

Phase solubility studies and stability of cholesterol/ β -cyclodextrin inclusion complexes

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Abstract

BACKGROUND: Cyclodextrins (CDs) are able to enhance the solubility, stability and bioavailability of several bioactive hydrophobic compounds by complex formation. They can also be used for removal of undesired components (such as cholesterol, off-flavors or bitter components) present in foods. Although many patents account for the use of cyclodextrins for removal of cholesterol from dairy foods, there is no available information on the effect of water on encapsulation efficiency and on the stability of sterols in CDs. The aim of this work was to study the inclusion properties and the factors affecting the encapsulation and stability of cholesterol in β -cyclodextrin (BCD). The optimum encapsulation conditions (ligand-CD molar ratio, stirring time and temperature), and stability of the complexes as a function of storage time and water content were analyzed.

RESULTS: Phase solubility study pointed out the formation of 1:1 stoichiometric complexes between cholesterol and β -cyclodextrin, which was influenced by temperature variations. The process was shown to be exothermic and energetically favored. The presence of cholesterol greatly modified the BCD water sorption curves, being the amount of adsorbed water smaller in the combined systems. The principal 'driving force' for complex formation is the substitution of the high-enthalpy water molecules by an appropriate hydrophobic ligand. The freeze-dried complexes probed to be stable at different storage conditions.

CONCLUSION: The phase solubility and stability data obtained could be essential for selecting the most suitable conditions when CDs are employed either for removing cholesterol or to incorporate functional ingredients (i.e. sitosterol) in the development of innovative food products.

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Keywords: cyclodextrin; cholesterol; stability constants; phase solubility

INTRODUCTION

Foods are now not only intended to provide necessary nutrients, but also to prevent nutrition-related diseases and improve physical and mental well-being of the consumers.¹⁻⁴ It can be assumed that cholesterol-lowering and cholesterol-free products or those containing phytosterol esters, which are considered to lower the cholesterol levels, will gain increasing relevance in the coming years.⁵ Food companies have developed many methods to reduce cholesterol in foods, such as blending in vegetable oils,⁶ extraction by organic solvents,⁷ cholesterol degradation by cholesterol oxidases⁸ and removal by supercritical carbon dioxide extractions.⁹ However, most of these are relatively non-selective methods, and when cholesterol is removed, flavor and nutritional components are removed as well. Moreover, some methods require high investment and have significant operation costs.

Encapsulation in cyclodextrins (CDs) is a method that has been used during the last few years in the cosmetics, drug and food industries for many purposes. As a result of their molecular structure and shape, CDs have the ability to act as molecular containers by entrapping guest molecules in their internal cavity. They are cyclic oligosaccharides composed of six, seven or eight

α -D-glucopyranose units with a relatively hydrophobic central cavity and hydrophilic outer surface.¹⁰ They are chemically and physically stable molecules, obtained by the enzymatic modification of starch.¹¹ The hydrophobic cavity forms inclusion complexes with a wide range of guest molecules.^{12,13} Non-covalent bonds are formed or broken during the complex formation, and in aqueous solution, the complexes readily dissociate remaining the released molecules in equilibrium with the molecules bound within the CD cavity. In the practical application of CDs, attention should be directed towards the dissociation equilibrium and

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stoichiometry of the inclusion complex. The stability constant (K_c) is a useful index to estimate the binding strength of the complex and the changes in the physicochemical properties of the guest molecule.¹⁴

The physical, chemical and biological properties of compounds encapsulated in CDs may be drastically modified.¹⁵ CDs act as flavor carriers, protect against oxidation, light-induced decomposition and heat-induced changes. Moreover, they improve shelf life of food products and mask or reduce undesired tastes.¹⁶ Encapsulation leads to enhancing dissolution rate, membrane permeability and bioavailability of nutraceuticals of low solubility such as sterols and others lipophilic compounds.^{12,17} In particular, β -cyclodextrin consists of seven α -1,4-linked D-glucopyranosyl units and provides advantages when used for removal of cholesterol from foods.^{18–22} It is non-toxic, edible, non-hygroscopic, chemically stable and is easy to separate from the complex.²³

The aim of this work was to study the inclusion properties and the factors affecting the encapsulation and stability of cholesterol in β -cyclodextrin. The optimum encapsulation conditions (ligand–CD ratio, temperature and stirring time), and the stability of the complexes as a function of storage time and water content were analyzed. The solubility of cholesterol was looked at by phase solubility studies and the thermodynamic parameters involved in the complex formation were calculated.

Although many studies and patents account for the use of CDs in removal of cholesterol from dairy foods,^{24,25} there is no available information on the effect of water on encapsulation efficiency and on the stability of sterols in CDs. Cholesterol serves also as a model ligand in order to understand inclusion, stability or controlled release of some additives and nutritional flavors of low solubility in polar solvents which could be of interest in food technology.

EXPERIMENTAL

β -Cyclodextrin (BCD) (containing eight water molecules/molecule of BCD, Mr 1135) and cholesterol (Cho) were from Sigma Chemical Co. (CAS No. 57-88-5, $C_{27}H_{46}O$, MW 386.65, boiling point 360 °C, melting point 140–150 °C, density 1.07 g mL⁻¹, vapor pressure 0.00 mm Hg at 25 °C). All other chemicals were of analytical grade and purchased from Mallinckrodt Chemical Works (St Louis, MO, USA).

Phase solubility studies

Phase solubility studies were carried out according to the method described by Higuchi and Connors.²⁶ An excess amount of cholesterol (5 mg L⁻¹) was mixed in an aqueous solution containing increasing amounts of BCD (0–15 mmol L⁻¹) using a laboratory shaker (Lab-Line® Orbit Environ-shaker; Lab-line Instruments, Melrose Park, IL, USA) at 28, 55, 60, and 65 °C during 48 h. In order to separate the excess of cholesterol, the samples were centrifuged at 15 500 × *g* for 20 min and the supernatants were filtered through 0.45 μ m PTFE filters. Then, the concentration of cholesterol in each aqueous solution or 'apparent solubility' (which comprises the free cholesterol plus the cholesterol complexed with BCD) was spectrophotometrically determined with a commercial enzymatic kit (Colestat; Wiener Laboratories, Rosario, Argentina) at 505 nm. The method was adapted to the experimental conditions, with a calibration curve made preparing adequate dilutions from a saturated Cho aqueous solution at 25 °C (5 mg L⁻¹). The experiments were carried out in triplicate at each temperature.

The stability constants, K_c , were calculated from the straight-line portion of the phase solubility diagram according to the Higuchi–Connors equation (Equation 1):

$$K_c = \frac{\text{slope}}{S_0 \times (1 - \text{slope})} \quad (1)$$

where S_0 is the ligand solubility in the absence of BCD.

Preparation of the solid inclusion complexes

Inclusion complexes of BCD with cholesterol (guest molecule) were prepared by the co-precipitation method.¹⁷ Saturated solutions of BCD (15 mmol L⁻¹) were prepared, heated at 50 °C and shaken until complete solubilization of the BCD. Cholesterol was dispersed in the BCD aqueous solution at suitable proportions for Cho/BCD molar ratios 1:1, 1:2 and 1:3 and the systems were stirred at a constant rate for 1 h at 50 °C. The experimental temperature for complex formation was selected taking into account that the solubility of Cho and BCD increases as increasing temperature but the encapsulation is an exothermal process and the degree of complexation decreases as increasing temperature.^{10,14,26,27} Thus, at 50 °C an adequate solubility for BCD and complexation is achieved. The obtained solutions were allowed to cool and maintained at 25 °C for 24 or 48 h under agitation. In order to favor the precipitation of Cho/BCD complexes the solutions were then stored overnight at 3 °C and filtered (PTFE filters of 0.45 μ m average pore diameter). The filtrates were frozen at –26 °C for 24 h and freeze dried in a Heto Holten A/S freeze dryer (operating at a condenser plate temperature of –111 °C, chamber pressure of 30 Pa, and shelf temperature of 25 °C). The secondary drying was also performed at 25 °C.

Once dehydrated, the systems were equilibrated to different relative humidities and water sorption isotherms and melting events in the samples were studied. Since the employed BCD was obtained as an octahydrate, it was dried in a vacuum oven (48 h at 90 °C) up to water content smaller than 3% dry basis (d.b.), before the experiments.

Preparation of the physical mixture

Binary systems consisting of Cho and BCD in Cho/BCD molar ratio 1:1 and 1:3 were obtained by physical mixture (PM). The corresponding amounts of the simple components for each molar ratio were admixed together in a mortar and pestle for 5 min to obtain homogeneous blends.

Determination of the water sorption isotherms

Sorption isotherms were determined by the standard isopiestic static gravimetric method. After freeze drying, samples of BCD or their complexes with Cho were distributed into glass 5 mL vials (around 200 mg vial⁻¹) and placed into vacuum desiccators containing saturated salt solutions which provide different relative humidities (RH): LiCl, KCOOCH₃·5H₂O, MgCl₂·6H₂O, K₂CO₃·2H₂O, MgNO₃·6H₂O, NaBr·2H₂O, NaCl, KCl, (NH₄)₂SO₄ and K₂SO₄ for 11, 22, 33, 43, 52, 57, 75, 84, 95 and 97% RH at 25 °C ± 1 °C, respectively.²⁸ The water content of the samples was determined as a function of storage time until reaching the equilibrium condition (mass differences lower than 0.0005 g). The longer time to reach this condition of constant mass was 2 weeks.

Determination of water content

The total water content of the samples was determined by difference in weight before and after drying in a vacuum oven at $96 \pm 2^\circ\text{C}$ during 48 h. These drying conditions had been proved to be adequate to assess constant weight after drying and were selected from previous studies. The determinations were performed in duplicate and the average value was reported. The calculated confidence interval for a 95% certainty was between 3% and 4% of the absolute values. Results were expressed as % dry basis (% d.b.).

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to verify the formation of the complexes and the release of the guest molecule during storage. A DSC, Mettler–Toledo equipment model 822 (Mettler Toledo AG, Greifensee, Switzerland), with a STARe Thermal Analysis System version 3.1 software (Mettler Toledo AG), was used for all the measurements. The instrument was calibrated with indium and zinc. All measurements were made at $10^\circ\text{C min}^{-1}$, using hermetically sealed aluminium pans (Mettler, 40 μL capacity), and an empty pan was used as a reference. An average value of two replicate samples was reported for each measurement. The confidence interval estimated for temperature values was 1°C .

The dynamic method was used to determine melting points (T_m) and heats of fusion (ΔH_m) of the pure cholesterol and of the ligand in the complexes. T_m for Cho was 150°C (Merck Index) taken as the onset of the melting peak. Each sample was heated at a rate of $10^\circ\text{C min}^{-1}$ from -20°C up to 180°C . The percentage of free Cho (ligand; that which has been released or that which was not encapsulated) in the BCD after humidification and during storage was determined as the ratio of the fusion enthalpy of cholesterol in the system at time, t (corrected according to the water content of the samples) and the fusion enthalpy of the pure compound. Then, the percentage of encapsulated ligand was expressed as indicated in the following equation:

$$\% \text{ encapsulated} = 100 \times \left(1 - \frac{\Delta H_L}{\Delta H_0} \right) \quad (2)$$

where ΔH_L is the heat of melting of Cho free in the systems and ΔH_0 is the heat of melting of pure cholesterol. Since the boiling point of Cho is 360°C the losses by evaporation were considered negligible.

Duplicate determinations were carried out for each sample and the average values were reported. The calculated confidence interval for a 95% certainty was between 4% and 6% of the absolute values.

Storage study

The % of encapsulated Cho in BCD after equilibration at different RH and after storage in the vacuum desiccators at 25°C was evaluated by DSC as indicated in Equation 2. Average values of duplicate analysis are reported.

RESULTS

Cholesterol complexes with β -cyclodextrin were generated in solution as well as in the solid state. Phase solubility studies were carried out in aqueous systems at different temperatures to calculate the stability constants (K_c) (or binding constants), the molar ratio of the complex Cho/BCD and the thermodynamic

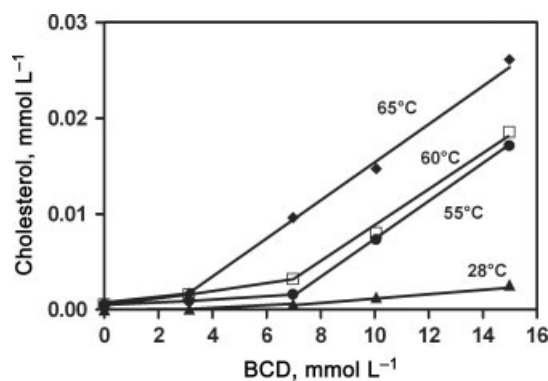


Figure 1. Phase solubility diagrams of cholesterol with β -cyclodextrin at 28, 55, 60 and 65°C .

Table 1. Aqueous solubility of cholesterol in absence of BCD (S_0), stability constants (K_c) obtained for the Cho/BCD complexes in the range 50 – 65°C calculated according to the Higuchi–Connors method²⁶ and the correlation coefficients (R^2)

Temperature ($^\circ\text{C}$)	S_0 (mmol L^{-1})	K_c (mol L^{-1})	R^2
55	0.0001	5214	0.9998
60	0.0005	3406	0.9829
65	0.0008	2637	0.9961

values for the formation of the complexes. The phase solubility diagrams made at 28, 55, 60 and 65°C are shown in Fig. 1. The plots showed that the solubility of Cho changes very slightly at 28°C in the whole BCD concentration range. At higher temperatures, Cho solubility increases markedly from a 7 mmol L^{-1} BCD concentration, with a linear dependence. Consequently, according to Higuchi and Connors²⁶ the plots can be considered as AL-type diagrams at concentrations from 7 mmol L^{-1} BCD, suggesting the formation of 1 : 1 complexes. As also shown in Fig. 1, the solubility of cholesterol in water increased as the temperature increased. Williams *et al.*²⁷ reported a similar dependence of Cho solubility in hydroxypropyl-BCD. At low BCD concentrations ($<7 \text{ mmol L}^{-1}$) the poor aqueous solubility of pure cholesterol makes the determination of solubility changes more difficult. Under these conditions, cholesterol determination may be also disturbed by self-association of cholesterol in the solution.^{27,29}

The stability constants, K_c , of the complexes at the studied temperatures were calculated from the straight-line portion of the phase solubility diagram using Equation 1. Table 1 shows the obtained values for S_0 (the solubility when BCD concentration is 0), K_c at the studied temperatures, and the correlation coefficients, R^2 . The data at 28°C were not included since the Higuchi–Connors method cannot be applied when no significant solubility change is observed as a function of BCD concentration.²⁷ The K_c values obtained for temperatures in the range 50 – 65°C , plotted in a Van't Hoff plot are shown in Fig. 2. The complex Cho/BCD showed a linear function between K_c and the inverse of the absolute temperature ($1/T$). The stability constants decreased with increasing temperature, as expected for an exothermic process. K_c values are useful indexes to estimate the binding strength of the ligand with the host:¹⁴ a small K_c value indicates a higher relative amount of free ligand due to a weak interaction between cholesterol and BCD. On the other side, a large K_c indicates that the equilibrium is displaced towards the complex formation and a

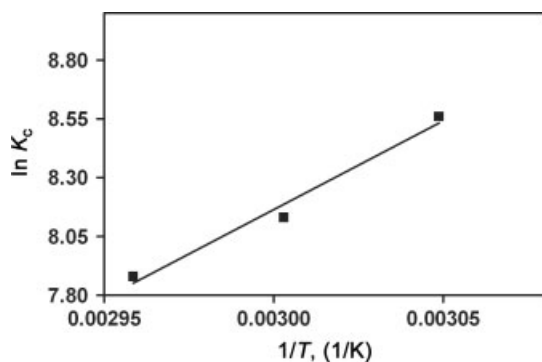


Figure 2. Van't Hoff plot of the formation of the complex between cholesterol and β -cyclodextrin.

Table 2. Thermodynamic values for complex formation of cholesterol with BCD, compared with the values obtained by other authors for terpenoids, such as thymol and geraniol,³² terpineol,³³ and for the flavonones hesperetin and naringenin¹⁴ and vanillin¹⁷

Ligand	Thermodynamic parameter			Molecular weight
	ΔH (kJ mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)	ΔG_{25} (kJ mol ⁻¹)	
Cholesterol	-32	-61	-14	387.7
Terpineol	-68	-197	-13	154.3
Geraniol	-40	-60	-22	56.0
Thymol	-47	-82	-22	150.2
Hesperetin	-35.5	-71.3	-14.3	302.3
Naringenin	-50	-120	-14	272.3
Vanillin	-7	-12	-3.5	152.2

limited release of ligand. Similar temperature effect on the stability constants are given by other authors working on the solubility of different compounds encapsulated in BCD.^{14,17,30}

As shown in Table 1, K_c values were lower at higher temperatures but the solubility of cholesterol (S_o) was higher at higher temperatures. It has to be noted that in the Higuchi and Connors method applied, K_c was obtained from the apparent cholesterol solubility, which comprises free cholesterol plus cholesterol encapsulated in BCD. Apparent solubility of cholesterol depends, among other factors, on temperature and on BCD presence, which forms soluble complexes with Cho. Increasing temperature, increases Cho solubility in water (increasing S_o), but it mainly favors the release of cholesterol from the internal cavity of BCD (decreasing K_c). The effect of temperature on S_o is less important than the increase of apparent solubility by the encapsulation process. Then, the global result of a temperature increase is the increase of total amount of cholesterol in aqueous phase.

The phase solubility data made at different temperatures allowed to obtain additional information such as the thermodynamic parameters involved in the formation of the complex. The integrated form of the Van't Hoff equation allows the calculation of the enthalpy values and of entropy changes, depending on the variations of the stability constants with temperature.^{14,31}

$$\ln K_c = -\frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R} \quad (3)$$

The relative thermodynamic parameters were calculated and are given in Table 2, compared with the values obtained by other

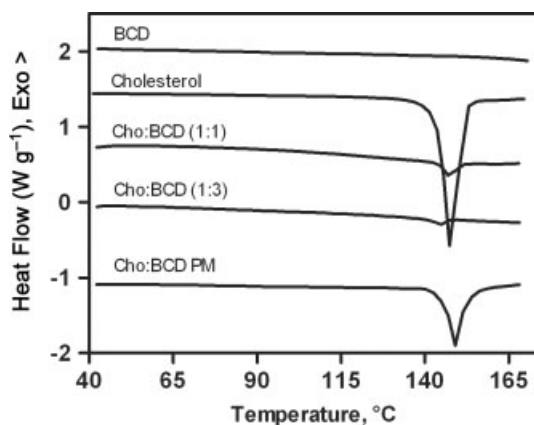


Figure 3. Differential scanning calorimetry (DSC) thermograms of cholesterol/ β -cyclodextrin systems (Cho/BCD) prepared by freeze drying (ligand:CD molar ratios, 1:1 and 1:3) and by physical mixture (PM) (ligand:CD molar ratio, 1:1), compared with the thermograms of pure cholesterol (Cho) and β -cyclodextrin (BCD). Scanning rate 10 °C min⁻¹.

authors for terpenoids such as thymol and geraniol,³² terpineol,³³ and for the flavonones hesperetin and naringenin,¹⁴ and vanillin.¹⁷ The negative value of the enthalpy change (ΔH) indicates that the interaction process of the ligands with BCD, leading to complex formation, is exothermic. The relatively small enthalpy change is typical of low energy interactions prevailing also in flavor-BCD complexes. They could derive from new interactions, like hydrophobic ones (due to the displacement of the water molecules from the cavity of the BCD by the more hydrophobic ligand), increase of Van der Waals interactions between the molecules, formation of hydrogen bonds and other low energy interactions.^{14,17}

The negative values observed for the entropy changes can be explained considering that inclusion moderately hinders the free rotation of the included molecule around its symmetry axis.^{14,17}

The Gibbs free energy change for the interactions that take place during the inclusion process was calculated using the following equation:

$$\Delta G_{25} = \Delta H - T\Delta S \quad (4)$$

The negative value of ΔG_{25} for cholesterol inclusion, as for the rest of the ligands (Table 2), indicates that the inclusion process in BCD is spontaneous.

Differential scanning calorimetry was used to verify the formation of the complex in the solid state.¹⁷ The total or partial disappearance of thermal events (melting point) corresponding to guest molecules, when they are examined as CD complexes, is generally taken as a proof of complex formation.^{27,34}

Thermograms obtained by DSC for cholesterol, BCD and for the freeze-dried Cho/BCD complexes, both in 1:1 and 1:3 ligand:CD molar ratios, are shown in Fig. 3. The thermogram for pure cholesterol shows an endothermic peak close to 150 °C, corresponding to its melting. However, for the combined systems the melting enthalpy value corrected by the mass of cholesterol in the system was very small indicating that most of the Cho was not free and was largely encapsulated in the host BCD. The thermograms obtained for systems prepared by physical mixture (shown in Fig. 3 for the 1:1 ratio), presented endothermic peaks indicating that cholesterol was not encapsulated in BCD by this procedure. At both molar ratios 1:1 and 1:3 the integration of these signals demonstrated that the remaining free Cho was 97 ± 2% (Equation 2).

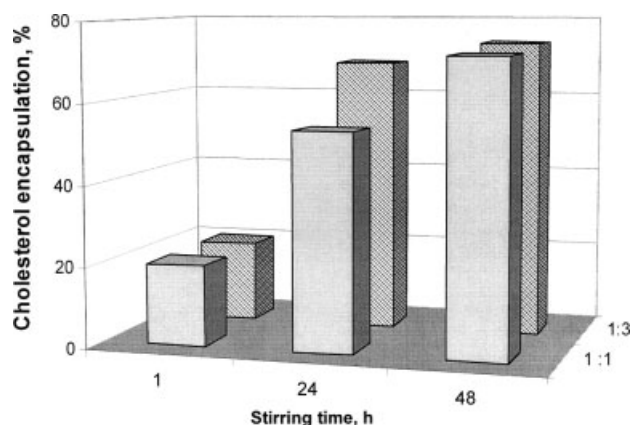


Figure 4. Effect of molar ratio (ligand : CD, 1 : 1 and 1 : 3) and stirring time on the percentage of cholesterol encapsulated in β -cyclodextrin solutions at 25 °C. The calculated confidence interval for a 95% certainty was between 4% and 6% of the absolute values.

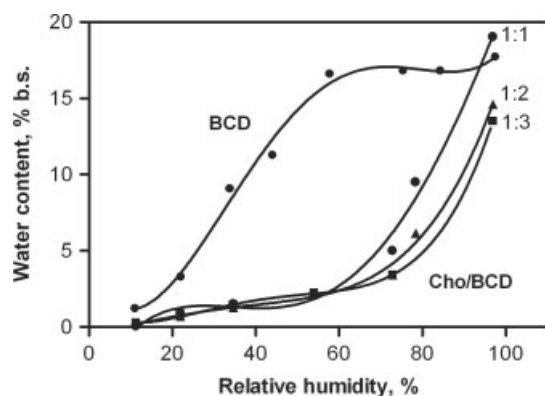


Figure 5. Water adsorption isotherms of β -cyclodextrin (BCD) and the complexes cholesterol/ β -cyclodextrin (Cho/BCD) in 1 : 1, 1 : 2 and 1 : 3 ligand : CD molar ratios.

When the complexes of CD and a convenient ligand are obtained by freeze drying, the whole ligand will be in the final system, either free or encapsulated. The complete disappearance of the endothermic DSC signal is a strong evidence of the total inclusion of the ligand inside the cavity of CD.^{27,34} If the disappearance of the thermal signal in the thermograms is only partial, the inclusion may not be completed. Therefore, the ratio between this signal and the theoretical value of the melting enthalpy of the ligand is an approximation to quantify the amount of free ligand in the system.³⁵

The effect of the preparation conditions, Cho/BCD molar ratio and stirring time, on the maximum Cho encapsulation in solid systems is shown in Fig. 4. There were no significant differences in the cholesterol encapsulation efficiency between 1 : 1 and 1 : 3 ratios that were both prepared by stirring for 48 h. It can be thus stated that the optimum encapsulation of cholesterol in BCD was achieved at Cho/BCD molar ratio 1 : 1 and stirring time of 48 h, as confirmed in the freeze-dried samples by DSC.

Considering the high influence of water interactions on complex formation and stability, when the dry formulations of CD containing the active components are stored, the analysis of the water adsorption behavior becomes of fundamental importance to define the appropriate storage conditions.³⁶ Fig. 5 shows the effect of complex formation on the water adsorption isotherms

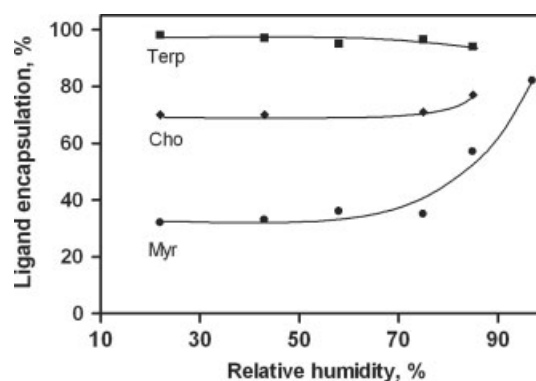


Figure 6. Percentage of encapsulated ligand in freeze-dried BCD systems stored at different RHs at 25 °C during 65 days. Data values determined previously for terpineol,³⁵ another terpenoid flavor compound, and myristic acid³⁹ were included for comparative purposes. Cho: cholesterol; Terp: α -terpineol; Myr: myristic acid. All systems were prepared in 1 : 1 ligand : CD molar ratio.

of β -cyclodextrin. The BCD isotherm does not show the typical sigmoid shape of amorphous food systems and reaches a plateau at 52% RH due to the formation of a stable crystalline hydrate.³⁷ The presence of cholesterol greatly modified the CD water sorption curves. The complexes adsorbed less water than BCD and did not show a plateau which indicates that no hydrates were formed. The water sorption isotherm for the complexes followed a sigmoid-type behavior, with the three characteristic adsorption stages,³⁸ with a marked increase of water content at relative humidities higher than 80%, indicating the beginning of the third sorption stage. The water content increase observed at RH 80% (Fig. 5) did not conducted to the release of cholesterol. On the contrary, the aqueous environment favored cholesterol encapsulation, as will be discussed latter.

Not only are the thermodynamic parameters of complex formation of interest, but also kinetics and stability aspects of the complexes as a function of relative humidity. Figure 6 shows the stability of the complexes Cho/BCD stored during 65 days at 25 °C at different RH. The values determined previously for a terpenoid flavor compound such as terpineol and for myristic acid,³⁹ were included for comparative purposes. The calorimetric studies performed to determine complex stability showed different types of behavior. As shown in Fig. 6, at RH values up to 75%, 70% of cholesterol remained encapsulated after 65 days at 25 °C. At 84% RH the amount of encapsulated increased up to 76%. Terpineol was completely encapsulated at the optimal molar ratio 1 : 1 and remained included even in systems stored at 80% RH. For more hydrophobic ligands than terpineol, like cholesterol and myristic acid, it can be observed that their inclusion was initially incomplete, but the encapsulation degree increased as increasing RH above 80% (Fig. 6) or the amount of CD in the system.³⁹ It is interesting to note that this RH is located in the third adsorption stage of the water sorption isotherm,³⁸ at which mobile water populations increase.^{40,41} The increase of cholesterol encapsulation during storage at high RH is the result of higher affinity of cholesterol for the hydrophobic inner BCD cavity than for the hydrophilic environment, where bulk water exists. During storage at 25 °C the amount of encapsulated Cho did not change at water contents lower than 80% (remaining at a level of about 70%), and a slight increase occurred at higher RH arriving to 75% of encapsulated Cho at 65 days.

DISCUSSION

Encapsulation improved the solubility of cholesterol in water. Phase solubility study pointed out the formation of 1 : 1 stoichiometric complexes between cholesterol and β -cyclodextrin, which was influenced by temperature variations. This process was shown to be exothermic and energetically favored. According to this, the equilibrium of complex formation was shifted towards the ligand release as increasing temperature.

The thermodynamic parameters obtained from the solubility data at different temperatures helped to explain certain phenomena regarding complex formation and BCD interactions with the ligands. Those data describe the dynamic equilibrium which is established between the ligand, the CD and the complex in aqueous media. The obtained entropy change value was negative, probably due to a decrease in translational and rotational degree of freedom for the complex, in comparison to the free molecules.

Once the complex is formed and dehydrated, the thermodynamic parameters obtained for aqueous systems are not enough to describe complex stability regarding relative humidity. Thus, the analysis of the water adsorption behavior becomes of fundamental importance to define the appropriate storage conditions of the dehydrated complexes. The 'driving force' for complexation is not yet completely understood,²⁵ but it seems that it is the result of various effects: substitution of water molecules from the inner cavity, which is energetically favored; the release of CD ring strain when the complex is formed; Van der Waals forces and hydrogen bond interactions, which are established when the complex is formed.^{14,36,39} The results presented support the idea of substitution of water molecules from the inner cavity by hydrophobic ligands. Furthermore, these results confirmed that the water molecules inside the cavity could be easily removed by compounds with the adequate size and hydrophobicity to occupy the BCD cavity, and thus, forming energetically favored inclusion complexes. In CDs aqueous solution, their non polar cavity is occupied by water molecules. This conformation is energetically unfavorable due to the existence of polar–non-polar interactions and therefore the water molecules can be really substituted by appropriate 'guest molecules' which are less polar than water.

The stability of the freeze-dried complex as a function of time was influenced by water content and storage temperature, and also by the nature of the ligand.

The obtained phase solubility and stability data may be useful to select the most suitable conditions in the production of special food products such as cholesterol-free or enriched with sterols (i.e. sitosterol) with health or medical benefits. These data could be essential in the development of innovative food products in which cyclodextrins are employed for removing cholesterol or when functional ingredients are incorporated.

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