ABSTRACT

A selection of curcuminoids has been synthesized and complexed to HPβCD, MβCD and HPγCD. The influence of concentration of cyclodextrin (CD), of ionic strength, choice of buffer salt and pH on aqueous phase solubility was investigated. In addition it was investigated if the use Mg²⁺ together with the CDs could increase the aqueous solubility of curcuminoids. Melting point and polymorphic forms of the curcuminoids were investigated using differential scanning calorimetry (DSC), and photochemical stability was investigated in hydrogen bonding organic solvent, EtOH, a mixture of this organic solvent and water and in aqueous solution of 10% HPβCD and HPγCD.

The ionic strength or addition of Mg²⁺ did not influence the solubility, nor did pH when kept at pH 5 or lower. The stoichiometry of the curcuminoids-CD complexes was not unequivocally determined, but some sort of higher-order complex or non-inclusion complexation seems to be present. Solubility was found to be best for curcumin in HPγCD and best for bisdemethoxycurcumin in the βCDs.

Different batches of the curcuminoids have formed different crystal forms, with slightly different melting points. This is assumed to have an effect on the aqueous solubility. Photochemical stability was found to be generally better for curcumin than for the other curcuminoids, presumably due to intramolecular bondings. The stability was best in hydrogen bonding organic solvent for all the curcuminoids.

An attempt was made to synthesize a curcumin galactoside, with postulated increased aqueous solubility. This was not successful.

\[
\text{Figure 1: These simple symmetrical curcuminoids were synthesized in the present work}
\]

<table>
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This project is a part of a collaborative work between the University of Oslo and the University of Iceland. Most of the lab work was performed in Iceland, where I stayed in the period January 2006 to July 2006. A small phase solubility experiment, DSC measurements and studies on photochemical stability was performed in Oslo, along with most of the literature search.

First and foremost I would like to thank my supervisors Hanne Hjorth Tønnesen and Mår Måsson for all the help they have given me on this project, for their interest and enthusiasm, and for the patience with my never ending questions. I am also very grateful for the opportunity to stay 6 months in Iceland.

I would like to thank PhD student Ögmundur for all the help on my syntheses and my fellow student Kjartan for showing me around the lab and with the use of the equipment. Thanks also to PhD student Kristjan and my fellow student Reynir for the help with the HPLC system and for help with computer issues in general.

In the University of Oslo I would like to thank Anne Lise for the help with the HPLC equipment and Tove for helping me with the DSC measurements.

Ragnhild, October 2006
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<td>CD</td>
<td>Cyclodextrin</td>
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<td>Deuterim-labelled chloroform</td>
</tr>
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<tr>
<td>DMSO</td>
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<td>DPPH</td>
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<tr>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>HPβCD</td>
<td>Hydroxypropyl-β-cyclodextrin</td>
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<tr>
<td>HPγCD</td>
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<td>HPLC</td>
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<td>MβCD</td>
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<td>Mass Spectrometry</td>
</tr>
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<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
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<tr>
<td>NMR</td>
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<tr>
<td>SPLET</td>
<td>Sequential proton loss electron transfer</td>
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<tr>
<td>ss</td>
<td>Solvent system</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>------------</td>
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<tr>
<td>UV/Vis</td>
<td>Ultraviolet radiation and visible light</td>
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The compounds synthesized in the present work are denoted RHC- and compounds previously synthesized by Marianne Tomren are denoted MTC-.
1 - AIM OF THE STUDY

Curcumin is a natural substance with many interesting properties and pharmacological effects. A major problem in formulation of curcumin is its low solubility in water at low pH and degradation under neutral-alkaline conditions. It is also rapidly degraded by light. The derivatives of curcumin are designated curcuminoids. There are two naturally occurring curcuminoids, demethoxycurcumin and bisdemethoxycurcumin, and different synthetic derivatives.

Use of cyclodextrins for solubilization of curcuminoids seems to improve aqueous solubility, but unfortunately also seems to have a photochemically destabilizing effect on the curcuminoids. Another way of increasing solubility in water is to make a polysaccharide derivative of the curcuminoids.

In the present work a few simple curcuminoids are synthesized and complexed with cyclodextrins. Aspects on the solubility and the influence of the used solvent system for these complexes are investigated. In addition investigations are performed on the photochemical stability and crystallinity of the curcuminoids.

It is also attempted to synthesize curcumin galactosides, and to investigate the same properties as for the cyclodextrin complex. The aim is to compare the curcumin-polysaccharides to the cyclodextrin-complexed curcuminoids, to see which is most suitable for making a stable aqueous pharmaceutical formulation.
2 – INTRODUCTION

2.1 Curcuminoids

2.1.1 Natural occurrence

Curcumin is the coloring principle of turmeric (*Curcuma longa* L.), which belongs to the Zingiberaceae family. Curcuminoids refer originally to a group of phenolic compounds present in turmeric, which are chemically related to its principal ingredient curcumin. Three curcuminoids were isolated from turmeric viz., curcumin, demethoxycurcumin and bismethoxycurcumin [1].

The “pure curcumin” on the market consists of a mixture of these three naturally occurring curcuminoids, with curcumin as the main constituent [2]. Turmeric has originally been used as a food additive in curries to improve the storage condition, palatability and preservation of food. Turmeric has also been used in traditional medicine. Turmeric is grown in warm, rainy regions of the world such as China, India, Indonesia, Jamaica and Peru [1].

2.1.2 Pharmacological effects

Several pharmacological effects are reported for curcumin and curcumin analogs making them interesting as potential drugs. This include effects as potential antitumor agents [3, 4], antioxidants [4-10] and antibacterial agents[11]. Inhibition of in vitro lipid peroxidation [4], anti-allergic activity [5] and inhibitory activity against human immunodeficiency virus type one (HIV-1) integrase [12] are also among the many effects reported. Curcumin has in addition been investigated as a possible drug for treating cystic fibrosis [13, 14]. Many of curcumin’s activities can be attributed to its potent antioxidant capacity at neutral and acidic pH, its inhibition of cell signaling pathways at multiple
levels, its diverse effects on cellular enzymes and its effects on angiogenesis and cell adhesion [15].

2.1.2.1 Antioxidant activity

The antioxidant compounds can be classified into two types: phenolics and β-diketones. A few natural products, such as curcuminoids, have both phenolic and β-diketone groups in the same molecule and thus become potential antioxidants [3]. Several studies have been performed with the aim to determine the importance of different functional groups in the curcuminoid structures on their antioxidant activity. The literature is somewhat contradictory on which of these is the most important structural feature, with some reports supporting phenolic –OH [4-6] as the group mainly responsible, while others reported that the β-diketone moiety is responsible for antioxidant activity [7, 8].

It has been suggested that both these groups are involved in the antioxidative mechanism of the curcuminoids [3, 9, 10], with enhanced activity by the presence and increasing number of hydroxyl groups on the benzene ring [3]. In the curcumin analogs that are able to form phenoxy radicals this is likely to be the basis of their antioxidant activity [10]. Investigations also indicate that curcuminoids where the methoxy group in curcumin is replaced by a hydroxyl group, creating a catechol system, have enhanced antioxidant activity [3, 16].

The differences in the results obtained in experiments performed may however be related to variables in the actual experimental conditions [17]. The “curcumin antioxidant controversy” was claimed to be resolved by Litwinienko and Ingold [17]. The antioxidant properties of curcumin depend on the solvent it is dissolved. In alcohols fast reactions with 1,1-diphenyl-2-picrylhydrazyl (dpph·) occur, and is caused by the presence of curcumin as an anion [17]. They introduce the concept of SPLET (sequential proton loss electron transfer) process, which is thought to occur in solvents ionizing the keto-enol moiety [17]. In non-ionizing solvents, or in the presence of acid, the more well-known HAT (hydrogen atom transfer) process involving one of the phenolic groups occur [17].
In a study performed by Suzuki et al [5] radical scavenging activity for different glycosides of curcumin, bisdemethoxycurcumin and tetrahydrocurcumin were determined. Based on their results, the authors states that the role of phenolic hydroxyl and methoxy groups of curcumin-related compounds is important in the development of anti-oxidative activities [5]. The findings in this paper also show that the monoglycosides of curcuminoids have better anti-oxidative properties than their diglycosides. Antioxidant activity of the diglycoside of curcumin compared to free curcumin was also investigated by Vijayakumar and Divakar. This experiment did however show that glucosidation did not affect the antioxidant activity [18].

Some information on which structural features are deciding antioxidant activity is important when formulating the curcuminoids. Since antioxidant activity of curcuminoids have been suspected to come from the hydroxyl groups on the benzene rings, and because these rings might be located inside the CD cavity upon complexation with CD, it is likely that complexation of the curcuminoids with CD will affect the antioxidative properties of the curcuminoids. Other antioxidants, like flavonols and carotenoids, have also been complexed with CDs in order to improve water solubility. The antioxidant effect of these compounds was changed due to the complexation [19, 20].

2.1.2.2 Pharmacokinetics and safety issues

Studies in animals have confirmed a lack of significant toxicity for curcumin [15]. Curcumin is approved as coloring agent for foodstuff and cosmetics, and is assigned E 100 [21].

Curcumin has a low systemic bioavailability following oral administration, and this seems to limit the tissues that it can reach at efficacious concentrations to exert beneficial effects [15]. In the gastrointestinal tract, particularly the colon and rectum, the attainment of such levels has been demonstrated in animals and humans [15]. Absorbed curcumin undergo rapid first-pass metabolism and excretion in the bile [15].
2.1.3 Chemical properties and chemical stability

Curcumin has two possible tautomeric forms, a β-diketone and a keto-enol, shown in figure 2.1. In the crystal phase is appears that the cis-enol configuration is preferred, due to stabilization by a strong intramolecular H-bond [22]. The enol group seems to be statistically distributed between the two oxygen atoms [22]. The keto-enol group does not, or only weakly, seem to participate in intermolecular hydrogen bond formation, with for instance protic solvents [23].

![Figure 2.1: The keto-enol tautomerization in curcumin](image)

The phenolic groups in curcumin are shown to form intermolecular hydrogen bonds with alcoholic solvents, and these phenolic groups show hydrogen-bond acceptor properties, see figure 2.2 [23]. The phenol in curcumin does also participate in intramolecular bonding with the methoxy group [23].

![Figure 2.2: The formation of hydrogen bonds between alcoholic solvent and phenolic groups in curcumin and bisdemethoxycurcumin](image)
In the naturally occurring derivative bisdemethoxycurcumin the situation is a little different, with the phenolic groups in bisdemethoxycurcumin acting as hydrogen-bond donors as it can be seen from figure 2.2 [24]. The difference between curcumin and bisdemethoxycurcumin is explained by Tønnesen et al [23] to come from the presence of a methoxy next to the phenolic group in curcumin. In addition, the enol proton in bisdemethoxycurcumin is bonded to one specific oxygen atom, instead of being distributed between the two oxygen atoms, like in curcumin [23]. The other oxygen is engaged in intermolecular hydrogen bonding [23].

The pKa value for the dissociation of the enol is found to be at pH 7.75-7.80 [25]. Curcumin also has two phenolic groups, with pKa values at pH 8.55±0.05 and at pH 9.05±0.05 [25]. Other authors have found these pKa values to be 8.38±0.04, 9.88±0.02 and 10.51±0.01 respectively [26].

Curcumin is in the neutral form at pH between 1 and 7, and water solubility is low [25]. The solubility is however increased in alkaline solutions, where the compounds become deprotonated and results in a red solution [26]. Curcumin is prone to hydrolytic degradation in aqueous solution, it is extremely unstable at pH values higher than 7 and the stability is strongly improved by lowering pH [25] [27]. Wang et al. suggest that this may be ascribed to the conjugated diene structure, which is disturbed at neutral-basic conditions [27]. The degradation products under alkaline conditions have been identified as ferulic acid, vanillin, feruloylmethane and condensation products of the last [28]. According to Wang et al the major initial degradation product was predicted to be trans-6-(4´-hydroxy-3´-methoxyphenyl)-2, 3-dioxo-5-hexenal, with vanillin, ferulic acid and feruloyl methane identified as minor degradation products. When the incubation time is increased under these conditions, vanillin will become the major degradation product [27].

The half-life of curcumin at pH > 7 is generally not very long [25, 27]. A very short half-life is obtained around and just below pH 8, with better stability in the pH area 8.10-8.50
[25]. Wang et al. [27] reports the half life to be longer at pH 10 than pH 8, but Tønnesen and Karlsen found the half-life at these pH values to be quite similar and very short [25].

2.1.4 Photochemical properties and photochemical stability

The naturally occurring curcuminoids exhibit strong absorption in the 420 nm to 430 nm region in organic solvents [23]. They are also fluorescent in organic media [23], and the emission properties are highly dependent on the polarity of their environment [29]. Changes in the UV-VIS and fluorescence spectra of the curcuminoids in various organic solvents demonstrate the intermolecular hydrogen bonding that occur [23].

Curcumin decomposes when it is exposed to UV/Vis radiation, and several degradation products are formed [24]. The main product results from cyclisation of curcumin, formed by loss of two hydrogen atoms from the curcumin molecule, and is shown in figure 2.3 [24]. The photochemical stability strongly depends upon the media it is dissolved in, and the half life for curcumin is decreasing in the following order of solvents methanol > ethyl acetate > chloroform > acetonitrile [24]. The ability of curcumin to form intra- and inter molecular bindings is strongly solvent dependant, and these bindings are proposed to have a stabilizing or destabilizing effect towards photochemical degradation [24]. For the naturally occurring curcuminoids the stability towards photochemical oxidation has been found to be the following: demethoxycurcumin> bisdemethoxycurcumin> curcumin [30].
Curcumin has been shown to undergo self-sensitized photodecomposition involving singlet oxygen [24]. Other reaction mechanisms, independent of the oxygen radical, are also involved [24]. The mechanisms for the photochemical degradation have been postulated by Tønnesen and Greenhill, and involves the β-diketone moiety [7].

2.2 Synthesis and analysis of curcuminoids

2.2.1 Synthesis

2.2.1.1 Simple symmetrical curcuminoids

In a method suggested by Pabon [31], shown in figure 2.4, curcumin is prepared when vanillin condenses with the less reactive methyl group of acetylacetone. In this synthesis vanillin reacts with acetylacetone/B₂O₃ in the presence of tri-sec. butyl borate and
butylamine. Curcumin is obtained as a complex containing boron, which is decomposed by dilute acids and bases. Dilute acids are preferred because curcumin itself is unstable in alkaline medium [31].

\[
\text{CH}_3\text{O} + \text{B}_2\text{O}_3 + \text{H}^+ \rightarrow \text{H}_3\text{C} - \text{O} - \text{O} - \text{B} - \text{O} - \text{B} - \text{O} - \text{CH}_2\text{O} + \text{BO}_2^- + \text{H}_2\text{O}
\]

![Curcumin synthesis by the Pabon method](image)

**Figure 2.4:** Curcumin synthesis by the Pabon method [31, 32]

Curcuminoids can also be prepared by treating vanillin, acetylacetone and boric acid in N,N-dimethylformamide with a small amount of 1,2,3,4-tetrahydroquinoline and glacial acetic acid [33, 34].
2.2.1.2 Galactosylated curcuminoids

Curcumin carbohydrate derivatives have been made by adding a glucose or galactose moiety on the phenolic hydroxyl groups of curcumin [5, 11, 18, 35, 36]. Synthesis of different glycosides and galactosides of curcumin have been performed by adding glucose or galactose to vanillin and 4-hydroxybenzaldehyde, which is further synthesized to different curcumin carbohydrate derivatives [36]. The synthesis of curcumin diglycoside has also been performed by addition of the glucose unit directly to the phenolic groups curcumin [11]. Curcumin glycosides have in addition been synthesized by enzymatic [18] and plant cell suspension culture [35] methods.

In the present work it was attempted to synthesize curcumin-digalactoside by the method reported by Mohri et al. [36]. By using this method it is possible to make the asymmetrical mono-derivative, with a carbohydrate moiety connected to the hydroxyl on only one of the aromatic rings of the curcuminoids, in addition to symmetrical derivatives [36].

Step 1: 2,3,4,6-tetra-O-acetyl-\(\alpha\)-D-galactopyranosylbromide is prepared by acetylation of galactose under acidic conditions, followed by generation of the bromide by addition of red phosphorus, \(\text{Br}_2\) and \(\text{H}_2\text{O}\) in a “one-pot” procedure [37, 38]. This reaction (figure 2.5) is essentially the preparation of D-galactose pentaacetate from D-galactose under acidic conditions, which yields the two anomic forms of the pentaacetate, followed by reaction with hydrogen bromide in glacial acetic acid with both anomers [38]. Both anomic forms of the product are expected to be formed, but tetra-\(O\)-acetyl-\(\beta\)-d-galactopyranosyl bromide will be converted to the more stable \(\alpha\)-anomer during the reaction or undergo rapid hydrolysis during the isolation procedure [38].
Figure 2.5: The synthesis of acetobromogalactose from galactose

The reaction product that is obtained is the tetra-O-acetyl-\(\alpha\)-D-galactosyl bromide, which is referred to as “acetobromogalactose” in the present work. The acetobromogalactose is reported to be unstable and will decompose during storage, probably due to autocatalysis [37].

Step 2: The acetobromogalactose is subsequently reacted with vanillin in a two-phase system consisting of NaOH solution and CHCl\(_3\) in the presence of Bu\(_4\)NBr, to yield tetra-O-acetyl-\(\beta\)-D-galactopyranosylvanillin (figure 2.6) [36]. Here Bu\(_4\)NBr is added as a phase transfer reagent [39].

Figure 2.6: The synthesis of vanillin galactoside from acetobromogalactose and vanillin

In tetra-O-acetyl-\(\alpha\)-D-galactosyl bromide (acetobromogalactose) there is a trans-relationship between the acyloxy protecting group at C-2 and the bromide at C-1. When there is a trans-relationship between these groups the reaction proceed by solvolysis with neighboring group participation [40]. The cation formed initially, when Br\(^-\) dissociates
from the acetylated galactose molecule, interacts with the acetyl substituent on C-2 in the same galactose molecule to produce an acetoxonium ion [41]. A “free” hydroxyl group, here in vanillin, approaches the acetoxonium ion from the site on the molecule opposite to that containing the participating neighboring group to produce a glycosidic linkage (figure 2.7) [41].

![Figure 2.7](image)

**Figure 2.7**: The proposed reaction mechanism for acetoxy group formation in galactoside formation [41]

Step 3: The vanillin galactoside formed in step 2 is further condensated with acetylacetone-B₂O₃ complex to give acetylated curcumin galactosides (figure 2.8) [36]. The reaction is a modified version of the Pabon method [31] previously employed to synthesize simple symmetrical curcuminoids. It is also possible to synthesize a mono-galactoside of curcumin from vanillin galactoside and acetylacetone [36].

![Figure 2.8](image)

**Figure 2.8**: The synthesis of curcumin galactoside octaacetate from vanillin galactoside and acetylacetone

Step 4: In the end, the acetoxy groups are removed by treatment with 5% NH₃-MeOH (figure 2.9), and the compounds are concentrated and purified by chromatography [36].
Curcumin galactoside octaacetate → Curcumin galactoside

Figure 2.9: Removal of the acetyl groups to yield curcumin galactoside

Glucose is used by some of the references for these reactions. The reactions are however assumed to be the same for galactose as for glucose, since the only structural difference between glucose and galactose is that the hydroxyl at the 4-position is axial in galactose and equatorial in glucose [42].

2.2.2 Chromatographic conditions

2.2.2.1 TLC

Different TLC systems have been reported for the separation of curcuminoids. In combination with a silica gel stationary phase a mobile phase consisting of CHCl₃:EtOH (25:1) or CHCl₃:CH₃COOH (8:2) have been used [43]. Different solvent systems for separation on silica gel 60 were investigated by Pèret-Almeida et al., and the use of CH₂Cl₂:MeOH (99:1) was reported to give the best separation [44]. Nurfina et al (1997) reported to have used CH₃OH:H₂O (7:3), but no information was given on the type of stationary phase [32].

2.2.2.2 HPLC

Baseline separation was achieved by Cooper et. al using THF/water buffer on a C₁₈ column [45]. The mobile phase used for this HPLC method consisted of 40% THF and 60% water buffer, containing 1% citric acid adjusted to pH 3.0 with concentrated KOH solution [45].
The keto-enol structures of curcuminoids are capable of forming complexes with metal ions [45]. Presence of such ions in the sample will give excessive tailing in HPLC chromatograms, when acetonitrile or THF are used in the mobile phase [45]. A better separation for compounds capable of complexion with metal ions can be achieved by using citric acid in the mobile phase [45]. Citric acid in the mobile phase can also reduce tailing from interaction between residual silanol groups on the C18 packing material with the keto-enol moiety, by competing for these active sites [45]. ACN as the organic phase gives better selectivity than methanol or THF [46]. The curcuminoids have previously been analyzed with a mobile phase consisting of 0.5% citrate buffer pH 3 and ACN [2, 47].

Although UV/Vis detection is mostly used, HPLC for the curcuminoids can also be interfaced to mass spectrometry (MS) [48]. Separation before MS has been reported using a mobile phase consisting of 50 mM ammonium acetate with 5% acetic acid and acetonitrile, on a octadecyl stationary phase [48]. Acetonitrile – ammonium acetate buffer was used because a volatile mobile phase is required for MS [48].

### 2.2.3 NMR properties

![Curcumin structure](image)

**Figure 2.10:** The hydrogen atoms in curcumin

Several papers on the synthesis of curcuminoids have reported \(^1\text{H}-\text{NMR}\) and \(^{13}\text{C}-\text{NMR}\) for these compounds [3, 32-34]. The solvents used in these investigations are CDCl\(_3\) [3, 32, 33] and CD\(_3\)OD [34]. \(\delta\) values given below are collected from these references. The hydrogen atoms are shown in figure 2.10. The obtained \(\delta\) values and splitting pattern are,
however, dependent on both which solvent is chosen and the equipment used for the NMR analysis. This explains the differences in the reports.

For the symmetrical curcumin molecule the following pattern seems to be obtained. At approximately 3.90-\(\delta\) 3.95\(\delta\) there are signals denoted to the singlet related to the 6 hydrogen atoms in the methoxy groups (-OCH\(_3\)). Aromatic hydrogen atoms usually give signals between 6.5 and 8.0\(\delta\), due to the strong deshielding by the ring [42]. The aromatic system in curcumin has three hydrogen atoms on each ring structure (figure 2.10), which gives signals in the area between 6.81\(\delta\) and 7.3\(\delta\). The splitting pattern reported differs, with the simplest obtained in CD\(_3\)OD [34]. Here, the three non-equivalent protons give two doublets for H\(_5\) and H\(_6\), and a singlet for H\(_2\). Other reports however, suggest that this pattern is more complex. Nurfin et al reported this as a multiplet at 6.91\(\delta\) [32]. Both Babu and Rajasekharan [33] and Venkateswarlu et al [3] reported this to be doublets for H\(_2\) and H\(_5\) and a double-doublet for H\(_6\), on the aromatic ring system. Spin-spin splitting is caused by interaction, or coupling, of the spins of nearby nuclei [42].

According to \(^1\)H NMR measurements, curcuminoids exist exclusively as enolic tautomers [34]. This proton, 4-H in figure 2.10, appears as a singlet in the area between \(\delta\) 5.79-5.96. The allylic protons closest to the aromatic ring (1, 7-H) gives a doublet in the area \(\delta\) 7.55-7.58\(\delta\) while the protons 2, 6 H appear as a doublet in the area \(\delta\) 6.43-6.66\(\delta\).

### 2.3 Preformulation and solubility

#### 2.3.1 General aspects on preformulation

Prior to development of dosage forms, it is essential that certain fundamental physical and chemical properties of a drug molecule and other derived properties of the drug powder should be determined. The obtained information dictates many of the subsequent events and approaches in formulation development [49]. This is known as preformulation.
During the preformulation phase a range of tests should be carried out, which are important for the selection of a suitable drug compound [50]. These include investigations on the solubility, stability, crystallinity, crystal morphology and hygroscopicity of a compound [50]. Partition and distribution coefficients (log P/log D) and pKa are also determined [50].

In the present work investigations on solubility, photochemical stability and crystallinity of a selection of curcuminoids and their complexation with three different cyclodextrins are carried out.

2.3.1.1 Solubility investigations

Before a drug can be absorbed across biological membranes it has to be in aqueous solution [51]. The aqueous solubility therefore determines how much of an administered compound that will be available for absorption. Good solubility is therefore a very important property for a compound to be useful as a drug [50]. If a drug is not sufficiently soluble in water, this will affect drug absorption and bioavailability. At the same time the drug compound must also be lipid-soluble enough to pass through the membranes by passive diffusion, driven by a concentration gradient. Problems might also arise during formulation of the drug. Most drugs are lipophilic in nature. Methods used to overcome this problem in formulation are discussed in the next section (section 2.3.1.2).

The solubility of a given drug molecule is determined by several factors, like the molecular size and substituent groups on the molecule, degree of ionization, ionic strength, salt form, temperature, crystal properties and complexation [50]. In summary, the two key components deciding the solubility of an organic non electrolyte are the crystal structure (melting point and enthalpy of fusion) and the molecular structure (activity coefficient) [52, 53]. Before the molecule can go into solution it must first dissociate from its crystal lattice [52]. The more energy this requires, depending on the strength of the forces holding the molecules together, the higher the melting point and the lower the solubility [52, 53]. The effect of the molecular structure on the solubility is described by the aqueous activity coefficient [52]. The aqueous activity coefficient can be
estimated in numerous ways, and the relationship with the octanol/water partition (log $K_{o/w}$) coefficient is often used [52]. If the melting point and the octanol/water partition coefficient of a compound are known, the solubility can be estimated [52]. This will also give some insight to why a compound has low solubility, and which physicochemical properties that limits the solubility [52, 53]. When the melting point is low and log $K_{o/w}$ is high, the molecular structure is limiting the solubility. In the opposite case, with a high melting point and low log $K_{o/w}$, the solid phase is the limiting factor that must be modified [52]. Compounds with both high melting points and high partition coefficients, like the curcuminoids [47], will be a challenge in development [52].

2.3.1.2 Enhancing the solubility of drugs

The solubility for poorly soluble drugs could be increased in several ways. The most important approaches to the improvement of aqueous solubility are given below [54].

- **Cosolvency**
  Altering the polarity of the solvent, by adding a cosolvent can improve the solubility of a weak electrolyte or non-polar compound in water.

- **pH control**
  The solubility of drugs that are either weak acids or bases can be influenced by the pH of the medium.

- **Solubilization**
  Addition of surface-active agents, which forms micelles and liposomes that the drug can be incorporated in, might improve solubility for a poorly soluble drug.

- **Complexation**
  In some cases it is possible for a poorly soluble drug to interact with a soluble material to form a soluble intermolecular complex. Drugs can for instance be
incorporated into the lipophilic core of a cyclodextrin, forming a water-soluble complex.

- **Chemical modification**
  Poorly soluble bases or acids can be converted to a more soluble salt form. It is also possible to make a more soluble prodrug, which is degraded to the active principle in the body.

- **Particle size control**
  Dissolution rate increases as particle size decreases, and the total surface area increases. In practice this is most relevant for solid formulations.

As previously mentioned, different polymorphs often have different solubilities, with the more stable polymorph having the lowest solubility. Using a less stable polymorph to increase the solubility is mainly a possibility in solid formulations, where the chance of transformation to the more stable form is much lower compared to solution formulations [53]. This can however only be done when the metastable form is sufficiently resistant to physical transformation during the time context required for a marketed product [53].

Curcumin is known to be highly lipophilic. In the present study cyclodextrins were used to enhance solubility of a selection of simple symmetrical curcuminoids. It was also attempted to synthesize the polysaccharide derivatives of curcumin, which are expected to have increased solubility in water.

### 2.3.1.3 Crystallinity investigations and Thermal analysis

Differences in solubility might arise for different crystal forms of the same compound, along with different melting points and infrared (IR) spectra [51]. For different crystal forms of a compounds one of the polymorphs will be the most stable under a given set of conditions, and the other forms will tend to transform into this [51]. Transformation
between different polymorphic forms can lead to formulation problems [51], and also differences in bioavailability due to changes in solubility and dissolution rate [51]. Usually the most stable form has the lowest solubility and often the slowest dissolution rate [51].

In addition to the tendency to transform into more stable polymorphic forms, the metastable form can also be less chemically and physically stable [53]. Care should be taken to determine the polymorphic forms of poorly soluble drugs during formulation development [51].

There are a number of interrelated thermal analytical techniques that can be used to characterize the salts and polymorphs of candidate drugs [50]. The thermo analytical techniques usually used in pharmaceutical analysis are “Differential Scanning Calorimetry” (DSC) or “Differential Thermal Analysis” (DTA) and “Thermo gravimetric Analysis” (TGA) [55]. Thermo dynamical parameters can be decided from DSC- and DTA-thermograms for a compound. They can give information on the melting point and eventual decomposition, glass transition, purity, polymorphism and pseudo polymorphism for a compound. Thermo analysis can also be used for making phase-diagrams, and for investigating interactions between the drug and formulation excipients [55].

2.3.1.4 Photochemical stability investigations

A wide range of drugs can undergo photochemical degradation. Several structural features can cause photochemical decomposition, including the carbonyl group, the nitroaromatic group, the N-oxide group, the C=C bond, the aryl chloride group, groups with a weak C-H bond, sulphides, polyenes and phenols [50]. It is therefore important to investigate the effect light has on a drug compound in order to avoid substantial degradation, with following loss of effect and possible generation of toxic degradation products, during shelf life of the drug.
2.3.2 Experimental methods for the present preformulation studies

2.3.2.1 The phase solubility method

The phase solubility method was used for the investigations on solubility of the curcuminoids in cyclodextrin (CD) solution.

The drug compound is added in excess to vials, and a constant volume of solvent containing CD is then added to each container. The vessels are closed and brought to equilibrium by agitation at constant temperature. The solutions are then analyzed for the total concentration of solubilized drug [56, 57]. A phase solubility diagram can be obtained by plotting molar concentration of the dissolved drug against the concentration of CD [56]. The phase solubility method is one of the most common methods for the determination of the association constants and stoichiometry of drug-CD complexes [56].

A system with a substrate S (the curcuminoid) and a ligand L (the cyclodextrin) is named $S_mL_n$. When $n=1$, the plot of the total amount of solubilized substrate, $S_t$, as a function of the total concentration of ligand, $L_t$, is linear. The solubility of the substrate without ligand, $S_0$, is the intercept [57]. The slope can not be more than 1 if only 1:1 complexation occurs, and is given by $K_{1:1}S_0/(1-K_{1:1}S_0)$ [57]. A linear phase solubility diagram can however not be taken as evidence for 1:1 binding [57]. If 1:1 complexation occurs the stability constant is given by

$$K_{1:1} = \frac{\text{slope}}{S_0(1-\text{slope})} \text{ (Equation 2.1 [57])}$$

For systems with $n>1$, the nonlinear isotherm with concave-upward curvature is characteristic [57]. For a system where $n=2$ the equation becomes $S_t-S_0/[L]=K_{1:1}S_0 + K_{1:1}K_{1:2}S_0[L]$. By approximating $[L] \approx L_t$ a plot of $(S_t-S_0)/L_t$ against $L_t$ can be made [57]. In reality, plotting these data is usually performed using a suitable computer program.
2.3.2.2 Photochemical stability investigations

Photochemical stability testing at the preformulation stage involves a study of the degradation rate of the drug in solution, when exposed to a source of irradiation for a period of time [58]. The rate at which the radiation is absorbed by the sample and the efficiency of the photochemical process determines the rate of a photochemical reaction [58]. An artificial photon source, which has an output with a spectral power distribution as near as possible to that of sunlight, is used for consistency [58]. The use of natural sunlight is not a viable option for studies on photostability because there are too many variables in the conditions that can not be accounted for, for instance in the intensity of the light that vary with weather, latitude, time of day and time of year [58].

At low concentrations in solutions, photodegradation reactions are predicted to follow first-order kinetics [58]. In preformulation studies of photodegradation it is recommended to conduct the studies with a solution concentration low enough to keep solution absorbance < 0.4 at the irradiation wavelength [58]. Then first order kinetics apply, and the reaction rate is limited by drug concentration rather than light intensity [58].

2.3.2.3 Differential Scanning Calorimetry (DSC)

DSC has been extensively used in polymorph investigations as a change in melting point is the first indication of a new crystal form [53]. The method will be used in this study for determination of the melting points of the compounds and investigations of polymorphism. DSC can also be useful for investigating possible incompatibilities between a drug and excipients in a formulation during the preformulation stage [59].

In the basic procedure of DSC [60] two ovens are linearly heated, one oven containing the sample in a pan and the other contains an empty pan as a reference pan. If changes occur in the sample as it is heated, such as melting, energy is used by the sample. The temperature remains constant in the sample, but will increase in the reference pan. There will be a difference in temperature between the sample and the reference pan. If no
changes occur in the sample when it is heated, the sample pan and the reference pan are at the same temperature. The temperature difference can be measured (heat flux-DSC, which is not very different from DTA), or the temperature can be held constant in both pans with individual heaters compensating energy when endothermic or exothermic processes occur [60]. Information on heat flow as a function of temperature is obtained. For first-order transitions, such as melting, boiling, crystallization etc, integration of the curve gives the energy involved in the transition [60].

In addition to the melting point, DSC curves can also provide more detailed information on polymorphism, pseudo polymorphism and amorphous state [60]. Information on the purity of a compound can also be obtained, with impurities causing melting point depression and broadening of the melting curve [60].

2.4 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligomers of glucose that can form water-soluble inclusion complexes with small molecules or fragments of large compounds [61]. The most common pharmaceutical application of CDs is to enhance drug solubility in aqueous solutions [62]. CDs are also used for increasing stability and bioavailability of drugs, and other additional applications [62].

2.4.1 Nomenclature

The nomenclature derives from the number of glucose residues in the CD structure, with the glucose hexamer referred to as α-CD, the heptamer as β-CD and the octomer as γ-CD [61]. These are shown in figure 2.11. CDs containing nine, ten, eleven, twelve and thirteen units, which are designated δ-, ε-, ζ-, η- and θ-CD, respectively, are also reported [62]. CDs with fewer than six units can not be formed for steric reasons [63].
2.4.2 Chemistry of cyclodextrins

CDs are cyclic (α-1, 4)-linked oligosaccharides of α-D-glucopyranose [62]. The central cavity is relatively hydrophobic, while the outer surface is hydrophilic [62]. The overall CD molecules are water-soluble because of the large number of hydroxyl groups on the external surface of the CDs, but the interior is relatively apolar and creates a hydrophobic micro-environment. These properties are responsible for the ability to form inclusion complexes, which is possible with an entire drug molecule or only a portion of it [61].

The CDs are more cone shaped than perfectly cylindrical molecules (figure 2.12), due to lack of free rotation about the bonds connecting the glucopyranose units [64]. The
primary OH groups are located on the narrow side, and the secondary on the wider side [64]. CDs have this conformation both in the crystalline and the dissolved state [63].

The CDs are nonhygroscopic, but form various stable hydrates [63]. The number of water molecules that can be absorbed in the cavity is given in table 2.1. The water content can be determined by drying under vacuum to a constant weight, by Karl Fischer titration or by GLC [63]. No definite melting point is determined for the CDs, but they start to decompose from about 200°C and upwards [63]. For quantitative detection of CD HPLC is the most appropriate [63]. CDs do not absorb in the UV/Vis region normally used for HPLC, so other kinds of detection are used [63].

The β-CD is the least soluble of all CDs, due to the formation of a perfect rigid structure because of intramolecular hydrogen bond formation between secondary hydroxyl groups [63]. In the presence of organic molecules the solubility of CDs is generally lowered, owing to complex formation [63]. The addition of organic solvents will decrease the efficiency of complex formation between the drug molecule and CD in aqueous media, due to competition between the organic solvent and the drug for the space in the CD cavity [65].
### Table 2.1: Physicochemical properties of the parent CDs

<table>
<thead>
<tr>
<th>CD</th>
<th>Mw</th>
<th>Number of glucose residues</th>
<th>Central cavity diameter (Å) and height of torus (Å)</th>
<th>Solubility in water (25°C, g/100 ml)</th>
<th>Molecules of water in the cavity</th>
<th>Number of hydroxyl groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-CD</td>
<td>927</td>
<td>6</td>
<td>4.7-5.3 and 7.9 [64]</td>
<td>14.5 [64]</td>
<td>6 [64]</td>
<td>18 [63]</td>
</tr>
<tr>
<td>β-CD</td>
<td>1135</td>
<td>7</td>
<td>6.0-6.0 and 7.9 [64]</td>
<td>1.85 [64]</td>
<td>11 [64]</td>
<td>21 [63]</td>
</tr>
<tr>
<td>γ-CD</td>
<td>1297</td>
<td>8</td>
<td>7.5-8.3 and 7.9 [64]</td>
<td>23.2 [64]</td>
<td>17 [64]</td>
<td>24 [63]</td>
</tr>
</tbody>
</table>

2.4.2.1 Cyclodextrin derivatives/chemically modified cyclodextrins

The aqueous solubility of the parent CDs is limited, which can partly be due to relatively strong binding of the CD molecules in the crystal state [62]. For β- and γ-CD intramolecular hydrogen bonds between secondary hydroxyl groups can be formed, reducing the ability to form hydrogen bonds with surrounding water molecules and therefore low aqueous solubility [62]. By adding substituents on these hydroxyl groups the water solubility can be dramatically increased [62]. For other common CD-derivatives the solubility enhancement comes from a transformation of the crystalline CD to amorphous mixtures of isomeric derivatives [62].

Another reason for making derivatives of the parent CDs is to improve safety. Two of the parent CDs, α- and β-CD, are unsafe when administered parenterally due to severe nephrotoxicity [66].
CDs have 18-24 sites (figure 2.13) where it is possible for chemical modification or derivatization to take place, two secondary hydroxyls at C-2 and C-3 positions (figure 2.13) and a primary alcohol at the C-6 position (figure 2.13) on each glucose unit [56]. Hydroxyl groups in the parent CDs are modified chemically, with a little difference in reactivity, where the C6-OH being most reactive and C3-OH the least reactive [63]. Reactivity does however depend on the reaction conditions [63]. Derivatives containing hydroxypropyl (HP), methyl (M) and sulfobutyl ether (SBE) substituents are commercially available and in the position to be used as pharmaceutical excipients [56].

![Figure 2.13: β-CD with possible derivatization sites (see table 2.2) [61]](image)

**Table 2.2: Substituents on different CD-derivatives**

<table>
<thead>
<tr>
<th>CD Substituent (R)</th>
<th>Substituent (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxypropyl β-CD (HPβCD)</td>
<td>CH₂CHOHCH₃ or H [61, 66]</td>
</tr>
<tr>
<td>Hydroxypropyl γ-CD (HPγCD)</td>
<td>CH₂CHOHCH₃ or H</td>
</tr>
<tr>
<td>Methyl β-CD (MβCD)</td>
<td>CH₃ or H [61, 66]</td>
</tr>
<tr>
<td>Dimethyl β-CD (DMβCD)</td>
<td>CH₃ or H [61, 66]</td>
</tr>
<tr>
<td>Randomly methylated β-CD (RMβCD)</td>
<td>CH₃ or H [61, 66]</td>
</tr>
<tr>
<td>Sulfobutylether β-CD (SBEβCD)</td>
<td>(CH₂)₄SO₃Na or H [61]</td>
</tr>
</tbody>
</table>
Both the degree of substitution and the location of the substituent groups on the CD molecule will affect the physicochemical properties of the derivatives, including the ability to form drug complexes [62]. A general tendency is that the most hydrophobic CD derivatives show the highest solubilizing power, but also the most severe unwanted effects [63].

2.4.3 Toxicology and pharmacokinetics

The toxicity of CDs is dependent on their route of administration. CDs do not produce an immune response in mammals [61].

2.4.3.1 Oral administration

The absorption from the GI tract following oral administration is generally poor, and the majority of the CD dose is metabolized by the GI flora [66]. The safety issues due to systemic absorption of CD themselves are therefore minimal, but secondary systemic effects through increased elimination of certain nutrients and bile acids from the GI tract may occur [66]. The increased elimination, however, has only been observed at very high oral doses of the CD [66]. Absorption of the guest molecule and of the CD are separate processes, the guest molecule is rapidly dissociated in the gastric juice [63]. The highest possible dose of CD does not result in mortality in the animals, and no definite acute toxicity can be determined after oral administration [63].

2.4.3.2 Parenteral administration

CDs injected intravenously are essentially excreted intact via the kidney, because they are resistant to degradation by human enzymes [61]. Some metabolism has been observed with γCD [56]. The safety in parenteral use differs for the different CDs and their derivatives, and can be seen from table 2.3.
Table 2.3: The safety of a selection CDs

<table>
<thead>
<tr>
<th>CD</th>
<th>Parenteral safety</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-CD</td>
<td>Nephrotoxic [56, 66]</td>
</tr>
<tr>
<td></td>
<td>Haemolytic [56]</td>
</tr>
<tr>
<td>β-CD</td>
<td>Nephrotoxic [56, 66]</td>
</tr>
<tr>
<td></td>
<td>Haemolytic [56]</td>
</tr>
<tr>
<td>γ-CD</td>
<td>Nephrotoxic, but less than α- and β-CD [56]</td>
</tr>
<tr>
<td></td>
<td>Haemolytic [56]</td>
</tr>
<tr>
<td>HPβCD</td>
<td>Generally found to be safe and well tolerated [56, 63, 66]</td>
</tr>
<tr>
<td>DMβCD</td>
<td>Causes substantial hemolysis [56, 63]</td>
</tr>
<tr>
<td></td>
<td>High surface activity and high affinity for cholesterol [63]</td>
</tr>
<tr>
<td></td>
<td>Renal nephrosis observed [56]</td>
</tr>
<tr>
<td>HPγCD</td>
<td>Little or no renal toxicity [56]</td>
</tr>
<tr>
<td>(SBE)7Mβ-CD</td>
<td>Safe [56, 66]</td>
</tr>
</tbody>
</table>

γ-CD has a lower ability to affect cellular lipids and is considerably less toxic than the other parent CDs [66].

2.4.4 Cyclodextrin/Drug complexes

Almost all pharmaceutical use of CDs involves formation of a complex between the CD and a drug molecule [63]. Not all drug molecules will form complexes with CD, and size and chemical structure of the drug will affect the complexation [64]. Only relatively apolar molecules, or a suitable apolar part of a molecule, of appropriate size will enter the cavity of the CD [64]. The size of the guest molecule, or suitable part of the molecule, must be compatible with the dimensions of the cavity for an inclusion complex to be formed [56, 63]. Electronic effects between guest and CD may also be important, especially for ionic CD derivatives [56].
Figure 2.14: Complexation of a drug by a cyclodextrin

The kinetics of formation and dissociation of the inclusion complexes between a drug molecule and CD molecule is fast and the complexes are continually being formed and broken down [56, 66]. There is a equilibrium between free CD, free drug and the formed complex, and no covalent bonds are formed or broken during complex formation (figure 2.14) [64].

At low concentration of CD and/or drug most drug-CD complexes have a 1:1 stoichiometry, even those that are of higher order stoichiometry at high CD and/or drug concentration [62]. The stoichiometry depends in many instances on the concentration ratios [63]. The stoichiometry and the value of their stability constant are considered to be the most important characteristics of the complexes [67]. For 1:1 complexes the complexation is defined by the binding constant $K_{1:1}$, given by equation 2.2 [66].

$$K_{1:1} = \frac{[\text{Drug}]_{\text{complex}}}{[\text{Drug}]_{\text{free}}[\text{CD}]_{\text{free}}}$$  \hspace{1cm} (Equation 2.2)

2:1 complexes can also be formed for molecules that are too large, and often long, to fit in one CD cavity, and in which the other end is also suitable for complex formation with CDs [63].

The driving forces responsible for complex formation have no simple explanation. It has been suggested come from release of water from the CD cavity [62]. Water molecules cannot fulfill their hydrogen-bonding potential inside the CD cavity, and replacement by suitable less polar guest molecules in the CD cavity is thermodynamically favorable for the system [62]. Elimination of water molecules from the cavity is also related to release of ring strain, which is thought to be involved in the driving force for the interaction between compound and CD [62]. Other forces like release of ring strain in the CD, van
der Waals interactions, hydrogen bonding, hydrophobic interactions and changes in solvent-surface tension may also be important [62].

The inclusion complexation process between a drug and CD is also affected by the temperature and the binding constant will in most cases decrease as the temperature increases [56]. The solubility of the drug in the CD solution may however increase, due to improved intrinsic solubility of the free drug [56].

Formation of a drug-CD complex results in different physicochemical properties of both the drug molecule and the CD, compared to their respective free molecules [67]. By incorporation in a CD molecule, various spectral properties of both the guest and the host can be changed [63]. At sufficiently high CD concentration the UV spectra of guests are similar to their spectra in ethanolic solution, and at low concentration of CD the spectra are between those obtained in ethanolic or pure water solution [63].

2.4.4.1 Drug stability

When CDs are complexed to labile compounds, drug degradation can be retarded or accelerated, or it can have no effect on stability [62]. The degree of stabilization/destabilization of the labile drug will depend on both the degradation rate inside the complex and also on the fraction of compound that is complexed to CDs [62]. In some ways the CDs mimic enzymatic catalysis or inhibition [62]. The CD molecule can also, at least partly, shield a drug molecule from attack by various reactive molecules [62]. Light sensitive compounds can also be protected against decomposition by CD complexation [63].

2.4.5 Applications and current use of cyclodextrins

CDs can be used to achieve enhanced solubility, enhanced bioavailability, enhanced stability, conversion of liquids and oils to free-flowing powders, reduced evaporation and
stabilized flavors, reduced odors and tastes, reduced hemolysis and prevention of admixture incompatibilities [61].

In oral formulations the use of CDs can increase bioavailability through increased rate and extent of dissolution of a drug [66]. Oral absorption of drugs may be altered by enhancing permeation through mucosal membranes [66]. CDs have also been used to modify the time of drug release during GI transit and decrease local irritation [66].

CDs might also be considered in development of formulations for other ways of administration than oral and parenteral. CDs have been used and marketed for both ophthalmic and nasal applications, and also in dermal, rectal, sublingual and buccal delivery of drugs [61].

The first marketed products using CDs were drug delivery forms of various prostaglandins [61]. Some drugs are marketed in Norway with CDs as an excipient, using different CDs [47].

2.5 Enhancing the solubility of curcuminoids

As discussed in section 2.3.1.1, making the curcuminoids sufficiently water soluble to be useful as drugs is a considerable challenge to the formulator. Different methods have been used and are discussed below.

2.5.1 Complexation of curcuminoids with cyclodextrins

Cyclodextrin (CD) complexes can be prepared of curcuminoids, in order to improve the water solubility and the hydrolytic and photochemical stability. A few papers have been published on the complexation of curcuminoids with cyclodextrins [2, 29, 47, 68-70]. The results are summarized below.
Tønnesen et al [2] reported an increase in water solubility at pH 5 by a factor of at least $10^4$, resulting from CD complex formation of curcumin, and strongly improved hydrolytic stability of curcumin under alkaline conditions. The highest concentration obtained in this study was $8 \times 10^{-4}$ M (approximately 290 μg/ml⁻¹) of curcumin, measured in 11% solution of RMBβCD. Solutions with a higher concentration of CD were not investigated in this study [2]. Compared to curcumin solution in organic solvent the photodecomposition rate was observed to increase, with a higher destabilizing effect for the β-CD derivatives than the α- and γ-CDs used in this study [2].

Szente et al [68] found that complexation of curcumin with β-CD gave increased thermal stability, increased stability against light and improvement of shelf life. The most potent solubilizing agent of the CDs used was found to be methylated β-CD [68]. HP-γ-CD was not included in this study [68].

Qi et al [69] found better complexation for β-CD than for α- and γ-CD, and greatly enhanced binding ability for a series of organoselenium-bridged bis(β-CD)s [69].

Baglole et al. [29] reports better solubilizing effect by hydroxypropylated derivatives of α-, β-, γ-CD than their corresponding parent CDs. Of the parent CDs, β-CD is reported to give the largest solubility [29]. For the hydroxypropyl derivatives used in this investigation HP-γ-CD gave the largest solubility [29], which is consistent with results obtained by Tønnesen et al. [2]. Tønnesen et al. found however RM-β-CD to give even better solubility [2].

Tomren [47] investigated a range of curcuminoids. The solubility was found to be best in combination with HPγCD for all curcuminoids with hydrogen binding possibilities. Some investigations were also performed to find the reasons for the observed differences in solubility compared to the study by Tønnesen et al. The hydrolytic stability of the curcuminoids was improved by CD complexation. On comparison between HPβCD, MβCD and HPγCD, hydrolytic stability was found to be decreasing in the order HPβCD>MβCD>HPγCD. Photochemical stability was investigated in MeOH, a 60:40
mixture of MeOH and aqueous buffer and in HPβCD, and found to be best in MeOH and generally better in CD solution than in the mixture of MeOH and buffer.

The UV/Vis absorption of curcumin increases with increasing concentrations of CD [69, 70]. Complexation with cyclodextrins is also reported to influence the fluorescence spectra of curcumin [29].

2.5.1.1 Stoichiometry of cyclodextrin-curcuminoid complexes

Considering the structures of symmetric curcuminoids, with two identical aromatic rings separated by a seven carbon spacer, it is a possibility that both 1:1 host-guest inclusion complexes and 1:2 guest:host inclusion complexes can be formed with CDs. The reports on the stoichiometry of the curcumin-CD complexes are somewhat contradictory, some indicating 1:1 stoichiometry [2, 69] and some 1:2 stoichiometry [29, 70].

Tønnesen et al. [2] reported 1:1 complex formation, since linear phase solubility diagrams were obtained for curcumin in CD solutions. The aqueous solubility of these compounds is very low and difficult to determine, and this makes it difficult to determine the exact stoichiometry. In this investigation the minimum stability constant was therefore estimated using the analytical detection limit (3x10^-8M) as the highest possible value for the solubility in pure water [2].

Tang et al. [70] came to the conclusion that a 1:2 β-CD-curcumin complex is formed, from their investigations on the apparent formation constant of this complex. In this complex each of the aromatic rings in curcumin are included in one β-CD cavity [70]. It was found that pK_{a1} and pK_{a2} of the curcumin phenolic groups were increased in the presence of β-CD, compared to the pKa values obtained in the absence of β-CD, and from this it was also concluded that the two phenolic hydroxyls are located inside β-CD cavities [70]. The dissociation of the enolic group was however not mentioned in this paper. The apparent formation constant was determined from changes in UV-absorption
spectra, where the absorbance at 431nm was plotted as a function of CD-concentration [70]. 1:2 complex gave the best fit to be obtained [70].

By plotting the changes in UV/Vis-absorption as a function of CD-concentration Qi et al. [69] however, determined the stoichiometry for the formation of curcumin with host CD to be 1:1, for α-, β- and γ-CD and a series of organoselenium-bridged bis (β-cyclodextrin)s.

Baglole et al. [29] found the complexation stoichiometry to be 1:2 for the CDs used in their study except for β-CD, where a 1:1 model fits the data just as well or even better than a 1:2 stoichiometry [29]. The change in fluorescence as a function of CD-concentration was used to determine association constants [29]. It was suggested that for α- and β-CD 1:1 complex is initially formed followed by complexation of a second CD molecule with increasing CD concentration [29]. The situation is somewhat more complicated for the γ-CDs with a suggested initial inclusion of a folded curcumin molecule, but still 1:2 inclusion is reported [29].

The methods used to determine the stoichiometry here are not identical, and might account for some of the observed differences.

2.5.2 Carbohydrate derivatives of curcuminoids

Water solubility is drastically increased by glucosidation of curcumin, with a $1.2 \times 10^7$-fold increase reported for curcumin diglucoside and a 230-fold increase reported for curcumin monoglucoside [35]. For curcumin gentiobiosides the solubility was even further increased [35].

Kaminaga et al. suggests that the soluble curcumin glycosides may also be useful prodrugs of curcumin, with increased absorption after oral administration [35]. This is supported by Mishra et al. [11] by investigations of curcumin-diglycoside together with other bioconjugates of curcumin. Glycine, D-alanine, acetic acid, glucose and piperic acid
are covalently attached to the phenolic groups, and it is indicated that antibacterial activity is increased [11]. Enhanced activity, compared to curcumin, might come from improved cellular uptake or reduced metabolism of the bioconjugates, and the concentration inside the infected cells is increased [11].

### 2.5.3 Comparison of enhancement of water solubility by cyclodextrin complexation and by carbohydrate derivatives

The reported solubility of the curcumin diglucoside, \(3.7 \times 10^2 \, \mu \text{mol/ml} [35]\), was extensively higher compared to the highest curcumin solubility reported in cyclodextrin solutions, approximately \(0.8 \, \mu \text{mol/ml}\) in 11% solution of \(\text{RM} \beta \text{CD} [2]\). For the monoglucoside the water solubility is reported to be \(7 \times 10^{-3} \, \mu \text{mol/ml} [35]\). This makes the synthesis carbohydrate derivatives a very interesting approach to increase the solubility of curcuminoids.

### 2.5.4 Other methods used to enhance water solubility

Incorporation of curcumin into micellar systems has also been used to improve water solubility [71, 72]. This resulted in an increase in water solubility by a factor of at least \(10^5\), improved hydrolytic stability under alkaline conditions and increased photodecomposition, compared to curcumin in hydrogen bonding organic solvents or aqueous solutions [72].

In a recent study by Tønnesen [73] the solubility, chemical and photochemical stability of curcumin in aqueous solutions containing alginate, gelatin or other viscosity modifying macromolecules was investigated. In the presence of 0.5% (w/v) alginate or gelatin the aqueous solubility of curcumin was increased by at least a factor \(\geq 10^4\) compared to plain buffer [73]. These macromolecules do, however, not offer protection against hydrolytic degradation, and it was postulated that formation of an inclusion complex is needed for stabilization towards hydrolysis [73]. Curcumin was also found to be photochemically more unstable in aqueous solutions in the presence of gelatin or alginate than in a
hydrogen bonding organic solvent [73].
3 - EXPERIMENTAL

3.1 Synthesis of curcuminoids

3.1.1 Synthesis of simple symmetrical curcuminoids

The first curcuminoids that were synthesized in the present work were simple symmetrical curcuminoids (figure 3.1). These differ from curcumin only by the substituents of the aromatic ring. They were synthesized my a method suggested by Pabon [31]. It was attempted to synthesize 5 curcuminoids, of which 3 have previously been made by M. Tomren [47]. One of the compounds, RHC-5, was previously attempted to be synthesized by the Pabon method, but the synthesis was not successful [47].

![Figure 3.1](image)

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<td>e)</td>
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**Figure 3.1**: These simple symmetrical curcuminoids were to synthesized in the present work: a) 1,7-bis(3,4-dimethoxyphenyl)-1,6-heptadiene-3,5-dione, b) 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, c) 1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione, d) 1,7-bis(4-methoxyphenyl)-1,6-heptadiene-3,5-dione and e) 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione.
3.1.1.1 Synthesis of 1,7-bis(dimethoxyphenyl)-1,6-heptadiene-3,5-one (RHC-1)

0.07 mole (5g) of boron oxide and 0.1 mole (10g) of acetylacetone were mixed in a round bottle and stirred for approximately 1 hour. The mixture became creamy white. 0.2 mole (33.2 g) of 3,4-dimethoxybenzaldehyde and 0.4 mole (92 g) of tributylborate were dissolved in 100 ml dried ethyl acetate (EtOAc). The solution of the aldehyde was added to the round bottle, which contained the boron complex, and stirred for 10 minutes. 2 ml of butylamine was mixed with 6 ml of dry EtOAc, to a total amount of 8 ml. 2 ml of this mixture was added drop wise to the round bottle every 10 minutes. The total amount of butylamine added was 2 ml, and the reason for diluting with EtOAc was to make these small amounts easier to handle. The color of the mixture changed when butylamine was added, first to orange and then red. The mixture was stirred for 4 hours, and was then allowed to stand overnight.

150 ml 0.4M HCl was now to be added. Due to a calculation error the dilution of the acid went wrong, and the acid that was added was more than tenfold too strong. After the acid was added the mixture was stirred for approximately 1 hour, and a dark red mixture with a lot of dark precipitation was obtained. This mixture was filtered and the dark precipitation was washed with EtOAc. A dark red liquid was obtained and this was washed with water. A little sodium chloride was added to separate the emulsion layer. A precipitate had now appeared in the red liquid, and this was filtered off and the remaining liquid phase was washed 3 times with 100 ml water in a separating funnel. A little amount of sodium chloride was added to the final washing, to separate the emulsion layer. The dark precipitation first obtained had some orange-yellow spots and seemed to contain some of the curcuminoid. This precipitate was washed twice more with 75 ml EtOAc, and these combined EtOAc phases were washed 3 times with 150 ml water. A little sodium chloride was added the last time. All ethyl acetate phases were combined and filtrated, and were then upconcentrated in a rotavapor until approximately 75 ml was left. 200 ml methanol (MeOH) was added and the mixture was stored in the freezer overnight for crystallization. The next morning an orange precipitation had appeared and the mixture was filtered. The precipitate was washed with 100 ml MeOH.
To increase the yield, the combined liquid after the filtration were upconcentrated in a rotavapor until just a little solvent was left. This was stored in the freezer, but did not seem to crystallize. It was attempted to evaporate both solvent and eventual residual starting material in a Glas Oven (Büchi Glas Oven B-580) at temperatures up to 200°C. At this temperature also the curcuminoid was melted, but not evaporated. This procedure was not successful.

The compound was recrystallized by adding 100 ml hot EtOAc, and 300 ml hot MeOH. The compound did not dissolve completely, but it was assumed that impurities in much lower concentration would dissolve. The mixture was stored in the freezer overnight and crystals were filtered off. The melting point analysis indicated that two different crystal forms of the compound had been formed, and the compound was therefore recrystallized again. The compound was this time dissolved in 200 ml hot EtOAc and stirred until everything was dissolved. 300 ml hot MeOH were added and the mixture was left to cool down at room temperature. Crystallization began already at room temperature, and the mixture was stored in the freezer overnight. Crystals were filtrated off and washed with MeOH.

3.1.1.2 Synthesis of 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-one (RHC-2, Curcumin)

0.07 mole (5g) of boron oxide and 0.1 mole (10g) of acetylacetone were stirred together in a round bottle for approximately 1 hour. The mixture became creamy white. 0.2 mole (30 g) of 4-hydroxy-3-methoxybenzaldehyde and 0.4 mole (92 g) of tributylborate were dissolved in 100 ml dry EtOAc. The solution of the aldehyde was added to the round bottle, which contained the boron complex, and stirred for 10 minutes. 2 ml of butylamine was mixed with 6 ml of dry EtOAc, to a total amount of 8 ml. 2 ml of this mixture was added drop wise to the round bottle every 10 minutes. The total amount of butylamine added was 2 ml, and the reason for diluting with EtOAc was to make these
small amounts easier to handle. The color of the mixture changed when butylamine was added, first to orange and then to dark red. The mixture was stirred overnight. The next morning 150 ml 0.4 M HCl was added and the mixture was stirred for 1 hour. The HCl was added to hydrolyze the boron complex. The mixture was filtered, and the precipitate was washed with EtOAc. There were some precipitation in the aqueous phase and the mixture was filtered again. This precipitate was also washed with a little EtOAc. Then the EtOAc phase and the aqueous phase were separated in a separating funnel, and the water phase was washed twice with 50 ml EtOAc. The water phase now seemed relatively clear. Then the combined EtOAC phases were washed with 200 ml water. A small amount of sodium chloride was added to separate the emulsion layer between the phases. The EtOAc was evaporated in a rotavapor until there was just a little left, approximately 75 ml. The mixture was now very viscous and something had crystallized. 200 ml MeOH was added and the mixture was stored in the freezer overnight for crystallization. The next morning the crystals were isolated by filtration and washing with cold MeOH.

The liquid was suspected to still contain some of the compound, and MeOH was evaporated off in a rotavapor and the remaining solution was stored in the freezer. After 5 days more curcumin had crystallized, and the mixture was again filtrated. The remaining liquid was evaporated in the rotavapor until just a little was left and stored in the freezer. The product filtered off from this liquid did not seem to be curcumin, after melting point analysis.

Recrystallization was attempted by dissolving in acetone and adding approximately 100 ml hot MeOH. The compound did not dissolve completely, but it was assumed that more soluble impurities and impurities at lower concentration would be removed. The recrystallization decreased the yield.
3.1.1.3 Synthesis of 1,7-bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-one (RHC-3, Bisdemethoxycurcumin)

The starting material for this curcuminoid was p-hydroxybenzaldehyde. The procedure was as described for RHC-2, except that the mixture was only filtrated once before it was transferred to the separating funnel. After transferring, a precipitate appeared in the aqueous phase. The phases were separated, and both had to be filtrated before they were washed with respectively EtOAc and water.

The mixture was stored in the freezer overnight and red crystals appeared. These were filtrated off and washed with MeOH. The remaining liquid was left at room-temperature, and after a while more crystals appeared. These were filtered off, and analysis by NMR, IR and melting point showed it was the same compound.

The compound was recrystallized by dissolving in approximately 75 ml acetone and adding approximately 50 ml hot MeOH. The compound did not dissolve completely in acetone, but was dissolved when hot MeOH was added. Red crystals could be filtered off, and the liquid was concentrated on a rotavapor until precipitation started. More red-orange powder was obtained.

3.1.1.4 Synthesis of 1,7-bis(4-methoxyphenyl)-1,6-heptadiene-3,5-one (RHC-4)

The starting material for this curcuminoid was p-anisaldehyde. The procedure was as described for RHC-2, except that the mixture was left in the freezer over the weekend.

Recrystallization was attempted by dissolving in approximately 10 ml acetone and adding approximately 10 ml hot methanol. The compound did not dissolve completely, but it was assumed that more soluble impurities and impurities at lower concentration would be removed.
3.1.1.5 Synthesis of 1,7-bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-one (RHC-5)

Method 1: First curcumin was synthesized the same way as for RHC-2, but not recrystallized or further purified. From melting point and TLC analyses this curcumin seemed pure enough for further synthesis. Without the recrystallization step the yield of curcumin was much better. 0.074 mole (11.22g) of vanillin was used as starting material, and this produced 0.0194 mole (7.13g) curcumin. 0.0176 mole (6.5g) was used for synthesis of RHC-5. 0.059 mole (8g) AlCl₃ was added to a stirred mixture of 0.0176 mole (6.5g) curcumin in 295 ml dry CH₂Cl₂. When AlCl₃ was added the mixture changed color from yellow to red. 19 ml dry pyridine was added drop wise, and the mixture was refluxed for 24 hours. The mixture was cooled on ice, and acidified with 1 M HCl until the pH was about 1-2. In total almost 200 ml of the acid was added. When the acid was added some precipitation appeared in the water phase and this was filtered off. The phases were separated in a separating funnel, and the organic phase was washed with 50 ml EtOAc five times. The combined organic phases was dried on dry Na₂SO₄, and filtrated. The solvents were evaporated to dryness in a rotavapor, and red-brownish crystals were left in the roundbottle. Analysis (NMR, melting point) showed that this was not a pure compound, but the compound was not further purified.

Method 2: Since the first attempt to synthesize this compound was not successful, another method [33] was tried instead. 0.0304 mole (4.2g) of 3,4-dihydroxybenzaldehyde was dissolved in 3.1 ml dry DMF, together with 0.0152 mole (1.52g) acetylacetone. 0.049 mole (3.04g) of boric acid was added and the mixture was heated at 100°C in a water bath for 5 minutes. When boric acid was added the mixture got a purplish-black dark color. 0.024 mole (3.1 ml of 1,2,3,4-tetrahydroquinoline and 0.92 ml glacial acetic acid was mixed in 3.1 ml DMF. This mixture was combined with the solution of the aldehyde and acetylacetone, and was heated for 4 hours at 100°C on a water bath. After cooling 150ml 20% acetic acid was added and stirred vigorously for 10 minutes. Some black precipitation was filtered off, and the remaining liquid was red-orange. The liquid was kept in the fridge over night, but no compound precipitated. The liquid was washed 4 times with approximately 150ml EtOAc, and dried over night with Na₂SO₄. The liquid
was filtered and EtOAc was upconcentrated in a rotavapor. A red liquid was left and did not evaporate. To this liquid was added approximately 50 ml diethyl ether in order to precipitate the red compound. A dark red compound was filtered off. NMR analysis indicated that the intended compound had been synthesized, but was too impure for further investigations. Further purification was not attempted.

3.1.2 Synthesis of curcuminoid galactosides

3.1.2.1 Step 1: Synthesis of Tetra-O-acetyl-α-D-galactopyranosyl Bromide
(Acetobromogalactose)

34.8 g acetic anhydride in a 250 ml three-necked roundbottle was cooled to 4°C. 0.16 ml HClO₄ was added in a drop wise manner, and the solution got a yellow-brownish color. After the mixture had been heated to 9-10°C 8 g galactose was added in portions at room temperature, and the temperature was kept at 20-40°C. The mixture was cooled when necessary. After adding the total amount of galactose the mixture was cooled to < 20°C and 2.5 g of red phosphorus was added. This was followed by addition of 4.8 ml Br₂ (14.8 g), which was added with a dropping funnel. The temperature was held < 20°C, and 2.9 ml water was added slowly and carefully in a drop wise manner. After 2 hours stirring 24 ml CH₂Cl₂ was added. The mixture was filtered twice through glass-wool to remove residues of phosphorus. The mixture was further washed twice with approximately 130 ml ice-cold water (approximately 0°C), and the CH₂Cl₂ phase was washed with 40 ml ice-cold saturated solution of NaHCO₃. The phases were separated in a separating funnel and some silica gel 60 was added to the organic phase. The mixture was stirred with a magnetic stirrer for 10 minutes and then filtrated. The solvents were evaporated in a rotavapor. When the solvents where evaporated a foam appeared and then the compound was left in the roundbottle as a gum. This was recrystallized from diethyl ether. The compound was dissolved in approximately 20 ml diethyl ether and left in the freezer overnight. The next morning a solid was filtered off and washed carefully with diethyl ether. The compound appeared as white crystals. To increase the yield the solution in diethyl ether was concentrated using a rotavapor, and left in the freezer for
crystallization. After approximately 1 hour more solid was filtered off and washed carefully with diethyl ether. The compound now appeared as white crystals with a shade of yellow.

After analysis by NMR, IR and melting point the compound was used for synthesis of vanillin galactoside.

3.1.2.2 Step 2: Galactosidation of vanillin

5.85g (0.0143 mole) acetobromogalactose, 4.35g (0.0286 mole) vanillin and 4.61g (0.0143 mole) Bu4NBr were dissolved in 50 ml CHCl3, and then 50 ml 1 M NaOH was added. Bu4NBr was used as a phase transfer agent in the reaction. The mixture was vigorously stirred at room temperature for 24 hours. The organic phase was washed 3 times with 100 ml 1 M NaOH solution, and then twice with 100 ml 5% NaCl-solution and once with 100 ml water and NaCl. After the washing procedure the organic phase was dried over anhydrous Na2SO4, and concentrated to dryness in a rotavapor. An attempt was made to crystallize the compound from approximately 20 ml EtOH in the freezer. The resulting solid was a sticky gum-like compound, which was not very suitable for filtration. The liquid was removed with a pipette and the rest of the solvent was evaporated using a rotavapor. This procedure was repeated twice, using 10 ml hot EtOH for recrystallization, in an attempt to improve purity and obtain a crystalline material. It was attempted to wash the still sticky gum-like substance with ice-cold EtOH, using cooled equipment. Some of the compound could be filtrated, but turned into the gum-like compound again when left at room-temperature. The compound was now in two fractions, and the following procedure was performed for both of these. The gum-like substance was dissolved in approximately 10 ml EtOAc, and washed once with 40 ml 1 M NaOH solution in a separating funnel. The organic phase was dried overnight with Na2SO4. The drying material was filtrated off and the solvent evaporated using a rotavapor. The compound was dissolved in approximately 20 ml hot EtOH and cooled down at room-temperature. The solution was left in the fridge for crystallization, and later moved to the freezer. The EtOH was then removed using a pipette and the rest of the
solvent was evaporated on a rotavapor. The resulting gum-like substance was analyzed by TLC and IR, and was decided pure enough for the next step of the synthesis. The compound in both of the fractions was dissolved in a small volume of EtOAc, and combined. When the solvent was evaporated in a rotavapor, the compound could be obtained as foam-like crystalline material, which was analyzed by NMR and melting point.

3.1.2.3 Step 3: Synthesis of Curcuminoid Galactoside Octaacetate

0.04g (0.5475 mmole) boron oxide and 0.08g (0.76 mmole) acetylacetone were stirred for 1 hour at room temperature. 0.73g (1.51 mmole) of vanillin galactoside was dissolved in 10 ml dry EtOAc. 0.76 ml tributylborate was added and the mixture was stirred for 15 minutes. The stirred mixture of boron oxide and acetylacetone was added, and the resulting mixture was stirred for 10 minutes. 15 μl butylamin was added, using a automatic pipette, and the mixture was stirred for 20 hours at room temperature. A while after the butylamine was added the mixture started to change color, becoming more yellow. When the mixture had been stirred for 20 hours, it was attempted to evaporate the mixture to dryness using a rotavapor. This turned out to be difficult and some relatively clear liquid was left and was poured out of the roundbottle. The compound was left in the roundbottle as a yellow-orange solid. This was refluxed in 60 ml MeOH for 4 hours. It was attempted to purify the compound by column chromatography. The column was prepared with silica gel as stationary phase, and a 3:2 mixture of EtOAc and hexane was used as mobile phase. No impurities could be detected by TLC of the combined eluate, using a mobile phase consisting of EtOAc:hexane (3:2) on silica gel 60 F254 (Merck) coated plates. NMR analysis showed that the compound was not pure enough.

3.1.2.4 Step 4: Ammonolysis of Acetoxy group

Not performed
3.2 Tests of identity and purity

3.2.1 TLC analysis

TLC analysis was performed on silica gel 60 F254 coated aluminum plates (Merck). The mobile phase used was chloroform:EtOH 25:1 [43]. For this system some problems with band-broadening for the curcuminoids occurred. Bisdemethoxycurcumin was too much retarded on the silica plates when this system was used. Dichloromethane:MeOH 99:1 as mobile phase [44] was tried out, but this did not improve the results. The problem was finally resolved by using glass plates instead of plates made of aluminum, with mobile phase chloroform:EtOH 25:1.

A UV lamp was used for detection at 254 and 365 nm.

3.2.2 Melting point analysis

The compound was filled in small capillary tubes and the melting point was measured using a Gallenkamp melting point apparatus, to investigate purity and identity. When more than one harvest was obtained the melting point for different harvests of one compound was compared to see if it was the same compound, in addition to TLC investigations.

3.2.3 IR analysis

Tablets consisting of KBr and a small amount of compound were made, and analyzed in the area between 4000 cm⁻¹ and 400 cm⁻¹ using an Avatar 370 FT/IR apparatus.

3.2.4 NMR analysis

Approximately 20mg of each sample was dissolved in 0.8 ml of the selected solvent. RHC-1 and RHC-2 were dissolved in CDCl₃, and RHC-3 and RHC-4 was dissolved in
DMSO for NMR analysis. The samples were analyzed by the Department of Chemistry, University of Iceland, using a 400 MHz Bruker AC 250 P Spectrometer.

Acetobromogalactose, vanillin galactoside and curcumin digalactosyl octaacetate were dissolved in CDCl₃ for NMR analysis.

### 3.2.5 UV/Vis analysis

All compounds were dissolved in acetonitrile, and diluted to a final concentration of 10⁻⁵ M. The analyses were performed on a Ultrospec 2100 UV/VIS spectrometer. The impure compound RHC-5 did not dissolve sufficiently in acetonitrile, and was not analyzed by UV/Vis.

### 3.3 HPLC analysis

A previously developed HPLC method for curcumin and its natural derivatives was used (see section 2.2.2.2). The method has been modified for use on different derivatives, including C-1 [47]. This method had to be tested and eventually further adjusted, and in addition modified for use on the compound C-4.

When the compounds were analyzed with HPLC some more information on the purity of the compounds were obtained.

#### 3.3.1 The HPLC method

The mobile phase consisted of a combination of acetonitrile and 0.026M (0.5%) citrate buffer pH 3 [2, 47]. The buffer was prepared by dissolving citric acid in a volumetric flask almost filled with water, and adjusting the pH to the desired value by adding 10% KOH solution. Water was then added to the mark in the flask.
All the compounds were first analyzed with a mobile phase made of 60% citric acid buffer and 40% acetonitrile (ACN). To obtain retention times lower than 15 minutes for all the compounds the amount of organic solvent was increased until the compounds were sufficiently retarded. The flow was kept at 1 ml/min, and the samples detected at 350nm. The equipment and chemicals used is presented in Appendix, A.1-A.2.

An attempt was made to find suitable mobile phase systems for the impure compounds RHC-5 and curcumin digalactoside octaacetate. The purpose of this was to get some more information on the identity and impurities, but the attempt was not successful.

The chromatographic conditions developed in Iceland were also valid for the system used in Oslo, except for the compound RHC-1. RHC-1 resulted in one peak that was not baseline separated from the main peak in the chromatograms. In order to avoid this problem, the mobile phase and flow was adjusted to achieve baseline separation and suitable retention time.

3.3.2 Validation of the HPLC method

3.3.2.1 Limit of detection (LOD)

No systematic investigation on the LOD was performed in Iceland, and the lower detection limit was determined by the range of the standard curve. The lowest concentration used for this gave peaks which were above or close to the detection limit.

In Oslo a more sensitive HPLC detection was used. LOD was defined as signal/noise ratio at approximately 5, and determined by injecting samples with decreasingly concentrations.
3.3.2.2 Linearity

Stock solutions were made at $10^{-3}$ M in MeOH and diluted to cover the range between $1.5 \times 10^{-4}$ M and $5.0 \times 10^{-6}$ M. The concentrations $1.5 \times 10^{-4}$ M, $7.5 \times 10^{-5}$ M, $5 \times 10^{-5}$ M, $10^{-5}$ M and $5 \times 10^{-6}$ M were used and each sample was injected 3 times. For quantification in the phase solubility study the concentrations $10^{-4}$ M, $5 \times 10^{-5}$ M and $10^{-5}$ M were re-run at certain time intervals during the measurements.

In Oslo the same procedure was repeated. Concentrations $5 \times 10^{-5}$ M, $10^{-5}$ M, $5 \times 10^{-6}$ M and $10^{-6}$ M were used. For quantification in the phase solubility and photochemical degradation studies the concentrations $10^{-5}$ M, $5 \times 10^{-6}$ M and $10^{-6}$ M were re-run at certain time intervals during the measurements.

3.4 Hydrolytic stability

It was attempted to find simplified conditions in which hydrolytic degradation of curcuminoids in CD solutions could be studied, in order to investigate the effect of different concentrations of CD on the hydrolytic stability. The curcuminoid dissolved in MeOH was added to a mixture consisting of 60% MeOH and 40% aqueous buffer at different pH values between 7.5 and 10. It was attempted to keep temperature constant at 30°C. No significant degradation was seen for any of the curcuminoids under these conditions except for curcumin at the highest pH values. Curcumin has been more thoroughly investigated before by Tønnesen et al [2].

I must be noted that the pH in the samples were lower than in the buffer alone, due to the dilution with methanol. The high MeOH concentration did in addition probably stabilize the compounds towards hydrolytic degradation.

No further studies on hydrolytic stability were carried out.
3.5 Phase solubility

The solubility of the complexes formed between 4 different curcuminoids and 3 different cyclodextrins was measured in citrate buffer. HPβCD, MβCD and HPγCD were used, and for HPβCD and HPγCD solutions the solubility was also determined with different CD-concentrations in order to get some information on the complex stoichiometry. The solubility of the curcuminoid-cyclodextrin complex in phosphate buffers, where the ionic strength was varied and adjusted with different salts, was also investigated. In phosphate buffer the ionic strength 0.085 and 0.15 were obtained by adjusting with NaCl and KCl.

In citrate buffer ionic strength was held at 0.15M, and NaCl or MgCl₂ were used to achieve this strength. The solubility was also investigated in citrate buffer where the concentration of MgCl₂, and therefore also the ionic strength, were substantially increased.

The influence of pH on the solubility of the curcuminoid-CD complex in citrate buffers with different pH value was measured at pH 4.5 and 5.5. The results were inconclusive, and the effect of pH was further investigated in similar buffers with pH 4 and 6 respectively.

3.5.1 Quantification and quality checks

Before the samples could be measured some quality control was preformed.

3.5.1.1 Equilibrium in the system

To check that equilibrium had been reached after one week, and that a sufficient amount of curcuminoid had been added to the samples, the following extra samples was made and analyzed as a quality check of the system (table 3.1).
Table 3.1: Samples for quality checks of the curcuminoids in 10% solution of HPβCD, in buffer I.

<table>
<thead>
<tr>
<th></th>
<th>RH-C-1</th>
<th>RH-C-2</th>
<th>RH-C-3</th>
<th>RH-C-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mg in 2 ml buffer</td>
<td>1 sample</td>
<td>1 sample</td>
<td>1 sample</td>
<td>1 sample</td>
</tr>
<tr>
<td>30 mg in 2 ml buffer</td>
<td>1 sample</td>
<td>1 sample</td>
<td>1 sample</td>
<td>1 sample</td>
</tr>
<tr>
<td>6 mg in 2 ml buffer,</td>
<td>1 sample</td>
<td>1 sample</td>
<td>1 sample</td>
<td>1 sample</td>
</tr>
<tr>
<td>ultrasound.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Added 6 mg more</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

One sample of 6mg of each curcuminoid was made with 10% HPβCD in buffer I.

One sample was made with 30 mg of each curcuminoid and 2 ml of buffer system I, with 10% HP-β-CD. The results are compared with the results for the samples made from 6 mg in 2ml to establish that sufficient amount of curcuminoid was added to the samples.

One sample was made with 6 mg of each curcuminoid and 2 ml of buffer system I, with 10% HP-β-CD. This was heated to 60-64°C in an ultrasound bath for 1 hour, and visually inspected. The samples were shaken for one day while they were cooled down. Approximately 6mg more of the compound was added, and was shaken together with the other samples.

These samples were filtrated and analyzed first for each of the compounds. If the results had indicated that equilibrium was not reached, the rest of the samples would have been shaken longer.
3.5.1.2 Absorption of the curcuminoids to the filters

Representative samples of each compound are filtered twice. The samples were analyzed by HPLC after one and two filtrations respectively, in order to determine whether the curcuminoids are adsorbed to the filter.

3.5.2 Phase solubility for all the curcuminoids in citrate buffer

Buffer I (see appendix A.3.2) with a 10% w/v CD concentration

Citric acid was dissolved in a volumetric flask filled with water almost to the mark, and pH was adjusted to 5 with a 10% NaOH-solution. Water was then filled to the mark in the flask. The ionic strength was calculated using equation 3.1, and adjusted to 0.15 M with NaCl. The water-content of the CDs was measured and corrected for, and the CDs were dissolved in buffer to obtain 100 ml buffer with 10% CD-concentration. pH was measured in the final solution. The exact compositions of the buffers used in the phase solubility studies are presented in appendix A.3.2.

\[ I = \frac{1}{2} \sum (mz^2) \quad (Equation \ 3.1 \ [51]) \]

where \( m \) = molarity of the solution and \( z \) = electric charge of the ion [51].

<table>
<thead>
<tr>
<th></th>
<th>RH-C-1</th>
<th>RH-C-2</th>
<th>RH-C-3</th>
<th>RH-C-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP(\beta)CD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>M(\beta)CD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>HP(\gamma)CD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Table 3.2: Samples for phase solubility of the curcuminoids in 10% solution of the given CDs, in buffer I.

6 mg curcuminoid was weighed in vials, and 2 ml buffer was added to the vial. The vials were sealed and shaken for one week or more (generally 7-8 days). The samples (table
3.2) were then filtered, diluted with MeOH if necessary, and analyzed by HPLC to find the concentration of the solution. Dilution was necessary for C-2 and C-3, and for C-1 in HPγCD. The first drops of the filtrated samples were discarded.

3.5.3 The effect of CD-concentration on phase solubility

Buffer I (see appendix A.3.2) with HPβCD and HPγCD.

Dilution of 10% CD solution: Citrate buffer I with 10% HPβCD or 10% HPγCD, as used in section 3.5.2, were diluted with buffer I to obtain the selected concentrations of CD.
7.5%: 75 ml buffer I with CD + 25 ml buffer I without CD
5%: 50 ml buffer I with CD + 50 ml buffer I without CD
2.5%: 25 ml buffer I with CD + 75 ml buffer I without CD

Table 3.3: Samples for phase solubility of the curcuminoids in 10%, 7.5%, 5% and 2.5% solutions of the given CDs, in buffer I.

<table>
<thead>
<tr>
<th>CD Concentration</th>
<th>RH-C-1</th>
<th>RH-C-2</th>
<th>RH-C-3</th>
<th>RH-C-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% CD</td>
<td>See 3.3.2</td>
<td>See 3.3.2</td>
<td>See 3.3.2</td>
<td>See 3.3.2</td>
</tr>
<tr>
<td>7.5% CD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>5% CD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>2.5% CD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.3) were otherwise performed as described in section 3.5.2. For 10% CD solution, results from section 3.5.2 were used.

3.5.4 The influence of ionic strength on the phase solubility experiments

Buffer III-VI (see appendix A.3.2) with a 10% w/v CD concentration

Dimethoxycurcumin (RHC-1) and curcumin (RHC-2) were used for this experiment.
Table 3.4: Samples for phase solubility of RHC-1 and RHC-2 in 10% solution of the given CDs, in buffers III-VI.

<table>
<thead>
<tr>
<th></th>
<th>RHC-1</th>
<th>RHC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>MβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>HPγCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.4) were otherwise performed as described in section 3.5.2.

3.5.5 Phase solubility for all the curcuminoids in citrate buffer when the ionic strength is adjusted with MgCl₂

Buffer system II (see appendix A.3.2) with a 10% w/v CD concentration

Table 3.5: Samples for phase solubility of the curcuminoids in 10% solution of the given CDs, in buffer II.

<table>
<thead>
<tr>
<th></th>
<th>RH-C-1</th>
<th>RH-C-2</th>
<th>RH-C-3</th>
<th>RH-C-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>MβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>HPγCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.5) were otherwise performed as described in section 3.5.2.

The reason for adding MgCl₂ was to investigate if this salt could contribute to increased solubility of the curcuminoids in the CD solutions. An additional experiment was performed when the first did not give increased solubility in the buffer containing MgCl₂. This is further discussed in section 4.4.6.

Buffer system IX (see appendix A.3.2) with a 10% w/v CD concentration
Dimethoxycurcumin (RHC-1) and curcumin (RHC-2) were used for this experiment.

**Table 3.6:** Samples for phase solubility of RHC-1 and RHC-2 in 10% solution of the given CDs, in buffer IX.

<table>
<thead>
<tr>
<th></th>
<th>RHC-1</th>
<th>RHC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% HPβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.6) were otherwise performed as described in section 3.5.2.

The experiments with increased MgCl₂ concentration in HPβCD buffer did not show increased solubility. If a complex is formed between the curcuminoid and Mg²⁺, HPγCD has got a large cavity and might encapsulate this potential complex better than the other CDs. The experiment was therefore repeated with HPγCD.

Buffer system X-XI (see appendix A.3.2) with a 10% w/v CD concentration Dimethoxycurcumin (RHC-1) and curcumin (RHC-2) were used for this experiment.

**Table 3.7:** Samples for phase solubility of RHC-1 and RHC-2 in 10% solution of the given CDs, in buffers X-XI.

<table>
<thead>
<tr>
<th></th>
<th>RHC-1</th>
<th>RHC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% HPγCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.7) were otherwise performed as described in section 3.5.2.
3.5.6 The effect of pH on the phase solubility

Buffer system VII-VIII (see appendix A.3.2) with a 10% w/v CD concentration

Dimethoxycurcumin (RHC-1) and curcumin (RHC-2) were used for this experiment.

100 ml 1% citrate buffer was made twice and pH is adjusted to 4.5 and 5.5 respectively by adding 10% NaOH solution. The ionic strength is calculated using equation 3.1, and adjusted with NaCl for buffer system VII. The water-content of the CDs was measured and corrected for, and the CDs were dissolved in buffer to obtain 25 ml with 10% concentration. pH was finally adjusted with NaOH solution or HCl solution to achieve the right pH. This could cause the ionic strength to be incorrect, but for this experiment it was more important to keep the right pH value.

Table 3.8: Samples for phase solubility of RHC-1 and RHC-2 in 10% solution of the given CDs, in buffers VII-VIII.

<table>
<thead>
<tr>
<th></th>
<th>RHC-1</th>
<th>RHC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% HPβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>10% MβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>10% HPγCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.8) were otherwise performed as described in section 3.5.2.

It was difficult to draw any conclusion from the results. The experiment was therefore repeated at two additional pH-values (4 and 6).

Buffer system XII-XIII (see appendix A.3.2) with a 10% w/v CD concentration
The buffers were made the same way as described above for buffer VII-VIII.
Table 3.9: Samples for phase solubility of RHC-1 and RHC-2 in 10% solution of the given CDs, in buffers XII-XIII.

<table>
<thead>
<tr>
<th></th>
<th>RHC-1</th>
<th>RHC-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% HPβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>10% MβCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>10% HPγCD</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
</tbody>
</table>

Preparation and analysis of the samples (table 3.9) were otherwise performed as described in section 3.5.2.

3.6 Differential Scanning Calorimetry

Approximately 1 mg of each curcuminoid was weighed in an aluminum pan. A hole was made in the lid and the pans were then sealed.

The temperature interval in which the samples were to be analyzed was estimated from the previously obtained melting point intervals. One sample was first analyzed to determine the exact experimental conditions (table 3.10).

Table 3.10: Time interval for analysis of the different compounds

<table>
<thead>
<tr>
<th>Temperature interval (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
</tr>
<tr>
<td>RHC-2</td>
</tr>
<tr>
<td>RHC-3</td>
</tr>
<tr>
<td>RHC-4</td>
</tr>
</tbody>
</table>

Samples were analyzed by DSC using a Mettler Toledo DCS822\textsuperscript{e}. The instrument was calibrated using Indium. The samples were scanned in the predetermined temperature interval at 10°C/min, in a nitrogen environment. The analyses were carried out in duplicate.
In addition to the simple symmetrical curcuminoids synthesized in the present work, demethoxycurcumin and bisdemethoxycurcumin synthesized by M. Tomren were analyzed by DSC. Curcumin synthesized by Tomren and Tønnesen had been analyzed before (unpublished results), and the results were also included in the present discussion.

**3.7 Photochemical stability**

The photochemical stability of the curcuminoids were analyzed in 4 different solvent systems: EtOH

- 40% EtOH + 60% citrate buffer pH 5 (I=0.152)
- 10% HPβCD in citrate buffer pH 5 (I=0.152)
- 10% HPγCD in citrate buffer pH 5 (I=0.152)

Buffers were prepared as previously described. The ionic strength was calculated using equation 3.1, and not further adjusted.

Stock solutions of the curcuminoids were prepared in MeOH to a concentration of $10^{-3}$ M. 200 μl of this stock solution was diluted to 20ml in the desired solvent system, to achieve the final concentration $10^{-5}$ M. This gave a 1% concentration of MeOH. For compound RHC-4 a $10^{-3}$ M solution could not be made, due to low solubility in MeOH. Instead a stock solution was prepared in EtOH to a concentration of $10^{-4}$ M. The compound was further diluted in EtOH or in EtOH and buffer, to achieve a $10^{-5}$ M concentration in the samples. For the sample with EtOH and buffer, 2 ml of the stock solution was mixed with 6 ml EtOH and 12 ml buffer, to keep a constant ratio between EtOH and buffer. Photochemical stability was not investigated in CD-solutions for RHC-4.
Table 3.11: Samples for studies of photochemical stability of the curcuminoids in 4 different solvent systems

<table>
<thead>
<tr>
<th></th>
<th>EtOH</th>
<th>60% EtOH 40% buffer</th>
<th>10% HPβCD</th>
<th>10% HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>RHC-2</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>RHC-3</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td>3 parallels</td>
</tr>
<tr>
<td>RHC-4</td>
<td>3 parallels</td>
<td>3 parallels</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Suntest apparatus was calibrated to irradiate at 1170x100 Lux (visible) and 13.7 W/m² (UV) (intensity 4).

The samples (table 3.11) were kept in closed cuvettes, and irradiated at “intensity 4”. After every 5 minutes of irradiation samples were withdrawn for analysis with HPLC. The samples were irradiated for a total of 20 minutes, unless the total amount of curcuminoids was degraded in a shorter time. Three parallels were made for each curcuminoid in each solvent system. The degradation of one of the parallels was also monitored by UV.

The log value of the average concentration from the three parallels was plotted against irradiation time, and the slope k was found using simple linear regression. The half-life was calculated using equation 3.2, where k is the slope of the regression line.

\[ t_{0.5} = \frac{0.693}{k} \quad (\text{Equation 3.2}) \quad [51] \]

Normalization of the results was performed by dividing the area under the curve (AUC) for the UV/Vis absorption, obtained for un-irradiated samples of each compound in each solvent system (ss), by a reference AUC value. The AUC value of curcumin in EtOH was used as a reference value, which is a rough approximation to simplify the calculations.
The $\frac{AUC_{\text{compound in ss}}}{AUC_{\text{curcumin in EtOH}}}$ obtained were further multiplied with the half-life values calculated from equation 3.2 to get the normalized half-life.

AUC was measured from 310nm and until the absorbance was zero.
4 – RESULTS AND DISCUSSION

4.1 Synthesis of curcuminoids

4.1.1 Yield

4.1.1.1 Yield of simple symmetrical curcuminoids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mw</th>
<th>Appearance</th>
<th>Yield before recrystallization (g)</th>
<th>Final yield (g)</th>
<th>Final yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>396.42</td>
<td>Orange powder</td>
<td>8.58g</td>
<td>7.38g</td>
<td>18.62%</td>
</tr>
<tr>
<td>RHC-2</td>
<td>368.37</td>
<td>Orange-yellow powder</td>
<td>12.82g</td>
<td>5.93g</td>
<td>16.1%</td>
</tr>
<tr>
<td>RHC-3</td>
<td>308.32</td>
<td>Red crystals/dark orange powder</td>
<td>12.03g</td>
<td>11.26g (3.52g + 7.74g)</td>
<td>36.52%</td>
</tr>
<tr>
<td>RHC-4</td>
<td>336.39</td>
<td>Yellow powder</td>
<td>1g</td>
<td>0.80g</td>
<td>2.38%</td>
</tr>
</tbody>
</table>

The compound RHC-5 turned out to be difficult to synthesize and purify. In some other previous publications where a range of curcuminoids have been synthesized this compound is excluded [32, 74]. The compound was also unsuccessfully attempted to synthesize by Tomren [47]. This indicates that the synthesis is generally difficult to perform. This synthesis has however been reported by different authors, by the use of other methods [3, 12, 34].
4.1.1.2 Yield of compounds in the curcumin galactoside synthesis

Table 4.2: Yield and physical appearance of the compounds synthesized in the 3 first steps in the synthesis of curcumin galactoside

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mw</th>
<th>Appearance</th>
<th>Yield</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobromogalactose</td>
<td>411.2</td>
<td>White/white-yellow</td>
<td>5.9g</td>
<td>32.3%</td>
</tr>
<tr>
<td>Vanillin galactoside</td>
<td>482.4</td>
<td>Colorless foam/needles</td>
<td>0.73g</td>
<td>10.58%</td>
</tr>
<tr>
<td>Curcumin digalactoside</td>
<td>1028.95</td>
<td>Yellow/orange</td>
<td>204.7mg*</td>
<td>13.21%</td>
</tr>
<tr>
<td>Curcumin digalactoside octaacetate</td>
<td>856.75</td>
<td>Not synthesized</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*the compound was not pure, so the actual yield of the compound itself is lower

4.2 Analysis of purity and identity

4.2.1 Analysis of simple symmetrical curcuminoids

4.2.1.1 TLC analysis

Due to some initial problems with the TLC method, this analysis was not performed until quite late in the project. Different solvent systems were tried out on silica plates, but all gave severe band broadening. TLC was successfully performed using silica on plates made of glass instead for aluminum.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>0.67</td>
</tr>
<tr>
<td>RHC-2</td>
<td>0.35</td>
</tr>
<tr>
<td>RHC-3</td>
<td>0.07</td>
</tr>
<tr>
<td>RHC-4</td>
<td>0.69</td>
</tr>
</tbody>
</table>

**Figure 4.1:** Thin Layer Chromatography (TLC) on Silica Gel 60, mobile phase CHCl$_3$:EtOH 25:1

No impurities can be seen from TLC

4.2.1.2 Melting point analysis

**Table 4.3:** Melting point of the simple symmetrical curcuminoids measured using a Gallenkamp melting point apparatus.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting point (°C)</th>
<th>Previously reported melting point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>112.5-123.3</td>
<td>128-130 [32], 128.8-131.5 [47], 135-138 [33]</td>
</tr>
<tr>
<td>RHC-2</td>
<td>179.5-181.5</td>
<td>177-178 [32], 178.3-182 [47], 176-178 [31], 182-184 [33]</td>
</tr>
<tr>
<td>RHC-3</td>
<td>226.3-228.8</td>
<td>209-211 [32], 225.6-227.5 [47], 220-224 [33]</td>
</tr>
<tr>
<td>RHC-4</td>
<td>154-158.8</td>
<td>154-155 [32], 166-168 [33]</td>
</tr>
</tbody>
</table>
Here melting points were measured to give an indication on the purity of the compounds. Exact melting points were determined by DSC, and are presented in table 4.21.

4.2.1.3 IR spectroscopy

IR spectra for the simples symmetrical curcuminoids are presented in Appendix, figure A.6.1-A.6.4

The IR spectra are suitable to give information on the identity of the compounds, and on which functional groups are present.

Structural features that are common for all the simple symmetrical curcuminoids, the aromatic rings, the carbonyls and the alkenes, show several bands in the region at approximately 1400-1700 cm\(^{-1}\).

In the spectra of all the compounds bands appear at 3400-3500 cm\(^{-1}\), in the area where hydroxyl groups normally show a broad and intense band [42]. For the compound RHC-1 this signal is small and might come from MeOH. The methanolic solvate of RHC-1 was investigated by DSC, and is further discussed in section 4.5.1.

One intense band appears at 3440 cm\(^{-1}\) in the spectrum of monomethoxy curcuminoid RHC-4, which is assumed to come from the enol. This molecule has no other structural feature that is likely to cause this band. As discussed in section 2.1.3 the hydrogen of the keto-enol group in curcumin is shown to be equally distributed between the two oxygens. This enol can not be seen in IR. Since this group can be seen for the compound RHC-4 it is concluded that the hydrogen is bonded to one of the oxygen atoms rather than being distributed between them. This is also supported by NMR (section 4.2.1.4). The enol in RHC-3 (bisdemethoxycurcumin) also seems to give a broad intense band in this area, which is not separated from the phenol band.
For all the compounds, except RHC-3 (bisdemethoxycurcumin), small bands are seen around 2900 cm\(^{-1}\), due to absorption of C-H. This is probably due to the methoxy groups in these compounds.

Small bands seen at approximately 2300 cm\(^{-1}\) is from CO\(_2\) in the air.

4.2.1.4 NMR

NMR spectra of the simple symmetrical curcuminoids are presented in Appendix, figure A.8.1-A.8.4

RHC-1 (in CDCl\(_3\)):

\[
\text{Figure 4.2: The hydrogen atoms in RHC-1 (dimethoxycurcumin).}
\]

\(^1\)H NMR: \(\delta 3.89\) and \(\delta 3.90\) (two singlets, –CH\(_3\), 12 H), \(\delta 5.79\) (singlet, enolic 4-H, 1 H), \(\delta 6.47\) (doublet, \(J= 15,8\), 2-H and 6-H, 2 H), \(\delta 6.84\) (doublet, \(J= 8,4\), aromatic H\(_5\), 2 H), \(\delta 7.04\) (doublet, \(J= 1.92\), aromatic H\(_6\), 2H),\(\delta 7.11\) (double doublet, \(J= 3.41\) (\(J=1.88\) and \(J=1.92\) respectively), aromatic H\(_2\), 2 H), \(\delta 7.57\) (doublet, \(J= 15.8\), 1-H and 7-H, 2 H)

Signals are also detected at \(\delta 3.4129\) and \(\delta 2.0944\). These are assumed to be solvents, MeOH from recrystallization and acetone from cleaning of the NMR tube respectively.
RHC-2 (in DMSO):

\[ \text{Figure 4.3: The hydrogen atoms in RHC-2 (curcumin).} \]

\(^1\)H NMR: \( \delta 3.98 \) (singlet, –CH\(_3\), 6 H), \( \delta 6.11 \) (singlet, enolic 4-H, 1H), \( \delta 6.75 \) (doublet, J= 15.8 Hz, 2-H and 6-H, 2 H), \( \delta 6.82 \) (doublet, J= 8.0 Hz, aromatic H\(_6\), 2 H), \( \delta 7.14 \) (doublet, J=8.0 Hz, aromatic H\(_5\), 2 H), \( \delta 7.32 \) (singlet, aromatic H\(_2\), 2 H), \( \delta 7.58-7.62 \) (doublet, J=15.8, 1-H and 7-H, 2 H), \( \delta 9.71 \) (singlet, -OH, 2 H)

RHC-3 (in DMSO):

\[ \text{Figure 4.4: The hydrogen atoms in RHC-3 (bisdemethoxycurcumin).} \]

\(^1\)H NMR: \( \delta 6.05 \) (singlet, enolic 4-H, 2 H), \( \delta 6.70 \) (doublet, J=15.9, 2-H and 6-H, 2 H), \( \delta 6.83 \) (doublet, J=8.6, aromatic H\(_3\) and H\(_5\), 4 H), 7.55 (multiplet, aromatic H\(_2\) and H\(_6\) and 1-H and 7-H, 6 H), \( \delta 10.06 \) (singlet, -OH, 2 H)

A signal is detected at \( \delta 3.37 \) was assumed to be water and signals detected at \( \delta 4.12 \) and \( \delta 3.18 \) to be methanol.
RHC-4 (in DMSO):

\[ \text{H}_3\text{C}-\text{O}_{\text{H}_5}\text{H}_6\text{H}_2\text{H}_4\text{H}_6\text{O}\text{O}_{\text{H}_3\text{H}_5\text{H}_2\text{H}_6\text{H}_2\text{H}_6\text{O}} \]

**Figure 4.5:** The hydrogen atoms in RHC-4 (monomethoxycurcumin)

\(^1\text{H} \text{NMR}: \delta \ 3.76 \text{ (singlet, } -\text{CH}_3, \ 6 \text{ H)}, \delta \ 5.78 \text{ (singlet, enolic } 4-\text{H, 1 H), } \delta \ 6.46 \text{ (doublet, } J= 15.8, \text{ H-2 and H-6, 2 H)}, \delta \ 6.85 \text{ (doublet, } J= 8.8, \text{ aromatic H}_3 \text{ and H}_5, 4 \text{ H), } \delta \ 7.46 \text{ (doublet, } J= 8.8, \text{ aromatic H}_2 \text{ and H}_6, 4 \text{ H), } \delta \ 7.50 \text{ (doublet, } J= 15.9, 1-\text{H and 7-H, 2 H)}

One signal is detected at \( \delta \ 7.59 \), representing 0.5 hydrogen atoms. This signal is assumed to come from the hydroxyl group in the keto-enol moiety.

One signal is also observed at \( \delta \ 3.28 \), which is assumed to be water.

The positions of the aromatic hydrogen atoms were estimated by the use of NMR spectra obtained by ChemDraw Ultra 10.0.

4.2.1.5 UV/Vis analysis

The UV/Vis spectra obtained in ACN gave a major absorption peak at about 420nm. UV spectra of the simple symmetrical curcuminoids are presented in appendix, figure A.7.1-A.7.4.
4.2.2 Analysis of compounds prepared for the curcumin galactoside synthesis

4.2.2.1 Melting point analysis

**Table 4.4:** The melting points of the compounds synthesized in the 3 first steps in the synthesis of curcumin galactoside measured using a Gallenkamp melting point apparatus.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting point (°C)</th>
<th>Previously reported melting point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetobromogalactose</td>
<td>79.5-86.4</td>
<td>74.4-76 [37], 83-84 [75]</td>
</tr>
<tr>
<td>Vanillin galactoside</td>
<td>53.6-73.2 (viscous, not properly melted)</td>
<td>124-125 [36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Softened at 177.4-198.5</td>
</tr>
<tr>
<td>Digalactosyl curcumin octaacetate</td>
<td>135.7-224.4</td>
<td>102-105 [36]</td>
</tr>
<tr>
<td>Curcumin digalactoside</td>
<td>Not obtained</td>
<td>155-159 [36]</td>
</tr>
</tbody>
</table>

4.2.2.2 IR spectroscopy

IR spectra are presented in Appendix, figure A.6.5-A.6.8.

Most of the bands for these compounds are seen in the fingerprint area from 400 cm\(^{-1}\) to 1500 cm\(^{-1}\), and are difficult to interpret. Some useful information can anyway be found in the IR spectra.

All compounds show strong bands at 1750 cm\(^{-1}\), for C=O in acetyl groups 2960–2980 cm\(^{-1}\).

The vanillin galacoside show additional peaks in the area around 1500-1600 cm\(^{-1}\) for the aromatic group of vanillin. A strong band around 1075 cm\(^{-1}\) is assumed to come from the
methoxy group on vanillin. The hydrogen group in vanillin where galactose is supposed to be attached is, as expected, not detected. The band at 1692 cm$^{-1}$ might result from the aldehyde group in vanillin, and this band disappears in the spectrum of curcumin galactoside octaacetate.

In the spectra for curcumin galactoside octaacetate a band is observed at 3438 cm$^{-1}$, which is a typical place for hydroxyl groups to show bands. The peak is not very strong, as would be expected for a hydroxyl group, and it is not known if this is caused by solvent residue or by deprotected hydroxyls on galactose.

4.2.2.3 NMR
NMR spectra for acetobromogalactose and vanillin galactoside are presented in Appendix, figure A.8.5 and A.8.6.

Acetobromogalactose (in CDCl$_3$):
$^1$H NMR: $\delta$ 1.95 (singlet, –CH$_3$ on the acetyl group, 3 H), $\delta$ 1.99 (singlet, –CH$_3$ on the acetyl group, 3 H), $\delta$ 2.05 (singlet, –CH$_3$ on the acetyl group, 3 H), $\delta$ 2.08 (singlet, –CH$_3$ on the acetyl group, 3 H), $\delta$ 4.02-4.14 (multiplet, 2 H), $\delta$ 4.42 (triplet, 1 H), $\delta$ 4.96-5.00 (multiplet, 1 H), $\delta$ 5.32-5.36 (multiplet, 1 H), $\delta$ 5.45-5.46 (double doublet, 1 H), $\delta$ 6.63 (singlet, 1 H)

Vanillin galactoside (in CDCl$_3$):
$^1$H NMR: $\delta$ 1.93-2.15 (multiplet, acetyl groups, 13 H), $\delta$ 3.78 (singlet, -OCH$_3$, $\alpha$-anomer, 0.5 H), $\delta$ 3.83 (singlet, -OCH$_3$, $\beta$-anomer, 3 H), $\delta$ 3.97 (triplet, 1 H), $\delta$ 4.00-4.20 (multiplet, carbohydrate, 3 H), $\delta$ 4.87-5.07 (multiplet, carbohydrate), $\delta$ 5.37-5.51 (multiplet, carbohydrate, 1 H), $\delta$ 7.34-7.37 (multiplet, 2 H), $\delta$ 9.83 (singlet, -CHO, 1 H)

Curcumin digalactoside octaacetate (in CDCl$_3$):
A complex pattern was obtained using $^1$H NMR, and the spectrum is presented in figure 4.6. The results are discussed in section 4.2.3.2
4.2.2.4 UV/VIS-absorption

Curcumin galactosides octaacetate was also analyzed with UV/Vis in ACN, and this compound had no major peak in the area around 420 nm

4.2.3 Purity

4.2.3.1 Purity of the simple symmetrical curcuminoids

RH-C-1: The compound looked pure from NMR and, although the melting point was low, was assumed to be pure. The deviation from references in melting point was assumed to result from a different crystal form being made. This is supported by the fact that the melting point was higher before recrystallization.
4.2.3.2 Purity of the compounds used in synthesis of curcumin galactoside

Step 1:
The acetobromogalactose was considered pure enough for further synthesis. Some minor impurities were seen in the NMR.

Step 2:
Some problems arose in the crystallization and purification of the vanillin galactoside (see section 3.1.2.2). From NMR results (figure 4.7) it seems to be possible that two anomeric forms of the carbohydrate might have been formed under these reaction conditions, resulting in a mixture of
different vanillin galactosides. This can, for instance, be illustrated by looking at the integral of the peaks for the methoxy group on the aromatic ring. The ratio between these peaks is approximately 4.5. This pattern can also be seen in the acetyl region (figure 4.8), but because there are so many peaks absorbing in the same area it is more difficult to interpret the spectrum in this area.

Figure 4.8: Acetyl groups in the region around δ 2 in the NMR spectrum of vanillin galactoside

Bu₄NBr is present in this reaction as a phase transfer reagent. The presence of a tetraalkylammonium halide at the time when the reaction between the carbohydrate halide and the alcohol is conducted can cause interconversion between α- and β-galactosyl bromides, and this is sometimes taken advantage of to make the α-galactosides [40, 41]. In the rapid equilibrium between α- and β-anomer, catalyzed by the halide ion, the β- halide usually has greater reactivity compared to the α-anomer[41]. The the α-anomer is stabilized by the anomeric effect [41]. Generally, displacement of a halogen atom from a β-halide will lead to the formation of a α-galactoside [41] by a reaction mechanism which is different from the mechanism discussed in section 2.2.1.2 for the α-halide [40, 41]. Therefore it is possible to assume that the presence of Bu₄NBr causes some of the acetobromogalactose to be converted to the reactive β-form, which will yield some α-galactoside in this reaction.
One textbook [42] states that this kind of reaction in the presence of silver salts, called Koenigs-Knorr reaction, yield only the \( \beta \)-glycoside. The use of silver oxide and silver carbonate results in the \( \beta \) product, but mercury salts can give either anomer [75]. When mercury-containing compounds are used as catalysts the reaction is often described as being conducted under “Helferich conditions” [41]. Reactions of phenols with \( \sigma \)-substituents on the ring, such as vanillin, with \( \alpha \)-acetobromoglucose is reported to not be successful under classical Koenigs Knorr reaction conditions or in the presence of Brønsted and Lewis acids [76]. From these results it can be assumed that the synthesis of vanillin galactoside under the reaction conditions chosen in the previous work would fail or give a low yield.

Step 3:
In spite of the lack of a pure compound from step 2, it was still attempted to do the next step to make the curcumin galactoside. The resulting product was yellow, a color that is characteristic for the curuminoids. The NMR spectrum of this product also indicates that a curuminoid derivative was present, however very impure. Compared to the NMR spectrum for vanillin galactose, additional signals in the area between \( \delta \) 6 and \( \delta \) 7 can be seen. This corresponds to the conjugated carbon-chain between the aromatic rings in the curuminoid structure. The spectrum also show a singlet at \( \delta \) 5.77, which is assumed to be the enolic hydrogen in curuminoids.

Under alkaline reaction conditions it also seems like there is some deprotection of the acetyl groups protecting the carbohydrate –OH groups. In alkaline medium some of the covalent links between the carbohydrate hydroxyl groups and the protecting

![Figure 4.9: Acetyl groups in the NMR spectrum of curcumin galactoside octaacetate](image-url)
acetyl groups are hydrolysed. That is probably the main reason why there are so many and uneven signals in the area around δ 2.

Step 4:
It did not seem reasonable to continue the synthesis

4.3 HPLC analysis

4.3.1 The HPLC method

**Table 4.5:** Mobile phase for HPLC system used in Iceland, with a Waters Nova-Pak® C<sub>18</sub> HPLC column

<table>
<thead>
<tr>
<th>Compound</th>
<th>% ACN in mobile phase</th>
<th>% buffer in mobile phase</th>
<th>Flow (ml/min)</th>
<th>Retention times (Iceland)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>50</td>
<td>50</td>
<td>1</td>
<td>10.63 min.</td>
</tr>
<tr>
<td>RHC-2</td>
<td>40</td>
<td>60</td>
<td>1</td>
<td>11.5 min.</td>
</tr>
<tr>
<td>RHC-3</td>
<td>40</td>
<td>60</td>
<td>1</td>
<td>10.17 min.</td>
</tr>
<tr>
<td>RHC-4</td>
<td>60</td>
<td>40</td>
<td>1</td>
<td>11.92 min.</td>
</tr>
</tbody>
</table>

**Table 4.6:** Mobile phase for the HPLC system used in Oslo, with a Waters Nova-Pak® C<sub>18</sub> HPLC column

<table>
<thead>
<tr>
<th>Compound</th>
<th>% ACN in mobile phase</th>
<th>% buffer in mobile phase</th>
<th>Flow (ml/min)</th>
<th>Retention times (Oslo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>45</td>
<td>55</td>
<td>1.4</td>
<td>17.4 min.</td>
</tr>
<tr>
<td>RHC-2</td>
<td>40</td>
<td>60</td>
<td>1</td>
<td>10.88 min.</td>
</tr>
<tr>
<td>RHC-3</td>
<td>40</td>
<td>60</td>
<td>1</td>
<td>8.6 min.</td>
</tr>
<tr>
<td>RHC-4</td>
<td>60</td>
<td>40</td>
<td>1</td>
<td>12.2 min.</td>
</tr>
</tbody>
</table>
4.3.2 Validation

Linearity was established in the range between $1.5 \times 10^{-4}$M and $5 \times 10^{-6}$M with a regression coefficient $>0.99$ for all the curves.

Limit of detection (LOD) was not determined accurately for the system used in Iceland, the lower range of the standard curves did denote the lowest concentration that could be measured accurately.

For the system used in Oslo linearity was established between $5 \times 10^{-5}$ M and $10^{-6}$ M with a regression coefficient $>0.999$ for all the curves. LOD was determined to be $10^{-7}$ M for the compounds C-2 to C-4, and $5 \times 10^{-7}$ M for C-1.

4.3.3 Purity of the curcuminoids

4.3.3.1 Simple symmetrical curcuminoids

The curcuminoids RHC-1 to RHC-4 were dissolved in MeOH and analyzed using HPLC. Some extra peaks were observed in all the chromatograms. These were much smaller than the main peak and had shorter retention times. This phenomena has been observed before, and concluded to be some unknown artifact generated in the HPLC system [47]. The possibility of complexation with citrate has been investigated, and it was assumed that this was not the explanation [47]. This was not further investigated in the present work.

For all the compounds, except RHC-4, these peaks were base-line separated from the main peak. For RHC-4 there was however, one small peak that was not fully baseline separated, and it is not known whether this peak is caused by an impurity or the postulated HPLC phenomena for these compounds. No major impurity can be detected from NMR or TLC. A new recrystallisation was not done, mainly due to the initial low yield of compound. The unknown phenomena were mainly causing problems in
quantification of the samples with the HPLC system used in Oslo, for samples with a concentration higher than $10^{-5}$ M.

When phase solubility studies were performed an extra peak also appeared in the chromatogram of RHC-1, which is not seen when the compound is dissolved in MeOH. This was observed in buffers containing HPβCD and MβCD, but not in buffer with HPγCD where the compound is more soluble. It is assumed that the peak is due to some minor impurity, which is present in such a small amount that it is not seen when the compound is dissolved in MeOH or detected in the NMR spectrum. This impurity has probably a better solubility than RHC-1 in the aqueous CD buffers. In buffers where RHC-1 has very low solubility this impurity becomes more important. In the HPLC system used in Oslo this peak was seen for all samples, and as previously discussed the mobile phase was modified to achieve baseline separation between this peak and the main peak.

The impure RHC-5 was also analyzed by HPLC. A peak was detected with retention time 5 minutes and 34 seconds, when a mobile phase consisting of 70% citrate buffer and 30% ACN was used. The compound RHC-5 is a more polar compound than RHC-2 and RHC-3, and a more polar mobile phase like this one is expected to be suitable for RHC-5. The fact that it can be detected at 350 nm also indicates that the curcuminoid might be present, but this could not be proved.

### 4.4 Phase solubility

#### 4.4.1 Experimental conditions

Since a smaller amount of curcuminoid per ml is added compared to previous experiments on similar compounds [47], a preliminary experiment was performed to show that the amount of curcuminoid used in the present work is sufficient and that equilibrium is reached within the system. This was confirmed.
For RHC-4 the sample that had been treated in the ultrasound bath had to be excluded because HPLC analysis indicated substantial degradation.

In a previous study by Tomren [47] it was suggested that the curcuminoid-CD complex might absorb to the filters that is used. The filters used in the present work were therefore checked for absorption by refiltration of samples. The HPLC analysis of the refiltrated samples showed that the compounds do not seem to adsorb to the filters used here.

**4.4.2 Phase solubility for all the curcuminoids in citrate buffer**

**Table 4.7:** Solubility of the curcuminoids in 10% solution of the given CDs, in 0.05M citric acid buffer at pH 5. Ionic strength was 0.15, adjusted with NaCl (n=3, average ± min/max). (Buffer I)

<table>
<thead>
<tr>
<th></th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>3.74x10⁻³M</td>
<td>3.02x10⁻³M</td>
<td>4.41x10⁻⁴M</td>
</tr>
<tr>
<td></td>
<td>(3.68-3.83)</td>
<td>(2.94-3.11)</td>
<td>(4.23-4.59)</td>
</tr>
<tr>
<td></td>
<td>14.8 µg/ml</td>
<td>11.96 µg/ml</td>
<td>174.64 µg/ml</td>
</tr>
<tr>
<td>RHC-2</td>
<td>1.77x10⁻⁴M</td>
<td>1.59x10⁻³M</td>
<td>2.34x10⁻⁵M</td>
</tr>
<tr>
<td></td>
<td>(1.72-1.85)</td>
<td>(1.50-1.65)</td>
<td>(2.15-2.52)</td>
</tr>
<tr>
<td></td>
<td>65.14 µg/ml</td>
<td>58.51 µg/ml</td>
<td>861.12 µg/ml</td>
</tr>
<tr>
<td>RHC-3</td>
<td>1.34x10⁻³M</td>
<td>9.42x10⁻³M</td>
<td>1.96x10⁻³M</td>
</tr>
<tr>
<td></td>
<td>(1.24-1.48)</td>
<td>(9.07-9.61)</td>
<td>(1.83-2.06)</td>
</tr>
<tr>
<td></td>
<td>412.72 µg/ml</td>
<td>290.14 µg/ml</td>
<td>603.68 µg/ml</td>
</tr>
<tr>
<td>RHC-4</td>
<td>1.83x10⁻⁵M</td>
<td>1.47x10⁻³M</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>(1.64-2.12)</td>
<td>(1.44-1.51)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.15 µg/ml</td>
<td>4.94 µg/ml</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.8: Previously reported solubility for curcuminoids in citrate buffered 10% CD solution pH 5, I=0.145 [47].

<table>
<thead>
<tr>
<th>Curcuminoids</th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin * (MTC-4)</td>
<td>2.08x10^{-4}M</td>
<td>1.68x10^{-4}M</td>
<td>3.62x10^{-3}M</td>
</tr>
<tr>
<td>Curcumin (MTC-4)</td>
<td>1.16x10^{-4}M</td>
<td>0.808x10^{-4}M</td>
<td>5.35x10^{-3}M</td>
</tr>
<tr>
<td>Dimetoksy-curcumin</td>
<td>1.51x10^{-5}M</td>
<td>0.818x10^{-5}M</td>
<td>2.24x10^{-3}M</td>
</tr>
<tr>
<td>(MTC-1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisdemetoksyscurcumin</td>
<td>1.22x10^{-3}M</td>
<td>9.63x10^{-4}M</td>
<td>2.39x10^{-3}M</td>
</tr>
<tr>
<td>(MTC-5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The experiment was performed using filters: Spartan 13/0.45RC, Schleicher & Schull.
For the other experiment other filters (Millipore, Millex®, pore size 0.45mm) were used.

The main trends seen by Tomren [47] for a range of curcuminoids is generally the same in the present work. The solubility is higher in HPγCD, except for RHC-4 which has only one methoxy substituent on each aromatic ring and is very lipophilic. This is also consistent with the previous work, where a very lipophilic derivative with no substituents on the ring was found to be insoluble in HPγCD [47]. The solubilities in HPβCD and MβCD are found to be relatively similar, with a slightly lower solubility in MβCD. The difference in solubility in solutions of different CDs is smallest for RHC-3, and larger for the derivatives with more substituents on the aromatic ring. This is also consistent with the work performed by Tomren [47].

Cyclodextrins form complexes with compounds of a suitable size that is compatible with the dimensions of the cavity [56]. The three parent CDs have different internal diameters and are able to accommodate molecules of different size [56]. The steric requirements for an acceptable guest molecule can in addition be impacted by the derivatization of the hydroxyls on the parent CD [56]. In both β-CDs the smaller RHC-3 (bisdemethoxycurcumin) is the most soluble curcuminoid, and in the larger cavity of HPγCD the larger molecule RHC-2 (curcumin) is most soluble. The effect of the size of the curcuminoids in HPβCD, MβCD and HPγCD gave similar results as reported.
previously in [47], and is important in order to explain the differences in solubility of the compounds in the different CD solutions.

The influence of cavity size versus molecular size is not the only factor that can determine the solubility of compounds in CD solutions. The ability to form hydrogen bonds between curcuminoid and CD on solubility in solutions of the CDs is also of great importance [47]. Curcuminoids with hydrogen bonding substituents (figure 3.1) on the aromatic ring have the ability to form hydrogen bonds with hydroxyl groups and substituents on hydroxyl groups in CDs, and also with the incorporated water molecules in the CD cavity. RHC-1 and RHC-4 have methoxy groups which can act as hydrogen bond acceptors, and are not as soluble as RHC-2 and RHC-3. RHC-2 and RHC-3 have phenolic hydroxyl groups with both hydrogen bond donor and acceptor properties, and these compounds have the highest solubility.

A previous experiment using citrate buffer [47], differing values on the solubility of curcumin was obtained. Reaction conditions were similar, apart from the choice of filters. It was suggested that the complex between curcumin and CD adsorbs to the filter [47]. In the present work one type of filters has been used in all the experiments, Spartan 13/0,45RC, Schleicher & Schull. Since a different filter was used for the experiments performed by Tomren [47], and since this was suspected to affect the solubility measurements, this might cause differences between the solubility values obtained in the two studies. The solubilities obtained for curcumin in the present work are somewhat in between the values Tomren [47] found by the use of different filters, except for solubility of curcumin-HPγCD which is generally lower here. As mentioned before, absorption of the complex to the filters was not assumed to be a problem in the present study. It should be kept in mind that CDs from the same batches was used for the two βCD-derivatives, but not for HPγCD as in [47]. Difference in type and origin of the CDs can give some variation in the degree and pattern of substitution. This may account for the lower solubility in HPγCD in the present work. Other explanations for the differences between the present results and the previously reported results are discussed below.
4.4.3 The effect of CD-concentration on phase solubility

Table 4.9: Solubility of the curcuminoids in 10%, 7.5%, 5% and 2.5% concentration of HPβCD, in 0.05M citric acid buffer at pH 5. Ionic strength was 0.15, adjusted with NaCl (n=3, average ± min/max).

<table>
<thead>
<tr>
<th></th>
<th>10% HPβCD</th>
<th>7.5% HPβCD</th>
<th>5% HPβCD</th>
<th>2.5% HPβCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>3.74x10^-5 M</td>
<td>2.03x10^-5 M</td>
<td>1.13x10^-5 M</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>(3.66-3.83)</td>
<td>(1.82-2.15)</td>
<td>(1.08-1.17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.81 μg/ml</td>
<td>8.04 μg/ml</td>
<td>4.47 μg/ml</td>
<td></td>
</tr>
<tr>
<td>RHC-2</td>
<td>1.77x10^-4 M</td>
<td>9.99x10^-5 M</td>
<td>4.41x10^-5 M</td>
<td>1.06x10^-5 M</td>
</tr>
<tr>
<td></td>
<td>(1.72-1.85)</td>
<td>(9.62-10.20)</td>
<td>(4.18-4.64)</td>
<td>(0.98-1.11)</td>
</tr>
<tr>
<td></td>
<td>65.14 μg/ml</td>
<td>36.76 μg/ml</td>
<td>16.23 μg/ml</td>
<td>3.90 μg/ml</td>
</tr>
<tr>
<td>RHC-3</td>
<td>1.34x10^-3 M</td>
<td>6.87x10^-4 M</td>
<td>2.89x10^-4 M</td>
<td>7.15x10^-5 M</td>
</tr>
<tr>
<td></td>
<td>(1.24-1.48)</td>
<td>(6.65-7.04)</td>
<td>(2.84-2.94)</td>
<td>(6.82-7.56)</td>
</tr>
<tr>
<td></td>
<td>412.72 μg/ml</td>
<td>211.60 μg/ml</td>
<td>89.01 μg/ml</td>
<td>22.02 μg/ml</td>
</tr>
<tr>
<td>RHC-4</td>
<td>1.83x10^-5 M</td>
<td>1.07x10^-5 M</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>(1.64-2.12)</td>
<td>(1.04-1.09)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.15 μg/ml</td>
<td>3.60 μg/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.10: Solubility of the curcuminoids in 10%, 7.5%, 5% and 2.5% concentration of HPγCD, in 0.05M citric acid buffer at pH 5. Ionic strength was 0.15, adjusted with NaCl (n=3, average ± min/max).

<table>
<thead>
<tr>
<th></th>
<th>10% HPγCD</th>
<th>7.5% HPγCD</th>
<th>5% HPγCD</th>
<th>2.5% HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>4.41x10^{-3}M</td>
<td>2.32x10^{-3}M</td>
<td>1.64x10^{-3}M</td>
<td>3.94x10^{-5}M</td>
</tr>
<tr>
<td></td>
<td>(4.23-4.59)</td>
<td>(2.02-2.74)</td>
<td>(1.51-1.79)</td>
<td>(2.73-5.20)</td>
</tr>
<tr>
<td></td>
<td>174.64 µg/ml</td>
<td>91.87 µg/ml</td>
<td>64.94 µg/ml</td>
<td>15.60 µg/ml</td>
</tr>
<tr>
<td>RHC-2</td>
<td>2.34x10^{-3}M</td>
<td>1.54x10^{-3}M</td>
<td>7.61x10^{-3}M</td>
<td>2.60x10^{-4}M</td>
</tr>
<tr>
<td></td>
<td>(2.15-2.36)</td>
<td>(1.51-1.59)</td>
<td>(7.31-7.84)</td>
<td>(2.56-2.63)</td>
</tr>
<tr>
<td></td>
<td>861.12 µg/ml</td>
<td>566.72 µg/ml</td>
<td>280.05 µg/ml</td>
<td>95.68 µg/ml</td>
</tr>
<tr>
<td>RHC-3</td>
<td>1.96x10^{-3}M</td>
<td>1.12x10^{-3}M</td>
<td>4.06x10^{-3}M</td>
<td>8.00x10^{-5}M</td>
</tr>
<tr>
<td></td>
<td>(1.83-2.06)</td>
<td>(1.10-1.17)</td>
<td>(3.93-4.23)</td>
<td>(7.82-8.10)</td>
</tr>
<tr>
<td></td>
<td>603.68 µg/ml</td>
<td>344.96 µg/ml</td>
<td>125.05 µg/ml</td>
<td>24.64 µg/ml</td>
</tr>
<tr>
<td>RHC-4</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
</tbody>
</table>

The obtained saturation concentration of the curcuminoids (M) was plotted as a function of the concentration of the CDs (%). Representative curves are presented below, in figure 4.10-4.15.
Figure 4.10: The saturation concentration of RHC-1 plotted as a function of HPβCD concentration

Figure 4.11: The saturation concentration of RHC-1 plotted as a function of HPγCD concentration
Figure 4.12: The saturation concentration of RHC-2 plotted as a function of HPβCD concentration

Figure 4.13: The saturation concentration of RHC-2 plotted as a function of HPγCD concentration
**Figure 4.14:** The saturation concentration of RHC-3 plotted as a function of HPβCD concentration

**Figure 4.15:** The saturation concentration of RHC-3 plotted as a function of HPγCD concentration
4.4.4 Stoichiometry of the curcuminoid-cyclodextrin complexes

As discussed in section 2.5.1.1 the reports on the stoichiometry of the curcumin-CD complex are somewhat contradictory. It is therefore of great interest to get some information on this matter for the different curcuminoids. The monomethoxyderivative did however not give any result, due to low solubility at lower concentrations of βCD and in HP-γ-CD generally.

Phase solubility diagrams for 1:1 complexation are linear, as discussed in section 2.3.3. The curves obtained here (figure 4.10-4.15) do not give a good linear fit and has a rather concave-upward curvature. A concave-upward curvature is characteristic for systems with a substrate S (the curcuminoid) and a ligand L (the cyclodextrin) $S_mL_n$ where $n>1$ [57]. This indicates that the curcuminoid:CD complexes are of higher order.

From the structures of the curcuminoids, with two aromatic rings, it is reasonable to suspect that 1:2 drug-CD complexes might have been formed.

To determine the binding constant for this system is problematic, because the solubility of the curcuminoids in water without CD is very low. Previously it has been shown that the solubility of curcumin is $< 3 \times 10^{-8}$ M [2]. By estimating $S_0$ to be $3 \times 10^{-8}$ M, a plot of $(S_t-S_0)/L_t$ against $L_t$ was obtained (see section 2.3.2.1), but this was not linear either. To get a more reliable investigation on the possible 1:2 stoichiometry the data should be plotted into a computer program designed to do this kind of calculations.

4.4.4.1 Suitability of the method

Phase solubility diagrams are often used to decide the stoichiometry and stability constant of CD complexes, but it has been argued that this method may not be appropriate [67, 77-79]. When using this method it is important to keep in mind that the method does not indicate whether an inclusion complex is formed between the drug and the CD, but only
how the CD influences the solubility of the drug [67]. The phase solubility method used on drug-CD interactions ignores the possibility of the complexes to interact with each other [79]. CDs are known to self-associate and form non-inclusion complexes with various types of compounds, and CD complexes also self-associate and form water-soluble aggregates or micelles [77, 79]. Formation of multiple inclusion or non-inclusion complexes frequently contribute to the solubilizing effects of CDs [67, 78]. This can complicate investigations performed in order to determine stoichiometry of the complexes [77].

4.4.5 Experiments performed to determine the influence of ionic strength on the phase solubility experiments

The solubility of curcumin has previously been measured, with inconsistent results [2, 47]. Different buffers have been used, and the influence of type of buffer that was used was therefore investigated. It was concluded that other factors must also influence the solubility [47]. Different results have also been obtained using the same kind of buffer, phosphate buffer. In the present work the object was to investigate the importance of ionic strength on the solubility measurements, and to which extent the measurements are influenced by type of salt that is used to adjust the ionic strength.
Table 4.11: The solubility of dimethoxycurcumin (RHC-1) in 10% CD solutions in different 0.05 M phosphate buffers, pH 5 (n=3, average ± min/max).

<table>
<thead>
<tr>
<th>Buffer system III</th>
<th>HPβCD (μM)</th>
<th>MβCD (μM)</th>
<th>HPγCD (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μ=0.15, NaCl)</td>
<td>4.61x10⁻⁵</td>
<td>3.96x10⁻⁵</td>
<td>9.74x10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>(4.38-4.92)</td>
<td>(3.72-4.10)</td>
<td>(5.98-11.74)</td>
</tr>
<tr>
<td>18.26 μg/ml</td>
<td>15.68 μg/ml</td>
<td>385.70 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Buffer system IV</td>
<td>4.45x10⁻⁵</td>
<td>3.89x10⁻⁵</td>
<td>1.09x10⁻³</td>
</tr>
<tr>
<td>(μ=0.085, NaCl)</td>
<td>(4.38-4.53)</td>
<td>(3.82-3.96)</td>
<td>(0.82-1.27)</td>
</tr>
<tr>
<td>17.62 μg/ml</td>
<td>15.40 μg/ml</td>
<td>431.64 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Buffer system V</td>
<td>3.95x10⁻⁵</td>
<td>3.22x10⁻⁵</td>
<td>8.75x10⁻⁴</td>
</tr>
<tr>
<td>(μ=0.15, KCl)</td>
<td>(3.94-3.97)</td>
<td>(2.93-3.44)</td>
<td>(8.10-9.81)</td>
</tr>
<tr>
<td>15.64 μg/ml</td>
<td>12.75 μg/ml</td>
<td>346.50 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Buffer system VI</td>
<td>3.94x10⁻⁵</td>
<td>3.29x10⁻⁵</td>
<td>7.78x10⁻⁴</td>
</tr>
<tr>
<td>(μ=0.085, KCl)</td>
<td>(3.77-4.05)</td>
<td>(3.12-3.41)</td>
<td>(6.40-8.75)</td>
</tr>
<tr>
<td>15.60 μg/ml</td>
<td>12.03 μg/ml</td>
<td>308.09 μg/ml</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.12: The solubility of curcumin (RHC-2) in 10% CD solutions in different 0.05 M phosphate buffers, pH 5 (n=3, average ± min/max).

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>III (μ=0.15, NaCl)</td>
<td>2.22x10^-4 M (2.19-2.27)</td>
<td>1.98x10^-4 M (1.85-2.09)</td>
<td>2.70x10^-3 M (2.56-2.79)</td>
</tr>
<tr>
<td></td>
<td>81.70 μg/ml</td>
<td>72.86 μg/ml</td>
<td>993.6 μg/ml</td>
</tr>
<tr>
<td>IV (μ=0.085, NaCl)</td>
<td>2.11x10^-4 M (2.03-2.16)</td>
<td>2.17x10^-4 M (2.07-3.30)</td>
<td>2.47x10^-3 M (2.31-2.71)</td>
</tr>
<tr>
<td></td>
<td>77.65 μg/ml</td>
<td>79.86 μg/ml</td>
<td>908.96 μg/ml</td>
</tr>
<tr>
<td>V (μ=0.15, KCl)</td>
<td>1.88x10^-4 M (1.79-1.99)</td>
<td>1.88x10^-4 M (1.83-1.98)</td>
<td>2.43x10^-3 M (2.29-2.57)</td>
</tr>
<tr>
<td></td>
<td>69.18 μg/ml</td>
<td>69.18 μg/ml</td>
<td>894.24 μg/ml</td>
</tr>
<tr>
<td>VI (μ=0.085, KCl)</td>
<td>2.01x10^-4 M (1.93-2.09)</td>
<td>1.86x10^-4 M (1.79-1.92)</td>
<td>2.35x10^-3 M (2.06-2.38)</td>
</tr>
<tr>
<td></td>
<td>73.97 μg/ml</td>
<td>68.45 μg/ml</td>
<td>864.80 μg/ml</td>
</tr>
</tbody>
</table>

Table 4.13: Previously obtained solubility results for curcumin in phosphate buffered CD solutions

<table>
<thead>
<tr>
<th>Reference</th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>[47]</td>
<td>2.69x10^-4 M</td>
<td>2.38x10^-4 M</td>
<td>3.74x10^-3 M</td>
</tr>
<tr>
<td>(phosphate buffer pH 5, 10% CD, μ=0.13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2]</td>
<td>1.22x10^-4 M</td>
<td>8.10x10^-4 M (RMβCD)</td>
<td>3.82x10^-4 M</td>
</tr>
<tr>
<td>(phosphate buffer pH 5, 11% CD, μ=0.085)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results obtained in the four different buffers in the present work are quite similar, and for HPβCD and MβCD it can not be seen any systematic influence of the ionic strength.
on the solubilities obtained. For HPγCD the average solubilities are generally lower where the ionic strength is lowered, but the measured concentration of curcuminoid in these solutions is within the same range. It is therefore concluded that the ionic strength does not seem to affect the solubility to a considerable extent.

The trends in solubility in the different CDs seen in citrate buffer (section 4.4.2) are reproduced here, with higher solubility in HPγCD and relatively similar solubility in HPβCD and MβCD. This is consistent with the solubilities obtained by Tomren [47], although the solubilities in the present study is somewhat lower. The solubilities obtained by Tønnesen et al. [2] are however quite different. The big difference in solubility in MβCD and RMβCD is most likely explained by the fact that this is not the same kind of CD derivative, although other factors may also have influenced the solubility. The solubility of curcumin in HPγCD in phosphate buffer is quite high in the present study compared to what is reported in [2]. The difference in solubility in a little lower in citrate buffer than in the phosphate buffer, which is consistent with results reported by Tomren [47].

In addition the dimethoxy derivative of curcumin, RHC-1, was included in this study. This derivative has not been investigated in phosphate buffer before. Also from these results we can conclude that ionic strength does not seem to affect the solubility considerably.

4.4.6 The effect of adding MgCl₂

For a sparingly soluble quinolone antimicrobial agent under development it has been found that the use of Mg²⁺ in the combination with HP-β-CD gave a remarkable increase in solubility [80]. The interaction of the metal ion is believed to be with the keto-carboxylic acid of the quinolone[80]. Curcumin has not such a group, but it is possible that the keto-enol group might have the ability to interact with Mg²⁺. In a docking study where the binding mode of curcumin to HIV integrase was computed, the keto-enol group of curcumin was found to form co-ordinate bonds to Mg²⁺ ion at the active site
It has also been shown that addition of a small amount of various additives, including metal ions, can increase the CD solubilization of drugs in aqueous media [79]. This enhancement is thought to come from formation of non-inclusion complexes of the additives with CDs and their complexes [79]. Magnesium ions have been shown to form such non-inclusion complexes with HPβCD and RMcβCD, and to enhance the solubility of other compounds in aqueous solutions of CD and PVP [79].

Magnesium is an essential body cation that is involved in numerous enzymatic reactions and physiological processes in the body [21]. A normal concentration of magnesium in plasma is from about 0.7 to 1.0 mmol/litre [21]. From this point of view it would be interesting to examine if Mg$^{2+}$ can increase the solubility of curcuminoids.

The solubility of the curcuminoid-CD complex was measured in a citrate buffer where a small amount of MgCl$_2$ had been added, and compared to buffers containing NaCl to find out if MgCl$_2$ increased the solubility.

Table 4.14: Solubility of the curcuminoids in 10% solution of the given CDs, in 0.05M citric acid buffer at pH 5. Ionic strength was 0.157 (MgCl$_2$) (n=3, average ± min/max).

<table>
<thead>
<tr>
<th></th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>3.36x10$^{-5}$M (2.93-3.71) 13.31 µg/ml</td>
<td>3.17x10$^{-5}$M (2.85-3.43) 12.55 µg/ml</td>
<td>4.88x10$^{-4}$M (4.01-5.94) 193.25 µg/ml</td>
</tr>
<tr>
<td>RHC-2</td>
<td>1.81x10$^{-4}$M (1.80-1.81) 66.61 µg/ml</td>
<td>1.67x10$^{-4}$M (1.62-1.72) 61.46 µg/ml</td>
<td>2.76x10$^{-3}$M (2.72-2.79) 1015.68 µg/ml</td>
</tr>
<tr>
<td>RHC-3</td>
<td>1.21x10$^{-3}$M (1.18-1.24) 372.68 µg/ml</td>
<td>9.15x10$^{-4}$M (8.78-9.33) 281.82 µg/ml</td>
<td>2.20x10$^{-3}$M (2.14-3.31) 677.60 µg/ml</td>
</tr>
<tr>
<td>RHC-4</td>
<td>1.80x10$^{-5}$M (1.65-1.88) 6.05 µg/ml</td>
<td>1.56x10$^{-5}$M (1.44-1.64) 5.24 µg/ml</td>
<td>&lt;LOQ</td>
</tr>
</tbody>
</table>
The buffer where MgCl₂ was added had an ionic strength of 0.157. The buffer was the same as in section 4.4.2, except for the ionic strength and the presence of Mg²⁺. Only a small amount of MgCl₂ was needed to adjust the ionic strength to 0.157, and the final concentration of MgCl₂ was 9.32x10⁻³M in the buffer. The curcuminoids solubility obtained in the two buffers is very similar, and addition of MgCl₂ does not seem to affect the solubility to a considerable extent. For curcumin the solubility is slightly higher in all three CDs, but the difference is so small that nothing can be concluded from this. This is not observed for the other curcuminoids.

The ionic strength is slightly different in the two buffers used here, but this is not expected to affect the solubility to a great extent. (Refer to section 4.4.5 for discussion on the effect of ionic strength).

Since such a small amount of the magnesiumsalt was used, it was also performed a minor experiment to decide if adding a larger amount of the magnesiumsalt would give a better effect on solubility.

In a similar buffer with 10% HPβCD the concentration of MgCl₂ was increased to 0.119 M. The ionic strength in this buffer was 0.5.

**Table 4.15:** Solubility of the curcuminoids in 10% solution of CD, in 0.05 M citric acid buffer at pH 5. The ionic strength was 0.5 (MgCl₂) (n=3, average ± min/max).

<table>
<thead>
<tr>
<th>HPβCD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>2.69x10⁻³M</td>
</tr>
<tr>
<td></td>
<td>(2.59-2.88)</td>
</tr>
<tr>
<td></td>
<td>10.65μg/ml</td>
</tr>
<tr>
<td>RHC-2</td>
<td>1.90x10⁻⁴M</td>
</tr>
<tr>
<td></td>
<td>(1.79-2.01)</td>
</tr>
<tr>
<td></td>
<td>69.92 μg/ml</td>
</tr>
</tbody>
</table>
Even with this high concentration of MgCl₂, no increased solubility could be observed. The conclusion is therefore that adding MgCl₂ to the curcuminoid-HPβCD complex does not improve solubility.

When HPγCD is used there is however a small increase in solubility for all the curcuminoids, except C-4 which is not soluble in this CD. This was further investigated by adding a significant amount of the magnesium salt to a buffer containing 10% HPγCD, in the same way as for HPβCD.

**Table 4.16:** Solubility of the curcuminoids in 10% solution of HPγCD, in 0.05 M citric acid buffer at pH 5. The ionic strength was adjusted with MgCl₂, to 0.15 and 0.5 respectively (n=3, average ± min/max).

<table>
<thead>
<tr>
<th></th>
<th>HPγCD, I=0.15</th>
<th>HPγCD, I=0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>6.19x10⁻⁴M (5.79-6.76)</td>
<td>5.91x10⁻⁴M (5.66-6.16)</td>
</tr>
<tr>
<td></td>
<td>245.12 μg/ml</td>
<td>234.04 μg/ml</td>
</tr>
<tr>
<td>RHC-2</td>
<td>2.58x10⁻³M (2.55-2.61)</td>
<td>2.58x10⁻³M (2.55-2.62)</td>
</tr>
<tr>
<td></td>
<td>949.44 μg/ml</td>
<td>949.44 μg/ml</td>
</tr>
</tbody>
</table>

Adding a larger amount of MgCl₂ does not improve the solubility of the HPγCD-curcuminoid complex, and it can therefore finally be concluded that Mg²⁺ does not increase the solubility of curcuminoids in these CDs.

**4.4.7 Experiments performed to determine the influence of pH on the phase solubility experiments**

So far in this work, the pH of the buffers has not been adjusted after correction of ionic strength and addition of CDs, in order to keep ionic strength constant. pH was, however, measured in the buffers and there was some deviation from the theoretical value pH 5.
Therefore it was interesting to investigate if this deviation has an influence on the phase solubility measurements.

The final pH values in all the buffers are presented in appendix table A.5.1.

**Table 4.17:** The solubility of dimethoxycurcumin (RHC-1) in 10% solution of different CD in different 0.05 M citric acid buffers. Ionic strength was 0.169M (n=3, average ± min/max).

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.5</td>
<td>2.10x10⁻⁵M*</td>
<td>1.99x10⁻⁵M*</td>
<td>6.82x10⁻⁴M</td>
</tr>
<tr>
<td></td>
<td>(1.99-2.20)</td>
<td>(1.91-2.06)</td>
<td>(6.35-7.35)</td>
</tr>
<tr>
<td></td>
<td>8.32 µg/ml</td>
<td>7.88 µg/ml</td>
<td>270.07 µg/ml</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>2.49x10⁻⁵M</td>
<td>2.17x10⁻⁵M</td>
<td>5.79x10⁻⁴M</td>
</tr>
<tr>
<td></td>
<td>(2.45-2.54)</td>
<td>(2.11-2.26)</td>
<td>(5.69-5.84)</td>
</tr>
<tr>
<td></td>
<td>9.86 µg/ml</td>
<td>8.59 µg/ml</td>
<td>229.28 µg/ml</td>
</tr>
</tbody>
</table>

* only 2 samples

**Figure 4.16:** The average solubility of RHC-1 in CD solutions in two different 0.05 M citric acid buffers
Table 4.18: The solubility of curcumin (RHC-2) in 10% solution of different CD in different 0.05 M citric acid buffers. Ionic strength was 0.169M (n=3, average ± min/max).

<table>
<thead>
<tr>
<th>Buffer system VII</th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.5</td>
<td>1.78x10⁻⁴M</td>
<td>1.55x10⁻⁴M</td>
<td>2.01x10⁻³M</td>
</tr>
<tr>
<td></td>
<td>(1.73-1.83)</td>
<td>(1.50-1.60)</td>
<td>(1.95-2.11)</td>
</tr>
<tr>
<td></td>
<td>65.50 μg/ml</td>
<td>57.04 μg/ml</td>
<td>739.68 μg/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer system VIII</th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.5</td>
<td>1.83x10⁻⁴M</td>
<td>1.83x10⁻⁴M</td>
<td>2.13x10⁻³M</td>
</tr>
<tr>
<td></td>
<td>(1.79-1.86)</td>
<td>(1.82-1.84)</td>
<td>(1.94-2.26)</td>
</tr>
<tr>
<td></td>
<td>67.34 μg/ml</td>
<td>67.34 μg/ml</td>
<td>783.84 μg/ml</td>
</tr>
</tbody>
</table>

Figure 4.17: The average solubility of curcumin in the different CD solutions in two different 0.05 M citric acid buffers

There seems like there might be a slightly higher solubility when the pH value increases. The results for C-1 in HP-γ-CD do however go in the opposite direction. In order to establish if there is a real difference the solubility was further investigated at pH 4 and 6.
Table 4.19: The solubility of dimethoxycurcumin (RHC-1) in 10% solution of different CD in different 0.05 M citric acid buffers. Ionic strength was 0.199M (n=3, average ± min/max).

<table>
<thead>
<tr>
<th></th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer system XII</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 4</td>
<td>2.56x10⁻⁵ M</td>
<td>2.04x10⁻⁵ M</td>
<td>6.81x10⁻⁴ M</td>
</tr>
<tr>
<td></td>
<td>(2.49-2.57)</td>
<td>(1.99-2.10)</td>
<td>(6.42-7.41)</td>
</tr>
<tr>
<td></td>
<td>10.14 µg/ml</td>
<td>8.08 µg/ml</td>
<td>269.68 µg/ml</td>
</tr>
<tr>
<td><strong>Buffer system XIII</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6</td>
<td>3.44x10⁻⁵ M</td>
<td>2.79x10⁻⁵ M</td>
<td>8.21x10⁻⁴ M</td>
</tr>
<tr>
<td></td>
<td>(3.39-3.52)</td>
<td>(2.75-2.83)</td>
<td>(8.01-8.60)</td>
</tr>
<tr>
<td></td>
<td>13.62 µg/ml</td>
<td>11.05 µg/ml</td>
<td>325.12 µg/ml</td>
</tr>
</tbody>
</table>

Figure 4.18: The average solubility of RHC-1 in 10% CD solutions in two different 0.05 M citric acid buffers (n=3, average ± min/max).
Table 4.20: The solubility of curcumin (RHC-2) in 10% solution of different CD in different 0.05 M citric acid buffers. Ionic strength was 0.199M (n=3, average ± min/max).

<table>
<thead>
<tr>
<th></th>
<th>HPβCD</th>
<th>MβCD</th>
<th>HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer system XII</td>
<td>pH 4</td>
<td>pH 6</td>
<td>pH 4 (C-2)</td>
</tr>
<tr>
<td>pH 4</td>
<td>1.74x10^-4 M (1.66-1.81)</td>
<td>1.45x10^-4 M (1.43-1.47)</td>
<td>2.71x10^-3 M (2.66-2.78)</td>
</tr>
<tr>
<td>64.03 μg/ml</td>
<td>53.36 μg/ml</td>
<td>997.28 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Buffer system XIII</td>
<td>pH 6</td>
<td>pH 6</td>
<td>pH 4 (C-2)</td>
</tr>
<tr>
<td>pH 6</td>
<td>2.15x10^-4 M (2.11-2.21)</td>
<td>1.99x10^-4 M (1.96-2.02)</td>
<td>2.68x10^-3 M (2.61-2.79)</td>
</tr>
<tr>
<td>79.12 μg/ml</td>
<td>73.23 μg/ml</td>
<td>986.24 μg/ml</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.19: The average solubility of curcumin in different CD solutions in different 0.05 M citric acid buffers (n=3, average ± min/max).

The solubility at pH 4 is not dramatically different from the values obtained at approximately pH 5 previously in this experiment. There seems however to be a small consistent increase in solubility at pH 6 for both the β-CDs, but no considerable difference in HPγCD for RHC-2.
4.5 Differential scanning calorimetry

In order to determine the accurate melting point of the curcuminoids and to investigate the possible different crystal forms, the curcuminoids was analyzed by differential scanning calorimetry (DSC). The onset temperature is taken as the melting point of the compound [60].

Table 4.21: The M\textsubscript{onset} and peak values obtained from DSC thermograms of the curcuminoids (n=2, min-max value).

<table>
<thead>
<tr>
<th></th>
<th>M\textsubscript{onset} (°C)</th>
<th>Peak (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>111.63-111.99</td>
<td>116.99-117.81</td>
</tr>
<tr>
<td></td>
<td>131.27-131.60</td>
<td>133.10-133.60</td>
</tr>
<tr>
<td>RHC-2</td>
<td>183.22-184.07</td>
<td>184.68-185.58</td>
</tr>
<tr>
<td>RHC-3</td>
<td>68.62-68.67</td>
<td>75.00-77.49</td>
</tr>
<tr>
<td></td>
<td>229.97-230.06</td>
<td>233.26-233.79</td>
</tr>
<tr>
<td>RHC-4</td>
<td>158.15-158.20</td>
<td>161.76-161.89</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>M\textsubscript{onset} (°C)</th>
<th>Peak (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin, Hanne Hjorth Tønnesen*</td>
<td>179.59-179.71</td>
<td>181.42-181.73</td>
</tr>
<tr>
<td>Curcumin (MTC-4), Marianne Tomren*</td>
<td>181.55-182.35</td>
<td>184.01-184.33</td>
</tr>
<tr>
<td>Dimethoxycurcumin (MTC-1), Marianne Tomren</td>
<td>131.32-131.50</td>
<td>132.87-132.92</td>
</tr>
<tr>
<td>Bisdemethoxycurcumin (MTC-5), Marianne Tomren</td>
<td>224.82-226.92</td>
<td>230.36-231.92</td>
</tr>
</tbody>
</table>

* previously analyzed by DSC at the Department of Pharmaceutics, University of Oslo (unpublished results)
4.5.1 Purity and solvates of the compounds

For RHC-1 two peaks were observed in the thermogram. It was suspected that methanol might be incorporated in the crystals, since MeOH was also seen in the NMR spectrum. It was therefore possible that the two peaks originate from the melting of the solvate followed by recrystallization into the anhydrous form [60].

This was further investigated by heating up to 130°C, which is just past the first peak in figure 4.20, and then cooling down to start temperature at 50°C again. When the sample was heated a second time, this time up to 160°C, no extra peak appeared at 112°C ($t_{onset}$). This indicates that the MeOH was not present anymore, and it was just the more stable form of RHC-1 left.

![Figure 4.20: DSC thermogram of the recrystallization of the postulated RHC-1 methanol-solvate](image)

RHC-3 had one extra peak at approximately 68°C. Also for this compound MeOH was seen in the NMR spectra. Boiling point for MeOH is reported to be 64.7°C [82]. It is
therefore assumed that this peak results from residue MeOH in the sample, but a solvate with MeOH is not formed. This is also seen in bisdemethoxycurumin synthesized by Tomren. In the previous work the peak is broader, and might come from more solvent residues than just MeOH. Another possible solvent from recrystallization is EtOAc, which has a boiling point at 77°C [82].

No extra peaks were seen for RHC-2 (curcumin) and RHC-4, and it is concluded that these two compounds do not have any impurities or solvates with melting points in the analyzed temperature interval.

4.5.2 Influence of crystal form on the solubility

Comparing the results obtained in the present work with previous results is a bit difficult, due to the inconsistency in experimental conditions and filters used. From the investigations so far it seems that choice of buffer salt, choice of filters and pH might influence the solubility values obtained. Ionic strength did not seem to be of major importance and pH was kept at pH 5, so these parameters can be neglected when comparing solubilities. The use of CD from different batches and producers can also cause differences in solubility. The influence of varying experimental conditions are not always very big, but make it difficult to use these solubilities to determine the correlation between solubility and crystal form, represented by different melting points.
Table 2.23: Solubilities obtained in citrate buffer pH 5, in the present study and previously reported [47].

<table>
<thead>
<tr>
<th></th>
<th>Present results (Spartan filters)</th>
<th>Previous results (other filters)</th>
<th>Previous results (Spartan filters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>HPβCD 3.74x10⁻⁵M</td>
<td>1.51x10⁻⁵M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MβCD 3.02x10⁻⁵M</td>
<td>8.18x10⁻⁶M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPγCD 4.41x10⁻⁴M</td>
<td>2.24x10⁻³M</td>
<td></td>
</tr>
<tr>
<td>RHC-2</td>
<td>HPβCD 1.77x10⁻⁴M</td>
<td>1.16x10⁻⁴M</td>
<td>2.08x10⁻⁴M</td>
</tr>
<tr>
<td></td>
<td>MβCD 1.59x10⁻⁴M</td>
<td>8.08x10⁻⁵M</td>
<td>1.68x10⁻⁴M</td>
</tr>
<tr>
<td></td>
<td>HPγCD 2.34x10⁻³M</td>
<td>5.35x10⁻³M</td>
<td>3.62x10⁻³M</td>
</tr>
<tr>
<td>RHC-3</td>
<td>HPβCD 1.34x10⁻⁴M</td>
<td>1.22x10⁻³M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MβCD 9.42x10⁻⁴M</td>
<td>9.63x10⁻⁵M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPγCD 1.96x10⁻³M</td>
<td>2.39x10⁻³M</td>
<td></td>
</tr>
<tr>
<td>RHC-4</td>
<td>HPβCD 1.83x10⁻⁵M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MβCD 1.47x10⁻⁵M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPγCD &lt; LOD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.21: The solubility of dimethoxycurcumin in citrate buffer pH 5, different filters (n=3, average ± min/max).
For dimethoxycurcumin (RHC-1) better solubility is observed in HPβCD and MβCD in 1% citrate buffer pH 5 (section 4.4.2), compared to results by Tomren [47]. The same conditions were used as in the study by Tomren [47], with similar buffer and CDs from the same batches. The observed solubility is better in the present work with the methanol solvate form of dimethoxycurcumin (RHC-1). A solvate formed from a non-aqueous solvent which is miscible with water, such as MeOH, is known to have an increased apparent solubility in water [53]. This might explain why the solubilities obtained for dimethoxycurcumin (RHC-1) are higher in the present work. The reason is that the activity of water is decreased from the free energy of solution of the solvent into the water [53].

![Curcumin in citrate buffer pH 5](image)

**Figure 4.22:** The solubility of curcumin in HPβCD, MβCD and HPγCD in citrate buffer pH 5, filtrated with Spartan filters (n=3, average ± min/max).

Phase solubility was examined for curcumin in citrate buffer pH 5, with the only difference being ionic strength. The same kind of filters was used. If melting points, representing different crystal forms, were to correlate to the solubility one would expect solubility to be decreasing with higher melting point. This is exactly what is seen. The
melting point is higher for the curcumin synthesized in the present work, and solubility is lower in all CDs.

4.6 Photochemical stability

Ideally the sample concentrations should be kept low enough to give absorbance < 0.4 over the irradiation wavelength interval, to be sure that first order kinetics apply [58] (see section 2.3.2.2). The maximum absorbance for the samples in this study is about 0.6 or lower in the samples before irradiation. This was considered sufficient to apply first order kinetics, and linear curves with regression coefficient of ≥ 0.98 were obtained. Before an unequivocal determination of the order can be made, the degradation reaction must be taken to at least 50% conversion [58]. The samples were irradiated for totally 20 minutes, and as we can see from the obtained half-lives, most of the reactions actually were brought to approximately or more than 50% conversion. For all the samples where more than 50% degradation occur neither zero-order nor 2.-order kinetics fit.

The stability in HPγCD was very low for C-1 and C-3, and UV/Vis absorption scans showed that all of the curcuminoid was degraded within 5 minutes. The samples were analyzed by HPLC, but the exact half-life could not be determined. The HPLC chromatograms did not look the “normal” chromatograms for these compounds, and are presented in appendix (A.12), together with UV/Vis absorption scan spectra (A.11).

Table 4.24: Photochemical stability of the curcuminoids, reported as half-life (minutes) when exposed to irradiation at 1170x100 Lux (visible) and 13.7 W/m² (UV).

<table>
<thead>
<tr>
<th></th>
<th>EtOH</th>
<th>EtOH + buffer</th>
<th>10% HPβCD</th>
<th>10% HPγCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>20,87</td>
<td>8,57</td>
<td>17,11</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>RHC-2</td>
<td>66,63</td>
<td>28,88</td>
<td>16,31</td>
<td>31,08</td>
</tr>
<tr>
<td>RHC-3</td>
<td>17,95</td>
<td>9,75</td>
<td>5,01</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>RHC-4</td>
<td>13,70</td>
<td>3,66</td>
<td>Not performed</td>
<td>Not performed</td>
</tr>
</tbody>
</table>
It is often neglected in photochemical studies to correct for the number of photons absorbed by the compound in the actual medium [83]. The number of molecules available for light abruption is essential in the study of photochemical responses [83]. The area under the curve (AUC) in the UV spectra was used as a measure on how many molecules are available for conversion and an approximate normalization has been performed (see experimental) to account for the different AUCs.

**Table 4.25:** Photochemical stability of the curcuminoids, reported as normalized values of half-life (minutes) when exposed to irradiation at 1.17x10^5 lux (visible) and 13.7 W/m^2 (UV). (Half-life, \( \frac{AUC_{std}}{AUC_{sample}} \))

<table>
<thead>
<tr>
<th></th>
<th>EtOH</th>
<th>EtOH + buffer</th>
<th>10% HP(\beta)CD</th>
<th>10% HP(\gamma)CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHC-1</td>
<td>27.34</td>
<td>10.37</td>
<td>20.87</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td>(1.31)</td>
<td>(1.21)</td>
<td>(1.22)</td>
<td></td>
</tr>
<tr>
<td>RHC-2</td>
<td>66.63</td>
<td>31.77</td>
<td>17.13</td>
<td>34.81</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(1.10)</td>
<td>(1.05)</td>
<td>(1.12)</td>
</tr>
<tr>
<td>RHC-3</td>
<td>23.69</td>
<td>13.26</td>
<td>6.26</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td>(1.32)</td>
<td>(1.36)</td>
<td>(1.25)</td>
<td></td>
</tr>
<tr>
<td>RHC-4</td>
<td>18.22</td>
<td>5.67</td>
<td>Not performed</td>
<td>Not performed</td>
</tr>
<tr>
<td></td>
<td>(1.33)</td>
<td>(1.55)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Normalization of the results gave the same trends, but the values for half-lives for the different compounds in different solvent systems are more even.

**Table 4.27:** Previously reported results for the half-life of curcuminoids [2], t_{1/2} (min) when exposed to irradiation at 1.4x10^5 lux (visible) and 18.6 W/m^2 (UV).

<table>
<thead>
<tr>
<th>MeOH</th>
<th>EtOH + phosphate buffer pH 5</th>
<th>5% HP(\beta)CD</th>
<th>5% HP(\gamma)CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>133,3</td>
<td>70,7</td>
<td>28,9</td>
</tr>
</tbody>
</table>
The polarity of the internal cavity in $10^{-2}$ M aqueous solution of $\beta$-CD has been estimated to be identical to the polarity of a 40% EtOH / water mixture [63]. This will not be exactly similar to the polarities of the 10% aqueous solutions of the CD derivatives used in this study, but represents an approximation.

For curcumin mostly the same trends are seen as in a previously performed study by Tønnesen et. al. [2]. Curcumin is more stable in the pure organic solvent and less stable in the 40:60 mixture of ethanol and buffer at pH 5. In CD solution curcumin is more stable in HP$\gamma$CD solution than HP$\beta$CD solution. In the previous study [2] the stability was found to be much better in ethanol/buffer mixture than in the solution of HP$\gamma$CD, but in the present work the stability is in fact slightly better in the HP$\gamma$CD solution. Previously phosphate buffer was employed instead of citrate buffer, and the CD concentration was held at 5%. For all the curcuminoids investigated in the present work the stability was found to be better in pure ethanol than in the mixture with buffer.

Tomren [47] investigated the photochemical stability in organic solvent, MeOH, in a 40:60 mixture of citrate buffer and MeOH and in 10% solution of HP$\beta$CD for a selection of curcuminoids. Because the organic solvent and the composition of this mixture was different from the solvents used in the present work it is difficult to compare the results. The investigations by Tomren [47] showed better stability for curcumin (MTC-4) than for the other curcuminoids. In the selection of curcuminoid derivatives investigated dimethoxycurcumin (MTC-1) was most stable, and bisdimethoxycurcumin (MTC-5) had the lowest stability.

The stability of RHC-1 and RHC-3 in EtOH obtained in the present work is lower than for curcumin, with the half-life of RHC-3 a little shorter, and the stability of RHC-4 is lowest of these curcuminoids. As mentioned above, curcumin was better stabilized by HP$\gamma$CD than of HP$\beta$CD. The opposite was seen for the other two curcuminoids investigated in CD solutions, the more hydrophilic RHC-3 and the more lipophilic RHC-1. Both of these were rapidly degraded in HP$\gamma$CD solution, with the entire amount of compound being degraded after the 5 minutes irradiation. RHC-3 seemed to be less
stable in HPβCD than in ethanol/buffer, while for RHC-1 the stability was better in HPβCD than in ethanol/buffer.

4.6.1 The importance of the keto-enol group for photochemical stability

From the mechanisms postulated by Tønnesen and Greenhill on the photochemical degradation of curcumin, the keto-enol moiety seem to be involved in the degradation process [7].

The photochemical stability is observed to be lowest for the monomethoxy derivative, RHC-4. In this derivative the enol is seen in both IR and NMR spectra, and the hydrogen of this group is therefore assumed to be bonded to one of the oxygens in the keto-enol unit. In curcumin (RHC-2), which is most stable, this hydrogen atom has previously been determined to be distributed between the two oxygens in the crystalline state, creating a aromatic-like structure [23]. Although these properties are not necessarily the same in solution, this kind of intramolecular bondings seems to be present and do probably contribute to the better photochemical stability of curcumin.

4.6.2 The importance of the substituents on the aromatic ring for photochemical stability

As mentioned above, the photochemical stability is generally best for curcumin (RHC-2). Curcumin is the only curcuminoid used in the present work in which intramolecular bonding can be formed between the substituents on the aromatic ring. The phenol can act as a hydrogen donor and the methoxy group can function as a hydrogen acceptor. In dimethoxycurcumin (RHC-1) there are two substituents, both methoxy groups with only hydrogen acceptor properties, and in bisdemethoxycurcumin (RHC-3) and monomethoxycurcumin (RHC-4) there are only one substituent on each ring. This intramolecular bonding is likely to contribute to the enhanced stability in curcumin, compared to the other curcuminoids.
Bisdemethoxycurcumin (RHC-3) and monomethoxycurcumin (RHC-4) has only one substituent in para-position on the aromatic ring. These two curcuminoids are generally most unstable, although it seems possible that bisdemethoxycurcumin might be partly protected in MeOH due to intermolecular binding to the solvent molecules.

In the mixture of EtOH and buffer the stability of RHC-3 is actually better than for RHC-1. In HPβCD solution on the other hand the stability of RHC-1 is much better than for RHC-3. This illustrates how a addition of a hydrogen bonding organic solvent can stabilize RHC-3.
5 - CONCLUSIONS

The solubility of curcuminoids in aqueous medium in the presence of cyclodextrins was investigated as a function of ionic strength and choice of salt to adjust this. The ionic strength in the range 0.085-0.15 does not seem to be the reason for the observed differences in solubility. pH may give increasing solubility when approaching close to neutral conditions (pH 6). In the further studies on the solubility, it is probably more important to keep pH constant than to keep ionic strength constant. A variation in pH does not, however, seem to influence the solubility when pH is kept at 5 or lower. Crystallinity, represented by different melting points, is most likely to have an influence on the solubility.

The stoichiometry for the curcuminoids-CD complexes was found to deviate from 1:1 stoichiometry in the phase solubility study. It seems like self-association and non-inclusion complexation of the CDs might contribute to increase the observed curcuminoids solubilities.

Photochemical stability for the curcuminoids in a hydrogen-bonding organic solvent is found to be than in an organic solvent/water mixture. The photostability is generally lower in cyclodextrin solutions, with the exception of curcumin in HPγCD. The other curcuminoids are either not soluble or very unstable in this cyclodextrin.

In total the most promising curcuminoids is curcumin itself, both with respect on solubility and photochemical stability. Bisdemethoxycurcumin is more soluble in βCDs, and curcumin is better solubilized by HPγCD. Curcumin also show better photochemical stability in HPγCD than in HPβCD and in the mixture of EtOH and aqueous buffer. Which of the curcuminoids is more promising as future drugs is of course also dependent on their pharmacological activities.

The di-hydroxycurcumin derivative and the curcumin galactoside turned out to be difficult to synthesize, and the synthesis was not successful.
5.1 Further studies

For the further studies of the curcuminoids and their complexation to CDs, it would be interesting to investigate the effect the CD complexation has on the pharmacological activities. Especially the antioxidant activity of the curcuminoids-CD complex is an important property.

Little work was done in the present study on the hydrolytic stability of the curcuminoids. Some investigations have been performed in previous studies, especially on curcumin. It would, however, be interesting to have more knowledge on the hydrolytic stability at different CD concentrations for all the curcuminoids.

The synthesis of a carbohydrate derivative of curcumin is still a promising way of increasing the solubility, and more effort on this synthesis and further investigations on the carbohydrate derivative would be of great interest.


21. **http://www.thomsonhc.com/hcs/librarian/PFPUI/8t1qVzY1wOQpAV.** [cited 2006 02.10].


47. Tomren, M., Curcumin and chemically related curcuminoids: Their synthesis, stability, activity and complexation with cyclodextrins, in Department of Pharmaceutics. 2005, University of Oslo / University of Iceland: Oslo / Reykjavík.


Appendix

A.1 Equipment

A.1.1 Equipment in the University of Iceland

TLC plates: Merck Silika gel 60 F$_{254}$ (aluminum)
Melting point apparatus: Gallenkamp melting point equipment
IR: Avatar 370 FT/IR
NMR: Bruker Avance 400 NMR
UV/Vis absorption: Ultrospec 2100 pro UV/Vis Spectrophotometer
HPLC: Pump: LDC Analytical ConstaMetric® 3200 Solvent Delivery System
  Detector: SpectroMonitor® 3200 variable wavelength detector
  Autosampler: Merck Hitachi AS-4000 Intelligent AutoSampler
  Column: Waters Nova-Pak® C$_{18}$ 60Å 4μm 3,9x150mm HPLC Cartridge Column
  Computer: Dell Optiplex GI
  Software: LabVIEW 7 Express National Instruments and Igor Pro 4.0.4.0.
  WaveMetrics Inc.
pH measurement: Corning pH meter
Moisture analysis: Scaltec SMO 01 Electronic Moisture Analyzer
Analytical Weight: Mettler Toledo AG285
Water purification system: Milli-Q Academic. Millipore
Filter for phase solubility experiment: Scleicher & Schull: Spartan 13/0,45 RC

A.1.2 Equipment in the University of Oslo

TLC plates: Merck Silika gel 60 F$_{254}$ (glass, 20x20cm)
UV lamp: Merck Eurolab Model UVGI -58
UV/Vis absorption: Shimadzu UV-2101 UV-Vis scanning spectrophotometer
HPLC: Pump: Shimadzu LC-9A Liquid Chromatograph
Detector: Shimadzu SPD-10A UV Spectrophotometric detector
Autosampler: Shimadzu SIL-9A Auto Injector
Column: Waters Nova-Pak® C$_{18}$ 60Å 4μm 3.9x150mm HPLC Cartridge Column
Printer: Shimadzu C-R5A Chromatopac
pH measurement: WTW pH 526 pH meter
Moisture analysis: Sartorius Moisture Analyzer MA30
Analytical Weight: Sartorius analytical weight
Water purification system: Aquatron water purification system
Filter for phase solubility experiment: Schleicher & Schull: Spartan 13/0,45 RC
Irradiation for photostability studies: Heraeus Suntest CPS, Original Hanau, Germany
Calibration of the Suntest apparatus: Hagner EC1 Lux photometer (visible region)
Hagner EC1 UV-A photometer (UV region)
DSC: Mettler Toledo DCS822®
Mettler Toledo STAR®e software

A.2 Reagents

A.2.1 Reagents used in synthesis

Reagents and solvents used for the syntheses were of synthesis grade or better, obtained from Sigma/Aldrich/Sigma-Aldrich or Merck. Exceptions are stated below.

Absolute ethanol – Riedel de Häen
Chloroform – Rathburn (also Sigma Aldrich)
Tributylborate – Fluka
p-hydroxybenzaldehyde – Eastman Organic Chemicals
Dichlormethane – Riedel de Häen
Galactose – ICN biomedicals Inr
Bromine – Fluka
Bu$_4$NI – Fluka
Anhydrous Na$_2$SO$_4$ – M&B lab chemicals
Hexane – Rathburn (95%)
A.2.2 Reagents used for NMR

DMSO (methyl sulfoxide)-d$_6$, 99.9 atom % D
Chloroform-d, 99.8 atom % D
Both obtained from Aldrich

A.2.3 Reagents used for HPLC (Phase solubility and photodegradation studies)

The reagents and solvents used for HPLC work were of analytical grade or better, obtained from Sigma-Aldrich or Merck. Exceptions are stated below.
KOH (used for the mobile phase) – Aldrich (A.C.S Reagent)
Acidum citicum anhydricum – NMD, Oslo
Buffers for calibration of pH-meter - Riedel de Häen
HP-β-CD: Kleptose ® HPB (molar substitution = 0.75-0.95) – Roquette
M-β-CD: Kleptose ® crysmeb Exp (molar substitution = 1.8) - Roquette
HP-γ-CD: Cavasol ® W8 HP Pharma (molar substitution = 0.5-0.7) - Wacker Fine Chemicals
Ethanol – Arcus (Oslo)

A.3 Buffers

A.3.1 Buffer for HPLC (mobile phase)

0.026 M (0.5%) citrate buffer pH 3

For 1000 ml buffer:

5.0g citric acid
pH adjusted to 3 with 10% KOH solution
5,47 g citric acid monohydrate
pH adjusted to 3 with 10% KOH solution

A.3.2 Buffers for phase solubility experiments

Buffer I: 0,05 M citrate buffer pH 5 (ionic strength 0,15)

For 1000 ml buffer:

9,6 g citric acid
10% NaOH solution to pH 5 (38,25 ml)
0,409 g NaCl

Buffer II: 0,05 M citrate buffer pH 5 (ionic strength 0,157)

For 1000 ml buffer:

9,6 g citric acid
10% NaOH solution to pH 5 (38,25 ml)
1,894 g MgCl₂ x 6 H₂O

Buffer III: 0,05 M phosphate buffer pH 5 (ionic strength 0,15)

For 1000 ml buffer:

7,72 g NaH₂PO₄ x 2 H₂O
0,0828 g Na₂HPO₄ x 2 H₂O
5,8 g NaCl
Buffer IV: 0.05 M phosphate buffer pH 5 (ionic strength 0.085)

For 1000 ml buffer:
7.72 g NaH$_2$PO$_4$ x 2 H$_2$O
0.0828 g Na$_2$HPO$_4$ x 2 H$_2$O
1.988 g NaCl

Buffer V: 0.05 M phosphate buffer pH 5 (ionic strength 0.15)

For 1000 ml buffer:
7.72 g NaH$_2$PO$_4$ x 2 H$_2$O
0.0828 g Na$_2$HPO$_4$ x 2 H$_2$O
7.4 g KCl

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

For 1000 ml buffer:
7.72 g NaH$_2$PO$_4$ x 2 H$_2$O
0.0828 g Na$_2$HPO$_4$ x 2 H$_2$O
2.536 g KCl

Buffer VII: 0.05 M citrate buffer pH 4.5 (ionic strength 0.169)

For 1000 ml buffer:
9.6 g citric acid
10% NaOH solution to pH 4.5 (27.55ml)
3.86 g NaCl
Buffer VIII: 0.05 M citrate buffer pH 5.5 (ionic strength 0.169)

For 1000 ml buffer:

9.6 g citric acid
10% NaOH solution to pH 5.5 (45 ml)

Buffer IX: 0.05 M citrate buffer pH 5 (ionic strength 0.5)

For 1000 ml buffer:

9.6 g citric acid
10% NaOH solution to pH 5.0 (38.25 ml)
24.19 g MgCl₂ x 6 H₂O

Buffer X: 0.05 M citrate buffer pH 5 (ionic strength 0.15)

For 1000 ml buffer:

10.5 g citric acid monohydrate
10% NaOH solution to pH 5 (38.8 ml)
0.305 g MgCl₂ x 6 H₂O

Buffer XI: 0.05 M citrate buffer pH 5 (ionic strength 0.5)

For 1000 ml buffer:

10.5 g citric acid monohydrate
10% NaOH solution to pH 5 (38.8 ml)
24.04 g MgCl₂ x 6 H₂O
Buffer XII: 0,05 M citrate buffer pH 4 (ionic strength 0,199)

For 1000 ml buffer:

10,5 g citric acid monohydrate
10% NaOH solution to pH 4 (21 ml)
7,02 g NaCl

Buffer XIII: 0,05 M citrate buffer pH 6 (ionic strength 0,199)

For 1000 ml buffer:

10,5 g citric acid monohydrate
10% NaOH solution to pH 6 (53 ml)

A.3.3 Buffer for photochemical degradation experiments

0,052 M (1%) citrate buffer pH 5 (ionic strength 0,152)

For 1000 ml buffer:

10,94 g citric acid monohydrate
10% NaOH solution to pH 5 (40,45 ml)
**A.4 Water-content of CDs**

**Table A.4.1**: CDs used for phase solubility studies (Iceland)

<table>
<thead>
<tr>
<th>Date</th>
<th>HP-β-CD</th>
<th>M-β-CD</th>
<th>HP-γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. June 2006</td>
<td>8,57%</td>
<td>11,88%</td>
<td>14,14%</td>
</tr>
<tr>
<td>8. June 2006</td>
<td>8,70%</td>
<td>12,12%</td>
<td>17,21%</td>
</tr>
<tr>
<td>28. June 2006</td>
<td>6,35%</td>
<td>12,71%</td>
<td>6,48%</td>
</tr>
</tbody>
</table>

* Due to some problems with the moisture analyzer, the previous values might be inaccurate. This is however the values used to calculate how much CD to add.

**Table A.4.2**: CDs used for phase solubility and photo stability studies (Oslo)

<table>
<thead>
<tr>
<th>Date</th>
<th>HP-β-CD</th>
<th>M-β-CD</th>
<th>HP-γ-CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>29. August 2006</td>
<td>7,02%</td>
<td>8,26%</td>
<td>5,45%</td>
</tr>
<tr>
<td>18. September 2006</td>
<td>5,76%</td>
<td>Not used</td>
<td>7,55%</td>
</tr>
</tbody>
</table>
### A.5 pH of the final solutions used in phase solubility study

**Table A.5.1**: pH in the buffers used in phase solubility studies after addition of CD and salts

<table>
<thead>
<tr>
<th>Buffer I, 10% CD</th>
<th>pH 5.17</th>
<th>pH 5.20</th>
<th>pH 5.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer I, 7.5% CD</td>
<td>pH 5.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer I, 5% CD</td>
<td>pH 5.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer I, 2.5% CD</td>
<td>pH 5.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer II, 10% CD</td>
<td>pH 4.94</td>
<td>pH 4.98</td>
<td>4.85</td>
</tr>
<tr>
<td>Buffer III, 10% CD</td>
<td>pH 4.86</td>
<td>pH 4.90</td>
<td>pH 4.91</td>
</tr>
<tr>
<td>Buffer IV, 10% CD</td>
<td>pH 4.99</td>
<td>pH 4.98</td>
<td>pH 4.99</td>
</tr>
<tr>
<td>Buffer V, 10% CD</td>
<td>pH 4.87</td>
<td>pH 4.90</td>
<td>pH 4.90</td>
</tr>
<tr>
<td>Buffer VI, 10% CD</td>
<td>pH 4.99</td>
<td>pH 4.96</td>
<td>pH 5.02</td>
</tr>
<tr>
<td>Buffer VII, 10% CD</td>
<td>4.51 (4.56 before adjustment)</td>
<td>4.51 (4.62 before adjustment)</td>
<td>4.49 (not adjusted)</td>
</tr>
<tr>
<td>Buffer VIII, 10% CD</td>
<td>5.51 (5.64 before adjustment)</td>
<td>5.50 (5.69 before adjustment)</td>
<td>5.50 (5.58 before adjustment)</td>
</tr>
<tr>
<td>Buffer IX, 10% CD</td>
<td>pH 3.96</td>
<td>Not used</td>
<td>Not used</td>
</tr>
<tr>
<td>Buffer X, 10% CD</td>
<td>Not used</td>
<td>Not used</td>
<td>pH 5.06</td>
</tr>
<tr>
<td>Buffer XI, 10% CD</td>
<td>Not used</td>
<td>Not used</td>
<td>pH 3.93</td>
</tr>
<tr>
<td>Buffer XII, 10% CD</td>
<td>4.01</td>
<td>3.98 (4.06 before adjustment)</td>
<td>4.03 (3.92 before adjustment)</td>
</tr>
<tr>
<td>Buffer XIII, 10% CD</td>
<td>6.01 (6.12 before adjustment)</td>
<td>5.99 (6.15 before adjustment)</td>
<td>6.03</td>
</tr>
</tbody>
</table>
A.6 IR Spectra

Figure A.6.1: IR spectrum of RHC-1

Figure A.6.2: IR spectrum of RHC-2
Figure A.6.3: IR spectrum of RHC-3

Figure A.6.4: IR spectrum of RHC-4
Figure A.6.5: IR spectrum of acetobromogalactose

Figure A.6.6: IR spectrum of vanillin galactoside
Figure A.6.8: IR spectrum of curcumin galactoside
Figure A.7.1: UV/Vis spectrum of RHC-1, with maximum absorption at 419.2 nm

Figure A.7.2: UV/Vis spectrum of RHC-2, with maximum absorption at 418.2 nm
Figure A.7.3: UV/Vis spectrum of RHC-3 with maximum absorption at 410.9 nm

Figure A.7.4: UV/Vis spectrum of RHC-4, with maximum absorption at 409 nm
A.8 $^1$H-NMR Spectra

Figure A.8.1: NMR spectrum of RHC-1
Figure A.8.2: NMR spectrum of RHC-2
Figure A.8.3: NMR spectrum of RHC-3
Figure A.8.4: NMR spectrum of RHC-4
Figure A.8.5: NMR spectrum of acetobromogalactose
Figure A.8.6: NMR spectrum of Vanillin galactoside
A.9 HPLC chromatograms

Figure A.9.1: HPLC chromatogram of RHC-1 (detected at 350nm)

Figure A.9.2: HPLC chromatogram of RHC-2 (detected at 350nm)
Figure A.9.3: HPLC chromatogram of RHC-3 (detected at 350nm)

Figure A.9.4: HPLC chromatogram of RHC-4 (detected at 350nm)
A.10 DSC thermograms

Figure A.10.1: DSC thermogram of RHC-1

Figure A.10.2: DSC thermogram of RHC-2

Figure A.10.3: DSC thermogram of RHC-3

Figure A.10.4: DSC thermogram of RHC-4
Figure A.10.5: DSC thermogram of curcumin previously synthesized by Hanne Hjorth Tønnesen

Figure A.10.6: DSC thermogram of curcumin previously synthesized by Marianne Tomren (MTC-4)

Figure A.10.7: DSC thermogram of dimethoxycurcumin previously synthesized by Marianne Tomren (MTC-1)

Figure A.10.8: DSC thermogram of bisdemethoxycurcumin previously synthesized by Marianne Tomren (MTC-5)
A.11 UV spectra for photochemical degradation

Figure A.11.1: Photochemical degradation of C-1 monitored by UV/Vis absorption spectrophotometry
Figure A.11.2: Photochemical degradation of C-2 monitored by UV/Vis absorption spectrophotometry
Figure A.11.3: Photochemical degradation of C-3 monitored by UV/Vis absorption spectrophotometry
Figure A.11.4: Photochemical degradation of C-4 monitored by UV/Vis absorption spectrophotometry
A.12 HPLC chromatograms from photochemical stability experiment

Figure A.12.1: C-1 as a standard in MeOH and C-1 in HPγCD solution (detected at 350nm)

Figure A.12.2: C-3 as a standard in MeOH and C-3 in HPγCD solution (detected at 350nm)