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ORIGINAL ARTICLE

Studies of curcumin and curcuminoids. XXXVI. The stoichiometry and complexation constants of cyclodextrin complexes as determined by the phase-solubility method and UV–Vis titration

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Abstract Cyclodextrin (CD) complex stoichiometry and complexation constant with two symmetric curcuminoids and two unsymmetric curcuminoid-like compounds were investigated and compared by two independent methods, the phase-solubility method and ultraviolet-visible absorption spectroscopy (UV–Vis) titration. Two different methods were applied in an effort to increase the apparent intrinsic solubility of the compounds and make the investigation of stoichiometry and complexation constants possible. The intrinsic solubility could be determined for all four compounds in aqueous 10% (v/v) ethanolic solutions. Higher order complexation or solubilization through complex aggregation was observed for the symmetric molecules, while 1:1 complexation was observed for the unsymmetric molecules in the phase-solubility diagram. The UV–Vis investigation showed 1:1 complexation for all compounds, with some indication of higher order complexation for the symmetric molecules. Thus the stoichiometry found with the two methods correlated well for the unsymmetric, but not for the symmetric compounds where the phase-solubility investigations clearly indicated higher order complexation and possible aggregation of complexes. There was also a difference between the 1:1 complexation constants found with the two methods, especially for the compounds with low intrinsic solubility (i.e. the symmetric curcuminoids). However, they agree in the ranking of complexes according to the strength of the association. The 1:2 complexation constant observed with the phase-solubility method was more than 100 times the complexation constant found with the UV–Vis method, which explains why solubility is poorly predicted from the UV–Vis data. This discrepancy may be explained by solubilization by aggregation of complexes or some phenomena other than inclusion complexation.

Keywords Cyclodextrins · Curcuminoids · Phase-solubility · UV–Vis titration · Complexation stoichiometry · Complexation constants

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Introduction

The curcuminoids belong to the group diarylheptanoids and are the coloring principles found in the rhizomes of the plant *Curcuma longa* L. (*Zingiberaceae*), also known as turmeric in dry powder form [1, 2]. There are three main curcuminoids present in *Curcuma longa*. The total amount and the ratio between them vary, but curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is usually the major constituent [1, 3]. Commercial available "pure curcumin" is produced from purified extract of *Curcuma longa.* This product also contains the other two naturally occurring curcuminoids which are demethoxycurcumin and bisdemethoxycurcumin, with respectively one or both methoxy groups absent (Fig. 1 a) [4].

Curcumin has been studied for its potential use against cancer [5–8], HIV [9–12], cystic fibrosis [13, 14], as an immunomodulating agent [15, 16] and as an anti-microbial agent [17, 18]. It is considered safe for human use, even at high doses, but has poor bioavailability [19]. The major limitations for the pharmaceutical formulation and biological investigation of potential benefits of curcumin and

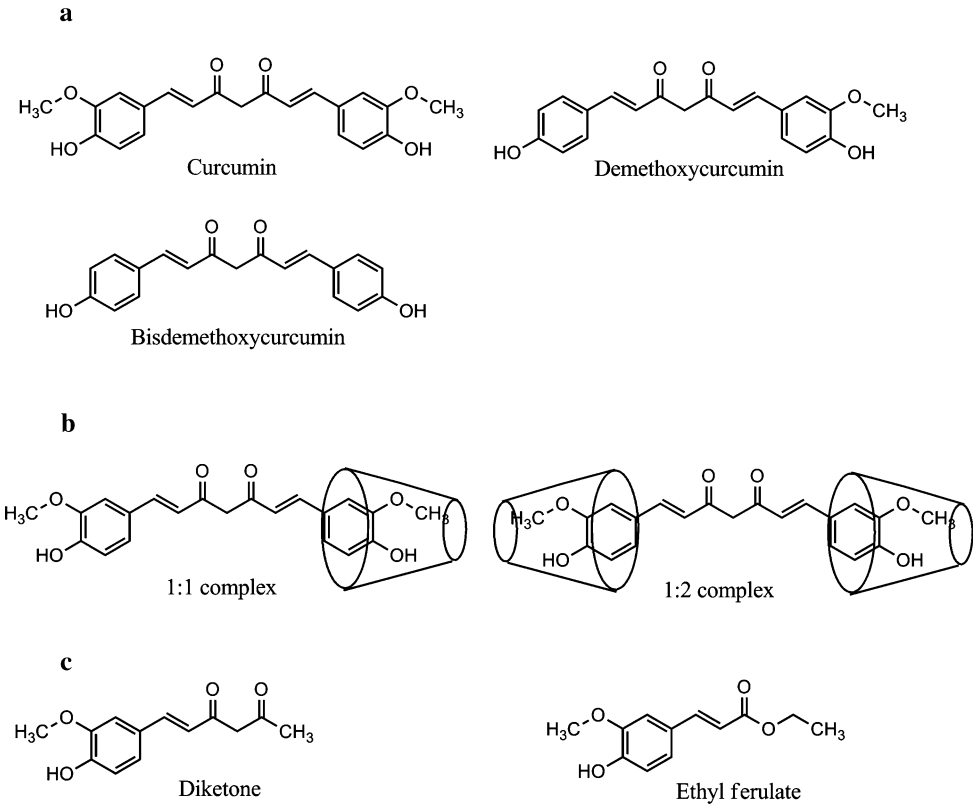

Fig. 1 The structure of the naturally occurring curcuminoids (**a**), curcumin-CD complex with 1:1 and 1:2 complex stoichiometry (**b**) and structure of the two unsymmetric curcuminoid-like compounds (diketone and ethyl ferulate) (**c**)

curcuminoids, are their low aqueous solubility [20, 21], and chemical [22] and photochemical instability [20, 23, 24].

Cyclodextrins (CDs) are cyclic oligosaccharides, composed of (α-1,4)-linked α-D-glucopyranose units, with a hydrophilic outer surface and a lipophilic central cavity. They can form water soluble inclusion complexes through inclusion of a lipophilic molecule or lipophilic moiety into the central cavity. Recently it has been reported that aggregation of CD molecules or CD complexes also contributes to solubilization [25–29]. The natural β-CD and γ-CD are composed of 7 and 8 glucopyranose units respectively. Hydroxypropyl derivatives of these CDs can form highly water soluble complexes and are therefore commonly used as pharmaceutical excipients. Previous studies have shown that inclusion into the CD cavity will enhance solubility and stability of curcumin [20, 30–32].

Studies on the complex stoichiometry have also been performed, and the complexation constant, or association constant, for the complex determined. These have been based on absorbance [33–35] or fluorescence methods [32, 33]. Curcumin is a symmetric molecule with two 3-methoxy 4-hydroxy substituted phenyl moieties that can be included in the CD cavity. The stoichiometry of the CD complex can therefore be 1:1, where one aromatic moiety is included in the CD cavity, or 1:2 where both aromatic moieties are include into CD cavities of two different CD molecules (Fig. 1b). Previous studies have indicated that curcumin/β-CD 1:2 complexes are formed [32, 34]. However, one of these studies showed also that the data fitted better to the 1:1 complex model than the 1:2 model [32]. The hydroxypropyl derivatives of β- and γ-CD (i.e. HPβCD and HPγCD) fitted to the 1:2 complex model [32].

In these studies of the interactions of curcumin with CDs either commercially available curcumin [32–35] or extracts from plant materials [31] were used. No analytical data on, for example, their purity was reported. The commercial curcumin and curcumin from plant extracts can contain significant amounts of demethoxycurcumin and bisdemethoxycurcumin [4, 36] which can also interact with CDs. The amount of these curcuminoid impurities can vary but in some products they are the major constituents [36]. The use of different formulation additives and buffer systems might also contribute to the discrepancies between these studies [37]. Since slight variation in the degree of substitution and the location of substituents in the CDs can influence the CD complexation efficacy, one can not exclude that the use of different batches of identical CD derivates may also result in variable results.

Previously we have studied the effect of various CD derivatives on the aqueous solubility, and chemical and photochemical stability of pure curcumin, obtained through chemical synthesis, and thus free of impurity from other curcuminoids [20]. In another study, we investigated the solubility and the stabilizing effect of 10% (w/v) solutions

of HPβCD and HPγCD on various synthetic curcuminoids. The study showed that, for curcumin, HPγCD is about 50 times better solubilizer than HPβCD [30]. This difference was only two fold for bisdemethoxycurcumin. And, in general, it has been shown that the curcuminoid without substituted phenyl rings preferred the β-CD cavity whereas the affinity for the γ-cavity increased with bulkiness of the substituents [30].

Analysis of spectral data to determine the complexation constant requires that a guest solution without CD (i.e. the host) is used as a reference. However, the solubility of curcumin in aqueous solutions without CDs is very low and below the limit of quantification (LOQ) for UV–Vis absorption spectroscopy [32] and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection [20]. Spectral data can therefore only be reported for a supersaturated pure aqueous curcumin solution, where the curcumin concentration is unstable. This limitation may also explain differences in the reported results for the curcumin complex stoichiometries and complex constants determined by the two methods.

Here we report investigations of complexation and complex stoichiometry for synthetic curcumin and bisdemethoxycurcumin, and two synthetic curcumin-like compounds with just one substituted phenyl moiety, 4-hydroxy-3-methoxyphenyl-5-hexene-2,4-dione (diketone) and ethyl ferulate, respectively (Fig. 1c). Further, the possibility of using ethanol and pH changes to increase the apparent intrinsic solubility of the guest compound, to allow for determination and verification of complex stoichiometry, as well as determination of the complexation constants with two independent methods, i.e. the phase-solubility method and UV–Vis titration method was investigated. Phase-solubility investigations require a significant amount of the guest compound and are time consuming. The UV–Vis titration method could therefore be an alternative and more efficient method than the phase-solubility method in the preformulation and formulation phase of drug development, when only small amount of compound is available and time and money are crucial factors for the developer [38].

Methods

Materials

The two cyclodextrins (CDs) used in the study were 2-hydroxypropyl-β-cyclodextrin (HPβCD) from either Wacker Fine Chemicals in Germany (Cavasol® W7 HP, with a molar substitution of 0.6–0.9), which was used in the solubility determinations, or from Roquette (France) with a molar substitution of 0.75–0.95(Kleptose® HPB), which was used to determine intrinsic solubility in 10% (v/v) ethanolic solutions and in the phase-solubility and UV–Vis titration methods, and 2-hydroxypropyl-γ-cyclodextrin with a molar substitution of 0.5–0.7 (HPγCD, Cavasol® W8 HP), from Wacker Fine Chemicals. Prior to preparation of CD solutions, the moisture content of the CDs was measured using a Scaltec SM 01 Electronic Moisture Analyzer (in the solubility determinations) and a Sartorius Moisture Analyzer MA30 (in the phase-solubility and UV–Vis titration methods).

Aqueous buffer solutions used were 0.05 M citrate buffer pH 5 (citric acid monohydrate), ionic strength adjusted to 0.15 by addition of sodium chloride (NaCl) and 0.1 M phosphate buffer pH 6.0, 7.4 and 8.5 (sodium diphosphate dihydrate and disodium phosphate tetrahydrate), ionic strength adjusted to 0.3 by adding NaCl.

Synthesis and characterization

General methods

Thin-layer chromatography (TLC) was carried out using Merck silica gel 60 F_{254} aluminium sheets. The UV–Vis spectra were recorded in acetonitrile (ACN) against blanks in the 200–600 nm range on a Shimadzu UV-2101 PC UV–Vis scanning spectrophotometer. Melting points (mp) were determined in open capillary tubes using a Gallenkamp melting point instrument and are uncorrected. Infrared (IR) spectra were obtained with a Avatar 370 FT/IR spectrophotometer using KBr disks. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained using a Brüker 400 MHz spectrometer, chemical shifts are expressed in ppm relative to tetramethylsilane (TMS) and coupling constants are given in Hertz.

1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin)

Curcumin was synthesized according to a previously reported method [39]. TLC gave one spot with a retention factor (R_f) of 0.14 (chloroform/ethanol 25:1). Mp 177–181 °C. IR (KBr) ν_{max} 3,503 cm^{-1} (OH), 1,627 cm^{-1} (CO), 400–1,600 cm^{-1} ("fingerprint area"). UV max –418 and 263 nm. ^{1}H NMR (dimethyl sulfoxide (DMSO)): δ 3.84 (6H, s), 6.06 (1H, s), 6.75 (2H, d, $J = 16.0$), 6.81 (2H, d, $J = 8.2$), 7.15 (2H, dd, $J = 8.2$ and 2.0), 7.32 (2H, d, $J = 2.0$), 7.55 (2H, d, $J = 16.0$), 9.45 (2H, s).

1,7-Bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (bisdemethoxycurcumin)

Bisdemethoxycurcumin was synthesized according to a previously reported method [40]. TLC gave one spot with an R_f of 0.03 (chloroform/ethanol 25:1). Mp 219–222 °C, which

is consistent with previously reported values [41]). IR (KBr) ν_{max} 3,220 cm^{-1} (OH), 1,627 cm^{-1} (CO), 400-1,600 cm^{-1} ("fingerprint area"). UV max −410 and 270 nm. ^{1}H NMR (DMSO): δ 6.04 (1H, s), 6.69 (2H, d, $J = 16$), 6.83 (4H, d, $J = 8.6$), 7.52–7.59 (6H, m), 10.06 (2H, s).

4-Hydroxy-3-methoxyphenyl-5-hexene-2,4-dione (diketone)

Diketone was synthesized according to a previously reported method [42]. TLC gave one spot with an R_f of 0.24 (chloroform/ethanol 25:1). Mp 142–147 °C, which is consistent with a previously reported melting point of 142–142.5 °C [43]. IR (KBr) ν_{max} 3,247 cm^{-1} (OH), 1,633 cm^1 (CO), 400–1,600 cm^{-1} ("fingerprint area"). UV max –361 nm. ^{1}H NMR ($CDCl_3$): δ 2.16 (3H, s), 3.93 (3H, s), 5.63 (1H, s), 5.88 (1H, bs), 6.32 (1H, d, $J = 16.0$), 6.92 (1H, d, $J = 8.0$), 7.02 (1H, d, $J = 2.0$), 7.08 (1H, dd, $J = 2.0$ and 8.0), 7.53 (1H, d, $J = 16.0$), 15.5 (1H, s). ^{13}C NMR ($CDCl_3$): δ 26.85, 55.98, 100.71, 109.52, 114.84, 120.37, 122.07, 127.70, 140.06, 146.81, 147.74, 177.97, 197.00.

Ethyl 4-hydroxy-3methoxy-cinnamate (ethyl ferulate)

Ethyl ferulate was synthesized and purified by the method reported by Quideau et al. [44]. TLC gave one spot with an R_f of 0.33 (chloroform/ethanol 25:1). Mp 59–61 °C. IR (KBr) ν_{max} 3,178 cm^{-1} (OH), 1,677 cm^{-1} (CO), 400–1,600 cm^{-1} ("fingerprint area"). UV max –281 nm. ^{1}H NMR ($CDCl_3$): δ 1.34 (3H, t, $J = 7.0$), 3.93 (3H, s), 4.25 (2H, q, $J = 7.0$), 5.85 (1H, s), 6.29 (1H, d, $J = 16.0$), 6.92 (1H, d, $J = 8.3$), 7.03 (1H, d, $J = 2.0$), 7.07 (1H, dd, $J = 2.0$ and 8.3), 7.62 (1H, d, $J = 16.0$).

Partition coefficient

The partition coefficent, given as CLog P, for diketone and ethyl ferulate was estimated using ChemBioDraw Ultra software 11.0.1.

Differential scanning calorimetry (DSC)

Samples were analyzed by DSC using Mettler Toledo DSC822^e to determine the melting point. The instrument was calibrated using indium. The samples were scanned in temperature intervals at 10 °C/min in the range 0–250 °C, starting at 0–50 °C and ending about 50 °C above the melting point.

HPLC analysis

The mobile phases were composed of 0.5% (w/v) citric acid buffer, pH 3 and ACN in appropriate ratios. Two different HPLC units were employed: *HPLC system I*: LDC Analytical ConstaMetric 3200 Solvent Delivery System pump with Merck Hitachi AS-4000 Intelligent auto sampler and Spectra-Physics SP8450 UV–Vis detector using Supelcosil® C_{18}, 4.6 × 150 mm, 5 µm particle column. *HPLC system II*: Shimadzu Liquid Chromatography LC-9A pump, Shimadzu Auto Injector SIL-9A auto sampler, Shimadzu UV–Vis Spectrophotometric detector SPD-10A using Waters Nova-Pak® C_{18}, 3.9 × 150 mm, 4 µm particle size column.

All the quantitative analyses were done in triplicate to reduce random error. Regression coefficients of the linear standard curves were >0.99. Limit of quantification (LOQ) was defined as the concentration that gave a signal to noise ratio of 10. Precision was measured by 6 injections of the same sample, at zero attenuation. The analytical conditions, retention times (Rt), relative standard errors and LOQ are given in Table 1.

Solubility investigations

The buffers were made by dissolving a given amount of the appropriate salt in distilled water using a volumetric flask. The pH was checked using Corning pH meter (solubility investigations) and WTW pH 526 pH meter (phase-solubility and UV–Vis titration methods). The ionic strength was fixed by adding a calculated amount of NaCl. For the ethanolic buffers, the given amount of ethanol was added to the citrate buffer pH 5.0 and then water was filled to the mark on the volumetric flasks. The reported pH is the pH of the aqueous buffer solution before addition of ethanol.

Aqueous solutions containing either 10% (w/v) HPβCD or 10% (w/v) HPγCD were prepared in the aqueous buffer solutions. A portion of the 10% (w/v) CD solution was then diluted with pure buffer solutions to get CD solutions containing less than 10% (w/v) CD. Aqueous buffer solutions for the phase-solubility investigations of diketone and ethyl ferulate, without ethanol, contained up to 15% (w/v) CD, and were prepared as previously described by dilution of buffer solutions containing 15% (w/v) CD.

Two milliliter of aqueous cyclodextrin containing buffer solutions were added to vials containing 6 mg of the compound to be tested. Then the sealed vials were agitated on an Edmund Bühler shaker for 7–8 days. In the case of ethyl ferulate more compound was added after the first few days when the initial portion had fully dissolved. The samples were withdrawn from the vials and filtrated (the first drops were discarded). The resulting clear solutions were diluted with methanol and analyzed on HPLC. And all measurements were done in triplicate.

Table 1 Chromatographic conditions

Compound HPLC system	Mobile phase (buffer-ACN)	Flow (ml/min)	Detection (nm)	Rt (min)	Relative standard deviation (%)	LOQ (M)
Curcumin						
System 1	50–50	1	350	7.6	2.6	1×10^{-6}
System 2	60–40	1.1	350	8.1	11.8	5×10^{-7}
System 2	60–40	1	420	9.0	1.0	5×10^{-8}
Bisdemethoxycurcumin						
System 1	50–50	1	350	6.4	5.9	5×10^{-6}
System 2	60–40	1.1	350	6.5	5.3	4×10^{-7}
System 2	60–40	1	420	7.5	NI*	NI*
Diketone						
System 1	50–50	1	350	5.2	8.1	5×10^{-6}
System 2	70–30	1	350	12.5	5.1	5×10^{-7}
Ethyl ferulate						
System 1	50–50	1	320	4.7	6.6	5×10^{-7}
System 2	70–30	1.1	320	8.1	2.3	5×10^{-7}

* The relative standard deviation and LOQ was not investigated (NI). However, all determinations were performed with a signal to noise ratio >10

UV–Vis Spectroscopy investigations

Spectrophotometric investigations were used to determine the complex stoichiometry and complexation constant.

Aqueous CD solutions were prepared in aqueous buffer, pH 5.0. A stock solution of each compound was prepared in ethanol. The ethanolic stock solution was diluted 10 times with the CD solution of appropriate concentration. The concentration of the compound in each sample was within Beers law limitation (usually <0.01 M), but high enough for spectrophotometric detection. All samples were made in triplicate.

Then the UV–Vis spectra of the samples were recorded against blanks in the 200–600 nm wavelength range on a Shimadzu UV-2101 PC UV–Vis scanning spectrophotometer.

Overlay was made of all the spectra obtained at different CD concentrations, in order to determine the isosbestic points and the point of largest spectral change.

Results and discussion

Synthesis and characterization

The compounds were synthesized according to previously reported procedures and the structure and purity were confirmed by TLC, melting point determination (including DSC), IR, UV, NMR and HPLC. The ^{1}H NMR spectra show only one proton in the central dione position of curcumin, bisdemethoxycurcumin and diketone showing that, due to resonance stabilization, the enol is the dominant tautomeric form (Fig. 2a). The resonance stabilization make the structures of these compounds relatively rigid, whereas ethyl ferulate lacks the dione structure and relatively free rotation around the ester and some of the C–C bonds can be expected (Fig. 2b). This is also shown by the large difference in crystal energy. The DSC onset temperatures are 180, 228 and 142 °C for curcumin, bisdemethoxycurcumin and diketone, respectively whereas the DSC onset temperature for ethyl ferulate is only 59 °C.

Solubility investigations

The reported curcumin pKa values are somewhat ambiguous due to the difficulty of investigating such poorly water soluble compound [30, 34, 45–49]. Studies have been based on mixtures of organic solvents and aqueous buffers [30, 34, 46, 48, 49] and plain aqueous buffers [47]. These studies have reported pKa values for the first deprotonation of curcumin in the range of 7.75–10.82. Ionization should improve the solubility of curcumin and curcuminoids. However, curcumin is very unstable in aqueous solutions at high pH, with half life ($t_{½}$) of less than 15 min at pH above 9 [46, 50]. Inclusion in the CD cavity does reduce the rate of hydrolysis of the curcuminoids. However, at pH 10 the $t_{½}$ is less than 24 h for 0.02 mg/ml curcuminoids in aqueous 10% (w/v) HPβCD or HPγCD solutions [30].

Previous solubility studies were performed at pH 5–6 [20, 24, 30], where curcumin is relatively stable. However curcuminoids will be partly ionized in slightly alkaline solutions and thus the solubility could be improved. The solubility of the four compounds was therefore determined

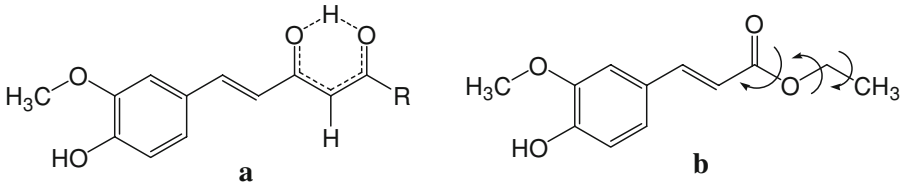

Fig. 2 The rigid enolic tautomer of diketone (**a**) and ethyl ferulate with relatively free rotation around the ester and some of the C–C bonds (**b**)

in phosphate buffer at pH 6.0, 7.4 and 8.5 and in the same buffers containing either 10% (w/v) HPβCD or 10% (w/v) HPγCD (Table 2).

The concentration of curcumin, bisdemethoxycurcumin and diketone were below the LOQ in pure buffer solutions. Increasing pH to 8.5 was not sufficient to increase the solubility of curcumin, bisdemethoxycurcumin and diketone above the LOQ. Ethyl ferulate is estimated to be more lipophilic (CLog P = 2.02) than the diketone (Clog P = 0.84). However, due to lower crystal energy the solubility of ethyl ferulate is much higher and could be determined also in the pure aqueous buffer solutions. The concentration of all compounds could be determined in the aqueous buffer solutions containing CD. However, the pH change and ionization did not significantly affect solubility to allow a more precise assessment of the complexation constant and complex stoichiometry. In addition, at higher pH additional peaks indicating degradation were observed in the HPLC chromatograms for all the compounds (except bisdemethoxycurcumin). Thus, due to the chemical instability of the compounds the maximum pH for the complexation studies was about 6.0. And hence it was not feasible to accurately determine the complex stoichiometry or complexation constants in pure aqueous buffer solutions.

Table 2 Solubility at different pH in phosphate buffer (0.1 M, ionic strength 0.3) with and without CD (10% w/v)

Compound CD	Solubility (mg/ml)		
	pH 6.0	pH 7.4	pH 8.5
Curcumin			
Without CD	<LOQ*	<LOQ	<LOQ
HPβCD	0.10	0.07 (degradation)	0.08 (degradation)
HPγCD	0.73	0.73	0.54 (degradation)
Bisdemethoxycurcumin			
Without CD	<LOQ	<LOQ	<LOQ
HPβCD	0.6	0.6	0.9
HPγCD	1.0	1.1	1.1
Diketone			
Without CD	<LOQ	<LOQ	<LOQ
HPβCD	0.4	0.3	0.6 (degradation)
HPγCD	0.1	0.1 (degradation)	0.1 (degradation)
Ethyl ferulate			
Without CD	0.36	0.37	0.50 (degradation)
HPβCD	6.82	7.03	7.83
HPγCD	6.18	6.88	6.33

**LOQ* is the limit of quantification

Curcumin is soluble in some water miscible organic solvents such as ethanol. Ethanol is also acceptable as an excipient in certain pharmaceutical formulations [51] and approved by the Food and Drug Administration (FDA) as an inactive ingredient [52]. The curcumin-CD complexation has been studied in solutions containing various alcoholic co-solvents and alginates [37]. Thus further investigations on the solubilization of the four compounds were carried out with ethanol as co-solvent in order to increase the intrinsic solubility.

Addition of organic co-solvents will in most cases increase the apparent intrinsic solubility of curcuminoids in pure water or pure buffer solutions. The same has not been observed in aqueous cyclodextrin solutions. Since the relative affinity of lipophilic guest molecules for the CD cavity is reduced as the medium becomes more lipophilic, the equilibrium of the complex will shift away from the complex and thus the value of the stability constant will decrease. Furthermore, the co-solvent molecules can form weak CD complexes and to some extent compete with more lipophilic guest molecules [37, 53–56]. Table 3 shows the solubility of the compounds at pH 5.0 in 5, 10 and 25% (w/v) ethanolic solutions in the absence and presence of CD. In 5% (w/v) and 10% (w/v) ethanolic solutions there is a significant reduction in CD complexation, the solubilising effect is decreased and solubility in CD solutions is reduced compared to solubility in CD solutions without ethanol. However, in solutions containing 10% (w/v) ethanol, the solubilising effect of ethanol alone is sufficient for determination of the intrinsic solubility for all compounds. In 25% (w/v) solutions the overall solubilising effect is significantly increased in the presence of CD, although the relative solubilising effect of the CD is decreased, showing that ethanol is interfering to a significant extent with the complexation. Thus 10% ethanolic solutions were used for determination and verification of complex stoichiometry, and for determination of the complexation constants by both the phase-solubility method and the UV–Vis titration method.

Phase-solubility investigations

The phase-solubility investigations were performed in aqueous buffer with and without 10% (v/v) ethanol, and

Table 3 Solubility in 0, 5, 10 and 25% (w/v) ethanol, pH 5.0 (0.05 M citrate buffer, ionic strength 0.15) without CD, and with 10% (w/v) HPγCD or HPβCD

Compound CD	Solubility (mg/ml)			
	Ethanol concentration (w/v)			
	0%	5%	10%	25%
Curcumin				
Without CD	<LOQ	<LOQ	<LOQ*	0.005
HPβCD	0.07	0.03	0.03	0.105
HPγCD	0.86	0.49	0.28	0.095
Bisdemethoxycurcumin				
Without CD	<LOQ	<LOQ	0.0028	0.03
HPβCD	0.90	0.41	0.32	0.35
HPγCD	2.17	1.92	1.64	0.19
Diketone				
Without CD	<LOQ	<LOQ	0.1	0.2
HPβCD	0.4	0.3	0.3	0.5
HPγCD	0.2	0.1	0.2	0.4
Ethyl ferulate				
Without CD	0.59	0.74	1.15	19.90
HPβCD	10.36	9.11	8.83	33.82
HPγCD	26.64	17.73	17.44	44.50

* The solubility of curcumin in 10% (v/v) ethanol could only be determined using HPLC with detection at 420 nm. This method would give a LOQ of 5 × 10−8

used to investigate both complex stoichiometry and the relative affinity of the guest molecules for the different CDs. Phase-solubility diagrams were obtained by plotting the concentration of dissolved solute (guest) on the vertical axis against the concentration of the CD on the horizontal axis. A linear phase diagram (A_L) is consistent with 1:1 complexation stoichiometry. Positive deviation from linearity (A_P curve) suggests higher order complexation with respect to the CD and it is also consistent with aggregation of the CD complexes [25–29].

In aqueous buffer without added ethanol the phase-solubility diagrams showed A_P type curves for the symmetric compounds, curcumin and bisdemethoxycurcumin with two aromatic moieties. For the two compounds with a single aromatic moiety (diketone and ethyl ferulate) A_L type curves were observed, consistent with 1:1 complex formation. The complexation constant could only be determined for ethyl ferulate because it was the only compound with sufficient intrinsic solubility [27].

Addition of ethanol did not significantly alter the shape of the phase-solubility diagrams, although some changes in the solubility were observed. The intrinsic solubility (S_0) could be determined for the four compounds in aqueous buffer solutions containing 10% (v/v) ethanol. The data could then be fitted to the 1:1 or the 1:2 solubility isotherm.

Figure 3 shows phase-solubility diagrams for all the compounds investigated.

The 1:1 solubility isotherm

In this case it is assumed there is only 1:1 complexation. Thus the equilibrium in the solution is:

$$C + CD \rightleftharpoons C/CD \tag{1}$$

where C is the curcuminoid or curcumin-like compound and C/CD is the 1:1 CD complex. The total solubility of the compound (S_t) and the total CD concentration are given by Eqs. 2 and 3:

$$S_t = [C] + [C/CD] = S_0 + [C/CD] \tag{2}$$

$$CD_t = [CD] + [C/CD] \tag{3}$$

The 1:1 complexation constant is then given as:

$$K_{1:1} = [C/CD]/([C][CD]) \tag{4}$$

And by combining Eqs. 2–4 the solubility isotherm can be obtained:

$$S_t = S_0 + K_{1:1} S_0 CD_t/(1 + K_{1:1} S_0) \tag{5}$$

The phase-solubility diagram will therefore be linear. Equation 5 can be rearranged to the equation used to calculate the complexation constant from the slope of linear phase-solubility diagrams:

$$K_{1:1} = \text{slope}/(S_0(1 - \text{slope})) \tag{6}$$

The 1:2 solubility isotherm

If a 1:2 complexation is also possible, then the equilibrium in the solution will be

$$C + CD \rightleftharpoons C/CD + CD \rightleftharpoons C/CD_2 \tag{7}$$

where C/CD_2 is the 1:2 CD complex.

Then S_t, CD_t and the complexation constant for the formation of the 1:2 complex can be given by:

$$S_t = S_0 + [C/CD] + [C/CD_2] \tag{8}$$

$$CD_t = [CD] + [C/CD] \tag{9}$$

$$K_{1:2} = [C/CD_2]/([C/CD][CD]) \tag{10}$$

From the equations above the non-linear solubility isotherm can be obtained as;

$$S_t = S_0 + K_{1:1} S_0[CD] + K_{1:2}K_{1:1}S_0[CD]^2 \tag{11}$$

Several ways have been devised to obtain the complexation constants from S_t and CD_t [57], but these equations are rather complex. However, if $S_t \ll CD_t$, as in the case of curcumin and bisdemethoxycurcumin, then $[CD] \approx CD_t$ and $K_{1:1}$ and $K_{1:2}$ can be obtained simply by nonlinear curve-fitting of the data to Eq. 11.

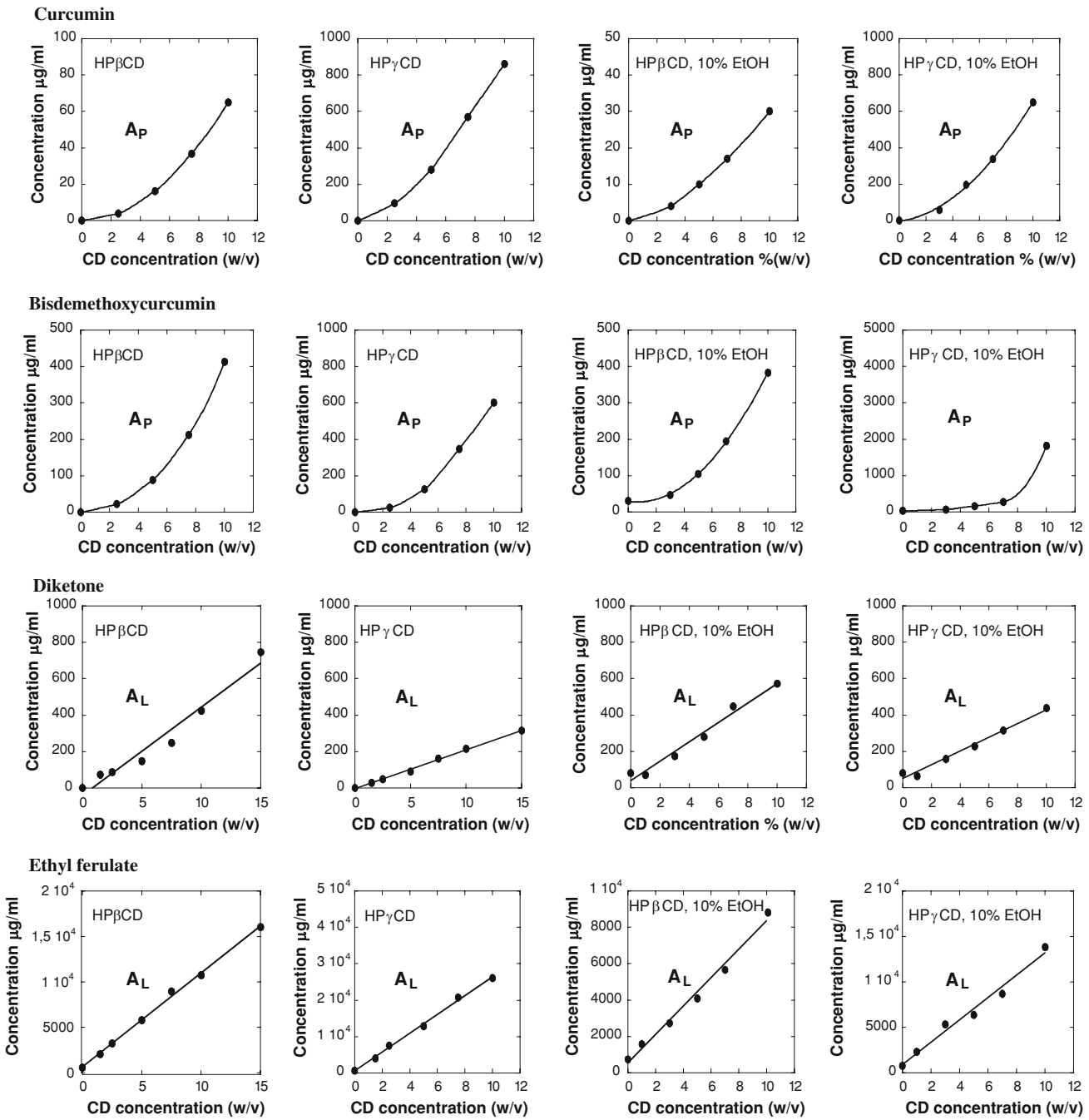

Fig. 3 Phase-solubility diagrams for the four compounds in HPβCD and HPγCD, with and without 10% (v/v) ethanol

UV–Vis investigations

Inclusion complex formation causes changes in the absorption spectra of substrates [58]. Spectral changes as a function of CD concentration can be used to analyse stoichiometry [59] and to calculate complexation constant [60], even though CD aggregates may also exist in the solution [25]. Figure 4 shows the UV–Vis spectra of the four compounds in aqueous 10% (v/v) ethanolic buffer solutions with different CD concentrations. In the case of curcumin and bisdemethoxycurcumin, the absorbtion at 280–390 nm (curcumin) and 270–370 nm (bisdemethoxycurcumin) is reduced as the CD concentration increases, whereas the absorption maximum at 430 nm (curcumin) and 415 nm (bisdemethoxycurcumin) is increased. The changes in the UV–Vis spectra of the diketone and ethyl

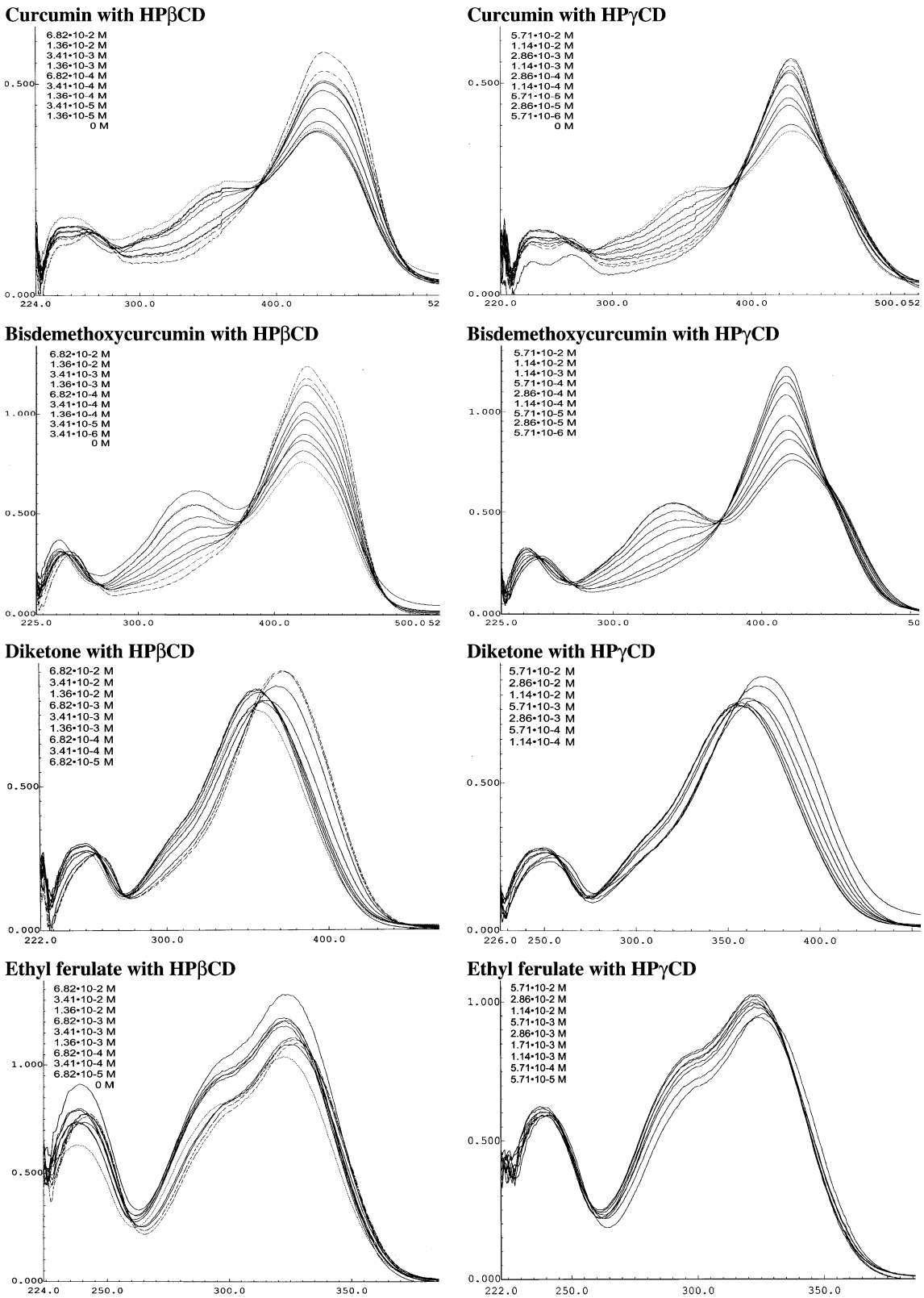

Fig. 4 The UV–Vis overlay absorption spectra of the four compounds with HPβCD and HPγCD (the CD concentrations are presented in the spectra) in 10% (v/v) ethanolic buffer solutions. The intensity of the main absorption maximum increases with increasing CD concentration

ferulate are much less marked, but in both cases there is a shift and increase in the main absorption maximum at 350 nm (diketone) and 320 nm (ethyl ferulate). The UV–Vis spectra of curcumin and bisdemethoxycurcumin show the presence of isosbestic points at 390 nm for curcumin and 370 nm for bisdemethoxycurcumin. Isosbestic points would indicate 1:1 complexation [58, 61]. Clearly distinguished isosbestic points were not observed for the diketone or ethyl ferulate.

The detection wavelength giving the greatest spectral change (i.e. largest ΔA values) was selected on the basis of the UV-scans at different concentrations of CDs. The absorption at the detection wavelenght increased when the CD concentration was increased. The calculated ΔA values were plotted against the logarithm of the CD-concentration (M) ($Mw_{HP\gamma CD} \approx 1{,}576$ g/mol and $Mw_{HP\beta CD} \approx 1{,}320$ g/mol) by use of Kaleidagraph 4.0 software and a non-linear curve fitting was used to determine the stoichiometry and complexation constant from the experimental data [59, 61] (Fig. 5). The data was fitted to the 1:1 binding isotherm given by Eq. 18. The data for curcumin and bisdemethoxycurcumin was also fitted to the 1:2 binding isotherm, given by Eq. 19, because the result showed some indication of systematic deviation from the 1:1 binding isotherm.

The 1:1 binding isotherm

Total absorbance (A) was determined by the following equation where the optical path length was 1 cm:

$$A = \varepsilon[C] + \varepsilon_{1:1}[C/CD] \quad (12)$$

where ε is the molar absorptivity. The CD absorption was not included in the equation because the molar absorptivity was very low at the given wavelength and the spectra were corrected for the CD absorption background signal. The total curcumin concentration is given by Eq. 13:

$$[C]_{total} = [C] + [C/CD] \quad (13)$$

Combining Eqs. 12 and 13 leads to Eq. 14:

$$A = \varepsilon[C]_{total} + \Delta\varepsilon_{1:1}[C/CD] \quad (14)$$

where $\Delta\varepsilon_{1:1}$ is:

$$\Delta\varepsilon_{complex} = \varepsilon_{1:1} - \varepsilon_c \quad (15)$$

$$A - A_0 = \Delta A = \Delta\varepsilon_{1:1} K_{1:1}[C][CD] \quad (16)$$

where A_0 is the absorbance of test compound when no CD is present. The concentration of the compound can be given as:

$$[C] = CD/(1 + K_{1:1}[CD]) \quad (17)$$

And thus,

$$\Delta A = \Delta\varepsilon_{1:1}[C]_{total}(K_{1:1}[CD])/(1 + K_{1:1}[CD]) \quad (18)$$

The 1:2 binding isotherm

A similar approach can be used to find an equation to describe the ΔA when also 1:2 complex formation is possible [61], giving the equation:

$$\Delta A = [C]_{total}\left(\Delta\varepsilon_{1:1} K_{1:1}[CD] + \Delta\varepsilon_{1:2} K_{1:1} K_{1:2}[CD]^2\right) \Big/ \left(1 + K_{1:1}[CD] + K_{1:1} K_{1:2}[CD]^2\right) \quad (19)$$

where $\Delta\varepsilon_{1:2}$ is the change in absorbtivity with the formation of 1:2 complex.

In all cases the data could be fitted to the 1:1 binding isotherm ($R^2 > 0.98$). Some deviation from the 1:1 binding curve was observed in the case of curcumin and bisdemethoxycurcumin and the data for these two compounds was therefore fitted to the 1:2 binding isotherm, but only a slight increase in the R^2 value was observed. However, a good fit to the 1:2 model required a very large $\Delta\varepsilon_{1:2}[C]_{total}$ value, which is unrealistic in this case. Thus, the UV–Vis titration data clearly showed 1:1 complexation with some indication of higher order complexation, although this could not be confirmed from the current data. The data for curcumin and bisdemethoxycurcumin are in contrast to the phase-solubility data where the curved A_p type diagram clearly indicates higher order complexation and possible aggregation of the complexes [25–29]. The UV–Vis data for the diketone and ethyl ferulate could be fitted to the 1:1 binding isotherm, with minimal deviation between the obtained data and the calculated curve. This result was consistent with the linear phase-solubility diagrams.

Complexation constants

The complexation constants were calculated by use of Kaleidagraph 4.0 software, by curve-fitting the experimental data points from the UV–Vis and phase-solubility investigations. The resulting complexation constants could then be compared for the two methods. The complexation constants obtained from the UV–Vis investigations and the observed intrinsic solubility could also be used to predict solubility at 10% (w/v) CD concentration. This value could be compared to the solubility at 10% (w/v) CD concentration determined by HPLC. The results are shown in Table 4.

The 1:1 complexation constants obtained for curcumin and bisdemethoxycurcumin by the UV–Vis titration method are in general 3-4 times less than the 1:1 complexation constants obtained by the phase-solubility method. In general, the predicted and determined solubility in 10% (w/v) CD are more consistent with the diketone and ethyl ferulate which apparently only form 1:1 complexes

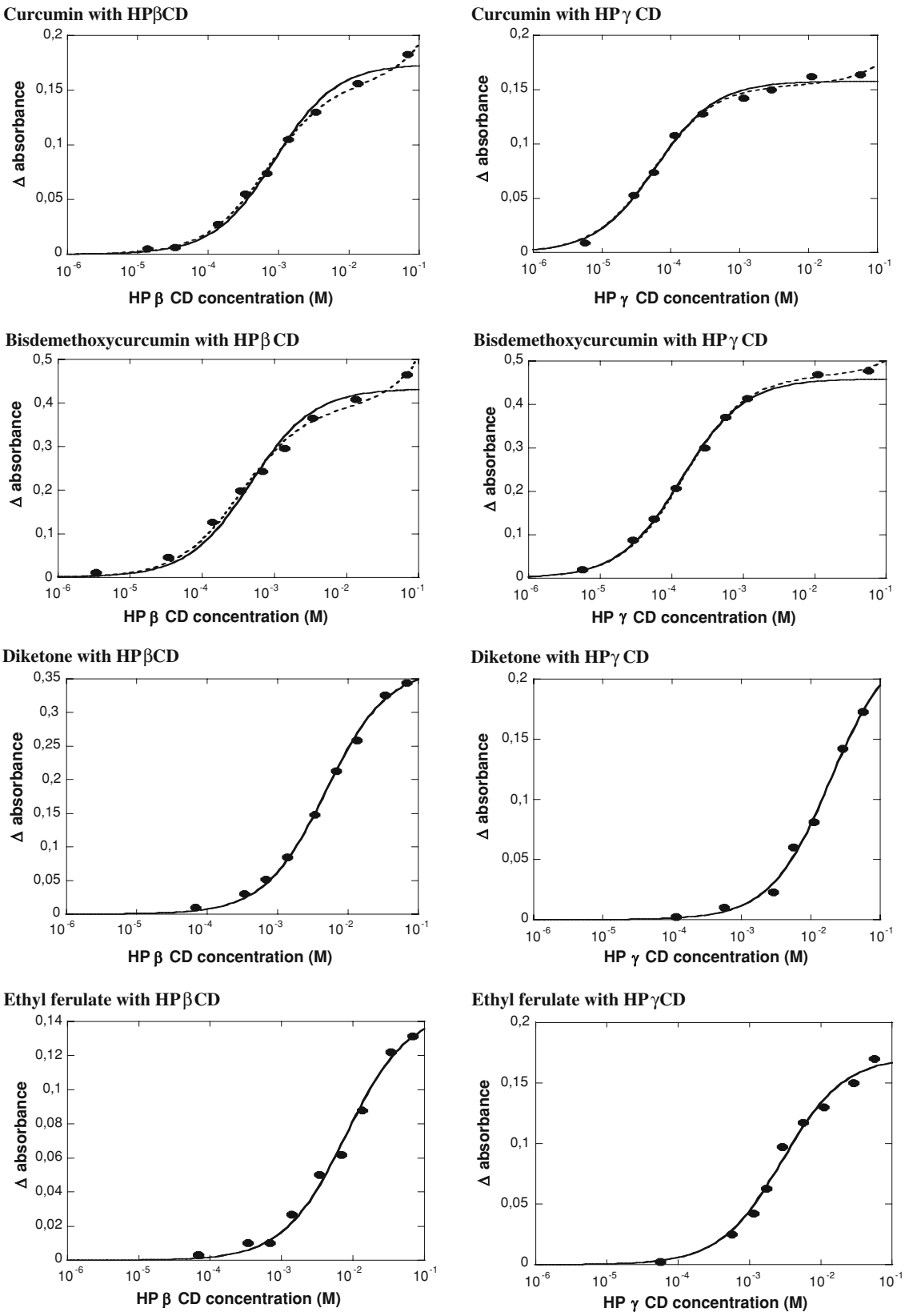

Fig. 5 The non-linear curve fitting of the data from the UV–Vis titration method, presented in Fig. 4, showing changes in the absorption at the detection wavelenght as a function of the cyclodextrin concentration. The solid line represents curve-fitting to the 1:1 binding isotherm, while the broken line represents curve-fitting to the 1:2 binding isotherm

Table 4 The complexation constants obtained from UV–Vis titration and phase-solubility (PS) investigation, intrinsic solubility in 10% (v/v) ethanol, the predicted solubility in 10% (w/v) CD concentrations (Calc.Sol. UV–Vis), based on the UV–Vis data, and the solubility in 10% (w/v) CD concentrations (Measured sol. PS) determined by HPLC

Compound	$K_{1:1}$ UV–Vis (M^{-1})	$K_{1:2}$ UV–Vis (M^{-1})	$K_{1:1}$ PS (M^{-1})	$K_{1:2}$ PS (M^{-1})	Intrinsic sol. (S_o) (M)	Calc. Slope UV–Vis	Calc. Sol. UV–Vis 10% CD (M)	Measured sol. PS 10% CD (M)
Curcumin					6×10^{-8}			
HPβCD	1.44×10^{3}	2.3×10^{-1}	4.42×10^{3}	4.0×10^{1}			6.74×10^{-6}	8.11×10^{-5}
HPγCD	1.75×10^{4}	7.6×10^{-2}	6.86×10^{4}	9.0×10^{1}			6.81×10^{-5}	1.77×10^{-3}
Bidsemethoxy-curcumin					1.71×10^{-7}			
HPβCD	2.87×10^{3}	2.7×10^{-1}	1.10×10^{4}	1.01×10^{2}			3.82×10^{-5}	1.24×10^{-3}
HPγCD	6.90×10^{3}	7.4×10^{-2}	No fit	No fit			9.04×10^{-5}	5.89×10^{-3}
Diketone					3.4×10^{-4}			
HPβCD	2.05×10^{2}		8.0×10^{1}			6.5×10^{-2}	5.3×10^{-3}	2.45×10^{-3}
HPγCD	5.20×10^{1}		6.8×10^{1}			1.7×10^{-2}	1.43×10^{-3}	1.87×10^{-3}
Ethyl ferulate					3.36×10^{-3}			
HPβCD	1.27×10^{2}		2.43×10^{2}			3×10^{-1}	3×10^{-2}	4×10^{-2}
HPγCD	3.49×10^{2}		2.39×10^{3}			5.4×10^{-1}	4×10^{-2}	6×10^{-2}

and also have higher intrinsic solubility than the curcuminoids. The exception is the HPγCD complexation of ethyl ferulate. In this case the 1:1 complexation constant predicted from the phase-solubility investigation (2,390 M^{-1}) is much larger than the constant predicted using the UV–Vis method. This may partially be explained by the high solubility of ethyl ferulate. A small variation in the slope of the phase-solubility diagram will, due to the high solubility, have significant effect on the calculated complexation constant. Generally, there is a significant difference in the 1:1 constants obtained by the two methods, especially for the low solubility compounds. However, the two methods in general agree in ranking of the complexes, i.e. the complexation strength and the relative difference between the complexation constants. The difference between the two methods is more distinct when 1:2 complexation is considered. In this case there is more than a 100 fold difference in the calculated 1:2 complexation constants, causing the discrepancy between the predicted solubility of curcumin and bisdemethoxycurcumin in 10% (w/v) CD, using the UV–Vis data, and the solubility determined by HPLC. While the UV–Vis data showed slight indication of 1:2 complexation, the phase-solubility diagram clearly showed positive deviation from linearity (A_P type phase-solubility diagram). Previously, we have proposed that some features in the phase-solubility diagrams of poorly soluble compounds strongly indicate aggregation of the CD complexes. Partial solubilization due to complex aggregation and/or some phenomena other than direct inclusion complex formation could therefore explain the current observations [25–29].

Conclusion

Complexation constants and stoichiometry for four compounds, two symmetric curcuminoids (curcumin and bisdemethoxycurcumin) and two unsymmetric curcuminoid-like compounds with just one substituted phenyl moiety (diketone and ethyl ferulate), were investigated by use of two independent methods, the phase-solubility method and the UV–Vis titration. The compounds were synthesised and purified in our lab to avoid contamination of other compounds, which might influenc the results. The compounds were identified and confirmed to be pure by use of TLC, melting point determination, IR, UV–Vis, NMR and HPLC. Ionization of the compounds by increasing the pH did not sufficiently increase solubility, and did in some cases lead to degradation. Thus pH 5.0 or 6.0 could be considered optimal for the study of CD complexation. The intrinsic solubility of all four compounds was increased to above LOQ by using 10% (v/v) ethanolic solutions, thus complex stoichiometry and complexation constants obtained by the two methods could be compared. Phase-solubility investigations showed that the symmetric compounds formed higher order complexes with possible aggregation of complexes, while the unsymmetric compounds formed 1:1 complexes. The UV–Vis investigation showed 1:1 complexation for all compounds confirmed by isosbestic points in the absorption spectra for the symmetric compounds. In the case of the symmetric compounds there was however some indication of 1:2 complexation. Thus the stoichiometry found with the two methods correlated well for the unsymmetric compounds,

but not for the symmetric compounds, where the phase-solubility investigation clearly indicated higher order complexation and possible aggregation of complexes. There was also a difference between the 1:1 complexation constants found with the two methods, especially for the compounds with low intrinsic solubility (i.e. the symmetric curcuminoids). The methods were consistent concerning the ranking of complexes, i.e. complexation strength and relative difference in complexation constants. The 1:2 complexation constants observed with the phase-solubility method was more than 100 times the complexation constant found with the UV–Vis method, which explains why the predicted solubility in 10% (w/v) CD, based on the UV–Vis data, is different from the solubility in 10% (w/v) CD determined by HPLC. This discrepancy may be explained by solubilization by aggregation of complexes or some phenomena other than inclusion complexation.

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