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***Solubilization of curcumin using a selected
cyclodextrin***

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Alexandra Rupp

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ABSTRACT

Working with curcuminoids, especially with the main curcuminoid named curcumin and formulate it to improve stability and solubility has been an ongoing ambition in the department of pharmacy, University of Oslo.

Curcumin has an exceedingly low solubility in water. The aim of the present study was to use a selected cyclodextrin to increase the solubility of curcumin in aqueous solutions. The complexation between curcumin and randomized-methylated- β -cyclodextrin was investigated in phosphate buffer at fixed pH 5. The influence of low amounts of alginate, HPMC (Hydroxypropylmethylcellulose) and ethanol on the complexation was then investigated.

All studies show non-linear A_P like curves, which indicates some kind of higher order complexation. The highest curcumin concentration obtained in the present study was a curcumin solubility of 1.77×10^{-4} M detected in 5% (w/v) RM β CD in phosphate buffer (1% (v/v) ethanol), which corresponds to an almost 6000x increase in curcumin solubility, which shows great promise.

To further enhance the solubility of curcumin in aqueous solutions, a solvent evaporation method was selected. For that, a solution of curcumin powder in methanol containing RM β CD was prepared. After evaporation by a rotavapor curcumin is significantly more soluble in its amorphous form than it is before the evaporation in its crystalline form.

An excess of this powder was then diluted with purified water and filtered. After filtration HPMC was dissolved in the filtrate and the solution was lyophilized by a freeze dryer. Here, the method of lyophilization was used to improve the physical and chemical stability of this solution. The drug load of this lyophilized product shows great promise for doing more investigations.

ABSTRACT

Die Arbeit mit Curcuminoiden, insbesondere mit dem Hauptcurcuminoid Curcumin, ist ein permanentes Bestreben der Mitarbeiter des Departments der Pharmazie an der Universität Oslo, Norwegen. Deren Arbeit besteht darin, dieses genannte Curcumin herzustellen und es in seiner Stabilität und Löslichkeit zu verbessern.

Curcumin besitzt eine äußerst geringe Wasserlöslichkeit. Das Ziel dieser Diplomarbeit besteht also darin, mit der Hilfe eines ausgesuchten Cyclodextrins die Löslichkeit von Curcumin in wässriger Lösung zu erhöhen. Die Komplexbildung des Curcumins mit dem randomisierten, methylierten β -Cyclodextrin wurde in Phosphatpuffer bei einem fixen pH von 5 durchgeführt. Weiters wurde der Einfluss von geringen Mengen von Alginaten und HPMC, als auch der Einfluss von Ethanol auf die Komplexbildung untersucht.

Alle Untersuchungen zeigten nicht lineare A_p Kurven, welche Komplexbildungen höherer Art bedeuten. Die höchste Curcumin-konzentration, welche in dieser ganzen Arbeit erlangt wurde, war eine Curcuminlöslichkeit von 1.77×10^{-4} M, die in 5% (w/v) RM β CD in Phosphatpuffer (1% (v/v) Ethanol) gefunden wurde. Dies bedeutet eine fast 6000-fache Erhöhung der Curcuminlöslichkeit, welches sehr vielversprechend scheint.

Um eine weitere Erhöhung der Löslichkeit von Curcumin in wässriger Lösung zu erzielen, wurde ein Lösungsmittelverdampfungsverfahren gewählt. Dieses Verfahren wurde mit einem Rotavapor durchgeführt. Hierzu wurde eine Lösung angefertigt bestehend aus Curcuminpulver und RM β CD, welche in Methanol gelöst sind. Curcumin zeigte nach diesem Verdampfungsverfahren eine deutlichere Erhöhung seiner Löslichkeit, was möglicherweise an seiner amorphen Form lag, im Unterschied zu davor, als Curcumin in seiner kristallinen Form vorlag.

Ein Teil dieses Pulvers wurde in destilliertem Wasser gelöst und filtriert. In diesem Filtrat wurde HPMC weitgehend aufgelöst und diese Lösung in einer Gefriertrocknungsanlage lyophilisiert. Die Wirkstoffbeladung dieses lyophilisierten Produktes scheint äußerst vielversprechend und ermutigt das Team in Oslo mit den Untersuchungen fortzufahren.

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ABBREVIATIONS

BC	before Christ
CD	Cyclodextrin
CDs	Cyclodextrins
CE	Complexation efficiency
Conc	Concentration
Cur	Curcumin
Cur : CD	Curcumin : Cyclodextrin complexation
D	Drug
DSC	Differential scanning calorimetry
e.g.	'exempli gratia', for example
EtOH	Ethanol
g	Gram
HP	Hydroxypropyl
HP β CD	Hydroxypropyl- β -cyclodextrin
HP γ CD	Hydroxypropyl- γ -cyclodextrin
HPLC	High performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
K	Association constant

$K_{m:n}$	Equilibrium constant
kV	Kilovolt
LOQ	Limit of quantification
m	Amount of substrate
M	Molar
M β CD	Methylated-beta-cyclodextrin
mg	Milligram
ml	Milliliters
min	Minute
$m:n$	Stoichiometry between substrate and ligand
n	Amount of ligand
nm	Nanometer
RM	Randomized methylated
RM β CD	Randomized methylated- β -cyclodextrin
S	Substrate
S.D.	Standard deviation
SEM	Scanning electron microscopy
S_{int}	Intercept
S_o	Intrinsic solubility
v/v	Volume divided by volume

w/v

Weight divided by volume

w/w

Weight divided by weight

μm

Micrometer

1 AIM OF THE STUDY

The aim of the present study was to improve the aqueous solubility and dissolution rate of curcumin by using a selected M β CD.

Further, to investigate the effect of low amounts of alginate, HPMC and ethanol on the Cur : CD complexation.

2 INTRODUCTION

2.1 CURCUMIN

2.1.1 Natural occurrences of curcumin

Curcumin is a naturally occurring compound and the commercially available curcumin is isolated from the plant *Curcuma longa* L., belonging to the Zingiberaceae family (Tomren, M.A., et al., 2007; Jayaprakasha, G.K., et al., 2005; Tønnesen H.H. et al., 2002a). This plant is widely cultivated in warm, rainy regions of the world such as China, India, Indonesia, Jamaica and Peru (Jayaprakasha, G.K., et al., 2005).

The coloring principle of turmeric was isolated in the 19th century and was named curcumin. Three Curcuminoids were isolated from turmeric viz., curcumin, demethoxycurcumin and bisdemethoxycurcumin (Jayaprakasha, G.K., et al., 2005).

The curcumin on the market consists of a mixture of these three naturally occurring curcuminoids, with curcumin as the main constituent (Tønnesen, H.H., et al., 2002a). However, to avoid interference from other curcuminoides (i.e. demethoxycurcumin and bisdemethoxycurcumin) the curcumin used in the present study is synthesized with the method of Pabon (Pabon, H., 1964). The purity of curcumin has previously been controlled with HPLC, TLC and DSC (Haukvik, T., et al., 2009).

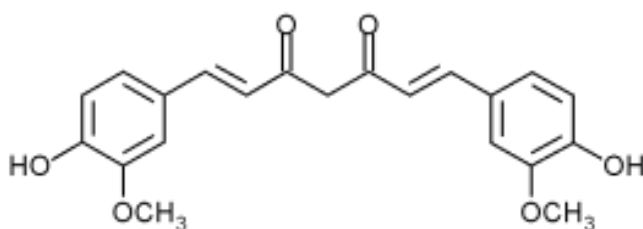


Figure 1: The chemical structure of curcumin

2.1.2 Use of curcumin

Curcuma has been used in Asian medicine since the second millennium BC. It also has been referred to the ancient Hindu scripture, the Ayurveda (Sharma, R.A., et al. 2007). There turmeric has been used internally as a stomachic, tonic and blood purifier and externally in the prevention and treatment of skin diseases (The Wealth of India, 2001; Jayaprakasha, G.K., et al. 2005).

Further, curcumin is used as a coloring agent in food, drugs and as well as in cosmetics and textiles. Curcumin is one of the constituents of curry powder (Govindarajan, V.S., 1980).

2.1.3 Physio-chemical properties of curcuminoids

2.1.3.1 Structure of curcumin

Curcumin is a symmetric molecule with two aromatic rings with ortho methoxy phenolic –OH groups and a diketone moiety (figure 1). The diketone moiety can undergo keto-enol tautomerization depending on the solvent and temperature as summarized in the reference (Priyadarsini, K.I., 2009). Figure 2 shows the keto-enol tautomerization of curcumin. In solution, the cis-enol configuration of curcumin is stabilized by a strong intramolecular H-bond. This intramolecular bonding gives the enol ring a pseudo aromatic character (Tønnesen, H.H., et al., 1982).

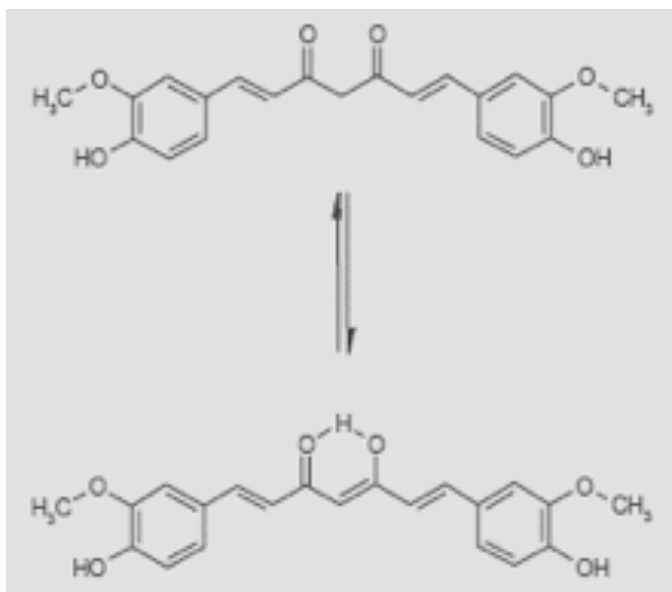


Figure 2: The keto-enol tautomerization of curcumin (Singh, R., 2008)

2.1.3.2 Pharmacological effects and Pharmacokinetics of curcumin

Curcumin has a number of pharmacological effects (Tønnesen H.H. et al., 2002a). In vitro studies show potential use against cancer, HIV-infections, cystic fibrosis and as an immunomodulating agent (Chattopadhyay, I., et al., 2004; Jayaprakasha, G.K., et al., 2005).

Other reported biological activities in vivo of curcumin include anti-inflammatory activity, antioxidant, antibacterial effect against Alzheimer's disease and antimicrobial activity, which is shown in vitro (Tønnesen, H.H., et al., 1987; Chattopadhyay, I., et al., 2004; Jayaprakasha, G.K., et al., 2005) Curcumin has a potential as a photosensitizer in antimicrobial photodynamic therapy (PDT) of localized superficial infections (Haukvik, T., et al., 2009; Hegge, A.B., et. al, 2010).

Pharmacologically curcumin is well tolerated even at a dose of 8 g/day orally.

2.1.3.3 Solubility and stability of curcumin – challenges of using curcumin

One of the challenges of making a formulation of curcumin is the compounds stability (Hegge, A.B., 2010). It is influenced by pH, light, temperature, chemical oxidants, metal ions, enzymes and solvents (Lauro, G.J., et al., 2000).

One of the main drawbacks is that curcumin is almost insoluble in water at acidic and neutral pH (pH 1-7). The lipophilic character of the compound is the reason of the low water solubility. This limits the use as a drug, as mentioned before (Tønnesen, H.H., Karlsen, J., 1985a,b; Lauro, G.J., et al., 2000; Tønnesen, H.H., 2006).

To date, curcumin is not approved as a drug. Probably one of the reasons is its very low water solubility at physiological conditions as mentioned before and a corresponding low absorption after oral or i.v. administration. Furthermore, curcumin undergoes fast metabolism, resulting in low bioavailability (Priyadarsini, K.I., 2009). Also animal studies have shown that curcumin is rapidly metabolized, conjugated in the liver, and excreted in the feces. Accordingly, the low bioavailability, rapid plasma clearance and conjugation may be important limiting factors for medical application of curcumin (Lin, Xie, et al., 2010).

On the other hand curcumin shows increased solubility in aqueous solvents with pH values over neutral, but is then highly susceptible to hydrolytic degradation, which means that it degrades under exposure to light (Tønnesen, H.H., Karlsen, J., 1985a,b; Lauro, G.J., et al., 2000; Tønnesen, H.H., 2002, Tønnesen, H.H., 2006). At pH 5 the hydrolytic degradation rate of curcumin is low (Tønnesen, H.H., Karlsen, J., 1985b). Curcumin in its solid state shows photodegradation. Radiation wavelengths from 280-450nm initiate singlet oxygen production, which cause degradation of the curcuminoids (Lauro, G.J., et al., 2000). Previous studies, which were performed to enhance the stabilization of curcumin against photochemical

degradation, have shown that it is a difficult task (Tønnesen, H.H., 2002; Tønnesen, H.H., et al., 2002a; Tomren, M.A., et al., 2007).

Summarized, the pH influences the ionization, the rate of degradation and the color of curcumin. In neutral form (pH 1-7) the color is yellow; pH below 1 and pH above 7.5 gives a red color (Tønnesen, H.H., Karlsen, J., 1985a,b; Lauro, G.J., et al., 2000; Tønnesen, H.H., 2006). The main degradation products are feruloylmethane, ferulic acid and vanillin. Alkaline degradation of curcumin also results in condensation products of feruloylmethane (Tønnesen, H.H., Karlsen, J., 1985a).

Accordingly, the main drawbacks for clinical applications of curcumin are its low solubility at acidic and neutral pH, its low photostability, its rapid plasma clearance and conjugation and as well its poor bioavailability.

Therefore, solubility and hydrolytic stability have been improved by making complex formation with cyclodextrins and micelles. Cyclodextrins are used in this thesis to increase the water solubility of curcumin in its neutral and most stable form (Tønnesen, H.H., Karlsen, J., 1985b). In fact, the solubility is increased by a factor of at least 10^4 at pH5 (Tønnesen, H.H., 2002; Tønnesen, H.H., et al., 2002a).

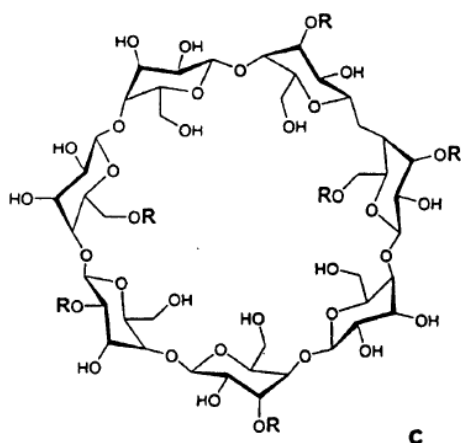
2.2 CYCLODEXTRIN

2.2.1 Structure and characteristics of cyclodextrin

As already mentioned above, the solubility of curcumin is an important drawback working with curcumin. To enhance the solubility of curcumin, CDs or different types of polymer can be used in order to improve the water solubility.

Researchers try to investigate the benefits of complexing curcumin with other substances to increase systemic bioavailability (Lin, Xie, et al., 2010). For example they increase the systemic bioavailability by encapsulation in polymeric nanoparticles (Bisht, S., et al., 2007), surfactants (micelles) (Tønnesen, H.H., 2002) or cyclodextrins (Tønnesen, H.H., et al., 2002a), which are utilized in this thesis.

The three most common naturally occurring CDs are α -cyclodextrin, β -cyclodextrin and γ -cyclodextrin. They are cyclic (α -1,4)-linked oligosaccharides of α -D-glucopyranose with six, seven and eight units, respectively (Loftsson, T., et al., 1998).



R: CH₃

Figure 3: chemical structure of randomly methylated- β -CD (Tønnesen, H.H., et al., 2002a)

CDs were first published in the year 1891 by the French scientist A. Villers. At this time he named them “cellulosine”. In 1903 the Austrian microbiologist Franz Schardinger published an article as well, where he describes two “crystalline dextrin”, which he had isolated from bacterial digest of potato starch. He identified his “crystalline dextrin” as Villers “cellulosine”. Later Schardinger changed the names to α -dextrin and β -dextrin (Loftsson, T., et al., 2007).

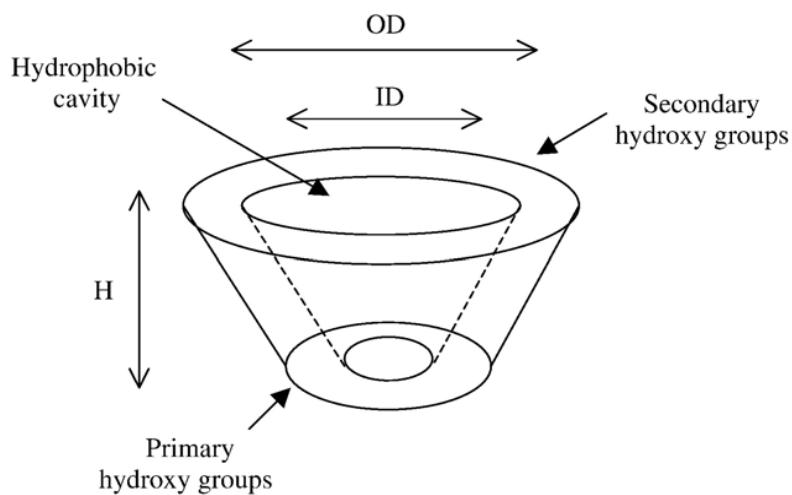


Figure 4: Cone shape of cyclodextrins (Brewster, M.E., et al., 2007)

Cyclodextrin molecules have a cylindrical, cone-like shape (Figure 4) with primary hydroxyl groups, which are located on the narrow side of the torus. They show a relative lipophilic central cavity while the secondary hydroxyl groups, which are located on the wider edge, having a hydrophilic outer surface (Loftsson, T., et al. 2005b; Loftsson, T., 1995). These hydroxyl groups on the outer surface are able to form hydrogen bonds with other molecules (Loftsson, T., et al., 2007a; Loftsson, T., et al., 2007b).

With their lipophilic cavity the CDs are able to form inclusion complexes with lipophilic compounds in aqueous solutions, which is discussed in detail in section 2.3.

Such inclusion complexation will influence the physiochemical properties of the drug, for example aqueous solubility and stability (Loftsson, T., et al., 2004a; Loftsson, T., et al. 2005b; Hegge, A.B., et al., 2008).

2.2.2 Physio-chemical properties of β -cyclodextrin

Even though the natural CDs are relatively hydrophilic, in particular β -cyclodextrin, they have limited aqueous solubility because of the relatively strong binding of the natural CD molecules in the crystal state. Therefore a huge number of cyclodextrin derivatives with high solubility in water have been synthesized. The main reason for the increased solubility is that the random substitution transforms the crystalline CDs into amorphous mixtures of isomeric derivatives (Szejtli, J., 1988; Loftsson, T., et al., 1996a; Loftsson, T., et al., 1998; Brewster, M.E., et al., 2007).

One of them is methyl- β -cyclodextrin. This selected CD was taken for the investigations in this thesis.

2.2.3 Application and current use of cyclodextrins

As pharmaceutical excipients, cyclodextrins are used to increase the aqueous solubility of poorly water-soluble, lipophilic substances (Loftsson, T., et al., 2002a; Loftsson, T., et al., 2007a).

Furthermore, they are used to increase the stability of drugs in aqueous preparations. The use of CDs is often preferred to organic solvents in parenteral and topical formulations, to enhance oral bioavailability, to reduce

or prevent gastrointestinal or ocular irritation, reduce or eliminate unpleasant smells or tastes, to increase dermal availability of drugs or even to convert oils and liquid drugs into microcrystalline or amorphous powders (Loftsson, T., et al., 1998; Loftsson, T., et al., 1996a; Loftsson, T., et al., 2002a; Loftsson, T., et al., 2005b; Loftsson, T., et al., 2007a; Brewster, M.E., et al., 2007).

Methyl- β -cyclodextrin is relatively lipophilic compared to the natural β -CD, which is able to permeate mucosa and to enhance drug delivery through biological membranes, such as through the nasal mucosa, by reducing barrier function of the membranes (Martini, E., et al. 1998).

Because of the limited water solubility, natural β -cyclodextrin can't be given parenterally. Also lipophilic CD derivatives, such as the methylated CDs, have been shown to be toxic after parenteral administration. The compound precipitate in the kidney and this can induce nephrotoxicity (Loftsson, T., et al., 1997; Loftsson, T., et al., 2005b).

The oral administration of methylated β -cyclodextrin or methylated β -cyclodextrin in topical formulations is somehow limited because of its potential toxicity but it is basically non-toxic when given in a low or moderate oral dosage. As a result β -cyclodextrin is being found in a number of oral, topical, buccal and rectal pharmaceutical formulations (Loftsson, T., et al., 2005b; Brewster, M.E., et al., 2007).

2.3 INCLUSION COMPLEXATION WITH CYCLODEXTRIN

2.3.1 Inclusion complexation

As already mentioned in 2.2.1, cyclodextrins are able to form inclusion complexes in aqueous solutions. The reason for this is the chair formation of the glucopyranose units. Lipophilic drug molecule or lipophilic moieties of the drug molecule are taken into the central cavity, where water-molecules are located and replace them (Loftsson, T., et al., 2004a; Loftsson, T., et al. 2005b; Hegge, A.B., et al., 2008).

Because of these characteristics CDs are able to interact through non-covalent interactions with poorly water-soluble substances and form a mixture of inclusion and non-inclusion complexes in aqueous solutions (Loftsson, T., et al., 2004a; Loftsson, T., et al. 2005b; Hegge, A.B., et al., 2008).

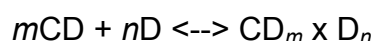
As well the size and the chemical structure of the guest molecule affect the complex formation. Only relative apolar molecules with an appropriate size, which is not too large, are able to enter the cyclodextrin cavity. But also the size of the cyclodextrin cavity is important (Loftsson, T., 1995).

The cyclodextrin molecules are relatively large (molecular weight ranging from almost 1000 to > 2000 Da) with a large number of hydrogen donors and acceptors and they are consequently poorly absorbed through biological membranes (Loftsson, T., et al., 2005).

2.3.2 Stoichiometry and association constant of compound-CD inclusion complex

Dependent on the type and amount of drug and cyclodextrin, a lot of different complexes of drug-cyclodextrin molecules can be formed, which have different CD : drug ratios or stoichiometry (Eq. 1).

$$K_{m:n}$$



Eq. 1 (Brewster, M.E., et al., 2007)

The association constant (K_c), also known as equilibrium constant ($K_{m:n}$) specifies the stoichiometric ratio of the complex. For explanation m is the amount of substrate and n is the amount of ligand. In this case ligand is the drug (D) and substrate is the cyclodextrin (CD). Thus $m:n$ is the stoichiometry between substrate and ligand. For complexation, the association constant can be written:

$$K_{m:n} = [\text{CD}_m \times \text{D}_n] / [\text{CD}]^m [\text{D}]^n$$

Eq. 2: (Brewster, M.E., et al., 2007)

In dilute solutions, the most common stoichiometry of drug-cyclodextrin complex is 1:1 complexation, where one drug molecule (substrate) forms a complex with one cyclodextrin molecule (ligand) (Eq. 1). This occurs if the drug fits entirely into the cyclodextrin cavity and/ or in dilute solutions. In many cases such as for curcumin it is only a part of the drug, which is probably included in the CD cavity. The aromatic group of curcumin is a typical group, which fits into the beta CD cavity. Other ratios are for example 1:2, 2:1 or 2:2. If the size of the drug is larger, two or more cyclodextrin molecules can enclose the drug and a 1:2 stoichiometry occurs or higher order complexes can be formed. If the cyclodextrin cavity incorporates 2 drug

molecules a formation of a 2:1 complex results (Connors, K.A., 1997; Frömring and Szejtli, 1994; Loftsson, T., et al. 2007b; Singh, R., 2008; Singh, R., et al. 2010).

2.3.3 Method to determine stoichiometry and association constant of the inclusion complex: The phase solubility method

The phase solubility method is a method to determine associations constants and stoichiometry of the equilibrium (Higuchi, T. and Connors, K.A., 1965). The exact procedure of the phase solubility method is described in section 3.2.4.

A phase solubility diagram (figure 5) is constructed by plotting the total molar concentration of dissolved solute, found on the vertical axis. On the horizontal axis there is the concentration of the complexing agent added (Higuchi, T. and Connors, K.A., 1965; Brewster, M.E., et al., 2007).

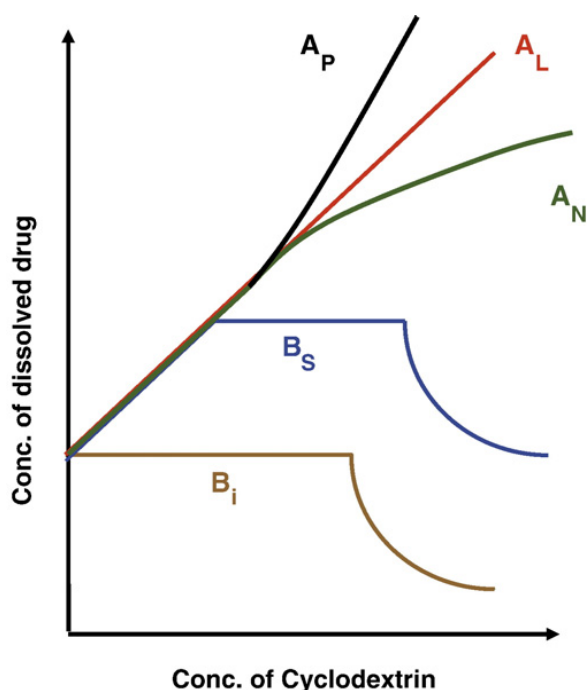


Figure 5: Phase solubility diagram (Brewster, M.E., et al., 2007)

In general, phase solubility diagrams have two major types: A- and B-types.

A-type phase-solubility profiles are obtained when soluble complexes are formed. The A-curves are again subdivided into A_L , A_P and A_N subtypes. A_L profiles indicate a linear increase in solubility as a function of cyclodextrin concentration. A_P profiles indicate positively deviating isotherm and A_N indicate a negatively deviating isotherm. A_L type diagrams are first order with respect to the cyclodextrin and may be first or higher order with respect to the drug, i.e., DCD, D_2CD , D_3CD , ..., D_mCD . If the slope of an A_L -type profile is greater than one, higher order complexes are assumed to be involved. Although a slope less than one, does not necessarily exclude higher order complexation. Often a one-to-one complex is assumed. A_P type diagrams suggest the formation of higher order complexes with respect to the cyclodextrin at higher cyclodextrin concentrations, i.e., DCD_2 , DCD_3 , ..., DCD_N (Brewster, T., et al. 2007; Loftsson, T., et al., 2005; Loftsson, T., et al., 2004b; Singh, R., 2008).

Complexation efficiency

A more accurate method for determination the solubilizing effect of cyclodextrins is to compare their complexation efficiency (CE). The complexation efficiency is the determination of the concentration ratio between cyclodextrin in a complex and free cyclodextrin. It is calculated from the slope of the phase-solubility diagram and is independent of both S_0 (intrinsic solubility) and S_{int} (intercept) (Loftsson, T., et al., 2007b).

Methods to enhance the complexation efficiency

There are several reasons for increasing the efficiency of cyclodextrin complexation of drugs. For example toxicological considerations, production costs, drug bioavailability and isotonicity are just a few reasons why to

include as little cyclodextrin as possible in pharmaceutical formulations (Loftsson, T., et al., 1999; Loftsson, T., et al., 2005a; Loftsson, T., et al., 2007a; Brewster, M.E., et al., 2007).

Several methods have been applied to enhance the complexation efficiency of cyclodextrins (Loftsson, T., et al., 1999). For example, addition of different types of polymer like HPMC or sodium alginate to the aqueous complexation media (Loftsson, T., et al., 2004a), addition of co-solvents and so on (Li, P., et al., 1998; Loftsson, T., et al., 1999; Brewster, M.E., et al., 2007).

2.4 INCREASING THE SOLUBILITY OF CURCUMIN

As mentioned above, the low solubility of curcumin limits its use as a drug. Cyclodextrins alone or in combination with water-soluble biopolymers is therefore used to enhance the solubility of curcumin.

Adding a small amount of water-soluble polymer can increase the complexing abilities of β -cyclodextrin in aqueous solution and thus improve the solubility (Loftsson, T., et al. 1996b; Loftsson, T., et al. 1994; Loftsson, T., et al. 1998).

In the present study sodium alginate and HPMC were chosen as polymer. Alginates are natural water-soluble polysaccharide polymers isolated from marine brown algae (Phaeophyceae) or fermentation of bacteria (Draget, K.I., et al., 2006). Alginic acid is a linear polymer consisting of D-mannuronic acid and L-glucuronic acid (Tønnesen, H.H., et al. 2002b). A number of different grades of sodium alginate, which have different solution viscosities, are available. Sodium alginate is practically insoluble in organic solvents and slowly soluble in water, forming a viscous colloidal solution. It is used in a variety of oral and topical pharmaceutical formulations, as stabilizing agent, suspending agent, viscosity-increasing agent and many more. In this study sodium alginate is used, which is probably the most frequently investigated one (Hegge, A.B., et al., 2008; Handbook of pharmaceutical excipients, 2003).

Hydroxypropylmethylcellulose (HPMC) also known under the trade name Hypromellose® is a partly O-methylated and O-(2-hydroxypropylated) cellulose. Depending upon the viscosity grade, Hypromellose is available in several grades. It is used as a viscosity-increasing agent, as well as stabilizing, suspending or coating agent. It is soluble in cold water, forming a viscous colloidal solution (Handbook of pharmaceutical excipients, 2003).

3 EXPERIMENTAL: MATERIALS AND METHODS

3.1 MATERIALS

Curcumin was synthesized according to the method of Pabon (Pabon, H., 1964).

Chemical	Lot Number	Supplier
Citric Acid monohydrate	08D150022	VWR International
Potassium hydroxide	11293	Eka Nobel AB/ Nobel Industrier
Sodium chloride	9D122/4	Norsk Medisinaldepot, Norway
Methanol Chromasolv® (HPLC-grade)	SZBA1195	Sigma-Aldrich Co./Chemie GmbH
Ethanol		Norsk Medisinaldepot, Norway
Sodium hydrogen phosphate dihydrate	K91380845 819	Merck KGaA
Disodium hydrogen phosphate dihydrate	K27618980 020	Merck KGaA
RM- β -cyclodextrin = Cavasol W7 M	71T051	Wacker Chemie AG
Hydroxypropylmethylcellulose	8028204	Shin-Etr Chemie. Lv.
Sodium Alginate (Protanal® LF 10/60 LS)	S17261	FMC BioPolymers/NovaMatrix

Table 1: all chemicals used in this thesis with Lot Number and Supplier

All chemicals used, were of analytical grade, except the polymers.

3.2 HPLC METHOD

The solubility of curcumin was quantified with a previously described HPLC quantification method (Hegge, A.B., et al., 2008). However, due to practical issues, one additional HPLC method was employed. The two methods are described below:

HPLC system I: system I consisted of a Waters Nova-Pak® C18, 3.9 x 150 mm, 4µm particle size column by use of a Shimadzu Liquid Chromatography LC-9A pump, a Shimadzu Auto Injector SIL-10 AD auto sampler, a Shimadzu UV-Vis Spectrophotometric detector SPD-10A, and a Shimadzu C-R3A integrator.

HPLC system II: system II consisted of a Waters Nova-Pak® C18, 3.9 x 150 mm, 4µm particle size column by use of a Shimadzu Liquid Chromatography LC-20AD pump, a Shimadzu Auto Injector SIL-20AC HT auto sampler, a Shimadzu UV-Vis Spectrophotometric detector SPD-M20A, a CBM-20A controlling unit and a CTO-20A oven.

3.2.1 Preparation of the buffers and the mobile phase

0.026 M (0.5%) Citric acid buffer pH3:

The citric acid buffer was prepared by dissolving a given amount of citric acid monohydrate in a volumetric flask almost filled with purified water and adjusted to pH 3 +/- 0.1 by adding 10% (w/v) KOH solution. The pH was checked using a WTW pH 526 pH meter. Purified water was then added to the mark in the flask before filtration.

The mobile phase was composed of 62 parts of MeOH to 38 parts of citric acid buffer for *system I*.

Phosphate buffer pH 5 (0.05 M; ionic strength 0.085):

The phosphate buffer was prepared by dissolving a given amount of the appropriate salt in purified water by using a volumetric flask. The ionic strength was fixed by adding a calculated amount of NaCl. The pH 5 +/- 0.2 was checked using a WTW pH 526 pH meter.

The exact compositions of the buffer used in the phase solubility studies are presented in appendix A.3.

3.2.2 Analytical conditions

The retention time for curcumin was at around 12 minutes.

All the quantitative analysis were done in triplicate to reduce random error.

3.2.3 Curcumin quantification

Linearity was investigated in the range 5×10^{-7} M and 1×10^{-5} M curcumin using a stock solution in methanol. Calibration of the instrument was performed with curcumin solutions in methanol (5×10^{-7} M, 1×10^{-6} M, 2×10^{-6} M and 1×10^{-5} M) for quantification of curcumin.

The quantification was performed using linear regression. The integrals were plotted on the x-axis and the concentration was plotted on the y-axis. This gave a straight line. The regression coefficient was > 0.99 .

$$y = ax + b$$

y is the concentration, x is the integral, a is the slope and b is the intercept.

3.2.4 Phase solubility studies

Phase solubility studies were performed by adding an excess amount of drug to several vials. A constant volume of solvent is added to each vial and also solvent containing successively larger concentrations of the complexing agent is then added. The vials are closed and the contents brought to

solubility equilibrium by shaking at a constant temperature. The suspensions are filtered and then analyzed for their total concentration of compound by HPLC.

In the present study an excess amount of curcumin is added to different solvents containing increasing concentration 0.1, 0.3, 0.5, 1, 2, 3 and 5 % (w/v) of RM β CD.

All investigations were performed at ambient temperature using three parallels. The samples were protected from light.

7 different solvents were taken to compare their solubilizing effect under the given conditions with RM β CD.

The samples were detected at the wavelength 420nm and 430nm.

	Solvent	crystalline curcumin	Curcumin in ethanol solution (1×10^{-3} M)
	purified water	x	
	purified water with 1% (v/v) EtOH	x	
	0,1% (w/v) sodium alginate in water + 1% (v/v) EtOH	x	
RM-β-CD in different ratio	0,1% (w/v) HPMC in water + 1% (v/v) EtOH	x	
	plain phosphate buffer (pH5)	x	
	phosphate buffer (pH5) + 1% (v/v) EtOH	x	
	phosphate buffer (pH5)	x	x

Table 2: this table shows the solvents, which were used.

Determination of the water content in the cyclodextrin:

It is important to determine the water content and correct it when preparing the cyclodextrin stock solution. The water content in the cyclodextrins was determined at the beginning of the studies of this thesis.

The measurement was carried out by use of approximately 2g of the cyclodextrin. A Sartorius Moisture Analyzer, MA 30 was used. The cyclodextrin was dried at 130°C for 60 minutes, respectively. The samples were weighted on an analytical balance before and after measurement. The water content of the samples was calculated, based on the weight loss. As well, the measurements were done in triplicates, and the value used for concentration corrections was the average of the three parallels.

3.2.4.1 Phase-solubility-studies with purified water and purified water containing 1% (v/v) EtOH

A 1% (w/v) cyclodextrin solution in water was prepared. The moisture content in the cyclodextrin was corrected for.

Vials were filled with an excess amount of compound and an appropriate amount of the cyclodextrin solution was diluted according to the different concentrations in each vial. The concentrations were 0.1, 0.3, 0.5, 1, 2, 3 and 5% (w/v) made from 1% stock solution of CD. The vials were sealed and were shaken for 1 day and protected from light. Undissolved curcumin was then removed by filtration 0.45 Spartan 13/0.45 RC before diluting the solution 1:1 with MeOH and analyzed by *HPLC system I* at 420nm wavelength to quantify the curcumin in the filtrate. The first drops of the filtrated samples were discarded.

3.2.4.2 Phase-solubility-studies with plain phosphate buffer and phosphate buffer containing 1% (v/v) EtOH

Three 1% (w/v) cyclodextrin solutions in phosphate buffer were prepared. The moisture content in the cyclodextrin was corrected for.

One solution was prepared only with plain phosphate buffer and two 1% (w/v) cyclodextrin solutions in phosphate buffer containing 1% (v/v) EtOH were prepared differently. One study was investigated with crystalline and solved curcumin (100µl of curcumin in ethanol solution 1×10^{-3} M) and the other one was investigated simply with crystalline curcumin.

Vials were filled with an excess amount of compound (and as well with 100µl of curcumin in ethanol solution). An appropriate amount of the cyclodextrin

solution was diluted according to the different concentrations in each vial. The concentrations were again 0.1, 0.3, 0.5, 1, 2, 3 and 5% (w/v) made from 1% stock solution of CD. The vials were sealed and were shaken for 1 day and protected from light. Undissolved curcumin was then removed by filtration 0.45 Spartan 13/0.45 RC before diluting the solution 1:1 with MeOH and analyzed by *HPLC system I* at 420nm wavelength to quantify the curcumin in the filtrate. The first drops of the filtrated samples were discarded.

3.2.4.3 Phase-solubility-studies with two different polymer in water containing 1% (v/v) EtOH

Two 1% (w/v) cyclodextrin solutions with 2 different polymers in water were prepared. The moisture content in the cyclodextrin was corrected for.

Vials were filled with an excess amount of compound and an appropriate amount of the cyclodextrin-polymer solution was diluted according to the different concentrations in each vial. The concentrations were 0.1, 0.3, 0.5, 1, 2, 3 and 5% (w/v) made from 1% stock solution of CD. The vials were sealed and were shaken for 1 day and protected from light. Undissolved curcumin was then removed by filtration 0.45 Spartan 13/0.45 RC before diluting 1:1 with MeOH and analyzed by *HPLC system I* at 420nm wavelength to quantify the curcumin in the filtrate. The first drops of the filtrated samples were discarded.

3.2.4.4 Quantification of curcumin in the freeze-dried product – drug load

An exact amount (approx. 0.005g) of the freeze dried product was added in 1ml MeOH and immediately filtered with BD Plastipak 5ml sterile syringe

from Becton Dickinson S.A., fitted with Spartan 13/0.45 RC, 0.45 μ m filters from Whatman. The first drops of the filtrated samples were discarded. The samples were diluted 1:1 with MeOH before quantification by the previously described reversed phase HPLC method with the following modifications: *HPLC system II* was used, the flow rate was 0.8ml/min and the detection wavelength was 430nm for the quantification of curcumin.

Degradation products were detected at different wavelength (350nm).

3.3 ROTAVAPOR or THE SOLVENT EVAPORATION METHOD

A homogenous drug-cyclodextrin system was prepared. Curcumin-cyclodextrin complex was set up in the molar ratio of 1:1, 1:2 and 1:3 using methanol as solvent. The solvent (0.1g curcumin in 10ml MeOH; 0,2g curcumin in 10ml MeOH; 0.3g curcumin in 10ml MeOH) was then evaporated for ½ hour at 25°C in a Büchi EL – 131 rotavapor. The resulting product was a film, which was obtained after scraping the film in the glass vial and was stored over night in an exsiccator.

The same procedure was repeated with curcumin in methanol (without CDs) as comparison.

3.4 DSC

A Mettler Toledo Stare System differential scanning calorimeter was used equipped with a DSC 822e Module.

Samples of 1-1.5 mg of crystalline curcumin, pure CD, curcumin in methanol after evaporation, curcumin with CD in methanol after evaporation and of the freeze-dried products were heated in an aluminum pan, an aluminum top was placed on the sample and crimped in place, under static atmosphere. An

empty pan was used as reference. The heating rate was 10°C/min and the temperature interval used was 5°C to 220°C, under a constant flow (100ml/min) of nitrogen gas. The equipment was calibrated with indium.

3.5 LYOPHILIZATION

Lyophilization is a very complex process (Patil, V.V., et al., 2010).

For Lyophilization Virtis 25ES from Virtis Company Inc. USA was used. A vacuum pump, named Leybold, Trivac D16B from Engineering Company Inc., USA was used.

An excess of cyclodextrin:curcumin powder (from the solvent evaporation) was dissolved in 10ml purified water by shaking (approximately 3min) and if necessary with the help of an ultrasonic bath (sonication). It was filtered with 0.45 Spartan 13/0.45 RC. After filtration 0.01g HPMC was dissolved in the filtrate. HPMC was chosen to carry out the following studies by lyophilization because the increased viscosity of sodium alginate complicated the filtration of the solutions prior to freeze dry and prior to quantification.

The vials were freeze-dried according to the following procedure:

Samples (approximately 10ml of the sample in 50ml glass vials with open plastic stopper) were put on the shelves and were frozen at -48°C for 24 hours. Meanwhile, the temperature of the condenser of the freeze dryer was allowed to reach at its minimum level of -50°C. Thereafter, the frozen samples are still placed in the drying chamber and then the vacuum pump and the heater were switched on. Vacuum was applied, and the samples were subjected to lyophilization for 72 hours in the freeze dryer.

Before initiating the second drying step and increasing the temperature for it, the vacuum should be below 100mbar first. If these conditions are

accomplished, the secondary drying step with 10°C for 2 hours, 20°C for 1 hour and 40°C for 2 hours was succeeded.

Finally, the vials were closed by pressing the plastic stopper.

3.6 SEM

The surface morphology of pure curcumin, pure RM- β -cyclodextrin, curcumin:cyclodextrin product after solvent evaporation and the freeze-dried product was investigated using a Hitachi S-4800 scanning electron microscope from Hitachi High-Technologies, Canada. The samples were spread on a brass stub using double-sided tape and then sputtered with a thin layer of gold platinum. The pictures were taken at an acceleration voltage of 1 kV.

4 RESULTS AND DISCUSSION

4.1 CURCUMIN COMPLEXATION WITH RM β CD

4.1.1 Phase solubility studies – the effect of solvents

Phase solubility studies were carried out according to the method described by Higuchi and Connors (1965).

Phase solubility studies were performed in different solvents to investigate if the medium affected the curcumin-cyclodextrin complexation.

Because of the symmetric appearance of curcumin with two identical aromatic rings, it is a possibility that both 1:1 guest-host inclusion complexes and 1:2 guest-host inclusion complexes can be formed with cyclodextrins. As seen in figure 6, 1:1 stoichiometry includes one aromatic moiety in the cyclodextrin cavity, 1:2 stoichiometry includes both aromatic moieties into the cyclodextrin cavities of two different cyclodextrin molecules (Singh, R., et al., 2010).

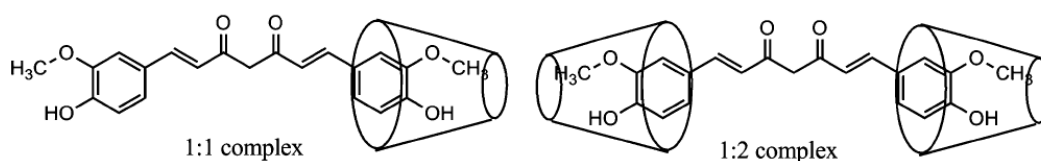


Figure 6: curcumin-cyclodextrin complexation (Singh, R., et al., 2010)

In the present study, non-linear, A_p-like curves for curcumin with RM β CD in water with and without ethanol were obtained (figure 7).

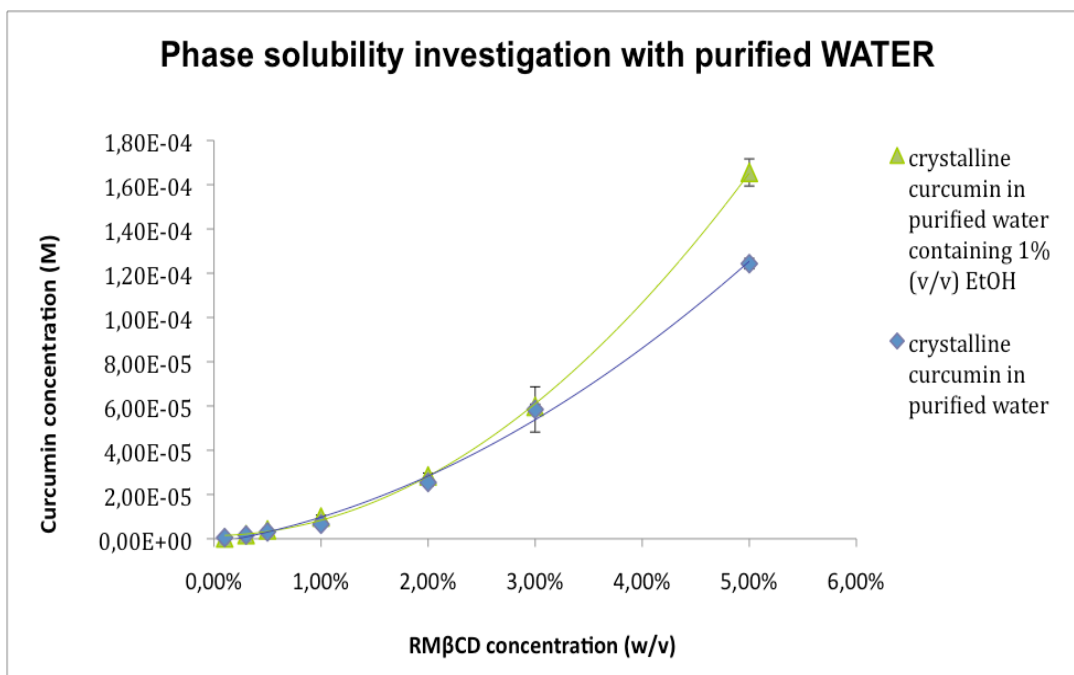


Figure 7 : Phase solubility diagram of curcumin in purified water showing A_p like curves. The curves show the solubility of curcumin at increasing concentrations (w/v) of RMβCD in purified water and purified water containing 1% (v/v) ethanol

RMβCD concentration	curcumin concentration (M) in purified water		Curcumin concentration (M) in purified water + 1% (v/v) EtOH	
	curcumin concentration (M)	+/- SD	Curcumin concentration (M)	+/- SD
0,10%	5.11x10 ⁻⁷	9.34x10 ⁻⁸	3.43x10 ⁻⁷	1.06x10 ⁻⁷
0,30%	1.66x10 ⁻⁶	3.51x10 ⁻⁷	1.85x10 ⁻⁶	7.20x10 ⁻⁷
0,50%	3.10x10 ⁻⁶	2.00x10 ⁻⁷	4.02x10 ⁻⁶	7.07x10 ⁻⁷
1%	6.55x10 ⁻⁶	4.76x10 ⁻⁷	9.81x10 ⁻⁶	8.89x10 ⁻⁷
2%	2.54x10 ⁻⁵	1.86x10 ⁻⁶	2.84x10 ⁻⁵	1.33x10 ⁻⁶
3%	5.84x10 ⁻⁵	1.02x10 ⁻⁶	5.98x10 ⁻⁵	1.08x10 ⁻⁶
5%	1.24x10 ⁻⁴	2.39x10 ⁻⁶	1.66x10 ⁻⁴	6.13x10 ⁻⁶

Table 3: The concentration of curcumin (M) expressed as the calculated mean +/- SD in different concentration (w/v) of RMβCD solution in purified water and purified water containing 1% (v/v) ethanol

An A_p-like curve suggests the formation of higher order complexes (Loftsson et al., 2004b). The curves appear similar at low concentrations of CDs, however, at a higher cyclodextrin concentration curcumin is more soluble in water containing 1% (v/v) ethanol (figure 7).

The highest solubility of curcumin obtained in the present study was 1.66×10^{-4} M as detected in 5% (w/v) RM β CD in purified water containing 1% (v/v) ethanol. In general, increased concentrations of cyclodextrin correspond to increased solubility of curcumin, that is, if the inclusion complex is formed, sufficiently soluble in water.

Several papers have been published on the complexation of curcumin with cyclodextrins.

Tønnesen, H.H., et al., 2002a reported an increase in water solubility at pH 5 by a factor of at least 10^4 , resulting from complex formation of curcumin and cyclodextrin. The highest concentration obtained in this study was 8×10^{-4} M of curcumin, measured in 11% solution of RM- β -cyclodextrin (Tønnesen, H.H., et al., 2002a).

Hegge, A.B., et al., 2009 reported a decrease in complexation and as well the overall solubility decreases in presence of ethanol in previously performed phase-solubility-studies with curcumin and HP β CD. These studies were obtained in phosphate buffer (pH 5) with 10% ethanol or in plain buffer. It might be that ethanol, being a small molecule, stabilizes the formations of higher order complexes, in particular 1:2 guest-host complexation (curcumin : CD).

Tang et al., 2002, as well, reported an 1:2 (guest:host) complex with β CD using spectrophotometric investigations.

The results of these previous studies of curcumin in CD solutions show that the complexing medium affected the stoichiometry of the curcumin-cyclodextrin complexation.

Figure 8 shows two A_p -like curves for curcumin with RM β CD in formulations with phosphate buffer containing 1% (v/v) ethanol and in plain phosphate buffer. The A_p -type of diagram of phosphate buffer containing 1% (v/v) ethanol was consistent with 2:1 or 3:1 complex formation between RM β CD and curcumin. As mentioned before, the formation of higher order complexes is suggested with an A_p -type of diagram, as well as with the symmetrical molecule appearance of curcumin with its two phenyl moieties. Both moieties are able to suit into the cyclodextrin cavity on each end (Loftsson, T., et al., 2004b; Hegge, A.B., et al., 2009).

In 11% (w/v) solution of RM β CD, the highest concentration, approximately 8×10^{-4} M of curcumin was measured in phosphate buffer by Tønnesen, H.H., et al. (Tønnesen, H.H., et al., 2002a).

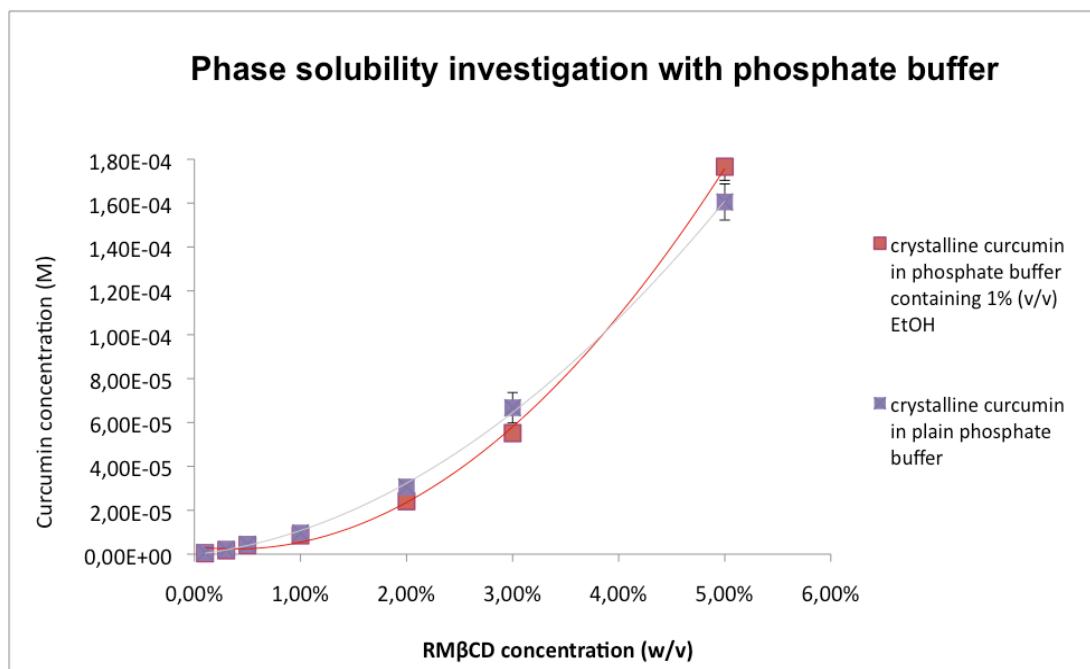


Figure 8: Phase solubility diagram of curcumin in phosphate buffer showing A_p like curves. The curves show the solubility of curcumin at increasing concentrations (w/v) of RM β CD with different solvents

RM β CD concentration	curcumin concentration (M) in plain buffer		curcumin concentration (M) in phosphate buffer containing 1% (v/v) EtOH	
	curcumin concentration (M)	+/- SD	EtOH	+/- SD
0,10%	7.57×10^{-7}	1.49×10^{-7}	4.28×10^{-7}	1.45×10^{-7}
0,30%	2.21×10^{-6}	3.48×10^{-7}	1.75×10^{-6}	4.54×10^{-7}
0,50%	4.51×10^{-6}	5.29×10^{-7}	4.12×10^{-6}	1.98×10^{-7}
1%	9.65×10^{-6}	6.17×10^{-7}	8.57×10^{-6}	1.11×10^{-6}
2%	3.06×10^{-5}	3.55×10^{-7}	2.41×10^{-5}	1.45×10^{-6}
3%	6.67×10^{-5}	6.93×10^{-6}	5.52×10^{-5}	2.48×10^{-6}
5%	1.61×10^{-4}	8.24×10^{-6}	1.77×10^{-4}	6.21×10^{-6}

Table 4: The concentration of curcumin (M) expressed as the calculated mean +/- SD in different concentration (w/v) of RM β CD solution in plain phosphate buffer and phosphate buffer containing 1% (v/v) ethanol

Previous investigations with another beta derivative i.e. HP β CD observed on curcumin-CD complexation showed 2:1 host-guest inclusion complex in distilled water (Baglolle et al., 2005) and a 1:1 host-guest inclusion complex in phosphate buffer (Tønnesen, H.H., et al., 2002b). Hegge, A.B., et al., 2008 observations with phosphate buffer ($\mu=0.085$) : ethanol (9:1) indicated mainly 1:1 stoichiometry. The phase solubility diagram of curcumin in a solution of HP β CD is close to linear and its slope is less than one, which indicates a 1:1 stoichiometry. But also a formation of higher order complexes is possible. The phase solubility diagram obtained in HP β CD/phosphate buffer without ethanol showed an A_p -like curve, which indicates higher order of complexation between the host and the guest molecule.

Hegge, A.B, et al., 2009 did again investigations with HP β CD and showed mainly 1:1 stoichiometry but also higher order complexes can't be eliminated. This study was investigated with the same solvent and the same synthesized curcumin but with a UV-VIS titration method. Due to different methods (Stoichiometry studies performed with HPLC can't be easily compared with UV-VIS titration method) a possibility of different reported curcumin-cyclodextrin stoichiometries could occur (Hegge, A.B., et al., 2009).

Figures 7 and 8 show non-linear A_p-type of phase solubility diagrams for both in the presence and absence of ethanol. In both systems the presence of ethanol enhances the overall solubilizing effect of CD. More precisely, in the presence of ethanol, the distinction in solubility is higher within the investigation with purified water than within the investigations with phosphate buffer. In fact, 1% (v/v) ethanol affects the complexation in both systems, in purified water and phosphate buffer. Compared to the investigations Hegge, A.B., et al., 2008 have performed on HPβCD. Here, in both systems (phosphate buffer and as well phosphate buffer with ethanol 9:1), the presence of ethanol decreases the overall solubilizing effect of CD (Hegge, A.B., et al., 2008). In this particular case the effect of ethanol may be caused by a competitive binding i.e. ethanol is able to displace curcumin in the cyclodextrin cavity (Loftsson, T., et al. 1999; Hegge, A.B., et al., 2008).

The phase solubility-curves obtained in Figure 9, when curcumin was added as a combination of crystalline curcumin (approximately 8mg) and of curcumin dissolved in ethanol (10⁻³ M) were compared to phase solubility curves showed in Figure 8. At CD concentration ≤ 2% the apparent solubility of curcumin is higher compared to the solubility detected, when only crystalline curcumin is added to the same solvent. The total concentration of ethanol in the phosphate buffer is 1% (v/v) EtOH. The increased solubility of curcumin observed in Figure 9 might be caused by curcumin in a supersaturated state. CDs are reported to be able to delay crystallization of drugs from supersaturated solutions (Brouwers, J., et al., 2009).

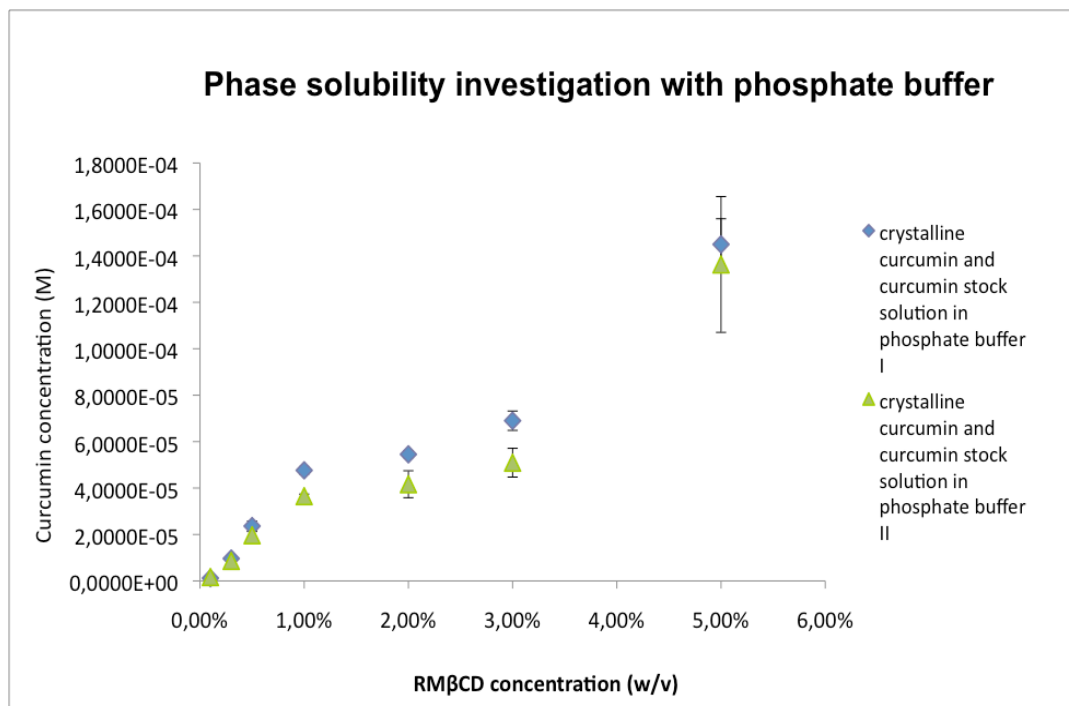


Figure 9: The diagram shows the solubility of curcumin at increasing concentrations (w/v) of RMβCD with different solvents

RMβCD concentration	curcumin concentration (M) of crystalline + solved curcumin in phosphate buffer I		curcumin concentration (M) of crystalline + solved curcumin in phosphate buffer II	
		+/- SD		+/- SD
0,10%	1.20×10^{-6}	4.21×10^{-7}	1.69×10^{-6}	5.89×10^{-7}
0,30%	9.65×10^{-6}	6.31×10^{-7}	8.63×10^{-6}	1.29×10^{-6}
0,50%	2.36×10^{-5}	2.15×10^{-6}	1.97×10^{-5}	1.60×10^{-6}
1%	4.76×10^{-5}	1.61×10^{-6}	3.65×10^{-5}	8.89×10^{-7}
2%	5.45×10^{-5}	1.42×10^{-6}	4.17×10^{-5}	5.80×10^{-6}
3%	6.90×10^{-5}	4.17×10^{-6}	5.09×10^{-5}	6.19×10^{-6}
5%	1.45×10^{-4}	1.10×10^{-5}	1.36×10^{-4}	2.92×10^{-5}

Table 5: Molar solubility of curcumin in different concentration (w/v) of RMβCD solution investigated with phosphate buffer.

The total concentration of ethanol is 1% (v/v) for all the samples. The concentration of curcumin (M) is expressed as well as the calculated mean +/- SD.

Thus, cyclodextrins can potentially stabilize a supersaturated state at low CD concentration. By adding dissolved curcumin and shaking the samples for one day a supersaturated system could occur. However, the supersaturated system may return to equilibrium if the shaking time would be enhanced to 1 week, for example. Further, investigations will be needed to clarify this.

The effect of small amounts of alginate and HPMC on the curcumin: cyclodextrin complexation was investigated. A_p-like curves indicating higher order complexation (Figure 8) were obtained in water containing 1% (v/v) ethanol. Compared to other investigations at same conditions but without polymers, alginate did not increase the complexation, however, HPMC appeared to decrease the complexation. 9.69×10^{-5} was the highest curcumin concentration as detected in 5% (w/v) RM β CD in HPMC in water containing 1% (v/v) EtOH compared to the highest curcumin concentration of 1.65×10^{-4} as detected in 5% (w/v) RM β CD in sodium alginate in water containing 1% (v/v) EtOH. Neither the presence nor the type of polymer did change the complexing abilities between curcumin and CD with 1% (v/v) ethanol in the complexing medium.

Loftsson, T. (Loftsson, T., et al., 1998) investigated the effect of complex formation between drug and β -cyclodextrin contained CMC, PVP or HPMC on the solubility. Adding different types of polymer resulted in a 3-10% increase in the total aqueous solubility of β CD but the drug- β CD complexation results in a 40% to >100% increase in solubility. The aqueous solubility of β CD is increased and it is caused by the polymers but its ability to form inclusion complexes isn't decreasing. In most cases, the polymers increase the complexing abilities of β CD (Loftsson, T., et al., 1998).

Hegge, A.B., et al., 2009 showed that the presence of various co-solvents, e.g. 0.1% (w/v) sodium alginate or propylene glycol alginate, did not affect the stoichiometry of the complexes formed. Neither the presence nor the type of alginate did change the complexation between curcumin and CD with 10%

(v/v) ethanol in the complexing medium. In the case of HP β CD a 30-90% increase in the association constant was observed in the presence of alginates (Hegge, A.B., et al., 2008).

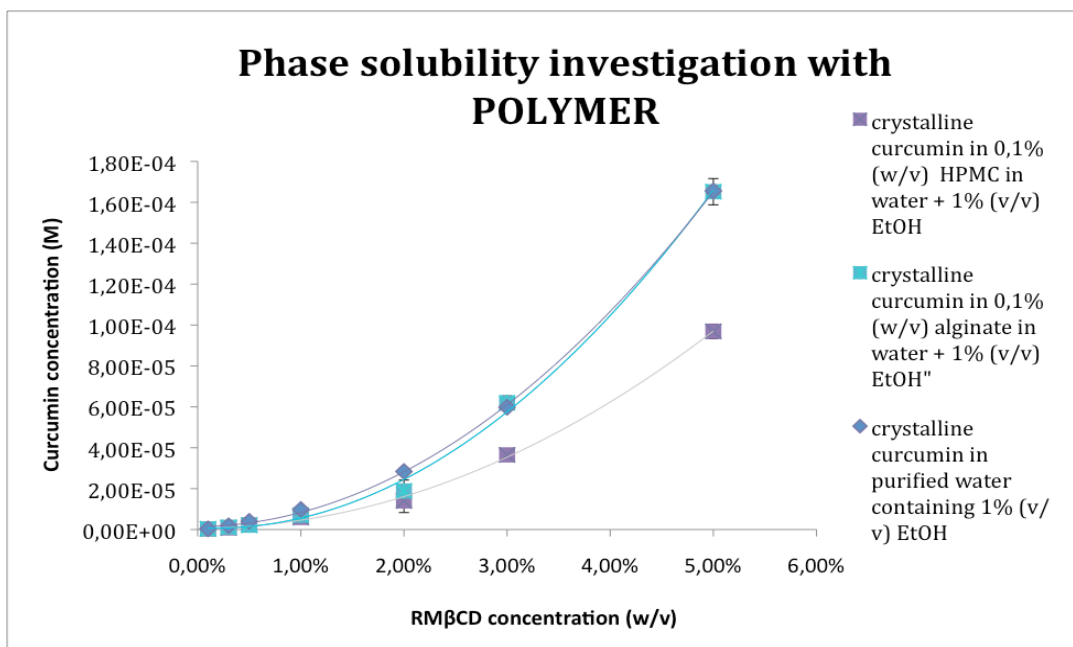


Figure 10: Phase solubility diagram of curcumin with different types of polymer showing A_p like curves. The curves show the solubility of curcumin at increasing concentrations (w/v) of RM β CD in purified water containing 1% (v/v) EtOH and/ or containing different types of polymer

RM β CD concentration	curcumin concentration (M) in 0.1% (w/v) HPMC in water + 1% (v/v) EtOH		curcumin concentration (M) in 0.1% (w/v) alginate in water + 1% (v/v) EtOH	
	mean	+/- SD	mean	+/- SD
0,10%	2.79×10^{-7}	5.79×10^{-8}	3.43×10^{-7}	7.33×10^{-8}
0,30%	7.89×10^{-7}	6.80×10^{-7}	1.15×10^{-6}	4.64×10^{-7}
0,50%	2.20×10^{-6}	6.90×10^{-7}	2.14×10^{-6}	1.07×10^{-6}
1%	5.90×10^{-6}	2.29×10^{-6}	7.04×10^{-6}	8.98×10^{-7}
2%	1.40×10^{-5}	5.70×10^{-6}	1.89×10^{-5}	5.36×10^{-6}
3%	3.65×10^{-5}	3.09×10^{-6}	6.19×10^{-5}	3.44×10^{-6}
5%	9.69×10^{-5}	3.54×10^{-6}	1.65×10^{-4}	6.41×10^{-6}

Table 6: The concentration of curcumin (M) expressed as the calculated mean +/- SD in different concentration (w/v) of RM β CD solution with 2 different polymers in purified water containing 1% (v/v) ethanol

4.1.2 Quantification of curcumin after freeze-drying – drug load

As seen in table 7, the averages of the curcumin load in the freeze-dried product obtained from four different runs of freeze drying. There is a clear variation between the different runs. It is caused by different reasons. One of the reasons is that the dissolution rate of the product after solvent evaporation wasn't controlled, which is an important factor to observe in the future. But also the freeze-dried process needs better technical preparation in the future.

	average curcumin load of the 1:1 product		average curcumin load of the 2:1 product		average curcumin load of the 3:1 product	
		+/- SD		+/- SD		+/- SD
1 run	0,0745%	0,0068%	0,2273%	0,0120%	0,2629%	0,0090%
2 run	0,1216%	0,0040%	0,1695%	0,0044%	0,3064%	0,0098%
3 run	0,0892%	0,0032%	0,2079%	0,0070%	0,2665%	0,0173%
4 run	0,2500%	0,0156%	0,2483%	0,0127%	0,3869%	0,0698%

Table 7: Curcumin load in % after freeze-drying
The average of the curcumin load of the freeze dried products in the ratio 1:1, 2:1, 3:1 (CD:curcumin), respectively, which were used to prepare the solution for the solvent evaporation

Ratio of CD:Cur in % (w/w), which was used to prepare the solution for the solvent evaporation for the freeze drying step	average curcumin load in % (w/w) of the freeze dried products	
		+/- S.D
1:1	0,1338%	0,0057%
2:1	0,2133%	0,0040%
3:1	0,3057%	0,0291%

Table 8: The average (of 4 runs) of the detected curcumin load and +/- standard deviation (SD) after freeze-drying

In Table 8 the averages of all the common products are summarized. Also the standard deviation is indicated. This table shows that the selected ratio of CD:curcumin influences the final drug load of the freeze dried product. A 3:1 (CD:Cur) resulted in the highest drug load detected of 0.31% (w/w) curcumin.

Hegge, A.B., et al., 2010 reported a theoretical curcumin load of 0.15% (w/w) in previous prepared alginate foams. Different ratios between CDs and curcumin were selected to investigate the effect of the CDs on the physical characteristics of the foams, the curcumin release and photostability (Hegge, A.B., et al., 2010).

The optimal ratio of CD:curcumin needs to be established. The importance of selection the optimal ratio of CD:curcumin to obtain the most efficient complexation in the solvent evaporation has been demonstrated in case of curcumin and HP γ CD (Singh, R., et al., in process). As well more importantly is the question, how it is possible to reach the highest possible curcumin load from the solvent evaporation product before freeze drying. However, in order to find the optimal ratio of M β CD:curcumin, further studies will be needed. It is essential to control the solubility of curcumin in the solution used for the freeze-dried process in order to control the drug load of the final product.

4.1.3 Degradation of the products after freeze-drying

The HPLC chromatograms indicate some degradation of curcumin, observed as several unidentified peaks. In Figure 11 only curcumin appears at 430nm and compared to the other figure 12 at a wavelength 376nm there are several unidentified peaks, which are not visible at 430nm. These unidentified peaks with retention time less than 4 min can be caused by impurities in the synthesized curcumin powder. In this thesis an excess of powder was used and it was filtered before freeze drying. Accordingly impurities might be visible in the HPLC chromatogram. Further investigations need to be done if these unidentified peaks are caused by degradation of curcumin or by impurities of the starting compound.

Feruloylmethane, ferulic acid, vanillin and also condensation products of feruloylmethane are some of the degradation products of curcumin, as already mentioned in 2.1.3.3 (Tønnesen, H.H., Karlsen, J., 1985a).

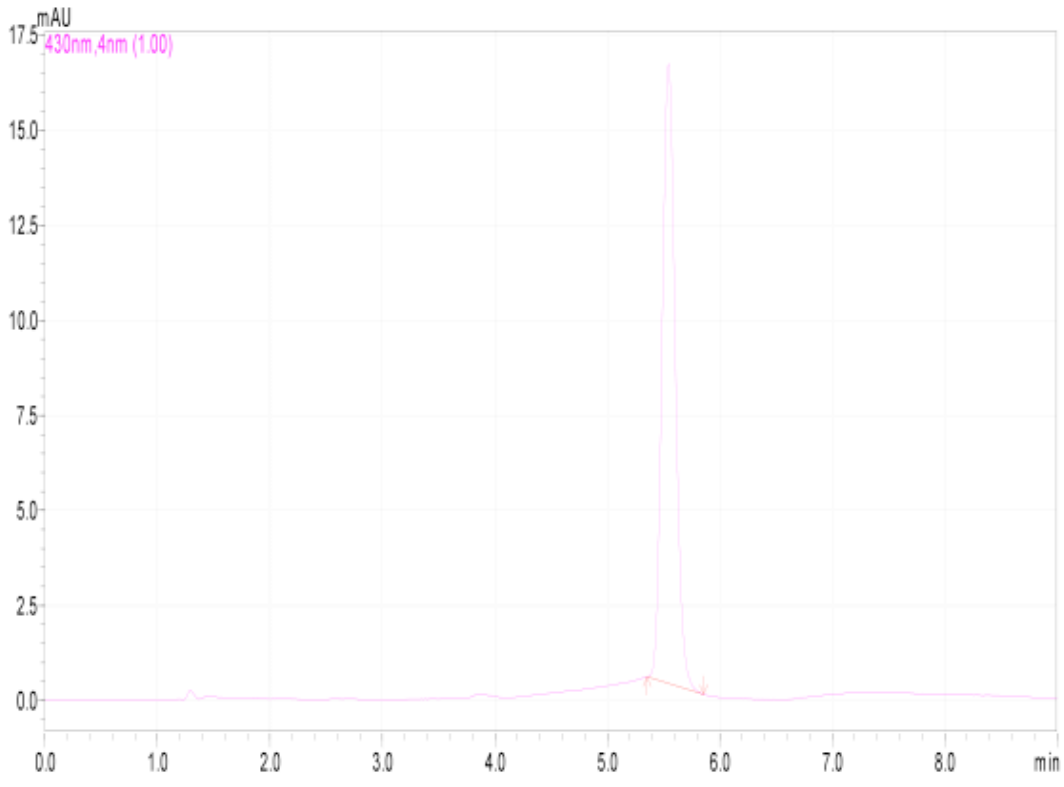


Figure 11: HPLC chromatogram of the product after freeze-drying at the wavelength 430nm

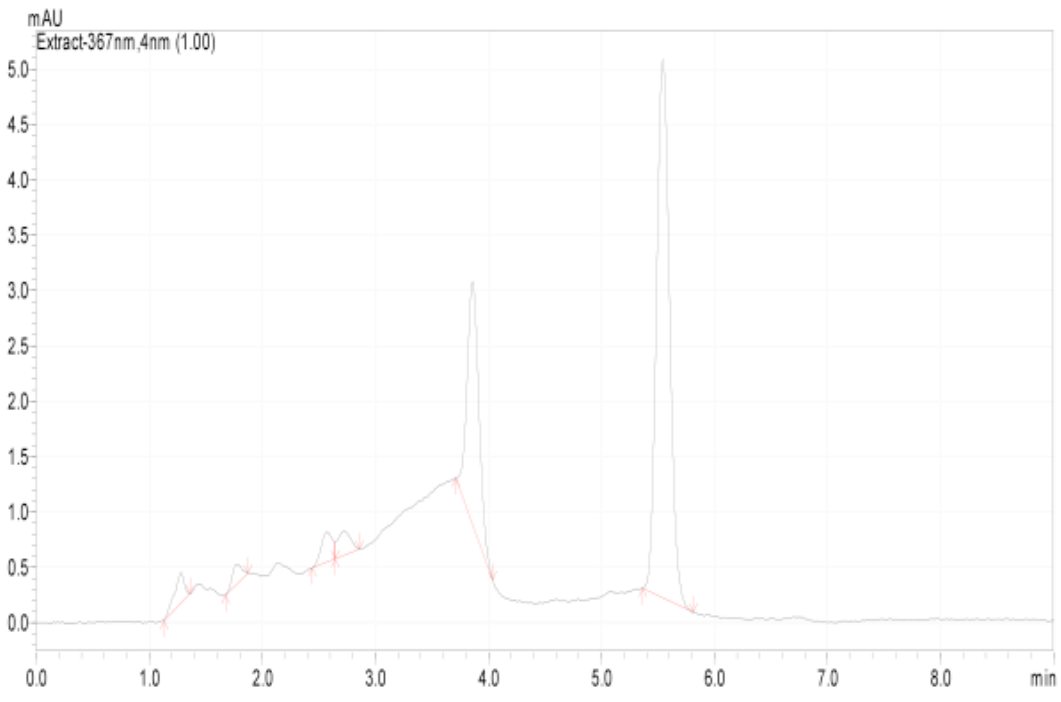


Figure 12: HPLC chromatogram of the product after freeze-drying at the wavelength 367nm

4.2 ROTAVAPOR

Two different systems were utilized. First system was curcumin dissolved in methanol. Second system was the combination of curcumin-cyclodextrin in methanol. Both systems were evaporated by a rotavapor. After evaporation both samples had the same powder-like visual appearance. Analyzed by DSC and SEM there are differences, which are mentioned in the section below.

4.3 DSC and SEM

Differential scanning calorimetry is a fast and relatively inexpensive technique to characterize the physical state of drugs or complexes of drugs (Bing, M., et al., 2010). DSC measures the energy change, which is the heat flow.

Scanning electron microscopy was used to imagine the sample surface by scanning it with high-energy beam of electrons in a raster scan pattern (Wikipedia, 2010).

DSC and SEM pictures of crystalline curcumin, pure RM β CD, curcumin with RM β CD in MeOH after evaporation and the freeze-dried product are shown in Figures 13-27. The SEM pictures below show the various components in their different appearance.

4.3.1 DSC and SEM of crystalline curcumin

The thermograms of pure curcumin in figure 13 show an intense melting endotherm at around 180°C corresponding to the melting point of pure

curcumin, which is specified in literature (Merck, 2001). In addition to this, a heating-cooling-heating cycle in exact temperatures from 25°C to 200°C back to 25°C and from 25°C to 300°C with the heating rate of 10°C/min was operated. This showed that curcumin doesn't recrystallize by cooling because exothermal crystallization could not be observed. It remains amorphous. By reheating it undergoes glass transition at around 70°C. This glass transition is indicated with the red box in figure 13.

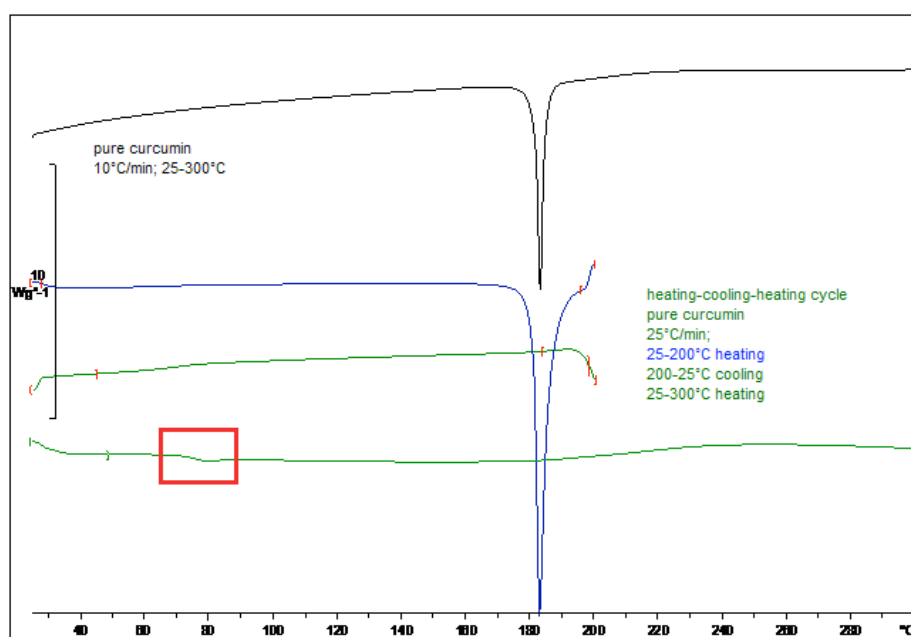


Figure 13: DSC thermograms of pure curcumin. The blue and green curves show the heating-cooling-heating cycle. The red box indicates the glass transition.

Figures 14 and 15 show two SEM pictures of pure curcumin with different magnification. Overall, it can be observed that curcumin appears as crystalline needles.

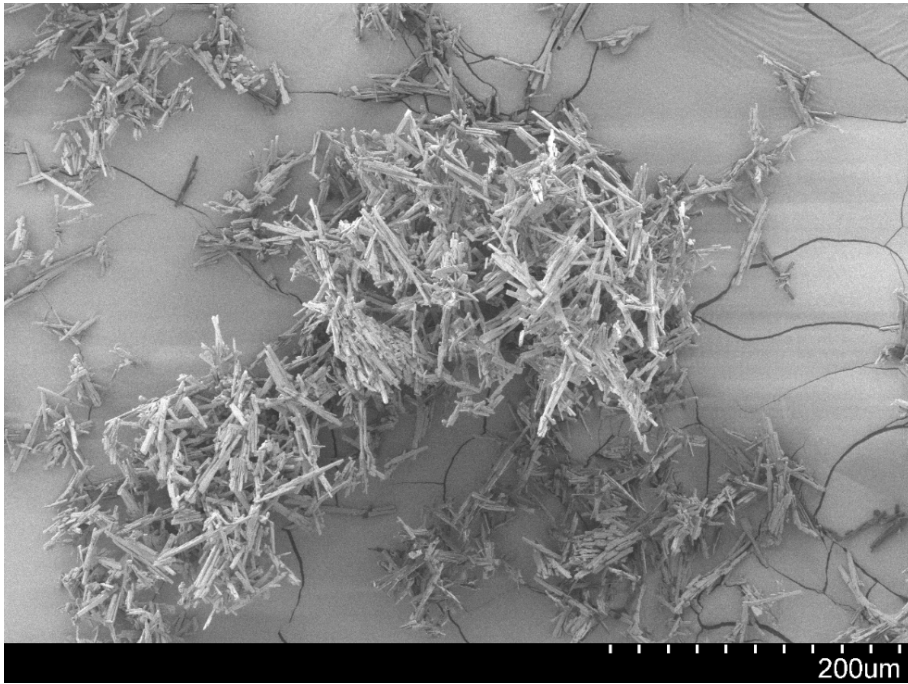


Figure 14: SEM picture of pure curcumin with a magnification of 200.

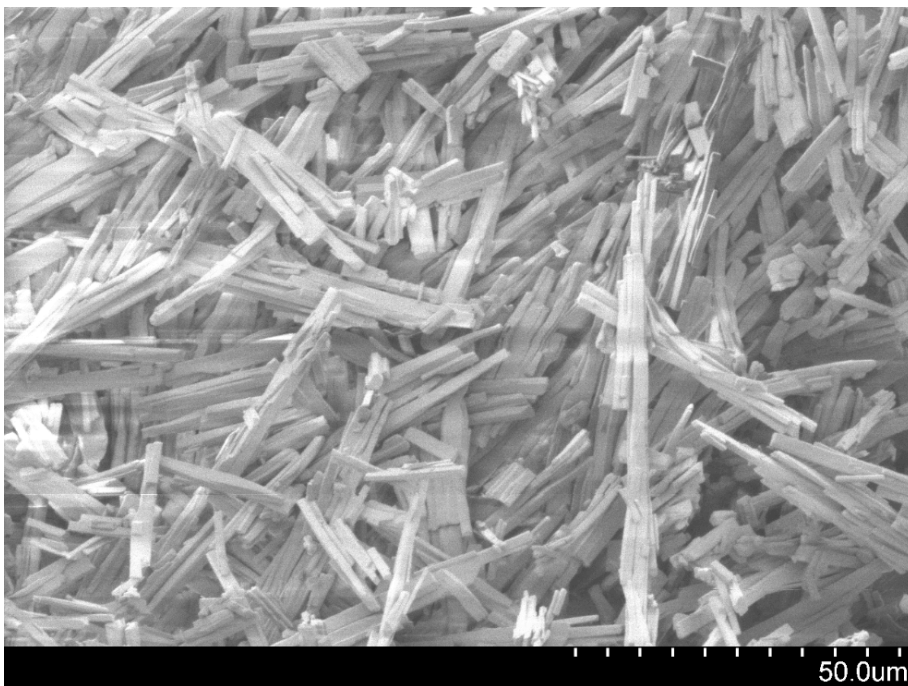


Figure 15: SEM picture of pure curcumin with a magnification of 900

4.3.2 DSC and SEM of RM β CD

The previous detected water content in RM β CD was 2.8%. The first endothermic peak in pure RM β CD in figure 16 is evaporation of residual water. Compared to pure curcumin, pure RM β CD undergoes glass transition at around 170°C which is again indicated with the red box. A heating-cooling-heating cycle was performed, which is seen in figure 17. The first heating from 25-110°C showed again evaporation of residual water which results in an endothermic peak. During the second heating from 25-220°C a glass transition at around 170°C appeared. In the producers technical sheet it is mentioned that the melting point of RM β CD is in the range of 165-172°C but according to another reference this selected cyclodextrin should be noncrystalline, which occurs in glass transition but in no melting (Froemming, K-H., et al. 1994).

The analyses in this present study show an amorphous state of the randomized-methylated- β -cyclodextrin due to the absence of a melting peak and the observed glass transition at around 170°C (figures 16 and 17).

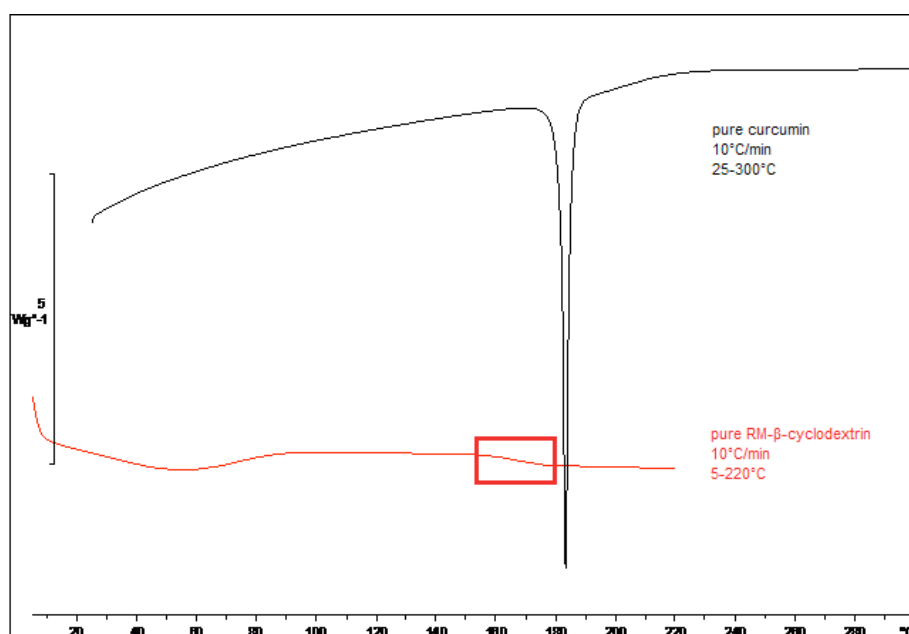


Figure 16: DSC thermograms of pure curcumin with its intense endothermic peak and pure RM β CD with its glass transition. The red box indicates the glass transition.



Figure 17: DSC thermograms of pure RM β CD and also its heating-cooling-heating cycle in the colors blue, green and violet.
The red box indicates the glass transition.

SEM pictures of pure RM β CD with different magnifications are shown in figures 18 and 19. With the results from DSC and the analyses with SEM it seems that this selected cyclodextrin is amorphous.

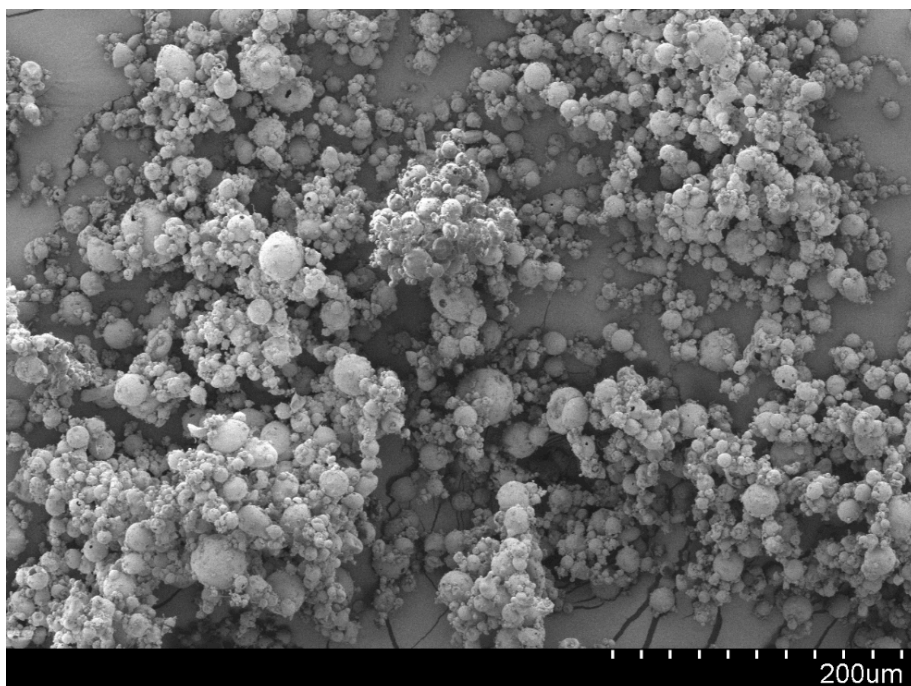


Figure 18: SEM picture of pure RM β CD with a magnification of 200

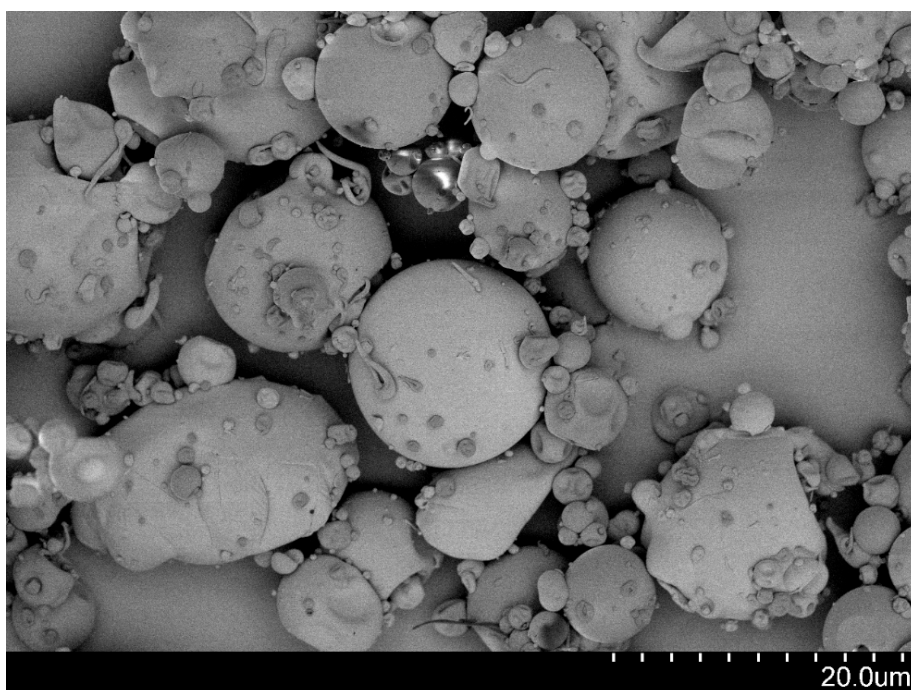


Figure 19: SEM picture of pure RM β CD with a magnification of 2000

4.3.3 DSC and SEM of cur : CD product after solvent evaporation

A solution of curcumin and cyclodextrins in methanol was prepared and the solvent was removed by solvent evaporation using a rotavapor. Analyses of the resulting product are shown in figure 20. The absence of an endothermic peak indicates that the product is not crystalline anymore but amorphous.

The DSC thermograms of curcumin + cyclodextrin in MeOH after evaporation (violet trace) show an endothermic peak in the beginning and as well glass transition occurred comparable to the pure β -cyclodextrin (red trace). A heating-cooling-heating cycle was operated, which shows glass transition at around 140°C during the second heating from 5-220°C. This indicates that curcumin with cyclodextrin after evaporation is turned amorphous compared to pure curcumin before evaporation, which has a melting point and is consequently crystalline.

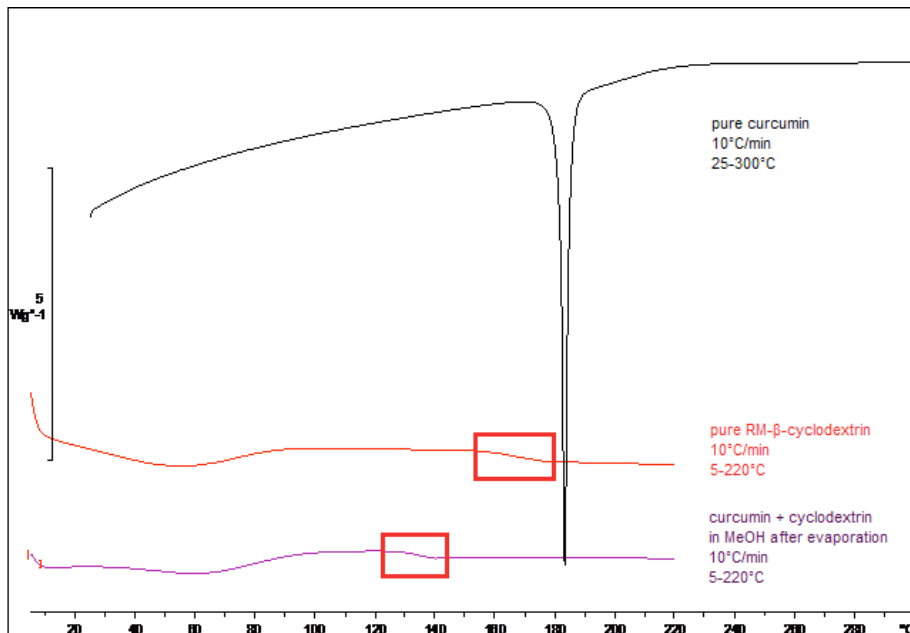


Figure 20: DSC traces of pure curcumin (black trace) and pure RMβCD (red trace) in comparison to curcumin + cyclodextrin in MeOH after evaporation (violet trace). The red box indicates the glass transition.

The SEM pictures demonstrate that curcumin with RM β CD in MeOH after evaporation have a flake-like structure throughout the sample as it is shown in figure 21 with a magnification of 150 and in figure 22 with a magnification of 6000. However, the prior morphology of the parent compounds disappeared and it is impossible to differentiate between the two initial components. A totally new appearance occurred.

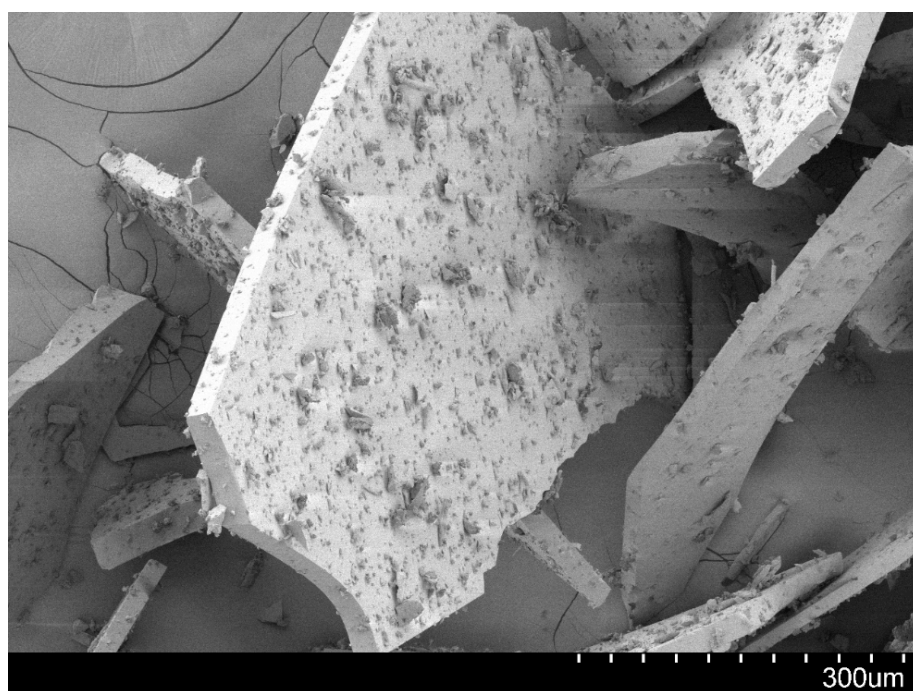


Figure 21: SEM picture of curcumin with RM β CD in MeOH with a magnification of 150

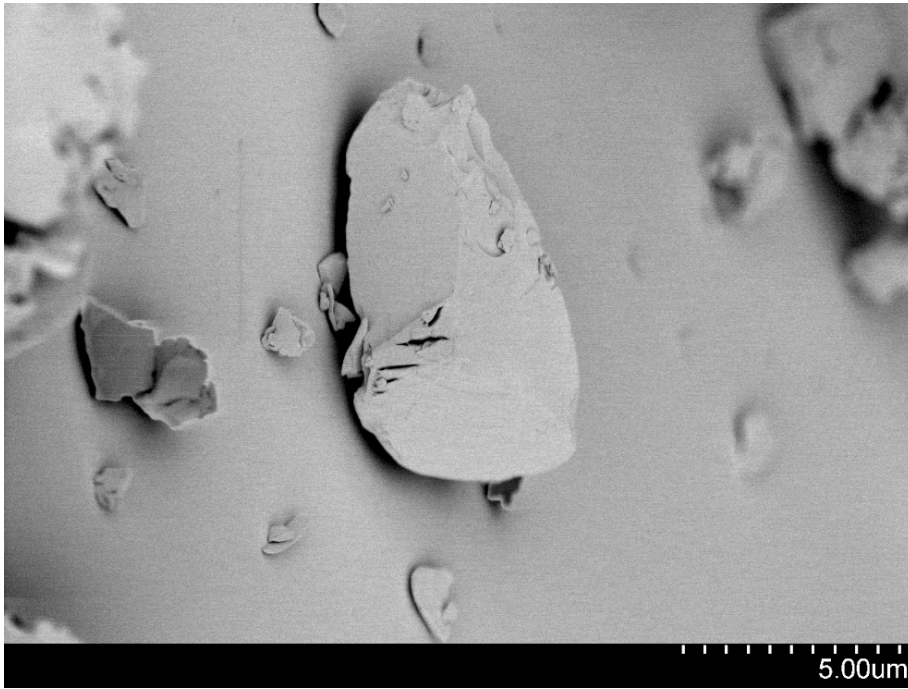


Figure 22: SEM picture of curcumin with RMβCD in MeOH with a magnification of 6000

The pure curcumin in methanol after evaporation was analysed as a control. Analyzing the product after evaporation by DSC with the heating rate of 10°C/min in the range of 5-220°C the same intense endothermic peak appears at around 180°C as seen in pure curcumin powder, which indicates the crystalline form of curcumin again (figure 23). The absence of a glass transition approves that curcumin in MeOH after evaporation is still crystalline, compared to the thermogram in figure 20 (with cyclodextrins), where glass transition appears at around 140°C, which indicates an amorphous structure.

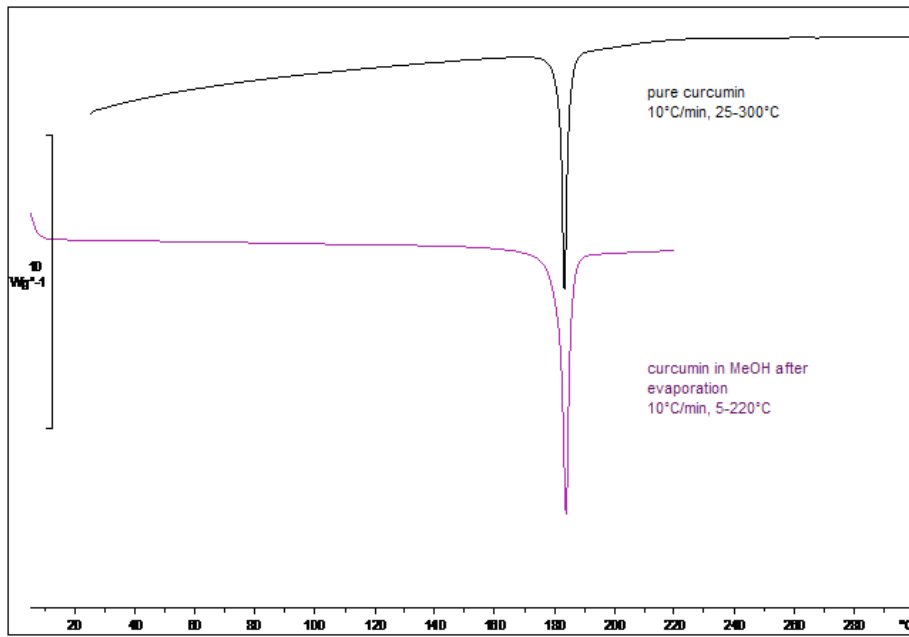


Figure 23: DSC thermograms of pure curcumin and the curcumin in methanol after evaporation

4.3.4 DSC of the freeze-dried products

The thermograms of three freeze-dried products with different CD:Cur ratios in figure 24 are similar in appearance. During heating from 5-220°C with the heating rate of 10°C/min glass transition appears in the range of 60-80°C. No melting endotherm was observed at around 180-190°C where pure curcumin is supposed to have its melting point. Consequently crystalline curcumin was not detected in the freeze-dried product.

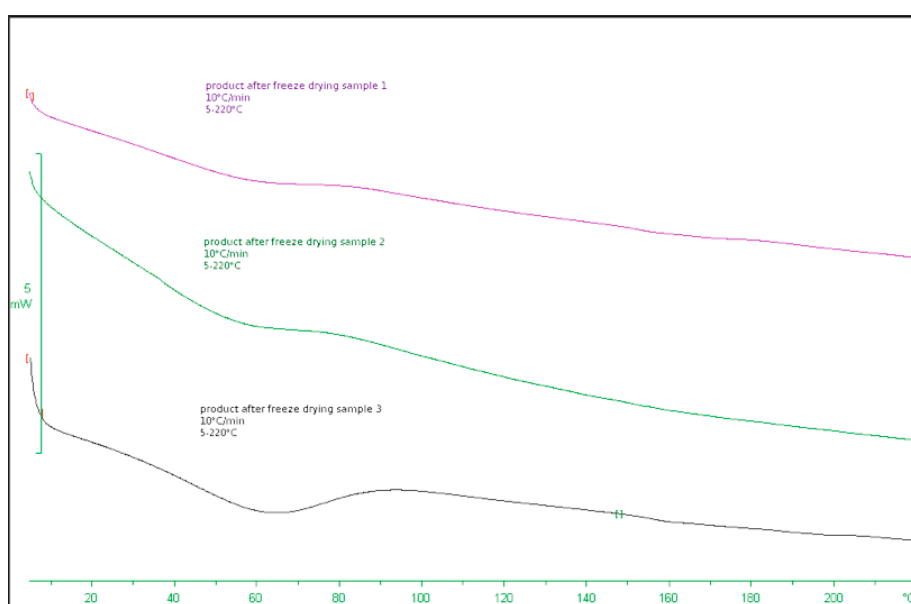


Figure 24: DSC thermograms of freeze-dried products with different ratios. Violet trace - CD:Cur ratio 1:1, green trace - CD:Cur ratio 2:1, black trace - CD:Cur ratio 3:1

Figure 25, 26 and 27 show the freeze-dried curcumin/ RM β CD/ HPMC system with its polymer structure with different magnification (45, 80 and 2200). The prior morphology of the parent compounds disappeared and a totally new appearance occurred. Ice crystals formed during the freezing process left behind the large pores.

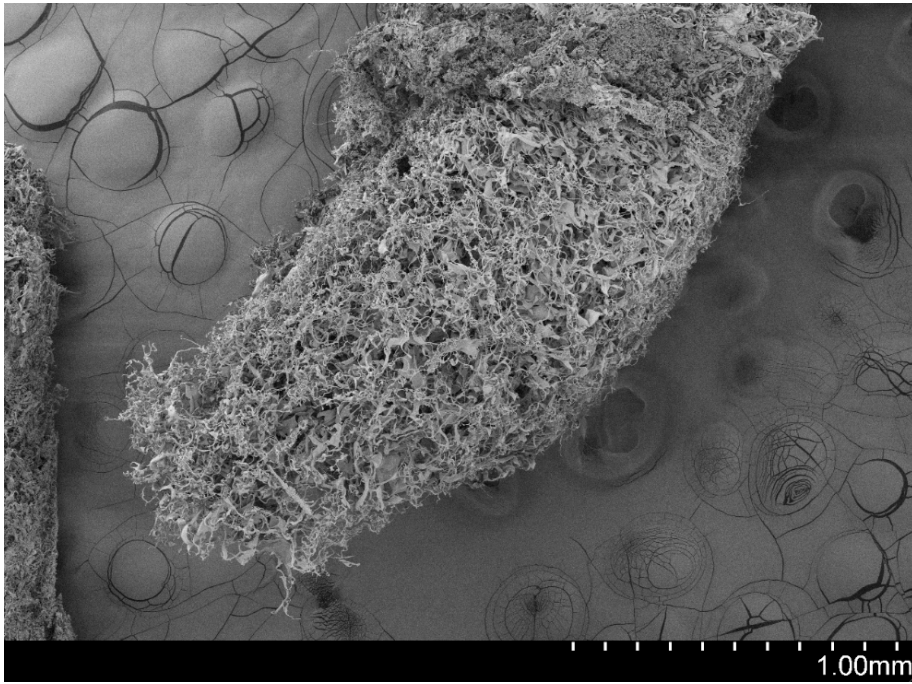


Figure 25: SEM picture of the freeze-dried curcumin/ RMβCD/ HPMC system with a magnification of 45

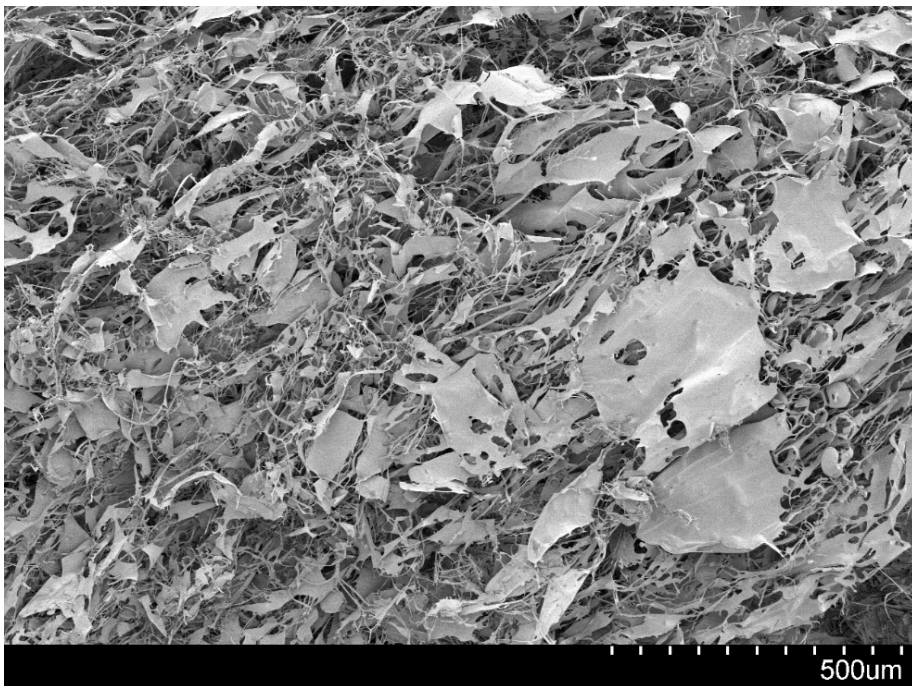


Figure 26: SEM picture of the freeze-dried curcumin/ RMβCD/ HPMC system with a magnification of 80



Figure 27: SEM picture of the freeze-dried curcumin/ RM β CD/ HPMC system with a magnification of 2200

4.4 LYOPHILIZATION

In order to improve the physical and chemical stability of the samples and to convert solutions or suspensions into solids, one of the most commonly used processes is freeze-drying (Patil, V.V., et al., 2010).

All freeze-dried products had a sponge like morphology. After lyophilization the products appeared like foam. Figure 28 shows a representative freeze-dried sample containing curcumin, CD and HPMC in a typical freeze-dried vial with plastic stopper.



Figure 28: one of the freeze-dried sample in a freeze-dried vial with plastic stopper

5 SUMMARY AND CONCLUSION

Curcumin has a wide range of pharmacological properties and is currently under intense investigation as a possible photosensitizer in a PDT. However, the extremely low aqueous solubility of curcumin and low stability at physiological conditions, solubilization and protection from degradation will be necessary in order to utilize this compound as a drug.

The aim of this master thesis was to improve the solubility and dissolution rate of curcumin by using a selected cyclodextrin. With the aid of phase solubility studies conducted by Higuchi and Connors (1965), the solubility of curcumin in 7 different solvents was investigated. All these studies show non-linear, A_p -like curves independent from the solvent used. This indicates some kind of higher order complexation.

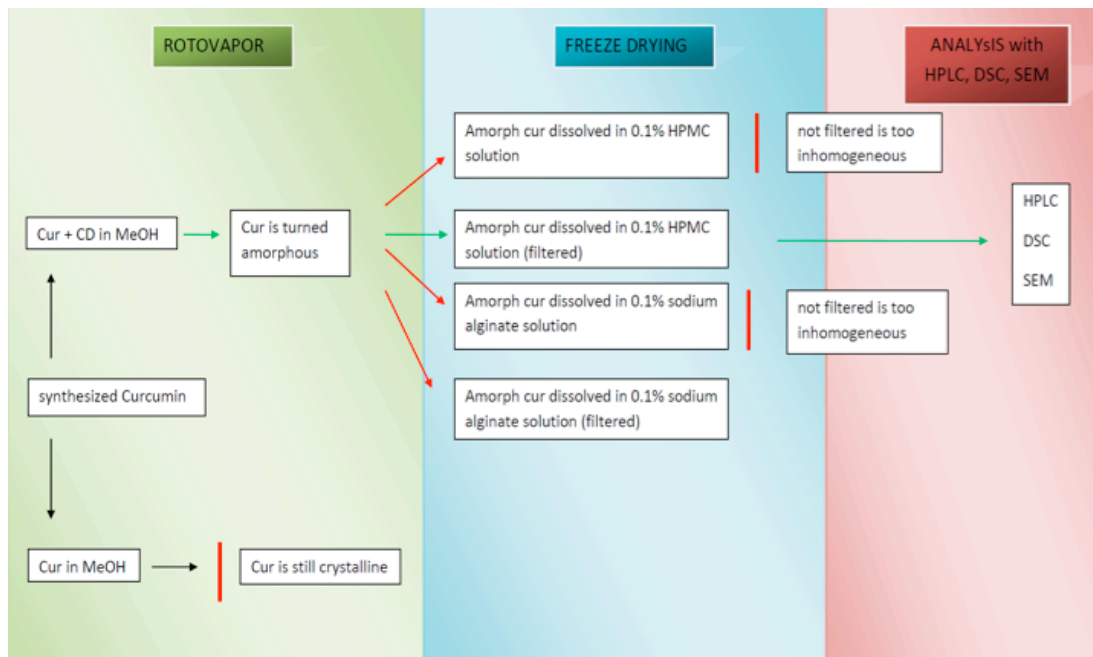
As described in chapter 4.1 the highest curcumin concentration obtained in the present study was 1.77×10^{-4} M as detected in 5% (w/v) RM β CD in phosphate buffer containing 1% (v/v) ethanol. Similar solubilities were detected independent of the solvent used and independent of the presence of 0.1% alginate. There is no increasing solubility by adding any polymer. In fact, less solubility occurred by adding HPMC.

Different solubility curves were found when curcumin was added as a combination of curcumin dissolved in EtOH (10^{-3}) together with crystalline curcumin to RM β CD in phosphate buffer. In the beginning the curves seemed to be linear but at higher cyclodextrin concentration the curves started being A_p -like. At CD concentration $\leq 2\%$ the apparent solubility of curcumin is higher compared to the solubility detected, when only crystalline curcumin is added to the same solvent. The increased solubility of curcumin might be caused by curcumin in a supersaturated state, which cyclodextrins

potentially stabilize. Further investigations under different buffer conditions will be needed to clarify this.

To further enhance the solubility of curcumin in aqueous solutions, a solvent evaporation method was selected. In fact, the product of curcumin with cyclodextrin after evaporation is turned amorphous compared to the pure curcumin before evaporation, which is crystalline. The prior morphology of the parent compounds disappeared after solvent evaporation and a totally new product appeared.

The figure below shows the series of tests, which were performed in this study to find the most promising product to enhance the solubility and stability of curcumin. Even though 0.1% (w/v) HPMC in water with 1% (v/v) EtOH didn't get as promising solubilizing effect as sodium alginate, HPMC was chosen to carry out the following studies. The increased viscosity of sodium alginate complicated the filtration of the solutions prior to freeze dry and prior to quantification.



The method of lyophilization was used to improve the physical and chemical stability of this new product. But at this point the procedure of lyophilization with a curcumin/ cyclodextrin/ HPMC solution is in its infancy. To find out the best conditions, a lot of measurements need to be taken.

Nonetheless, some analyses of the lyophilized products were performed but the products haven't been fully characterized yet.

By checking the freeze-dried products with DSC, a glass transition occurred, which indicates that the product after lyophilization remains amorphous.

The drug load of the freeze-dried products shows great promise as well. 0.31% (w/w) curcumin load was detected in 3:1 CD:Cur ratio. Unfortunately it's a clear variation between the different runs. It is caused by different reasons. It is essential to control the solubility of curcumin in the solution used for the freeze-dried process in order to control the drug load of the final product. Also the lyophilization process needs better technical preparation in the future.

The specific degradation products haven't been investigated so far. The HPLC chromatograms indicate some degradation of curcumin, observed as several unidentified peaks. Further investigations need to be done if these unidentified peaks are caused by degradation of curcumin or by impurities of the starting compound.

Furthermore, to find the exact water content of the lyophilized product, a Fischer titration could be carried out in the future.

However, a porous freeze-dried product will probably increase the dissolution rate and solubility of curcumin markedly. After all it will still be able to protect curcumin from degradation during storage. Such product has a potential use as a topical drug delivery system. This interesting and promising project has just started. More investigations need to be conducted in the future.

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Appendix:

A.1 Equipment

HPLCs:

HPLC system I: system I consisted of a Waters Nova-Pak ® C18, 3.9 x 150 mm, 4µm particle size column by use of a Shimadzu Liquid Chromatography LC-9A pump, a Shimadzu Auto Injector SIL-10 AD auto sampler, a Shimadzu UV-Vis Spectrophotometric detector SPD-10A, and a Shimadzu C-R3A integrator.

HPLC system II: system II consisted of a Waters Nova-Pak ® C18, 3.9 x 150 mm, 4µm particle size column by use of a Shimadzu Liquid Chromatography LC-20AD pump, a Shimadzu Auto Injector SIL-20AC HT auto sampler, a Shimadzu UV-Vis Spectrophotometric detector SPD-M20A, a CBM-20A controlling unit and a CTO-20A oven.

Rotavapor: Büchi EL -131

Differential scanning calorimeter: Mettler Toledo Stare System DSC822^e and Mettler Toledo STARE software

Lyophilization: Virtis 25ES from Virtis Company Inc. USA.

Vacuum pump: Leybold, Trivac D16B from Engineering Company Inc., USA

Scanning electron microscope: Hitachi S-4800 scanning electron microscope

Moisture analyzer: Sartorius Moisture Analyzer MA30

Analytical weight: Sartorius analytical weight

A.2 Reagents

Chemical	Lot Number	Supplier
Citric Acid monohydrate	08D150022	VWR International
Kaliumhydroxid	11293	Eka Nobel AB/ Nobel Industrier
Natriumchlorid	9D122/4	Norsk Medisinaldepot, Norway
Methanol Chromasolv® (HPLC-grade)	SZBA1195	Sigma-Aldrich Co./Chemie GmbH
Ethanol		Norsk Medisinaldepot, Norway
Natriumhydrogenphosphatdihydrat	K91380845 819	Merck KGaA
Di-natriumhydrogenphosphatdihydrat	K27618980 020	Merck KGaA
RM- β -cyclodextrin = Cavasol W7 M	71T051	Wacker Chemie AG
Hydroxypropylmethylcellulose	8028204	Shin-Etr Chemie. Lv.
Sodium Alginate (Protanal® LF 10/60 LS)	S17261	FMC BioPolymers/NovaMatrix

A.3 Buffers and mobile phase

A.3.1 Buffer for HPLC (mobile phase)

0.026 M (0.5%) Citrate buffer pH 3:

For 1000ml buffer:

5.47 g citric acid anhydrate

pH adjusted to 3 with 10% KOH solution

A.3.2 Buffer for phase solubility experiments

Buffer: phosphate buffer pH 5 (0.05 M; ionic strength 0.085):

For 1000ml buffer:

7.8 g $\text{NaH}_2\text{PO}_4 \times 2 \text{H}_2\text{O}$

0.089 g Na₂HPO₄ x 2 H₂O

2g NaCl

A.3.3 Mobile Phase for HPLC system I

62 parts MeOH

38 parts citric acid buffer

A.3.4 Filtration

BD Plastipak 5ml sterile syringe from Becton Dickinson S.A., fitted with Spartan 13/0.45 RC, 0.45µm filters from Whatman.

A.3.5 Detection of the water content in RMβCD:

The water content of the CDs (ca. 2.8% w/w) was determined by heating the CDs to 130°C on a Sartorius analytical weight for 60 minutes, respectively, and calculating the water loss.

The water-content was calculated according to the equation:

$(W1 - W2) / W1 \times 100\%$, where *W1* and *W2* are the weights of the CDs before and after drying, respectively.

The measurements were done in triplicates and the value used for concentration corrections was the average of the three parallels.

1 parallel:

$W1: 1.94478$ $W2: 1.89092$ \rightarrow $x=2.769465\%$

2 parallel:

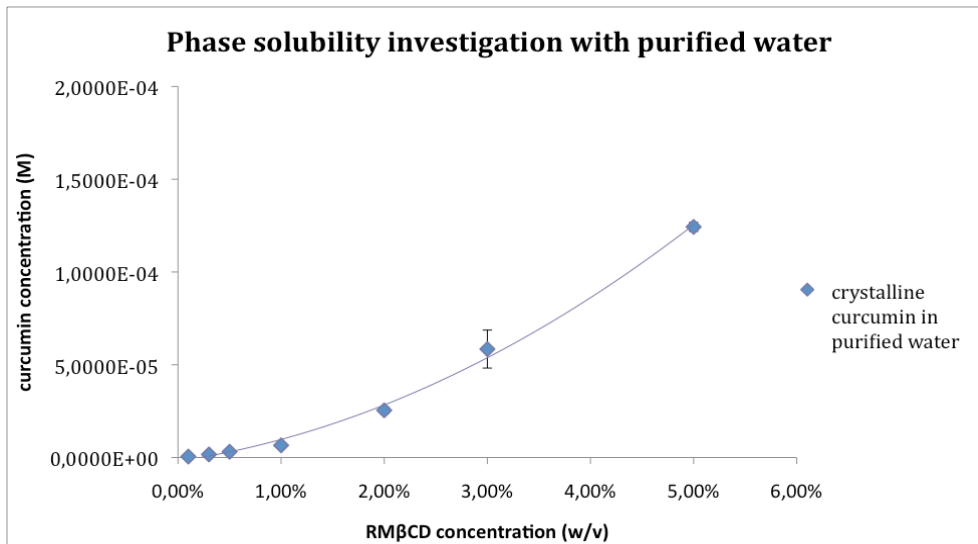
$W1: 1.73229$ $W2: 1.68409$ \rightarrow $x=2.782444\%$

3 parallel:

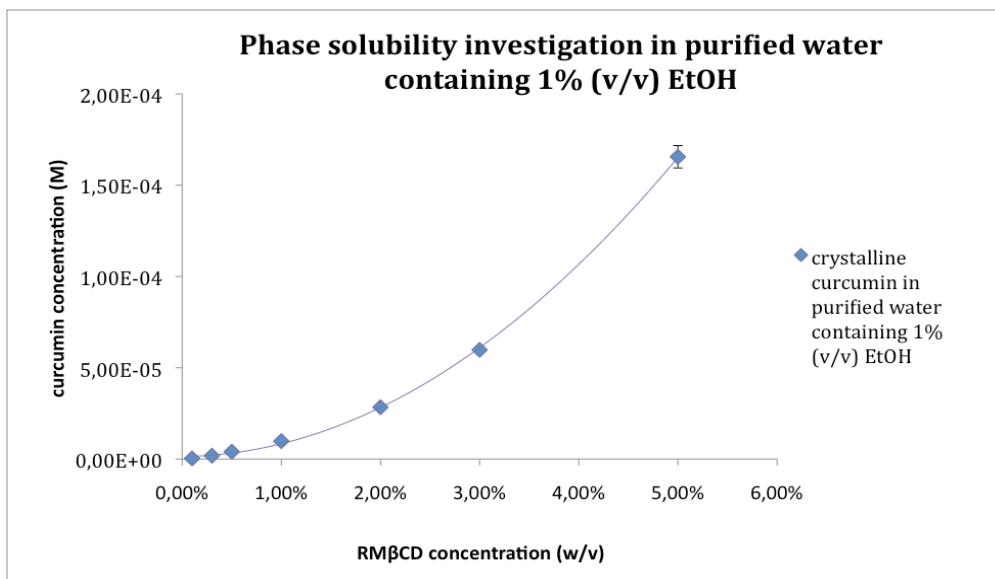
$W1: 1.98667$ $W2: 1.933$ \rightarrow $x=2.701506\%$

A 4 All Solubility investigations

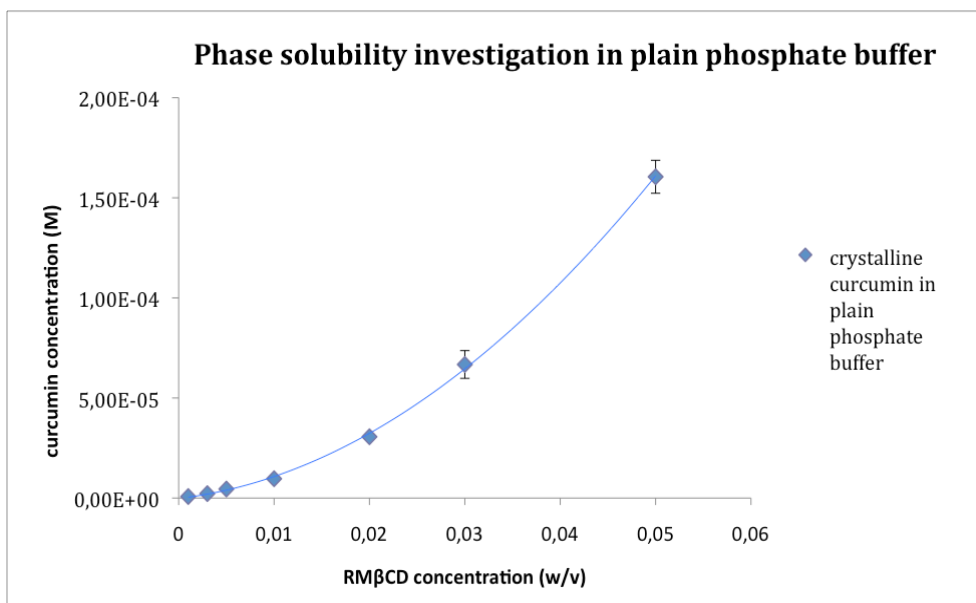
A.4.1 Solubility of crystalline curcumin in 0.1-5% (w/v) of RM β CD, purified water



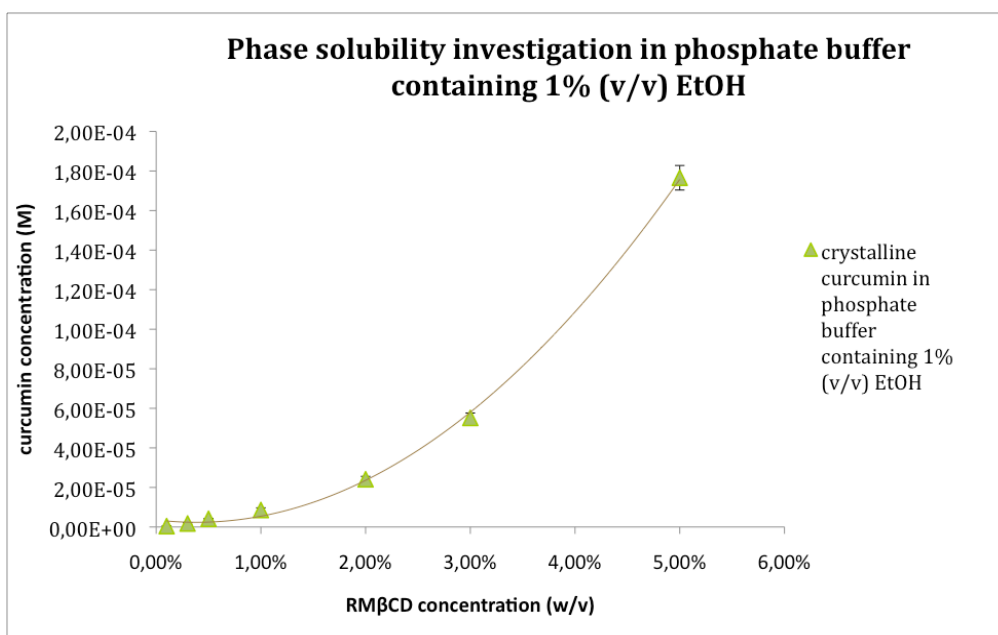
A.4.2 Solubility of crystalline curcumin in 0.1-5% (w/v) of RM β CD, purified water containing 1% (v/v) ethanol



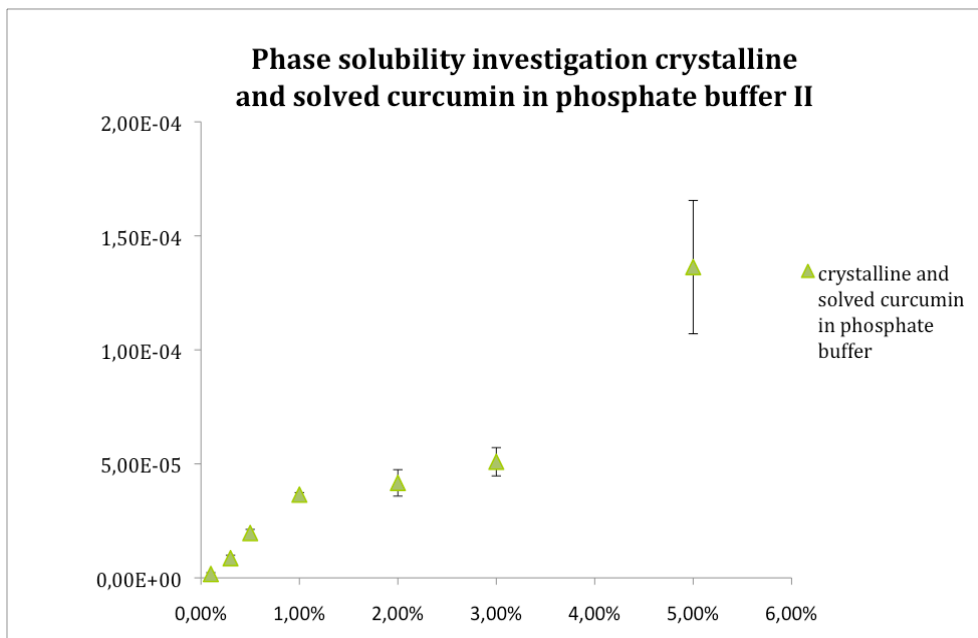
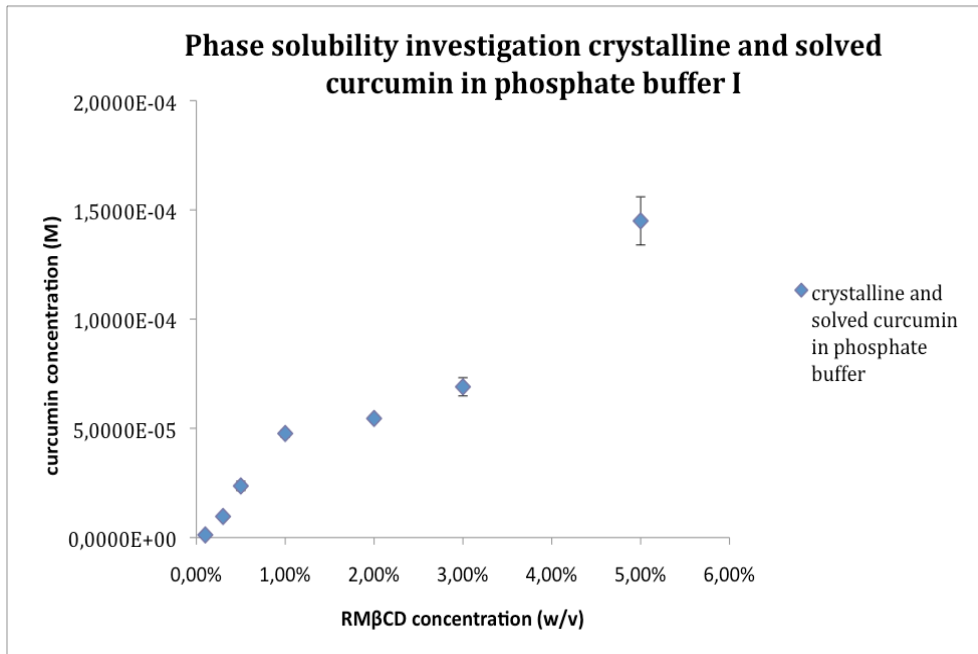
A.4.3 Solubility of crystalline curcumin in 0.1-5% (w/v) of RM β CD, phosphate buffer pH5 (0.1 M, ionic strength 0.085)



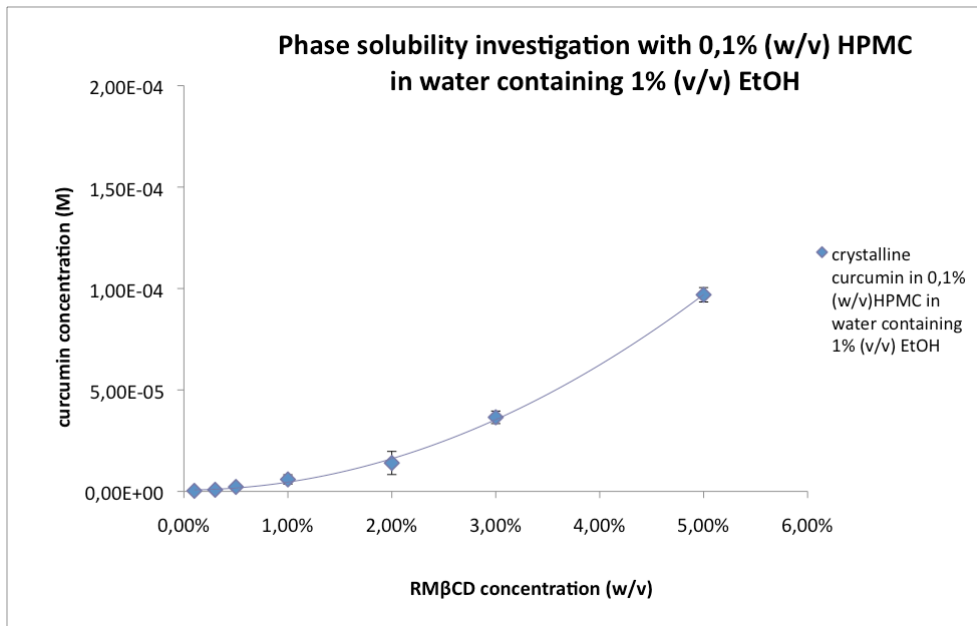
A.4.4 Solubility of crystalline curcumin in 0.1-5% (w/v) of RM β CD, phosphate buffer pH5 (0.1 M, ionic strength 0.085) containing 1% (v/v) ethanol



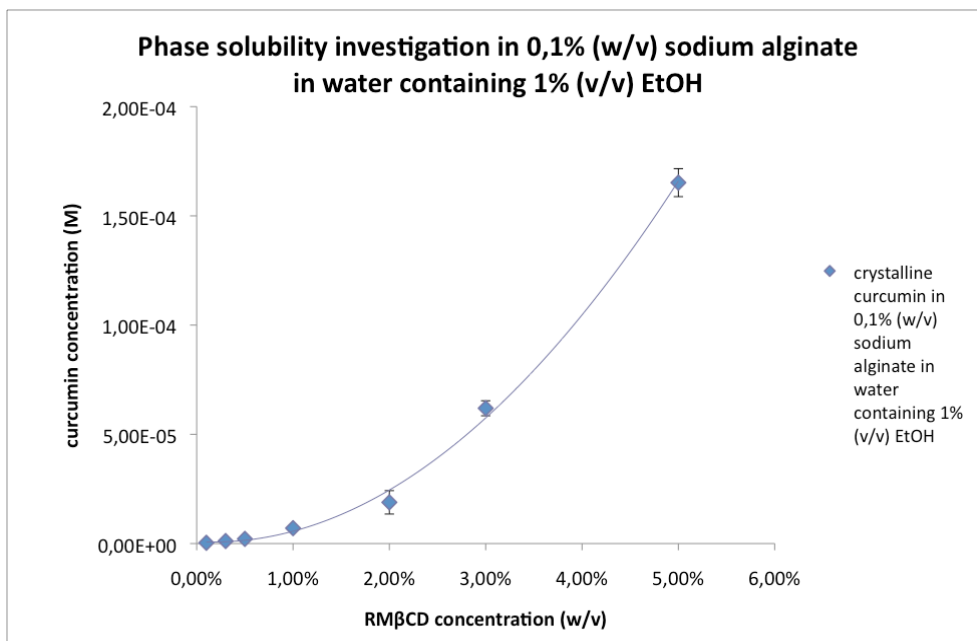
A.4.5. Solubility of crystalline curcumin and solved curcumin in 0.1-5% (w/v) of RM β CD, phosphate buffer pH5 (0.1 M, ionic strength 0.085)



A.4.6 Solubility of crystalline curcumin in 0.1-5% (w/v) of RM β CD, 0.1% (w/v) HPMC in water containing 1% (v/v) ethanol



A.4.7 Solubility of crystalline curcumin in 0.1-5% (w/v) of RM β CD, 0.1% (w/v) sodium alginate in water containing 1% (v/v) ethanol



Alexandra
Rupp



Berggasse 20/2/24
1090 wien
Österreich
Tel.: 0680/1172441
E-mail: alex.rupp@gmx.at

Curriculum Vitae

Alexandra Rupp

Geboren am 19.09.1983 in Bregenz, Vorarlberg, Österreich
Hauptwohnsitz in 6972 Fussach, Kanalstrasse 8

Ausbildung

seit 2002 Pharmaziestudium an der Universität wien

1990-1994
1994-2002
2002

volksschule Fussach in Fussach, Vorarlberg
Bundesgymnasium Blumenstraße in Bregenz, Vorarlberg
Matura erfolgreich bestanden

Persönliche Fähigkeiten

Muttersprache: Deutsch
Gute Englischkenntnisse
Gute Computerkenntnisse
Schulniveau Französisch

Interessen

Badminton, Kultur, Segeln, Kochen

Arbeits- erfahrung

Juli-August 2003
Juli 2004
Juli 2005
August 2006
August 2007
August 2008

Juli 2009
August 2010
Juli 2011

Braun Apotheke und Drogerie, Lustenau, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich

Seeapotheke Hard, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich
Seeapotheke Hard, Vorarlberg, Österreich

Curriculum Vitae

- okt.2006 - Feb.2008 Ich durfte an der Universität Wien als Tutor im Praktikum 'Allgemeine Mikrobiologie und Hygiene' in der Zeit von Okt. 2006 bis Feb. 2008 mitwirken.
- Feb. 2004 Um mein Wissen über die Pflanzen des Regenwaldes zu erweitern absolvierte ich für 1 Monat eine Forschungsreise der Universität Wien nach Costa Rica.
- okt.2005 - 2010 während des Studiums arbeitete ich geringfügig in einem Pharmazeutischen Unternehmen Mundipharma Ges.m.b.H. in Wien, in der Medizinischen Abteilung und in der Registrierungs-Abteilung.
- Sept.2010 - Dez. 2010 Zwischen September und Dezember 2010 hatte ich die Möglichkeit die praktische Arbeit meiner Diplomarbeit und das Schreiben der Diplomarbeit an der Universität Oslo zu absolvieren.