskan Spectrophotometer. The same procedure was repeated for analyses of PM and IC. All analyses were conducted triplicate, and the average values were plotted.

In vitro release of free and complexed rutin from silk fibroin based material (SBM) was studied with the help of Thermo Varioskan Spectrophotometer and 12 well plates which was a system -developed by E.Altiok- comprising a 12 well plate and perforated cartridges within each wells. Specifically, SBM were put into a well, containing release medium (PBS at pH 7.3 or Acetate buffer at pH 4.0). The whole

apparatus was placed in spectrophotometer with horizontal shaking at 100 rpm and thermostated at 37°C. At set time intervals, spectroscopic readings were done for blank sample solutions at 255 nm. All experiments were carried out with three samples, and the average values were plotted with standard error bars.

## 4.9. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) (Philips XL 30S FEG) analyses were performed to detect the morphological changes in the films. Films were cut into 1 x 1 cm pieces using a sharp razor for the cross-section observation. The surface of films were coated with gold-palladium (100-200 Å thickness) prior to imaging to eliminate charge effect with Emitech K550X. Also Rt, IC, PM and BCD were analyzed without coating with gold-palladium at X 1,000 X 3,000 and X 5,000 magnification. Pictures at X 1,000 magnification were found sensible and that's why only they were evaluated.

# CHAPTER 5

# RESULTS AND DISCUSSION

## 5.1. Determining Aqueous Solubility of the Free and Complexed Rutin

Firstly, maximum wavelengths were determined for Rt (255 and 351 nm) and IC (260 and 355 nm) with spectroscopic scannings. No maximum wavelength was observed in the spectrum scanning of BCD, see Appendix B.

Maximum solubility of rutin at 25°C is 0.0462 g/L which was compatible with the results in literature and maximum solubility of its IC reached 15.252 g/L. Aqueous solubility of rutin at room conditions is increased ~330 fold via inclusion complexation.

### 5.1.1. Calculation of Dissolution Energy with Respect to Temperature

Dissolution energy values supported the fact that free rutin needs more energy for dissolution as compared to the IC and PM of rutin, which proves the occurence of inclusion complex formation. Results were compatible with the findings of Taneri et al. who used the same method for calculation of solubility energies.

Table 5. 1. Dissolution energy values for free Rt,PM and IC.

| **Form of the compound** | | $\delta Q_{sol}$ **(kJ/mol)** | | |
|---|---|---|---|---|
| | **t (h)** | **25-45° C** | **45-65° C** | **25-65° C** |
| **Free Rutin** | 6 | 4898.46 | 7283.97 | 6016.22 |
| | 24 | - | - | - |
| **PM** | 6 | 4521.56 | 6573.63 | 5483.09 |
| | 24 | - | - | - |
| **IC** | 6 | 1737.46 | 473.15 | 1145.05 |
| | 24 | 468.98 | 127.67 | 309.05 |

## 5.2. Analysis of Inclusion Complexes via Phase Solubility Method

*Rutin-BCD Complexes*

The formation constants for rutin-BCD inclusion complexes were calculated via the UV-visible spectrophotometer; Spectrophotometric readings were done by Thermo-Varioscan Multiplate reader and with Thermomicrotiter 96-well plates.

The initial solubility of rutin in pure water is observed as 0.02 mM at 25ºC reaction temperature.

Statistical evaluation for the effect of BCD on rutin solubility; as shown at the Table 5.2.; since $p<0.05$ in all of the situations, we can state that there's a significiant effect of CD addition on solubility value of rutin in its aqueous solution.

Table 5. 2. Statistical evaluation for the effect of BCD on rutin solubility

| Anova: Single Factor | |
|---|---|
| Groups | Variance |
| **RT-CD** | 0.25191 |
| **CD** | 0.00067 |
| Source of Variation | **P-value** |
| Between Groups | 0.00035 |
| | |

| Anova: Single Factor | |
|---|---|
| Groups | Variance |
| **RT-CD** | 0.25191 |
| **RT** | 0.1201 |
| Source of Variation | **P-value** |
| Between Groups | 0.02892 |
| | |

| Anova: Single Factor | |
|---|---|
| Groups | Variance |
| **RT-CD** | 0.25191 |
| **CD** | 0.00067 |
| **RT** | 0.1201 |
| Source of Variation | **P-value** |
| Between Groups | 0.00032 |

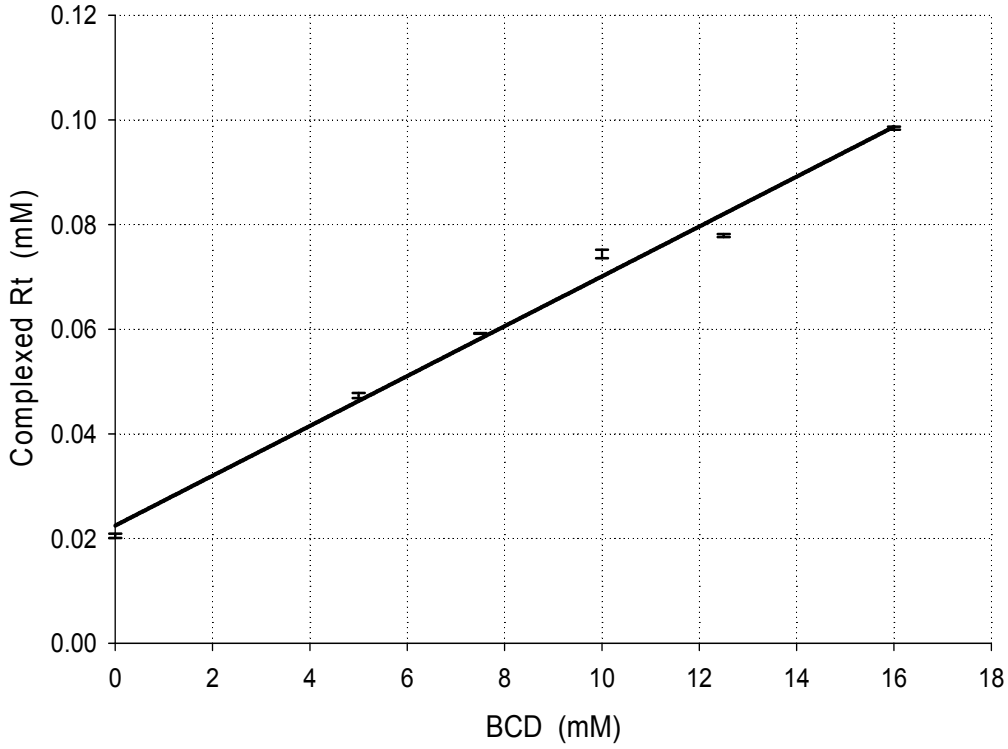


Figure 5. 1. Phase solubility chart for Rutin-BCD complexes

The solubility of rutin increased linearly as a function of BCD concentration and the solubility curve was found to be $A_L$ type. The apparent stability rate constant, calculated from the slope and interscept of phase solubility curve, via equation 4.2. and was found to be 262 $M^{-1}$ at temperature of 25 ºC in deionized water assuming 1:1 complex. The correlation coefficient was found to be 0.9987. Phase solubility graph shown in Figure 5.1.

## 5.3. Characterization

Characterization results supported formation of inclusion complexes which were compatible with the previous literature studies.

### 5.3.1. FTIR

Rutin crystals show a characteristic carbonyl absorption band at 1654.4 $cm^{-1}$, assigned to aromatic ketonic carbonyl stretching. In the case of IC in particular, the

characteristic aromatic carbonyl-stretching band of rutin shifted to 1651 $cm^{-1}$ for rutin-β-CD complexes respectively, along with reduced intensity and sharpness of the same band. Changes in the characteristic bands of rutin confirm the existence of the complex as a new compound with different spectroscopic bands.

In PM a sharp peak observed at 1654 $cm^{-1}$ as in the BCD's proving existence of Rt and BCD seperately within the physical mixture.

Both of β-CD and IC show a characteristic secondary cyclic alcohol band at 1024 and 1026 $cm^{-1}$ assigned to C-OH stretching vibrations. This band proves Rt is almost fully embedded in CD cavity.

If all of the spectra are analyzed in binary groups as Rt-PM and BCD-IC ;

Between 400-1700 cm-1 wavenumbers general profile of Rt and PM are very similar, as in the CD and IC (except some shifts or existence of some diffrent peaks mentioned above). Data showing that optical properties of IC is very similar BCD in fact proving Rt is embedded within the BCD's cavity.

The FTIR spectra of IC and PM were compared with those of β-CD and rutin are presented in Figure 5.2. See Appendix C. for characteristic frequency range on FTIR spectra for different groups.

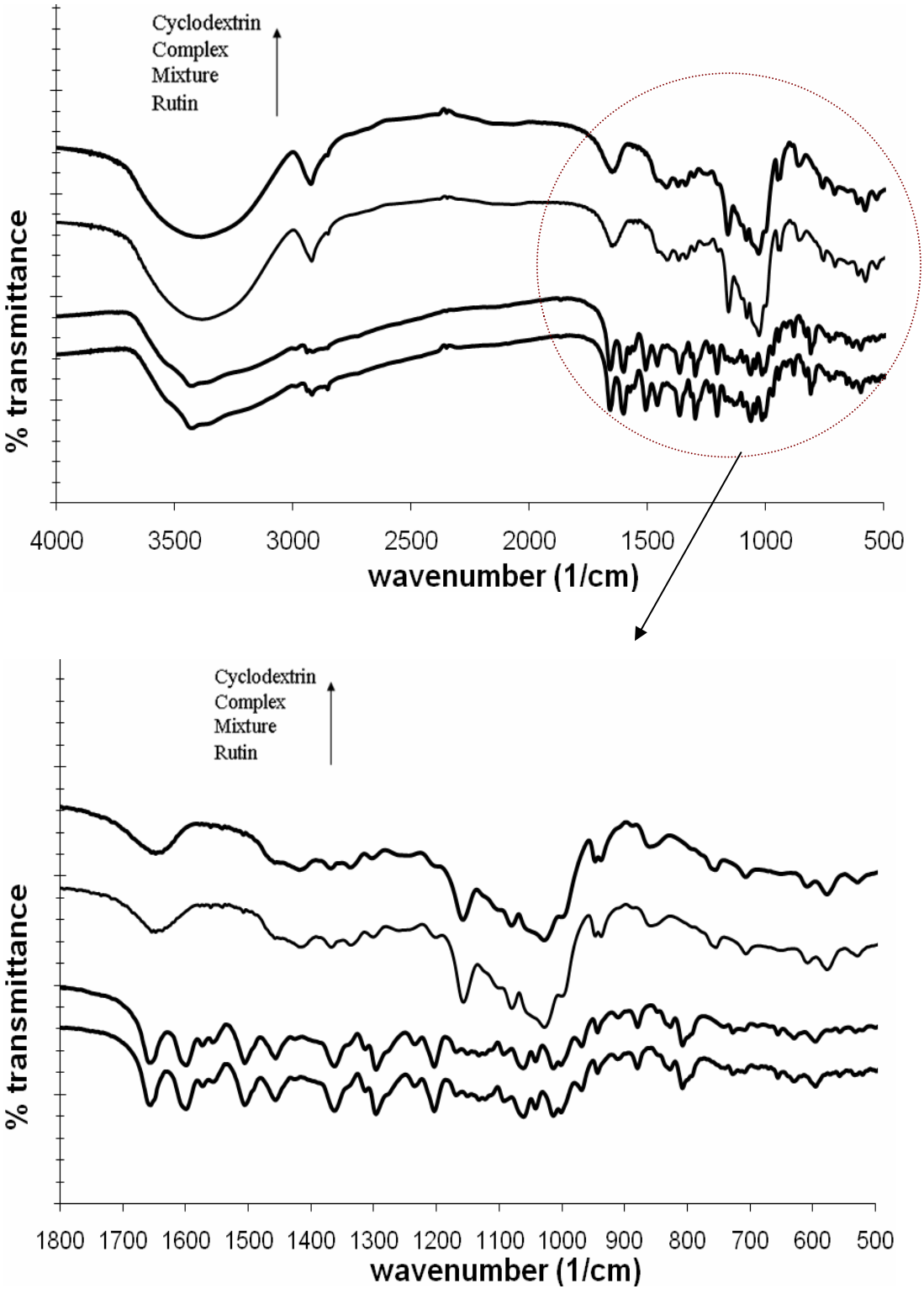


Figure 5. 2. FTIR spectrum of BCD, Rt, IC, PM in the form of ( % ) transmittance

## 5.3.2. XRD

Powder X-ray diffractometry is a useful method for the detection of cyclodextrin complexation in powder or microcrystalline states. The diffraction pattern of the complex is supposed to be clearly distinct from that of each of the components. Crystallinity is determined by comparing representative peak heights in the diffraction patterns.

The powder XRD pattern of rutin shows (Fig. 5.4.) highly crystalline nature as evident from the sharp peaks observed at diffraction angle of 14.79°, 16.62°, 22.01°, 26.09° and 26.62° of 2θ values. Crystallinity peaks were still detectable in the physical mixture with β-CD at 12.32°, 17.63°, 22.61°, 26.65° and 34.54° of 2θ values (Fig.. 5.3.).

A total drug amorphization was observed in XRD patterns of Rt-BCD (1:1) were characterized only by large diffraction peaks in which it is no longer possible to distinguish the characteristic peaks of the flavonoids and also the peaks observed were not sharp and had lower intensity when compared with the others' spectrum. These results, confirm that rutin doesn't present as a crystalline material and its complex exist in the amorphous state.

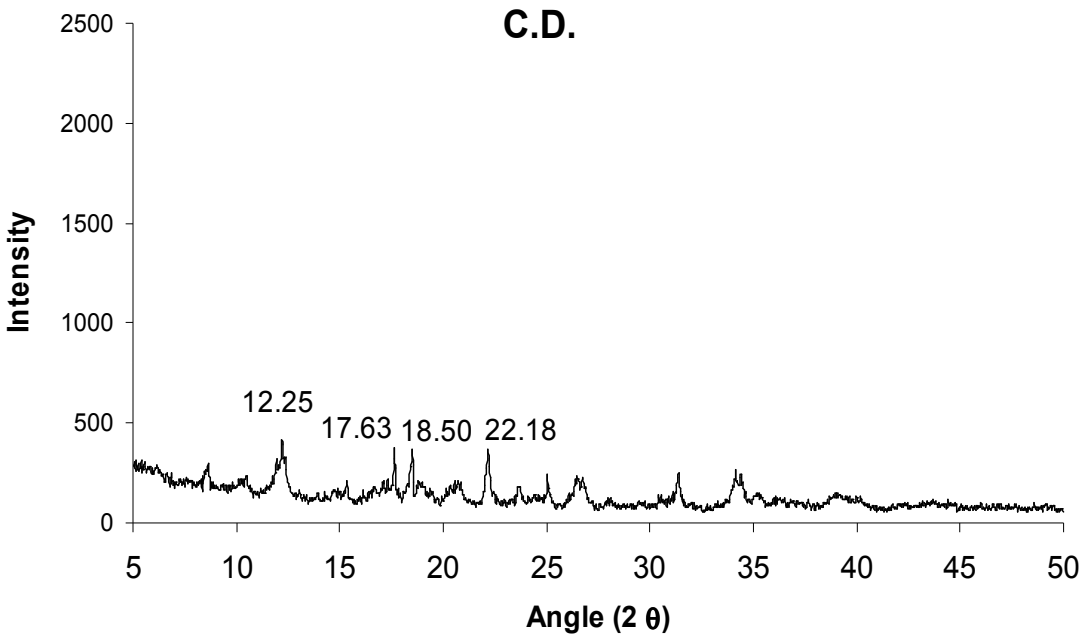


Figure 5. 3. X-Ray diffractogram of BCD

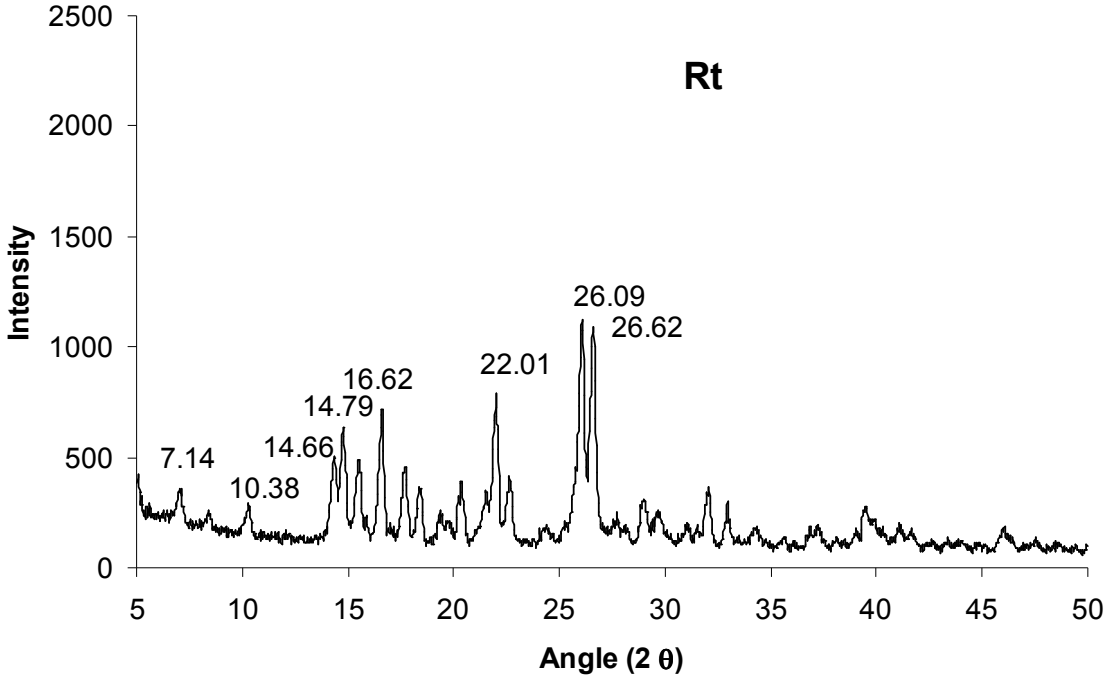


Figure 5. 4. X-Ray diffractogram of Rt

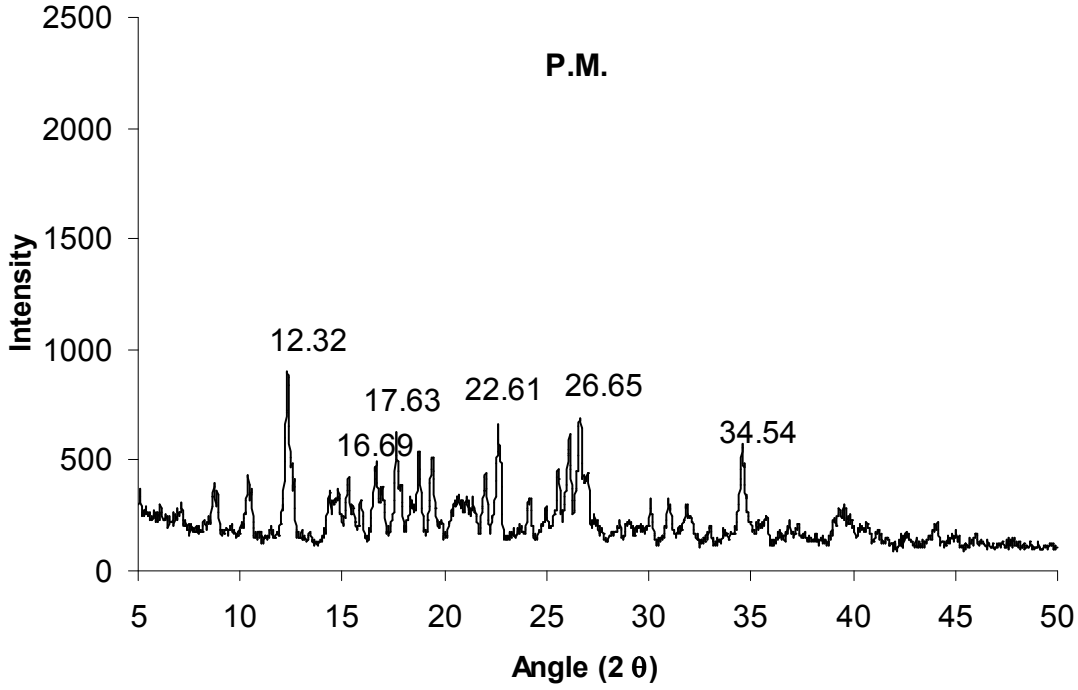


Figure 5. 5. X-Ray diffractogram of P.M.

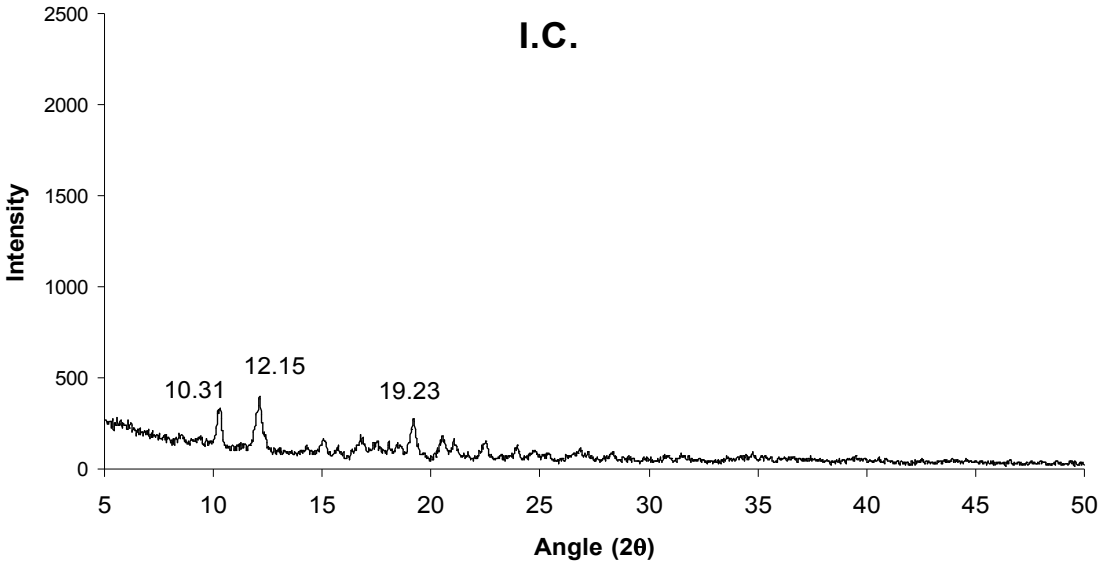


Figure 5.6. X-Ray diffractogram of IC

Evidently, the X-ray diffraction pattern of the complex is remarkably different from that of β-CD, rutin, or their mixture. The complex has a new solid phase. The result is consistent with that obtained by differential scanning calorimetry analysis given in section 5.3.3.1.

## 5.3.3. Thermal Analysis

Thermal analysis of CD complexes has been used first to differentiate between inclusion complexes and physical mixtures and second to characterize the special thermal effects due to molecular entrapment, upon a well defined, standard heating process. Only such complexes can be studied by these methods which have a guest substance having a melting or boiling point below the thermal degradation range of the cyclodextrin or which are volatile in the temperature range of 60-250°C (Fromming, et al. 1994).

### 5.3.3.1. DSC

Rutin presented a different calorimetric behavior, where several phase transition were observed. The DSC curves reveal the presence of rutin polymorphism (Merck 1996). The stability of the inclusion complex is largely governed by the hydrophobic

nature of the included molecule. The included molecule can be replaced by a relatively more hydrophobic molecule. For studying such replacement reaction, isolation of the complex is often needed. A simple DSC method was proposed here to study such reaction without the isolation of the complex. DSC provides the melting points of the compounds, and from the variation of the melting points the nature of the included molecule can be obtained. When guest molecules are embedded in CD cavities or in the crystal lattice, their melting, boiling or sublimation points generally shift to a different temperature or disappear, *see* Figure 5.7. and Table 5.3. $\beta$-cyclodextrin molecule gives a peak at 147 ºC and in endothermic region. This gives us the melting point at crystalline reagon. In the DSC diagram of the complex, we can see a shift in B-CD's melting point (from 147 ºC to 149 ºC). If we compare DSC diagram for rutin molecule with IC we can see that the rutin's character does not match with the complex's character. This shows us the existance of a newly occurred compound 'inclusion complex', which is more similar to rutin and less similar to B-CD**.** The physical mixture of $\beta$-cyclodextrin and rutin gave two peaks at 143 ºC and 180ºC in endothermic reagon. This result may arouse many alternative evaluations. Two seperate peaks might have occurred from existence of two different compounds in sample or the second peak might indicate decomposition reactions.

For DSC results of SF film and P.M., Rt, I.C. loaded SF films *see* Figure 5.8. and Table 5. 4. There are no slight difference in the calorimetric profile of SF films and Rt and PM loaded SF films, but in the IC loaded SF film there is a little shift in the last exothermic peak. This shift can be due to the Rt embedded in CD's cavity, that's why showing similar character to CD and enhancing thermal stability of Rt.

Table 5. 3. Melting ($T_m$ ) and Glass transition ($T_g$) Temperatures of Rt, BCD, PM and IC.

| Sample | Tm (°C) | Tm (°C) | Tg (°C) |
|---|---|---|---|
| CD | 147.12 | | |
| I.C. | 149.4 | 294.19 | 109.97 |
| P.M. | 143.37 | 179.88 | 118.14 |
| Rt | 182.34 | | 117.15 |

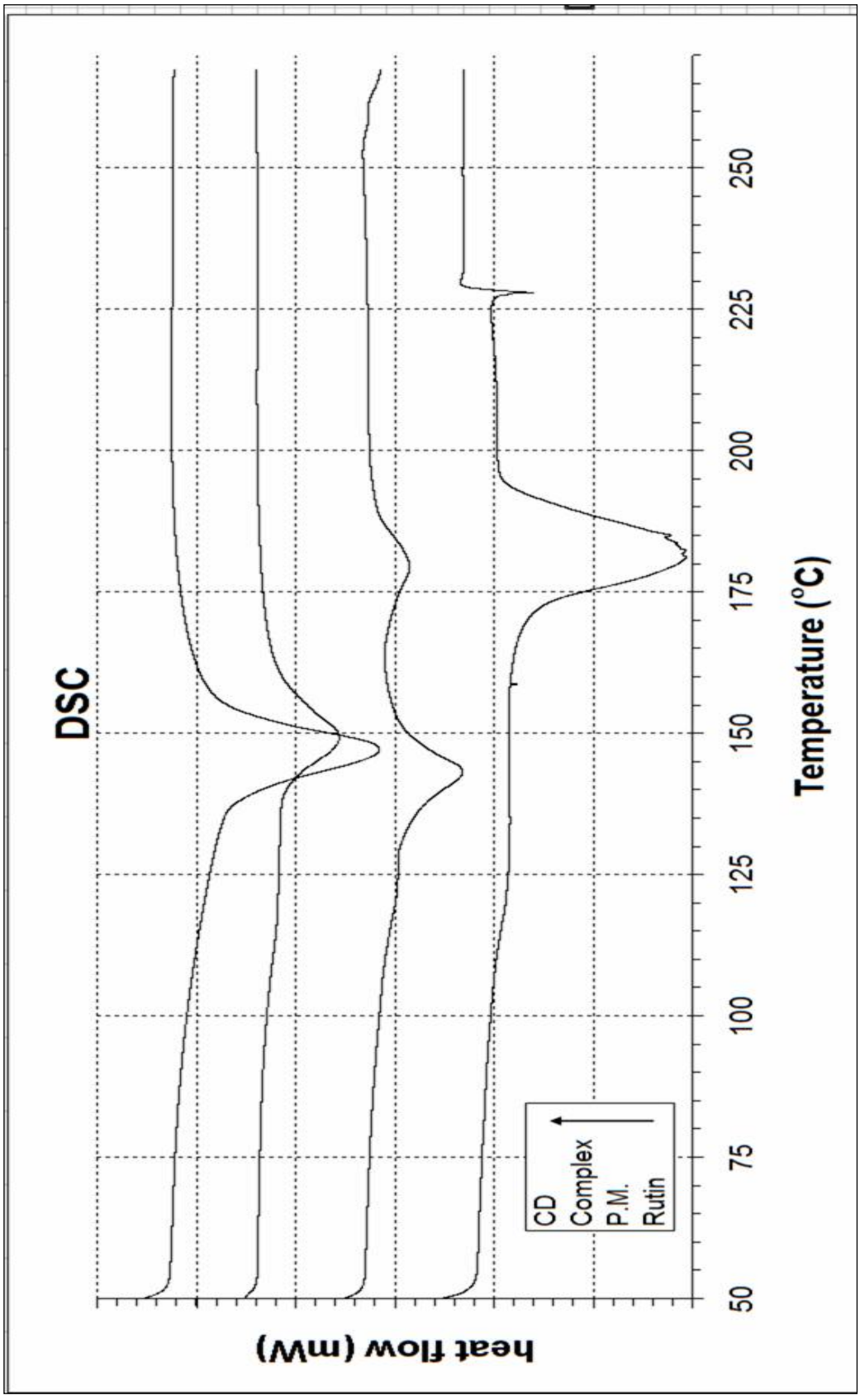


Figure 5. 7. DSC curves of the compounds; Rt, PM , IC, BCD

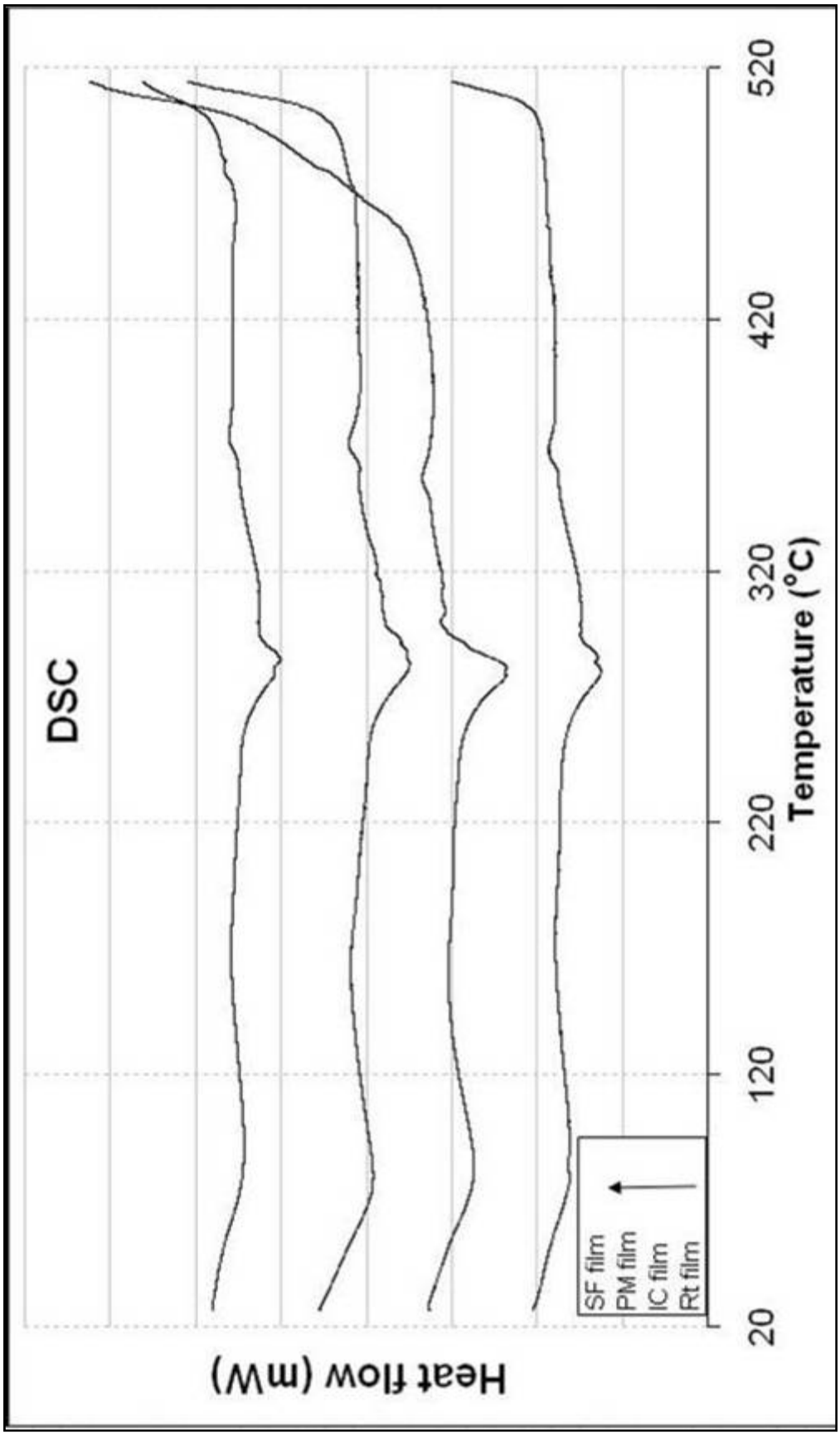


Figure 5.8. DSC curves of SF film and P.M., Rt, I.C. loaded SF films.

Table 5. 4. DSC peaks of SF film and P.M., Rt, I.C. loaded SF films

| DSC peaks | IC loaded | Rt loaded | PM loaded | SF film |
|---|---|---|---|---|
| Exothermic | 93 °C | 89 °C | 89 °C | 100 °C |
| | 283-286 °C | 285 °C | 287 °C | 284 °C |
| | 307°C | 289 °C | 292 °C | 289 °C |
| Endo | 359°C | (exo) 365-377 °C | 360-380 °C | 375 °C |

### 5.3.3.2. Thermal Degredation with TGA & dTG

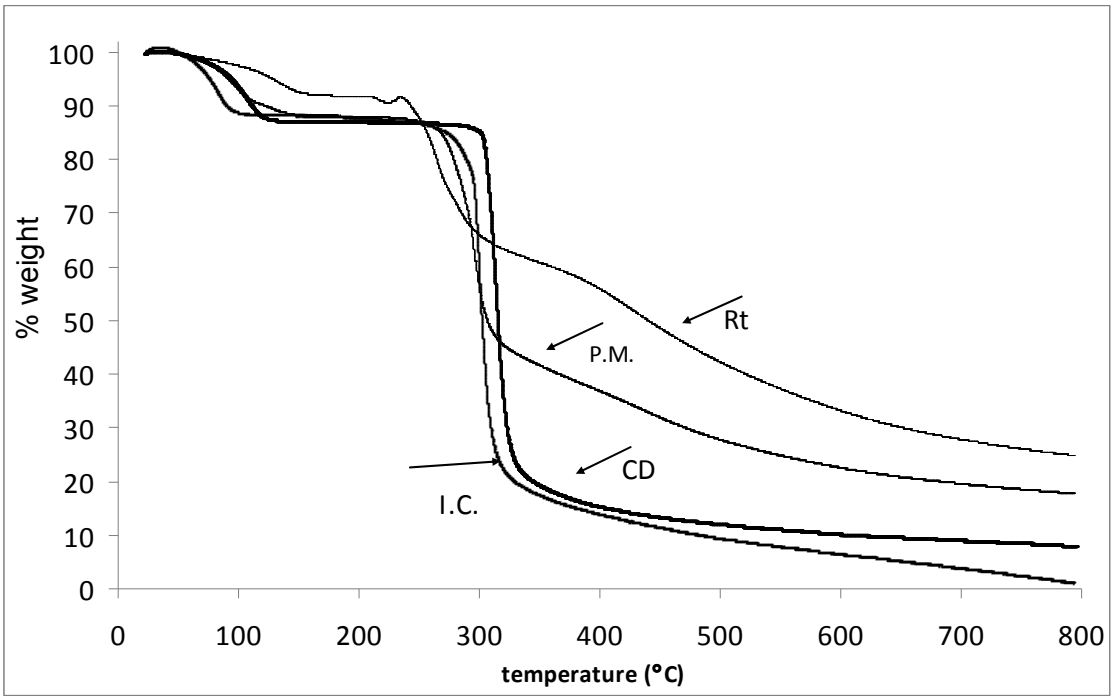


Figure 5. 9. TGA curves of P.M., Rt, I.C. and CD

The process of weight loss of the analized cyclodextrins consists of three stages:

- The first at a temperature lower then 100°C, probably due to loss of absorbed water (≤10%);
- The second stage develops in the temperature range between 250°C and 350°C and it is associated to a weight loss of 70-80%, with formation of residue ("char");
- The last stage is concerned with a relatively slow process of thermal degredation of the char (Trotta, et al. 1998).

The TG curve of rutin showed that thermal decomposition for this substance occurred in four stages, and whose mass loss depended on the gaseous atmosphere used and the heating rate (Costa, et al. 2002).

The comparison of the TG and DSC curves with the DSC–photovisual picture of the rutin (see Appendix E.) reveals that the rutin dihydrate suffers molecular transformation with a mass loss in the first two stages (TG curve) which are related to the loss of two water molecules to become anhydrous (Merck 1996). This fact can be an example for rutin trihydrate's thermal manner. Another aspect observed was that the stoichiometry for decomposition reaction, where the mass losses in the first three stages for the rutin 30.5%. This corresponds to 62.2% of the rutin are decomposed in the fourth stage (see Table 3.2.).

The TG isothermal profile for rutin flavonoid presented two decomposition stages as determined by the tangent TG analysis (Costa, et al. 2002).

The rutin presented in the photovisual system the first peak of the phase transition (DSC curve) at 177 °C and picture B at 180 °C confirms the change in the sample behavior that should be related to the molecular rearrangement of the rutin polymorph in a plastic substance. The subsequent peaks of phase transition at 214, 230, 240, 248 °C (DSC curve) and picture C at 214 °C provide evidence for the boiling process with chemical reaction without mass loss in the TG curve. The data obtained in the DSC curve shows the decomposition of rutin occurring with effervescence (see Appendix E).

As we seen from dTG results; P.M. 's melting behavior is more similar to Rt's melting behavior, besides that I.C. shows a similar melting pattern more similar to the cylodextrin's. Dtg results of IC- and Rt shows that thermal properties of Rt enhanced via inclusion complexation. Refer to figures 5.9, 5.10 and 5.11. In Table. 5.5. approximate thermal degredation temperatures can be observed.

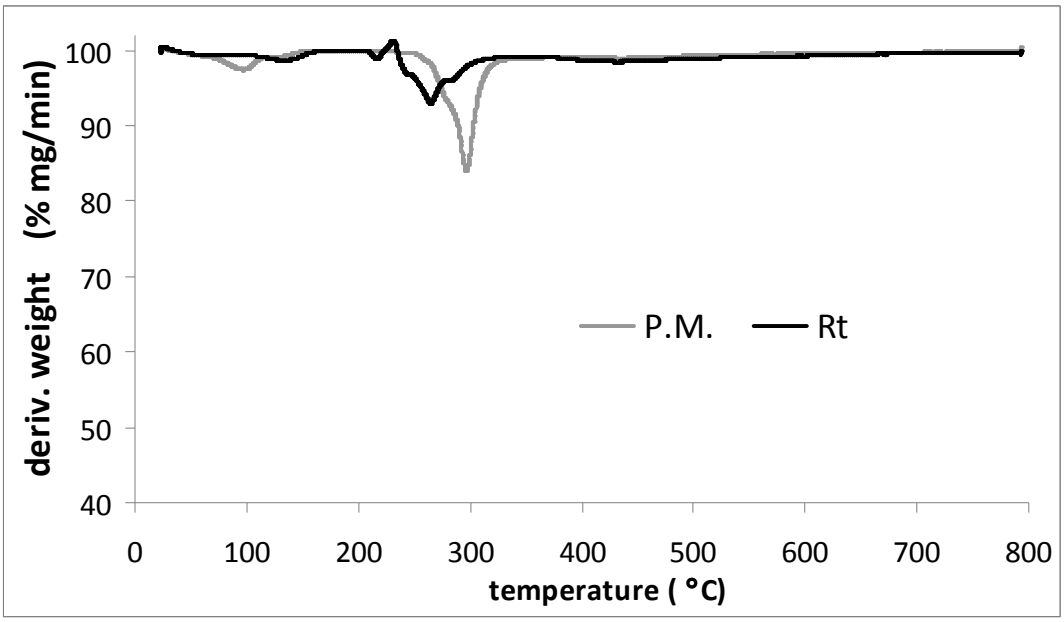


Figure 5.10. dTG curves of P.M. and Rt

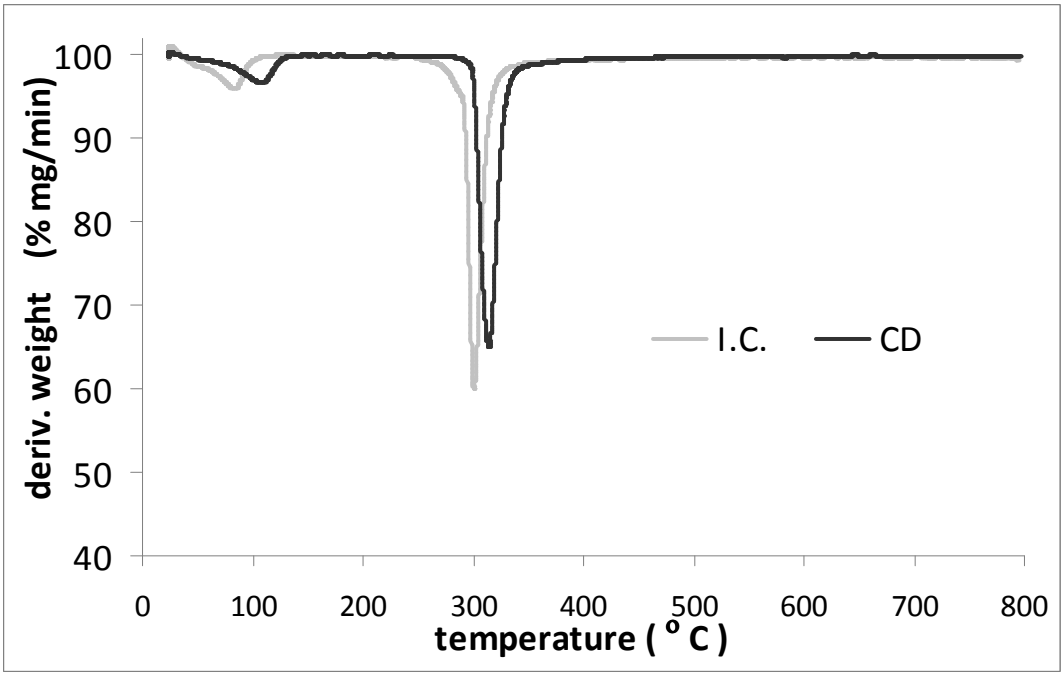


Figure 5. 11. dTG curves of I.C. and CD

Table 5.5. Thermal degredation temperature (°C) values

| I.C. | BCD | Rt | P.M. |
|---|---|---|---|
| 310 | 317 | 269 | 301 |

Table 5.6. dTG temperature($^{o}$C) peaks

| Dtg temperature peaks ($^{o}$C) | IC loaded | Rt loaded | PM loaded | SF film |
|---|---|---|---|---|
| | 79.23 | 74.1 | 80.46 | 83.81 |
| | 322.47 | 314.82 | 320.68 | 316.6 |
| | 688.78 | 576.36 | 570.43 | 562.7 |
| | | 610.71 | 630.22 | 625.1 |

For observing thermal behaviour of SF films see figures 5.12, 5.13 and 5.14.; also in Table 5.6. approximate thermal degredation temperatures can be observed. A negative shift was observed in the degredation peaks Rt loaded SF films proving presence of rutin. Also lowering effect of rutin on thermal stability of SF films was smaller in PM loaded SF film and smallest in IC loaded SF films showing that presence of CD was enhancing Rt's thermal stability.

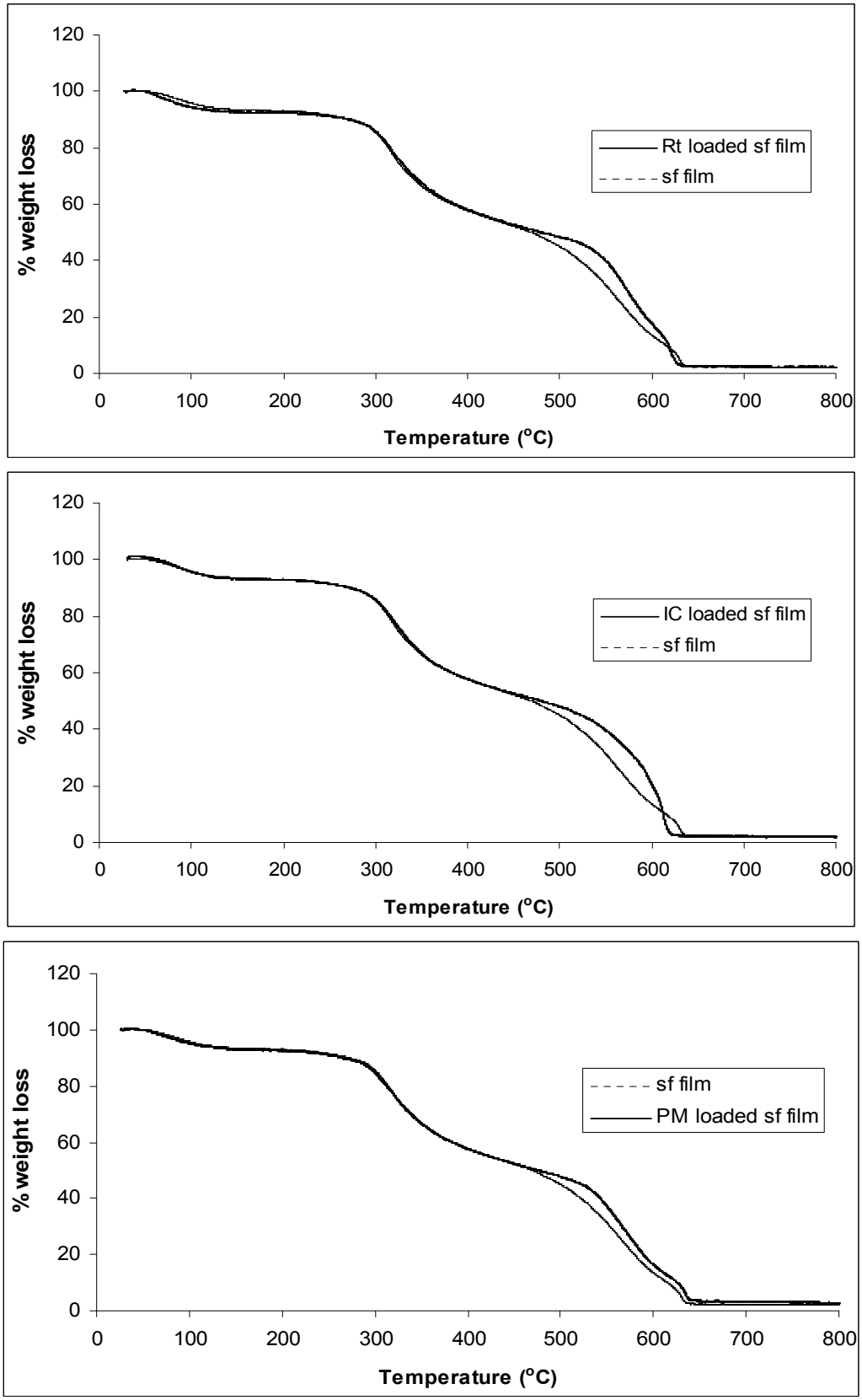


Figure 5. 12. TGA curves of SF film and P.M., Rt, I.C. loaded SF films

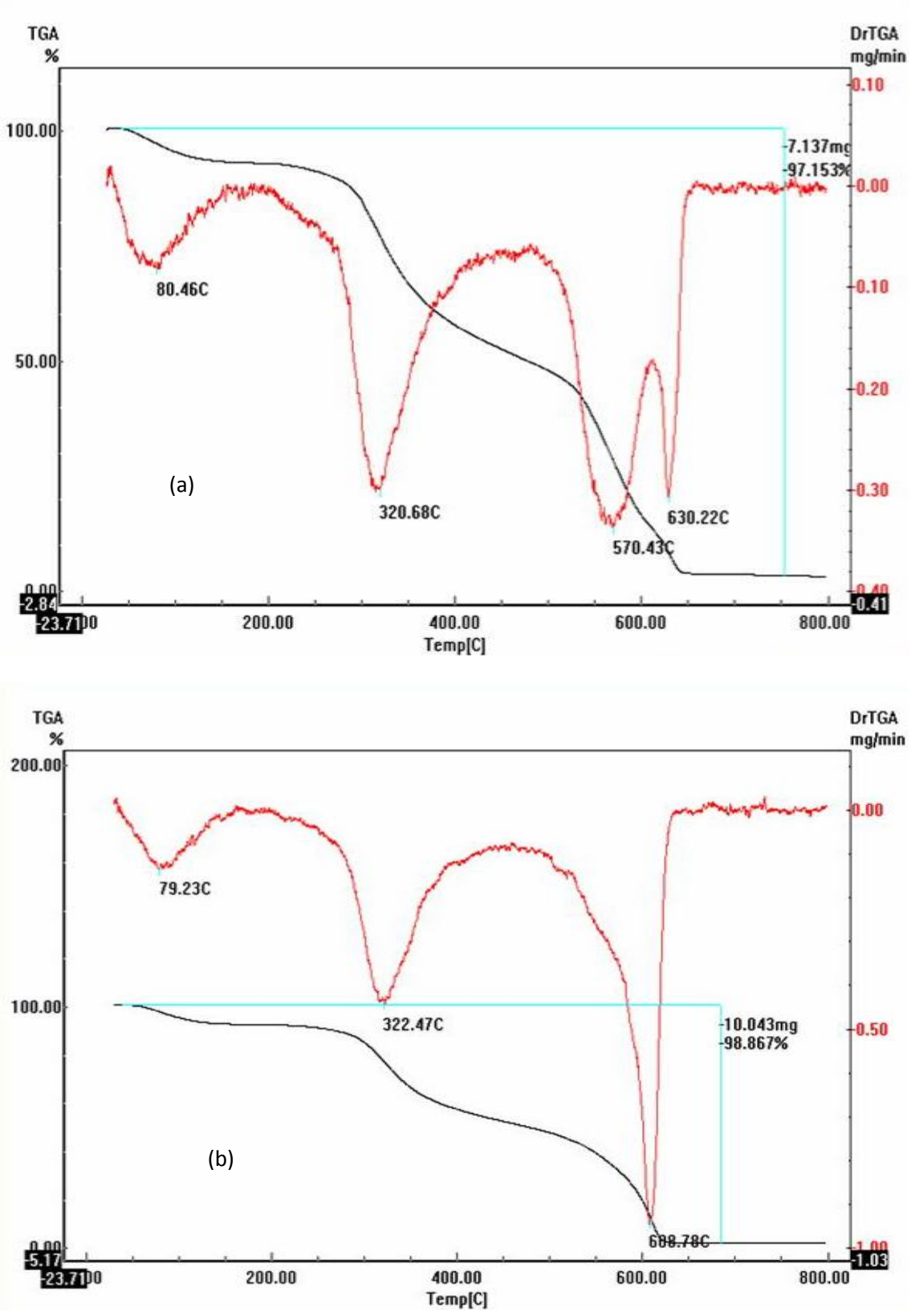


Figure 5. 13. TGA-DTG curves of P.M. (a) and I.C. (b) loaded SF films

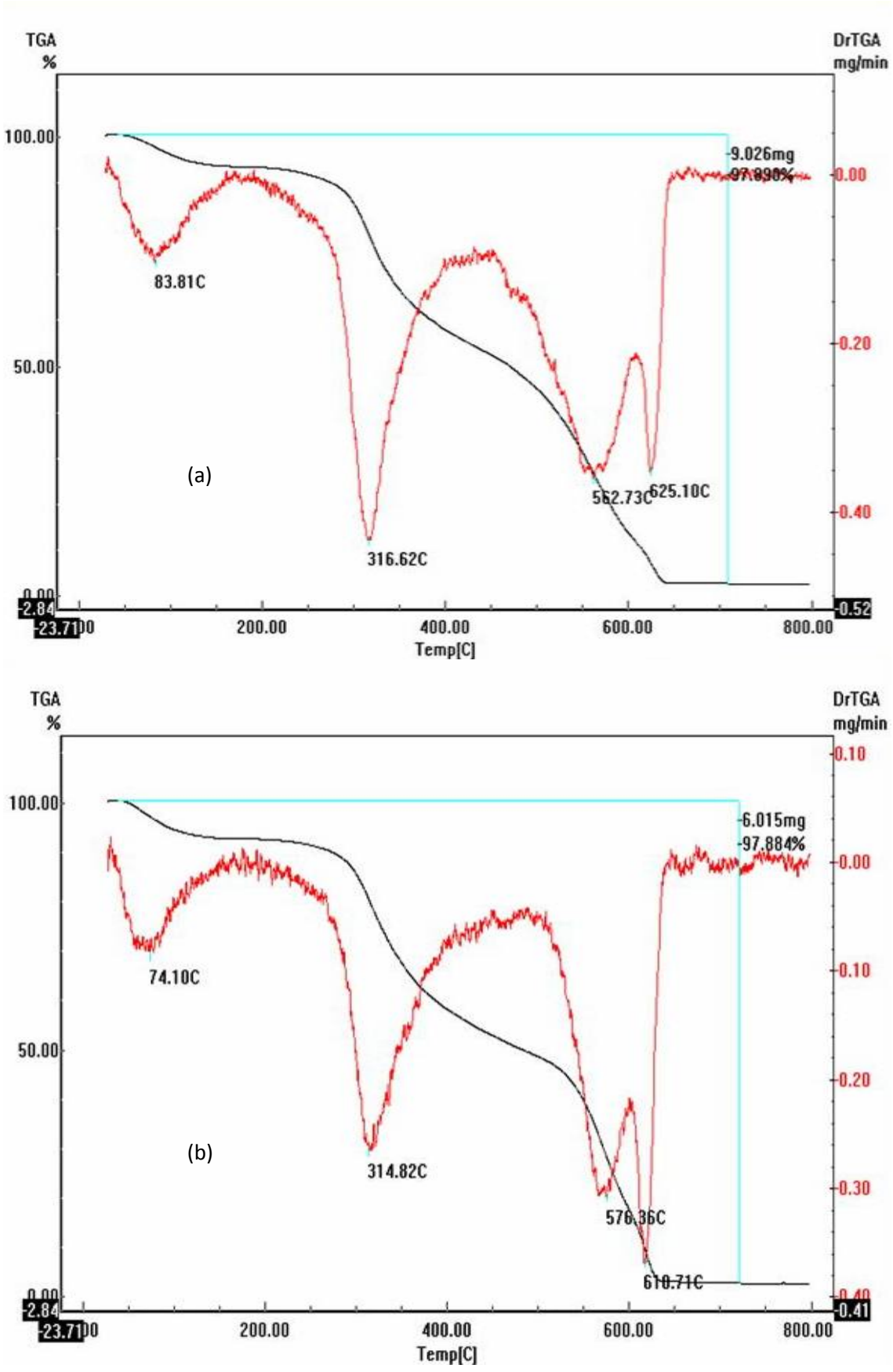


Figure 5. 14. TGA-DTG curves of SF film (a) and Rt loaded SF films (b)

## 5.4. Dissolution Profile and *in vitro* Drug Release

All analyses were conducted triplicate. The graphic is plotted with the average of these 3 values.

*Dissolution Profile*

The dissolution characteristics of free Rt and IC of Rt are given in Table 5.5. More than 70% of drug was released within 20 min of the starting of dissolution when ß-CD was the complexing agent. These values were much higher than those for physical mixtures as well as for free Rt. Dissolution efficiency at 40 min were calculated. The $DE_{40}$ (%) values also support the above results i.e., IC of rutin-ß-CD in 1:1 molar ratio showed higher dissolution efficiency (Table 5.7.) than the physical mixtures.

The graphic (Figure 5.15) shows how solubility of rutin compound changes in the presence of BCD in the aqueous solution or when it is in the form of cyclodextrin complex (at 37°C).

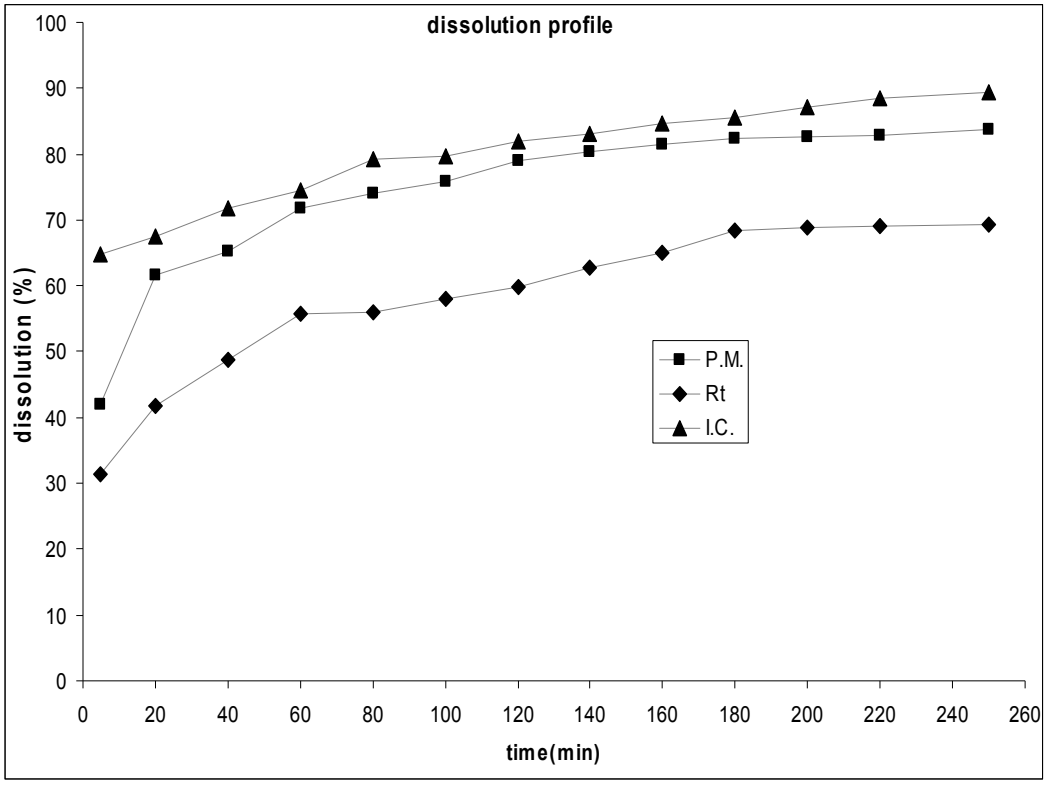


Figure 5. 15. Dissolution Profiles of free Rt, PM of Rt and IC of Rt

Table 5.7. $DE_{40}$ for Rt and Rt-CD binary systems (Mean ± SD Values; n=3)

| Product | $DE_{40}$ (%) |
|---|---|
| **Rt** | 48.65 ± 0.037 |
| **PM of Rt** | 65.33 ± 0.144 |
| **IC of Rt** | 71.90 ± 0.068 |

*Drug Release from Silk Fibroin Films*

*Release Profile for IC of Rt and Free Rt from SF Film*

As we see from the graphics (Figures 5. 16. ; 5. 17. and 5.18.) almost % 50 of rutin is released from SF film within the 5th hour. The rest of the active component is released within 24 hour completely at a slower rate. Release behaviour of active compound was mostly the same in both acidic and neutral medium (acetic acid buffer at pH= 4.0 and PBS at pH=7.3; 10 mM; 37°C; 100 rpm shaking). It can be observed in Figure 5.18. that a burst release occurs for IC loaded SF film at the beginning in physiological conditions due to high dissolution rate and ratio of IC in both neutral and acidic medium.

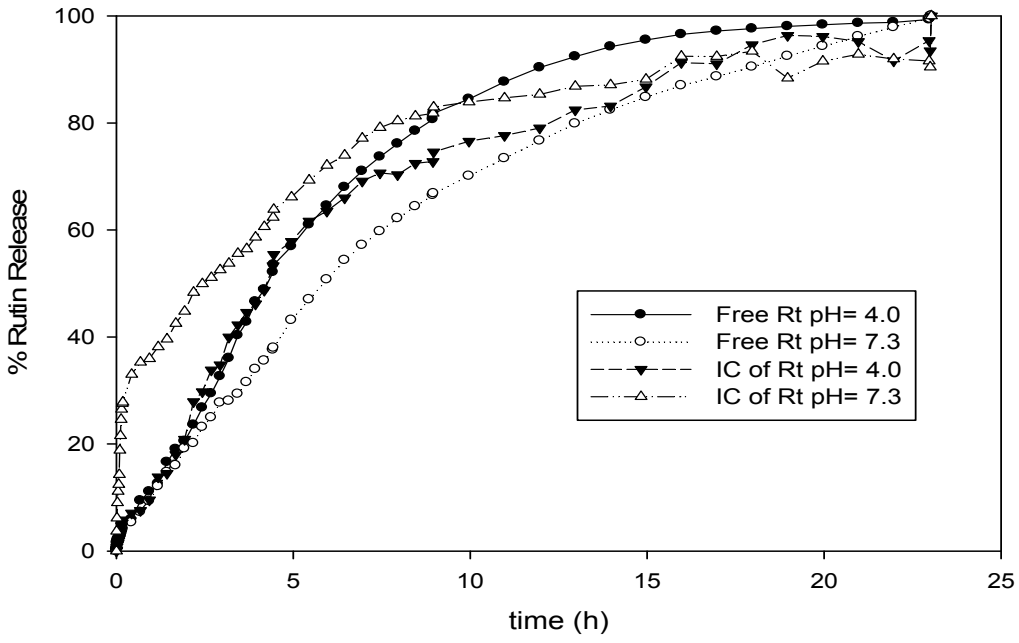


Figure 5. 16. Release profile for IC of Rt and free Rt from SF film

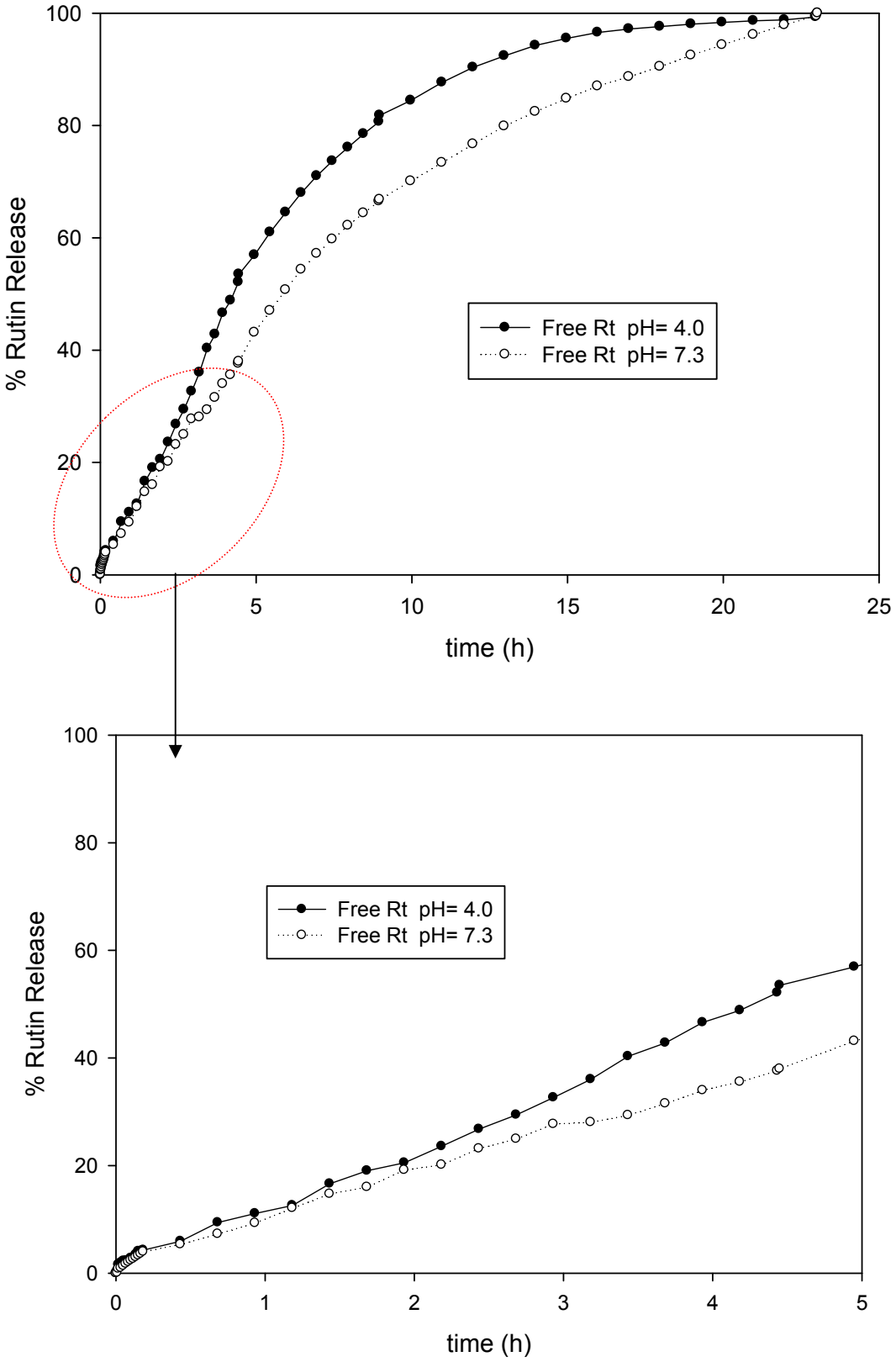


Figure 5. 17. Release profile for Rt from SF film

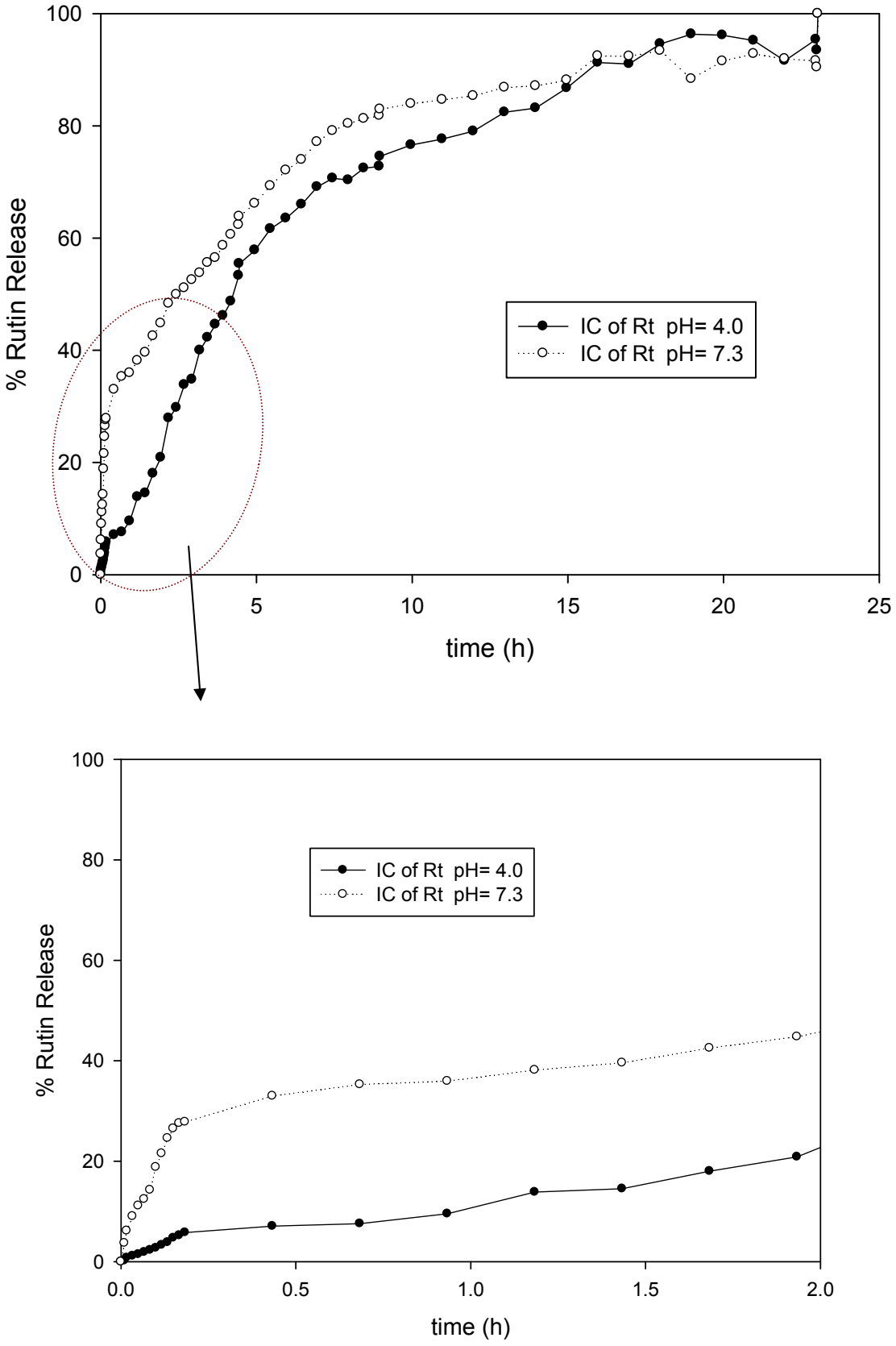


Figure 5. 18. Release profile for IC of Rt from SF film

## 5.5. SEM

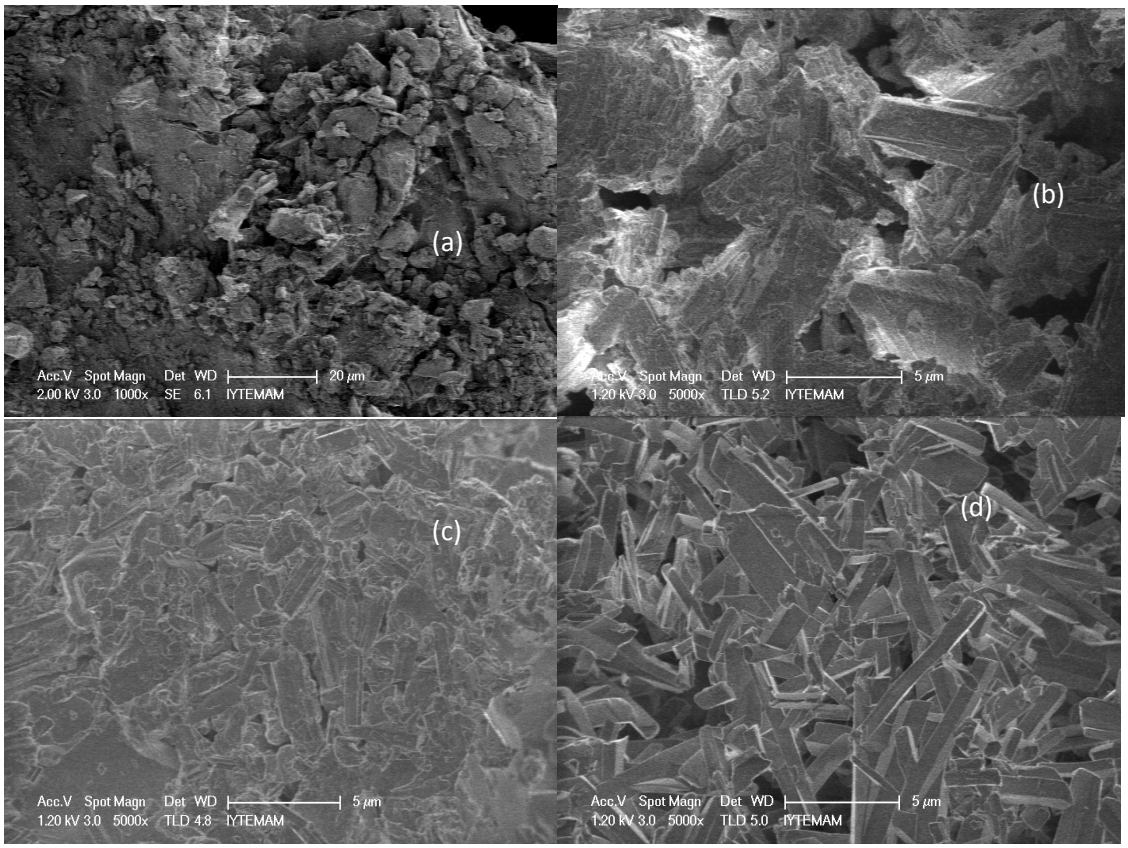


Figure 5. 19. SEM image of BCD (a) Rt (b) P.M. (c) IC (d)

The photomicrographs of the samples obtained by SEM are shown in the Figure 5.15. BCD particles Figure 5.19.(a) presented a parallelogram shape, whereas Rt figure 5.19.(b) presented columnar crystals. PM figure 5.19.(c) did not suggest an interaction between both molecules,because rutin crystals simply covered surface of BCD particles. IC presented amorphous particles with shrunken spherical shape figure 5.19.(d), a finding that is in accordance with the studies conducted by Haiyun et. al. and Sri et al. , which employed XRD to demonstrate that Rt/CD complexes exist in amorphous state (Borghetti, et al. 2009). The physical mixtures showed particles of BCD embedded with Rt particles and a comparable morphology with pure compounds taken separately. In contrast, a drastic change in the morphology and shape of particles was observed in 1:1 freeze-dried products revealing an apparent interaction in the solid-state (Pralhad, et al. 2004).

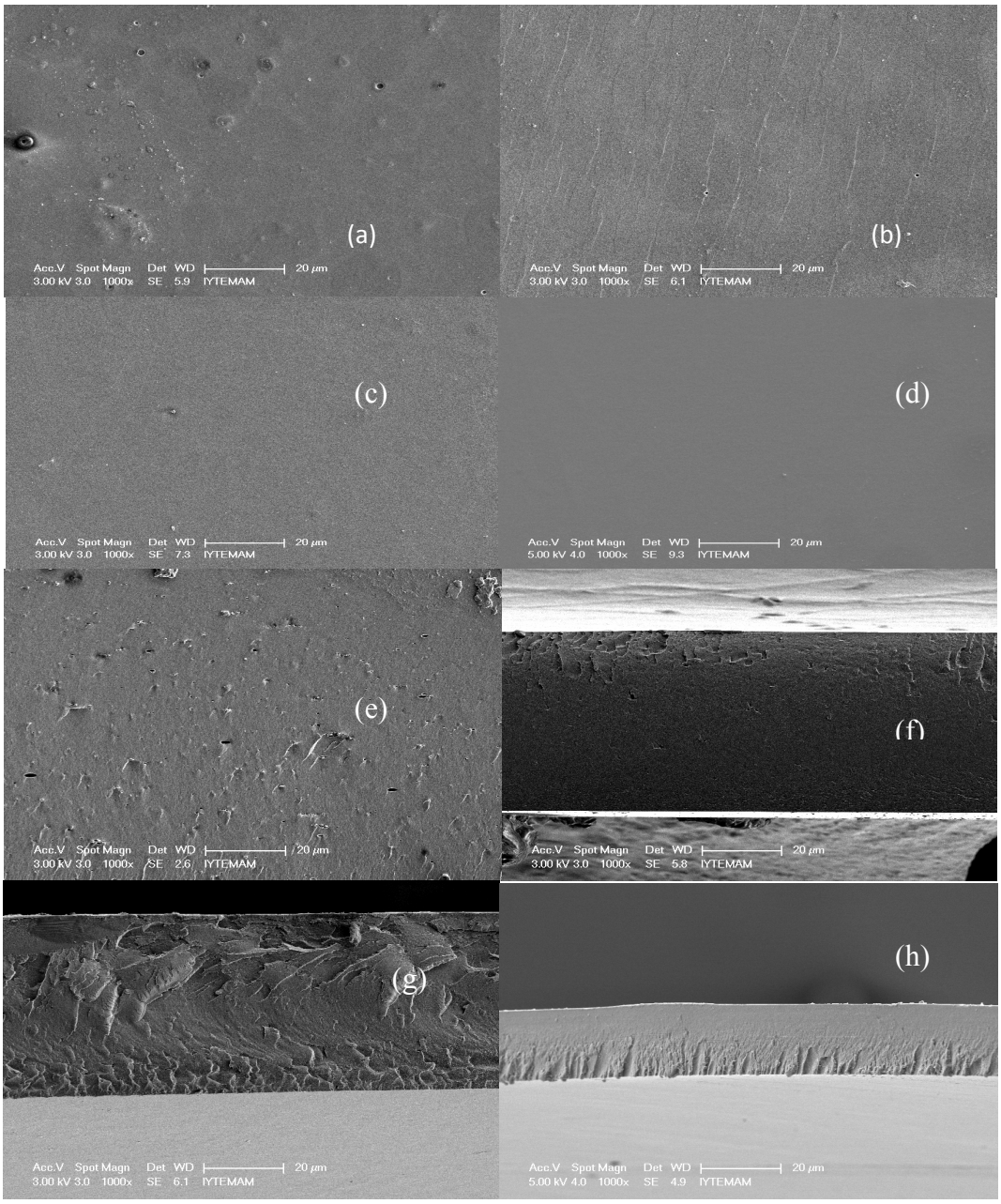


Figure 5. 20. Surface image of a) Control film, b) Rt film, c) IC film, d) PM film ; cross section image of e) Control film f) Rt film g) IC film h) PM film with SEM

Surface images of SF films with and without Rt were obtained by SEM magnified at 1000x (a) Control film, (b) Rt, (c) IC and (d) PM loaded film see Figure 5.20. Also cross section images of the SF films obtained by SEM magnified at 1000x; e) Control film, f) Rt, g) IC and h) PM loaded film see Figure 5.20. From SEM observations, it could be concluded that at high concentrations of Rt, semi-uniform distribution was observed in SF films while totally uniform distribution was observed in IC loaded SF films. Microscopic phase separation and cracks were not occurred in these films. Cross section images of SF films revealed dense film structure.

# CHAPTER 6

# CONCLUSION

Among all of cyclodextrins, BCD is preferred because of its suitable cavity sizes and low price. Hence, in our present work, Rutin-β-CD solid complex was prepared. The stability constant of BCD complex was calculated as 262 $M^{-1}$. Aqueous solubility of rutin was increased via inclusion complexation. Improvement in the hydrophilicity of rutin by CD might have contributed to the enhancement of dissolution rate. An increasing effect of temperature on aqueous solubility of free rutin, and its complex were also observed. The calculated solubility energies showed the energy needed to solubilize in water was highest in free Rt and lower for PM and IC. Characterization analyses were applied successfully. Analysis of the complexes by X-ray diffraction, DSC and FT-IR methods showed considerable interaction of BCD with rutin. Results of dissolution profile of Rt, PM and IC showed that addition of CD has an increasing effect on solubility rate and amount. Solid inclusion complexes exhibited higher dissolution efficiencies than their corresponding physical mixtures. Silk fibroin based films were prepared and loading of Rt, PM and IC into silk fibroin based films was applied successfully. By release tests it was investigated that most of the Rt – *independent from the form, whether free or complexed*- released from SF films within the first 5 hours (burst release occurs) and the rest of it released slowly within 24 hours. Electron microscobe analyses showed that films have a homogenous and dense morphology. Consequently, silk fibroin is useful to load natural compounds into silk fibroin films in order to modify their release period within physiological conditions. In the next coming studies it is planning to test antioxidant,antimicrobial activity, toxicity of flavonoid or IC loaded SF films and the antioxidant activity of the flavonoids released from the silk fibroin films in 24 or 48 hours.

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# APPENDIX A

# CALIBRATION PLOT FOR RUTIN

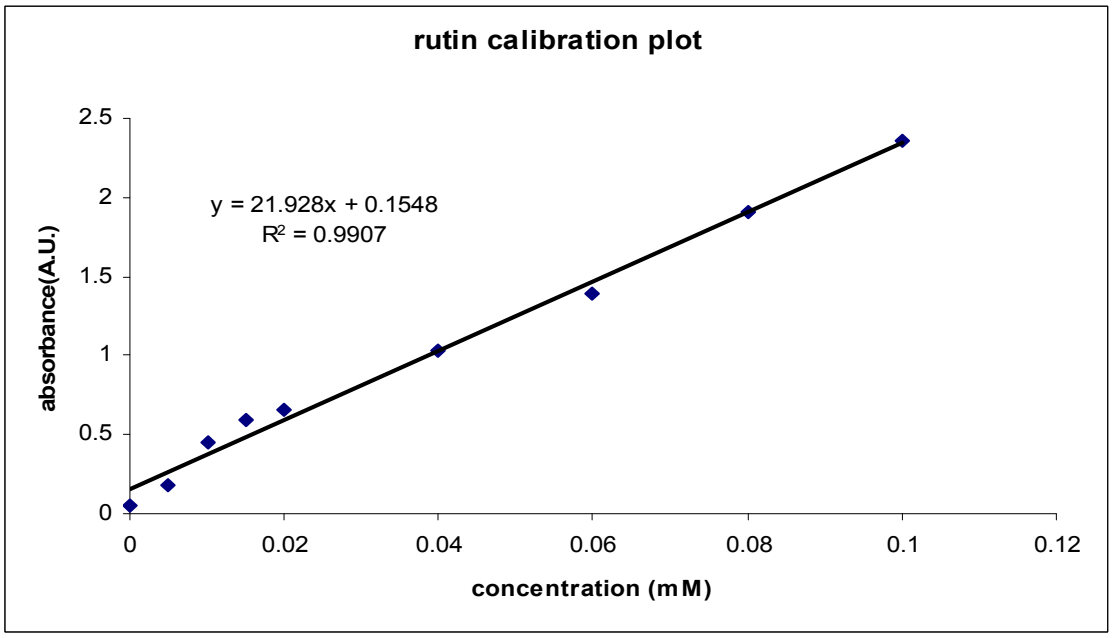


Figure A. 1. Calibration curve for rutin

# APPENDIX B

# SPECTRUM SCANNING OF COMPOUNDS USED

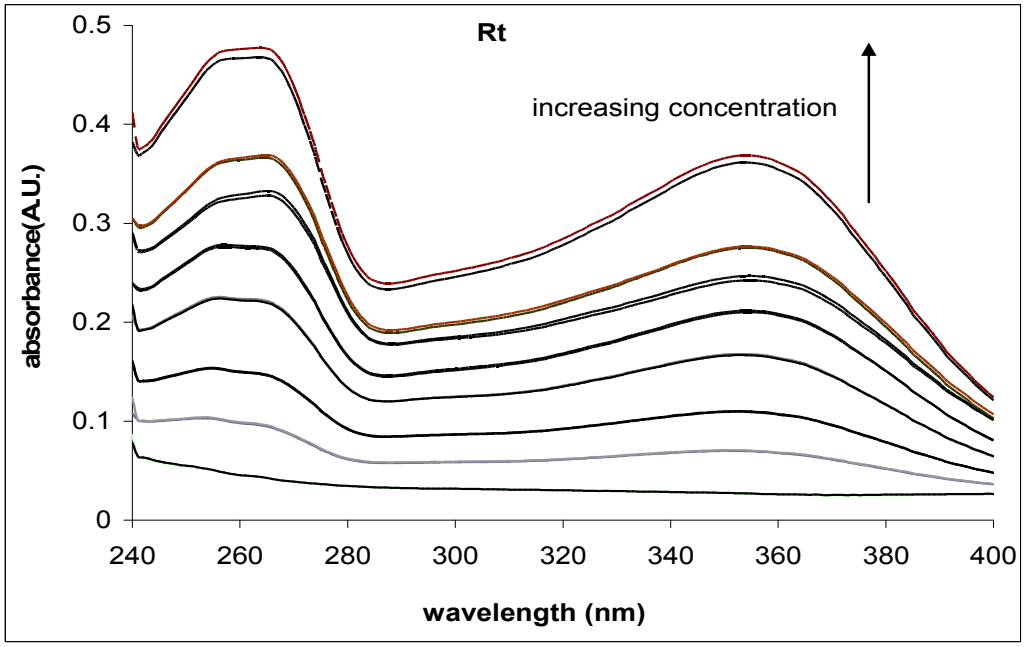


Figure B. 1.UV- Spectrum Scanning of rutin at different concentrations maximum wavelength for rutin is determined as 255; 351 nm from the graphic.

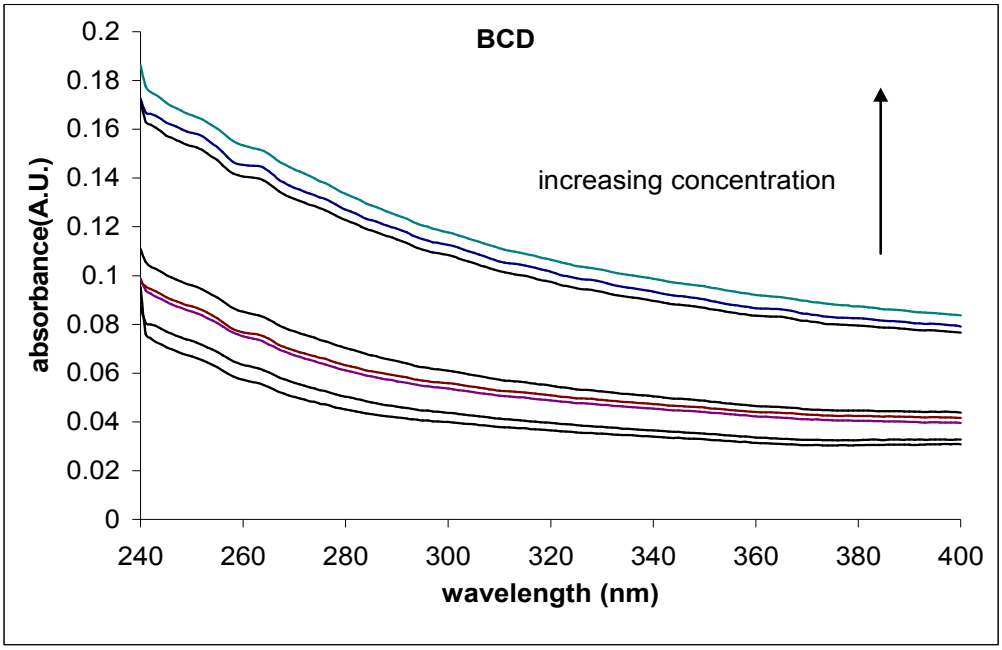


Figure B.2.UV- Spectrum Scanning of BCD at different concentrations it does not have a maximum absorbance value at a specific wavelength.

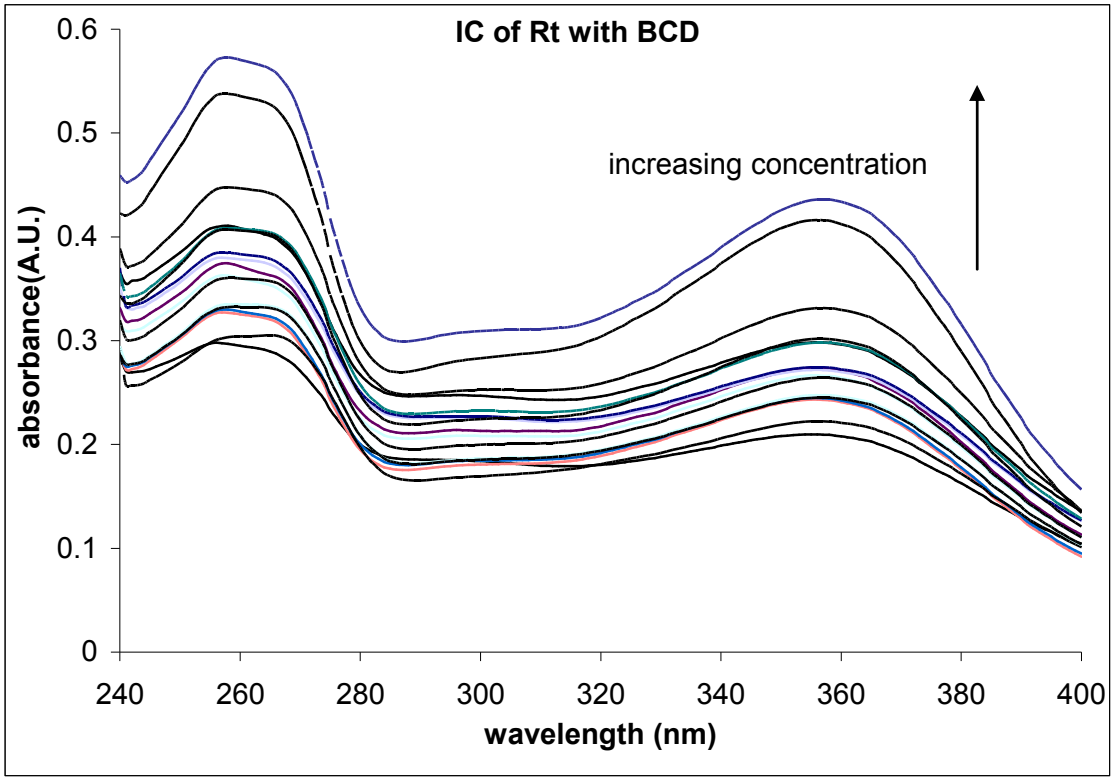


Figure B.3.UV- Spectrum Scanning for IC of rutin at different concentrations the maximum wavelengths of the I.C. has shifted towards 260 and 355 nm.

# APPENDIX C

# INFRARED SPECTROSCOPY WAVELENGTH TABLE

Table C. 1. Characteristic Frequency range on FTIR Spectra for Different Groups (Source: Infrared 2010)

| Group | Frequency Range ($cm^{-1}$) |
|---|---|
| **OH stretching vibrations** | |
| Free OH | 3610-3645 (sharp) |
| Intramolecular H bonds | 3450-3600 (sharp) |
| Intermolecular H Bonds | 3200-3550 (broad) |
| Chelate Compounds | 2500-3200 (very broad) |
| **CH Stretching vibrations** | |
| =-C-H | 3280-3340 |
| =C-H | 3000-3100 |
| C-CH3 | 2862-2882, 2652-2972 |
| O-CH3 | 2815-2832 |
| CH2 | 2843-2863,2916-2936 |
| CH | 2880-2900 |
| **C=-C Stretching Vibrations** | |
| C=-CH (terminal) | 2100-2140 |
| C-C=-C-C | 2190-2260 |
| C-C=-C-C=-CH | 2040-2200 |
| **C=O Stretching Vibrations** | |
| Nonconjugated | 1700-1900 |
| Conjugated | 1590-1750 |
| Amides | ~1650 |
| **C=C Sretching Vibrations** | |
| Nonconjugated | 1620-1680 |
| Conjugated | 1585-1625 |
| **CH Bending Vibrations** | |
| CH2 | 1405-1465 |
| CH3 | 1355-1395, 1430-1470 |
| **C-O-C Vibrations in Esters** | |
| Formates | ~1175 |
| Acetates | ~1240, 1010-1040 |
| Benzoates | ~1275 |
| **C-OH Stretching Vibrations** | |
| Secondary Cyclic Alcohols | 990-1060 |
| **CH out-of-plane bending vibrations in substituted ethylenic systems** | |
| -CH=CH2 | 905-915, 985-995 |
| -CH=CH-(cis) | 650-750 |
| -CH=CH-(trans) | 960-970 |
| C=CH2 | 885-895 |

# APPENDIX D

# PICTURES OF MATERIALS PREPARED

## D1. Rt and IC

Figure D. 1. Picture of IC (left) and Rt (right)

## D2. SF Solution and SF Foam

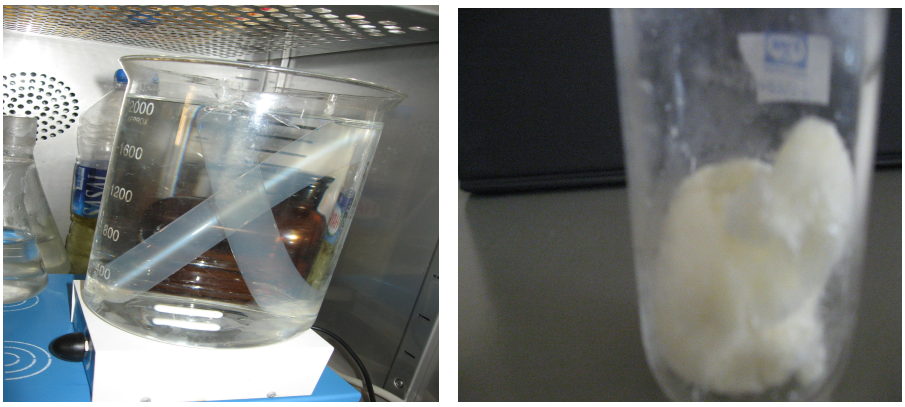

Figure D. 2. Picture of aqueous SF solution in dialysis bag (left) and SF foam (right)

## D3. SF Films

Figure D. 3. Picture of SF film in formic acid (a) Rt loaded SF film (b) IC loaded SF film (c)

# APPENDIX E

# THERMAL ANALYSIS OF RUTIN

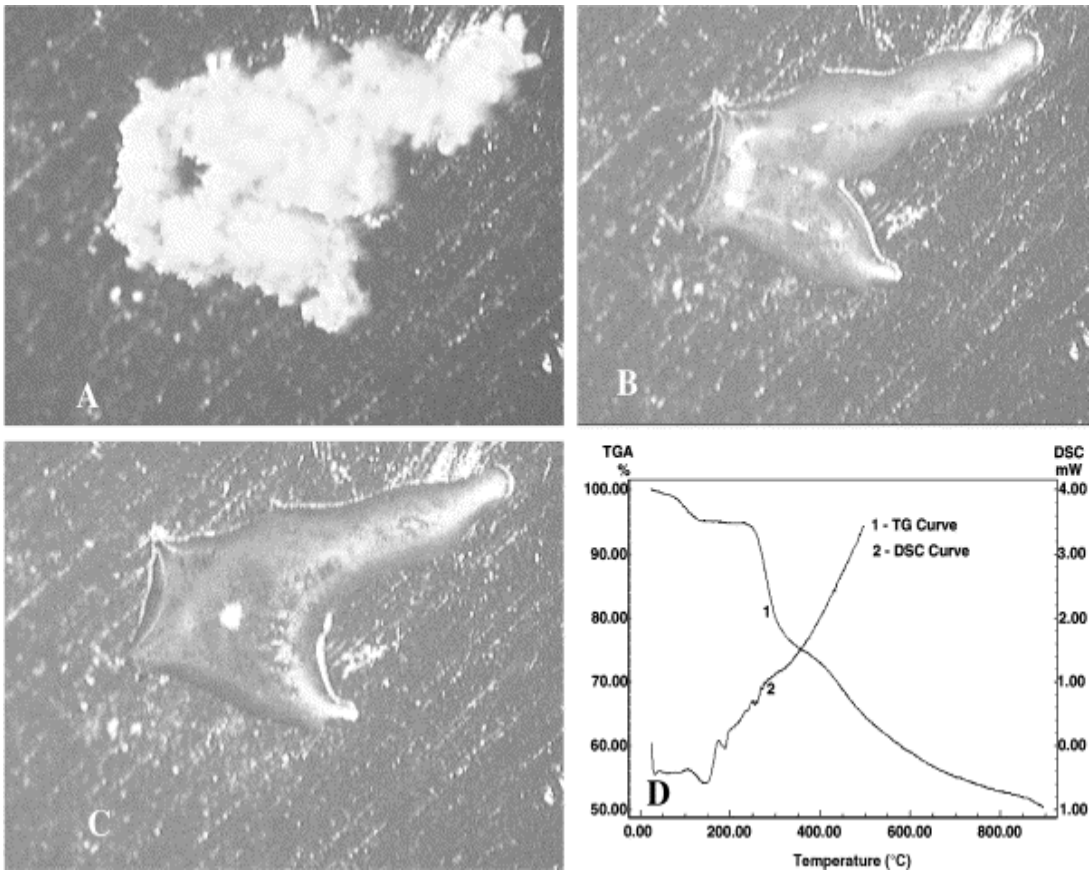


Figure E.1 .Pictures: (A) room temperature; (B) 180 °C; (C) 214 °C and (D) TG/DSC curve of the rutin under nitrogen atmosphere (Source : Costa, et.al. 2002)