Chapter 13

Obtention and Characterization of Cyclodextrins Complexes for the Development of Food Ingredients

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Abstract

Encapsulation is an interesting and feasible strategy to increase natural compounds solubility and stability, improving their application as food ingredients. In this sense, encapsulation in cyclodextrins emerges as a promising tool for the food industry. This chapter describes practical methods and protocols we have optimized for the obtention and characterization of inclusion complexes of different compounds such as α-terpineol, myristic acid, and cholesterol in β-cyclodextrin (BCD) or in a modified BCD as 2-hydroxypropyl-β-cyclodextrin (HPBCD). We show with these examples of relevance in foods, how the spatial configuration, size, and polarity of the ligand and of the cyclodextrin could significantly affect the degree of encapsulation, water interactions, thermodynamics parameters, and complex structure. We explain how the optimal encapsulation conditions (ligand:cyclodextrin molar ratio, temperature, and stirring time), water solubility, and complexes stability could be evaluated. Protocols about how to verify and study the encapsulation through spectrophotometry, water sorption, phase solubility, and differential scanning calorimetry (DSC) techniques are presented. The chapter provides information about methods of encapsulation of natural compounds in cyclodextrins and its analysis to develop ingredients for functional food formulations or nutraceuticals.

Key words Encapsulation, Cyclodextrins, Ultrasound, Food ingredients, DSC, Phase solubility studies

1 Introduction

Food processing has a particular interest in technologies of preservation, transformation, and extraction [1]. Nowadays, there is a growing consumer demand for foods formulated with natural compounds to replace synthetic additives or provide nutraceutical and/or nutritional value [2]. These compounds are frequently susceptible to unfavorable environmental, processing and/or gastrointestinal conditions. In this sense, encapsulation is an interest-

Andrea Gomez-Zavaglia (ed.), *Basic Protocols in Encapsulation of Food Ingredients*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-1649-9_13,

ing way to preserve them during processing and storage [3]. Among the methods, molecular encapsulation in cyclodextrins is an innovative and attractive option for the food industry [4].

1.1 Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of D-glucopyranose linked by α-1,4 glycosidic bonds, synthesized by enzymatic (cyclodextrin-glycosyltransferase) degradation of starch. α-CDs, β-CDs, and γ-CDs are natural cyclodextrins with six, seven, and eight glucose units, respectively [5]. They present a truncated cone spatial shape with hydrogen bonds between hydroxyl groups (HO) of glucose molecules. Cyclodextrin outer surface is hydrophilic, while the inner cavity is hydrophobic [6]. This spatial geometry gives them the possibility of interacting with a wide variety of compounds through noncovalent interactions and form host–guest complexes including molecules within their cavity [7]. The inclusion of these molecules, commonly called "ligands," changes their physicochemical properties. In this way, CDs have been used to increase compounds' solubility in aqueous medium and to improve the stability of bioactive compounds against degradation agents [3]. Studies for their oral administration have shown that natural CDs are not toxic due to their low or no absorption in the human intestine [8]. They are accepted as food additives in the United States and Japan and are listed by the Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) [7]. CDs can be used in many food products as carriers of polyunsaturated fatty acids (PUFA), vitamins, polyphenols, among others [9]. Currently, β-cyclodextrin (BCD) is the most commonly used in food and pharmaceutical formulations due to its low price, good availability, and proper cavity size allowing the inclusion of a wide range of compounds [10]. However, compared to other CDs, it has the lowest water solubility (*see* Table 1) due to its higher intramolecular interactions [11]. Some modifications are usually made on the natural BCD molecules by substituting hydroxyl groups of the

Table 1
Characteristics of α-, β-, γ-, and 2-hydroxypropyl-β cyclodextrins

CD type	Glucose units	Molecular weight (g/mol)	Cavity diameter (Å)	Water solubility at 25 °C (g/100 mL)
α-CD	6	972	4.7–5.3	14.5
β-CD	7	1135	6.0–6.5	1.85
γ-CD	8	1297	7.5–8.3	23.2
2-hydroxypropyl- βCD (HPBCD)	7	1400	6	>60[a]

Source: [14–16]
[a]The water solubility depends on the degree of substitution and location of the substituents

glucoses with hydroxypropyl or another residue to increase its water solubility. The different substituent groups and the degree of substitution conduct to notable changes in BCD properties and on its complexes [12]. The most used and low-cost modified cyclodextrin (CD) is the 2-hydroxypropyl-β-cyclodextrin (HPBCD). The number of hydroxypropyl groups (DS) in the HPBCD molecule is an important parameter, and the manufacturer usually provides this data. HPBCD (DS ≈ 6–7) has been approved by the European Medicine Agency (EMA) and the FDA as safe for humans due to its lower toxicity, higher water solubility nearly tenfold more soluble, and for certain types of ligands stronger inclusion ability than BCD [13].

Cyclodextrins have also the advantage of being neutral in terms of odor and taste which extend their possible applications [17]. For an efficient encapsulation, it is essential to select the appropriate type and concentration of CD and the right solvent. A dynamic equilibrium is established in the solution inclusion process, through noncovalent interactions and with a defined ligand:CD stoichiometry (Fig. 1). Different factors can promote the displacement of the equilibrium towards complex formation [18]. To reach that goal, it is necessary to define process variables such as time, temperature, and ligand:CD ratio. Complexes in solid state are commonly obtained through dehydration by freeze-drying or spray drying of the ligand:CD aqueous solutions, however these processes can affect the encapsulation. Therefore, complexes must be characterized/verified not only in solution but also in solid state.

When the solid CD complexes are hydrated under stirring, the equilibrium is reestablished since it is a reversible process [7]. This particular aspect of CD complexes allows obtaining systems where CDs could act as delivery agents, controlling the release, the stability, and the concentration of the ligand in the solution.

For the encapsulation process, ligands could be added to the CD solution as a liquid, solid, or dissolved in aqueous solution or in organic solvent [19, 21]. The selection of the type of CD, the solvent, and the encapsulation method depends on the properties of the ligand and the CD, the available equipment, and the costs. If the CD is dissolved in an aqueous-based solution, the solubility of

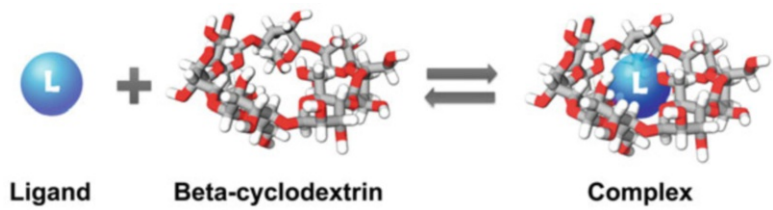

Fig. 1 Schematic representation of the dynamic equilibrium established in the inclusion process of a ligand in BCD with a stoichiometric 1:1

the ligand in this medium must be analyzed [22, 23]. There is no ideal technique to obtain solid-state complexes with CDs, but a method that maximizes the encapsulation must be sought. The most frequently used are [17]:

1. *Coprecipitation*. In this technique, the ligand is added to a CD aqueous solution, stirring at constant temperature long enough to favor the inclusion process and establish an equilibrium between the free and associated species. Stirring speed and time as well as temperature and ligand:CD molar ratio are essential factors in this process. Then, the precipitation of the complexes is promoted by keeping the solution under refrigeration temperatures. Solvent removal can be performed by different methods as freeze-drying, oven drying, or adding a matrix and proceeding with spray drying to obtain dehydrated systems.
2. *Freeze-drying or spray drying of solutions or suspensions*. It is a modification of the coprecipitation method. The step of cooling to favor the complexes precipitation is avoided, and the complete system is freeze-dried, or spray dried.
3. *Kneading or grinding methods*. In the kneading method, solid CDs and ligands are mixed with a small amount of solvent. In the grinding, the solvent is avoided. In both techniques, the preparation of the complex is by mechanochemical activation through manual kneading using a mortar or mechanical grinding using a ball or vibrating mills. This mechanical mixing provides the energy to favor the encapsulation. They are fast and eco-friendly methods to obtain inclusion complexes directly in the solid state but are not recommended for sensitive ligands that could be degraded or evaporated during the process [14].

1.2 Ultrasound Assistance in the Encapsulation Process

Ultrasonication (US) is an environmentally friendly technique as it is relatively safe and nontoxic, with low energy consumption and high efficiency [24]. High-intensity ultrasound is an efficient physical approach for preparing complexes faster, with high yield and reduced purification steps. When cavitation and collapse of bubbles occurs during the ultrasonic irradiation, energy is released and converted to high pressure and temperature. This process could accelerate dissolution of compounds breaking their intermolecular interactions, favoring the inclusion in the CD [25]. The US is currently being studied to reduce BCD encapsulation times of compounds like fatty acids or essential oils [26]. On the other hand, the application of ultrasound is also an ecological alternative to increase the extraction of bioactive compounds from agro-industrial by-products. Particularly, when aqueous solutions of CD are used as a solvent to increase the solubility and extraction

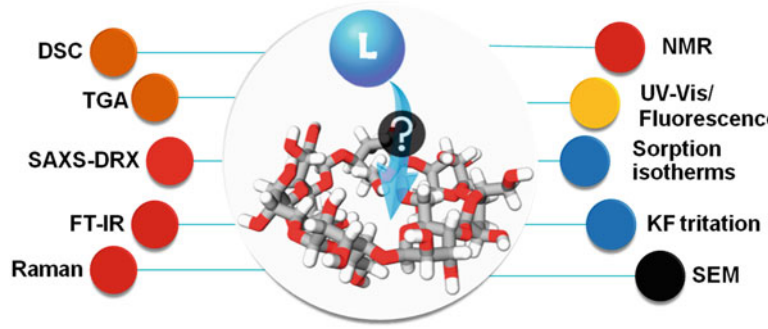

Fig. 2 Schematic representation of different techniques used to verify encapsulation, characterize inclusion complexes, or study stability and water sorption of the complexes. Differential scanning calorimetry (DSC), thermogravimetry analysis (TGA), x-rays (SAXS and DRX), infrared, Raman, and nuclear magnetic resonance spectroscopies (FTIR, Raman, and NMR), spectrophotometry (UV-Vis, fluorescence), water sorption properties (isotherms, Karl Fischer titration), and scanning electron microscopy (SEM)

of hydrophobic compounds, the application of US favors the inclusion of such compounds in the cyclodextrin [19]. The optimal parameters such as power, frequency, sample volume, and sonication time must be studied to obtain the desired effect without affecting the compounds of interest [27].

1.3 Verification and Characterization of the Encapsulation

Molecular encapsulation produces modifications in the physicochemical properties of both the cyclodextrin and the ligand. The study of these changes allows us to determine the stoichiometry and stability of the complexes, the thermodynamic of the inclusion process, and the type and intensity of interactions between the compounds. The characterization of the ligand:CD complexes in solution or in solid state is essential to select the optimal formulation that suits the desired purposes [28]. Figure 2 shows different techniques commonly used to verify and characterize CD complexes. The selection of the adequate method depends on the type of ligand, solvent and CD, the costs, the available equipment, and the future application of the complexes. Also, an essential aspect is whether the complexes are in solution or solid state since there are suitable techniques for each case.

This chapter describes the fundamentals of some of the most used techniques shown in Fig. 2 and the results we obtained of their application for studying BCD and HPBCD complexes with ligands of food interest (cholesterol, myristic acid, and α-terpineol).

1.3.1 Spectrophotometric Methods

They are widely used to determine the concentration of a ligand that absorbs in UV or visible or presents fluorescence. They are generally based on determining the change in the absorption or emission of the compound when it is encapsulated. These

techniques are simple, versatile, fast, and accurate, providing very reliable data. However, if the aqueous solubility of the ligands is low, the classical quantification techniques must sometimes be modified to improve the sensitivity [29].

1.3.2 Phase Solubility Study

The phase solubility methods developed by Higuchi and Connors [30] are commonly used to study the ligand-CD equilibrium in solution. They are also employed to calculate the thermodynamics parameters of the inclusion process and the stoichiometry of the complexes. They consist of studying the effect of the variation of CD concentration on the solubility of the ligand, whose initial concentration remains constant, forming a supersaturated solution (over its solubility). As ligand is added, a shift in equilibrium occurs towards the formation of the inclusion complex. The concentrations of the complex or of the ligand in equilibrium are determined by evaluating a suitable physical or chemical property as a spectrophotometric one. Then, the ligand molar concentration in solution is plotted as a function of the CD concentration at the equilibrium. The graphs obtained are called phase solubility diagrams (Fig. 3).

Higuchi and Connors [30] classified the systems according to the type of empirical phase diagram.

1. Type A_L: indicates the formation of soluble complexes. In this system, the increase in the solubility of the ligand is linear with the increase in CD concentration. The stoichiometric ratio in these cases is 1:1.

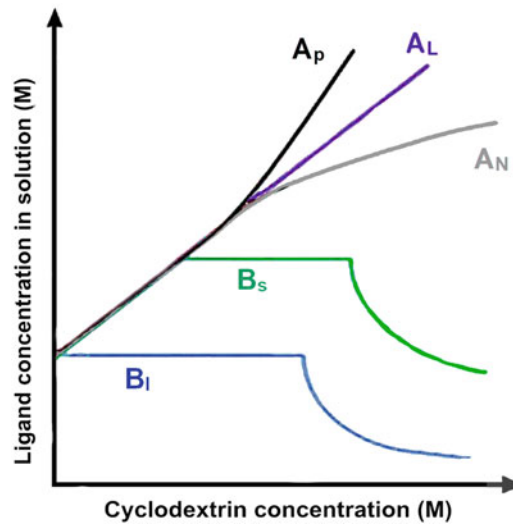

Fig. 3 Phase equilibrium solubility diagrams adapted from Higuchi and Connors (1965)

2. Type A_P: the positive curvature of the diagram indicates that the stoichiometric ratio is not 1:1, but rather that the order of the CD is greater than that of the ligands.
3. Type A_N: is difficult to interpret since the negative deviations can be associated with many reasons as guest molecular associations.
4. Type B: suggests the formation of poorly soluble (B_S) or insoluble (B_I) in aqueous medium complexes.

Most of the ligand:CD complexes present 1:1 stoichiometry. In this case, stability constants (K_s) of complexes in solution at a specific temperature can be obtained from the linearization of the A_L type phase diagrams. The following equation can be used to fit the phase diagram data:

$$K_s = K_{1:1} = \frac{[L - CD]}{[L][CD]} = \frac{\text{Slope}}{1 - \text{Slope}} \quad (1)$$

where [L], [CD], and [L−CD] are the equilibrium concentrations of the ligand, the cyclodextrin, and the complex formed, respectively. The intercept (S_0) can be estimated as the solubility of the ligand in water [15].

The thermodynamic parameters associated with the molecular encapsulation of the ligand, such as the variation of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG), can be obtained by studying the dependence of the stability constants of the complex with temperature [31]. This method determines K_s at different temperatures using the phase solubility method, as previously described. Then, the integrated equation of van't Hoff (Equation 2) could be applied:

$$\ln \; K_s^T = -\frac{\Delta H}{R}\frac{1}{T} + \frac{\Delta S}{R} \quad (2)$$

where K_s^T is the stability constant calculated at temperature T (in kelvin, K). With the data of enthalpy and entropy changes, the variation of free energy can be evaluated by applying the following equation (Equation 3):

$$\Delta G^0 = \Delta H - T\Delta S \quad (3)$$

where ΔG^0 is the free energy variation at 298 K. The analysis of the entropy, free energy, and enthalpy variations allows us to conclude whether a particular molecular encapsulation process is exothermic or endothermic, spontaneous, and whether it gives rise to more or less ordered systems.

1.3.3 Differential Scanning Calorimetry (DSC)

DSC is a frequently used method to verify and study solid-state complex formation [29, 32]. The total or partial disappearance of a characteristic transition of the guest molecule (e.g., endothermic

melting) is a strong evidence of complete or partial inclusion in the CD [23, 33], and encapsulation efficiency can be calculated. DSC also allows determining the glass transition temperature of the system, which occurs in a temperature range that depends on the rate of heating, the thermal history of the product, molar mass, and water content. BCD has a glass transition temperature difficult to determine as it is mostly a hydrate, while HPBCD, being an amorphous derivative, present a clear glass transition [22]. DSC can also be used to analyze oxidative stability of ligands and complexes by determining the oxidation initiation temperature.

1.3.4 Water Sorption Studies

Water sorption depends on solid–solid and solid–water interactions in the system and is affected by the physical state, the chemical composition, and the physical structure of the sample. Considering the strong influence of water on complex formation and stability, the analysis of water content in ligand:CD systems not only can give evidence of the complexation but also becomes of fundamental importance to define the appropriate storage conditions of the powder formulations [20]. If the equilibrium water content of the samples is plotted as a function of a_w, water sorption isotherms could be obtained.

2 Materials and Methods

2.1 Preparation of CD Solutions

To regularly prepare a saturated solution of BCD (15 mM), we weigh 1.85 g of the solid CD (Table 1) and add 100 ml of distilled water. Due to its difficulty to dissolve, BCD must be incorporated under stirring and heating at a temperature between 45 and 50 °C for 30 min. With this procedure, we obtain transparent solutions, stable at room temperature (20–25 °C). To prepare solutions with different concentrations of CD, it is convenient to make dilutions from the saturated solution (*see* **Note 1**).

2.2 Encapsulation with CD by Coprecipitation

2.2.1 Regular Protocol

In the coprecipitation method, the ligand is dispersed or dissolved in the CD aqueous solution in a suitable ligand:CD molar ratio (*see* **Note 2**). Depending on the properties of the ligand, it could be added directly in solid state or dissolved in water or aqueous ethanol. Controls of CD and ligand solutions must be prepared in the same conditions as complexes for comparison and for verifying complexation. Characteristics of the ligands, such as solubility, size, structure, spatial geometry, and polarity, are important to properly design the encapsulation experiment. To promote ligand–CD interactions, the system is stirred under heating at a constant rate for a certain time and then for a period of time at room temperature (*see* **Note 3**). For thermolabile ligands,

complexes can be prepared at room temperature optimizing stirring conditions. For example, to maximize the encapsulation of α-terpineol, we obtained an optimized time of 3 h at 50 °C and then 3 h at room temperature [20] and for cholesterol, 1 h at 50 °C followed by 23 h at 25 °C [21]. After stirring, the next step is to store the obtained suspensions overnight at 3 °C to promote the precipitation of the complexes (*see* **Note 4**). Then, the suspensions are filtered (PTFE filters of 0.45 μm average pore diameter), and the filtrates are dehydrated in a vacuum oven (50 °C) until constant weight, or frozen at −26 °C for 24 h and freeze-dried (operating at a condenser plate temperature of −111 °C, chamber pressure of 30 Pa, and shelf temperature of 25 °C).

2.2.2 Modifications to Regular Protocol

This protocol is recommended to study complexes of high aqueous solubility or that are part of a mixture, such as essential oils or vegetable extracts, and when a powder with all compounds present in the solution is required. As in the regular coprecipitation technique, CD solution is mixed with the ligand at the selected molar ratio (*see* **Note 5**). The combined solution is stirred during the selected time and temperature as previously described (*see* **Note 3**). The difference with the regular protocol is that, in this case, precipitation and filtering are not carried out, and the complete solution/suspension is frozen at −26 °C for 24 h and then freeze-dried in the same conditions explained for the coprecipitation method (Subheading 2.2.1).

Freeze-Drying of the Complete Solution/Suspension

Ultrasound Assistance

Protocols for ligand:CD complexation could be improved using ultrasound assistance. In this case, the mixture is ultrasonicated (US) (in an ice bath to avoid changes in temperature due to the process) for a time in the order of minutes (*see* **Note 6**) and then is stirred (*see* **Note 3**). To obtain complexes in solid state from aqueous solution, the dehydration of the systems is carried out by freeze-drying as explained in Subheading 2.2.1.

For achieving higher yields of extraction and encapsulation, we optimized a protocol using both modifications mentioned above to extract and encapsulate bioactive compounds from olive pomace, a by-product of the olive oil industry. In this experiment, the optimum sonication time was 10 min (100 W power, 30 Hz frequency, continuous pulse, titanium probe M2), and stirring during 21 h at 60 °C [19].

Hence, before selecting a protocol for encapsulation, it is essential to define and be clear about the optimization's goal and, also, how to follow the encapsulation of the ligand.

2.3 Study of Inclusion Complexes in Liquid Systems

2.3.1 Spectrophotometric Determination

Spectrophotometric techniques are commonly used for studying complexes in solution. The steps to quantify the ligand are listed below:

1. Obtention of the spectra (UV-Visible or fluorescence) of the ligand in the required solvent.
2. Analysis of the spectral data to obtain the maximum absorption UV-vis wavelength, absorptivity coefficient, fluorescence absorbance, etc.
3. A calibration curve must be done with different ligand concentrations, determining the absorbance at the maximum UV-Visible absorption wavelength. The range of ligand concentrations selected for the calibration curve should include the concentration of ligand used to prepare the combined ligand: CD solutions. In this way, the free ligand could be determined in the system.
4. In fluorescence studies, it is common to use standard reference solutions of compounds with high quantum yield, such as quinine sulfate (QS) (*see* **Note 7**). Solutions of different concentrations of the ligand of interest are then referenced to the calibration curve of QS. The results are commonly expressed in QS counts. Fluorescence is more sensitive than UV or visible techniques but is limited only to fluorescent ligands.

Figure 4 shows the absorption spectrum we obtained for an aqueous solution of α-terpineol (TER) 5 mM at 27 ± 2 °C. TER absorbs between 260 and 240 nm without a defined maximum. This type of spectra is typical of terpenoid compounds. The insert

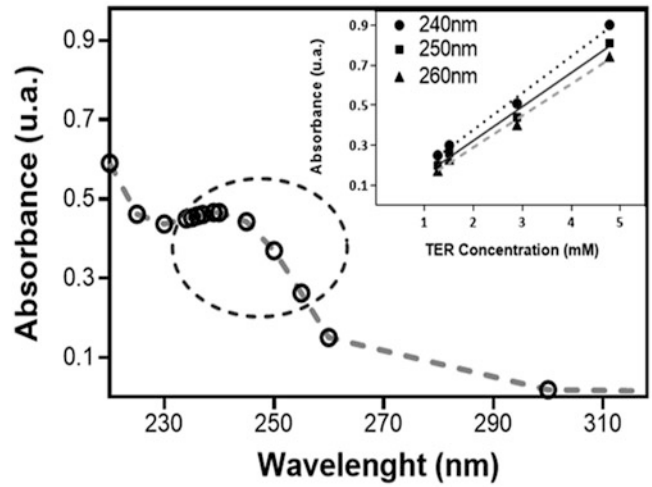

Fig. 4 UV absorption spectrum obtained for a 5 mM α-terpineol (TER) aqueous solution at 27 ± 2 °C. Insert graph shows the calibration curves made considering the absorbance at different wavelengths (240, 250, and 260 ± 4 nm) for 1.2 to 5 mM TER solutions

Table 2
Data of linearization of the TER calibration curves in the range 1.2–5 mM at 240, 250, and 260 nm. Limits of detection (LD = 3StDv$_0$) and quantification (LQ = 10StDv$_0$) values of TER were evaluated in saturated aqueous solutions of BCD (15 mM). (StDv$_0$ = the standard deviation)

(nm)	R^2	LD (mM)	LQ (mM)
240	0.994	0.34	2.89
250	0.991	0.21	1.27
260	0.993	0.38	2.36

in Fig. 4 shows the calibration curves performed considering the absorbance of standard solutions at different wavelengths within the TER absorption range (240, 250, and 260 nm).

The concentration range of TER standard solutions was 1.2 to 5 mM. All the linearizations presented a similar slope and a good regression coefficient. We performed a statistical analysis of the linearization at each wavelength, using the limits of quantification (LQ) and detection limit (LD) as selection criteria (*see* **Note 8**). In our experiments, we used a saturated solution of BCD (15 mM) without ligand as blank. Definitions: LQ = 10 StDv$_0$ and LD = 3 StDv$_0$. In Table 2, we show the results of this analysis.

Considering the results, we decided to perform the TER determinations at 250 nm as it maximized the sensitivity of the technique (lower LQ and LD).

In the introduction, we explain that one of the principal disadvantages of spectrophotometric determinations is that, in general, the solubility of the ligands in water is low, and the classical quantification techniques must be modified to improve its sensitivity. We determined a ligand as cholesterol with a very poor water solubility by modifying a commercial enzymatic kit test (COLESTAT, Wiener Laboratory, Rosario, Argentina) [21]. The determination of the chromophore product was made at 505 nm. By comparing the spectrophotometric absorption data of the standard ligand solutions with those of ligand:CD solutions, it is possible to know if the complex was formed and to quantify the ligand in solution during inclusion without modifying the equilibrium process. When the ligand is included in the inner cavity of the cyclodextrin, its absorption or emission signals could be modified in the spectra [34] due to electronic interactions. These spectrophotometric techniques allow not only to quantify but also to study the noncovalent ligand–CD interactions.

2.3.2 *Phase Solubility*

To determine stability constants (K_s) of the complexes, a phase solubility study could be carried out according to Higuchi and Connors method [30], as described in the introduction. Figure 5 shows an example of phase solubility diagrams we obtained for

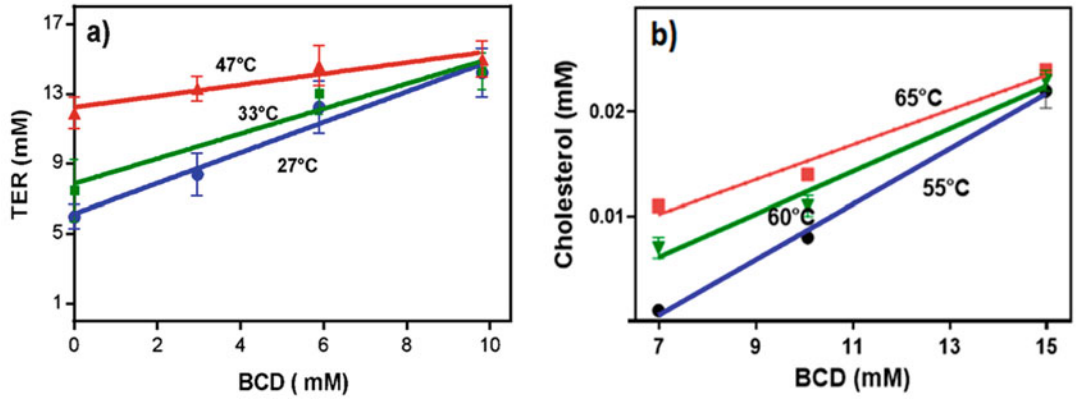

Fig. 5 Phase solubility diagrams for α-terpineol:BCD (**a**) and for cholesterol:BCD (**b**) at 27, 33, 47 °C and 55, 60, 65 °C, respectively

complexes of α-terpineol and cholesterol (CHO) with BCD (TER:BCD and CHO:BCD, respectively). These complexes were prepared by mixing an excess amount of ligand (TER 5 mM or CHO 5 mg/L) in an aqueous solution containing increasing amounts of the CD (0 to 15 mM for BCD) [20, 21]. After equilibration for 24 h, we determined the concentration of the ligands in the solutions spectrophotometrically (*see* **Notes 9** and **10**). We recommend to carry out the phase solubility experiments at least in triplicate. If the idea is to calculate thermodynamic parameters such as enthalpy or entropy changes, the K_s value must be obtained at three or more temperatures.

To characterize the complexes, we calculated K_s for each temperature from the fitted curve over the linear portion of the phase solubility diagrams using Eq. (1). Figure 5a shows that TER solubility increases linearly along with the assayed concentrations of BCD showing typical A_L-type diagrams that suggest the formation of 1:1 complexes. On the other hand, CHO solubility does not show typical linear phase solubility diagrams with all BCD concentrations. CHO water solubility changed very slightly with BCD concentrations less than 7 mM, regardless of temperature. Then, the solubility increased linearly with BCD concentrations higher than 7 mM (Fig. 5b). So, we consider CHO:BCD phase diagrams as type A_L from 7 mM CD concentration. The small change in cholesterol solubility at low BCD concentrations could be explained by the self-association of its molecules in aqueous solution. This particular characteristic of CHO led us to add an extra step of centrifugation at low temperature in the coprecipitation method (Subheading 2.2.1) [21]. This example clearly shows the need to adapt the methods to each ligand:CD system considering its particular and characteristic physicochemical properties.

Through the van't Hoff equations (Eqs 2 and 3), thermodynamic parameters (ΔH, ΔS, and ΔG^0) involved in complex formation can be calculated using the K_s values obtained at the studied

Table 3
Thermodynamic parameters of TER:BCD and CHO:BCD complex formation considering 1:1 molar ratio

System	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG_{25} (kJ mol^{-1})
TER:BCD	−124 ± 5	−355 ± 17	−18 ± 1
CHO:BCD	−43 ± 3	−65 ± 9	−23 ± 1

temperatures. Table 3 shows the thermodynamic parameters we obtained for the systems presented in Fig. 5 (TER:BCD and CHO:BCD). The negative values for ΔH indicate that both ligand inclusion processes are exothermic.

The greater the negative enthalphy change value, the more stable will be the ligand:CD system and more efficient is the inclusion. The relatively small and negative values of the entropy changes (ΔS) confirm that the main interactions that stabilize these complexes are of noncovalent type. The negative ΔS value is related to ligand:CD interactions that energetically favors the exit of water molecules from the inner cavity of the CD [21]. The rotational and translational degrees of freedom in the complex decrease with respect to the free molecules, giving rise to more ordered systems [21, 35]. The negatives values of ΔG indicate that the inclusion process of both ligands was spontaneous.

These thermodynamics studies provide useful information on suitable conditions for encapsulation and storage of inclusion complexes in cyclodextrins with potential application to the development of functional or nutraceutical foods.

2.4 Study of Inclusion Complexes in Solid State

We refer to complexes in solid state as those obtained in solution and then dehydrated by freeze-drying, as described in Subheading 2.2. These studies involve the verification of complexes formation and their characterization. The analyzes require comparing the results of the freeze-dried complexes with those obtained for systems prepared by physical mixture (PM). To prepare the physical mixture, accurately weighed quantities of the CD and the ligand (in the desired molar ratio) should be mixed in a mortar for a certain time (usually 5 to 10 min), enough to achieve a homogeneous powder. The PM is considered as control since no interactions are established between the ligand and the CD during the mixing process, and therefore the ligand remains free.

2.4.1 Differential Scanning Calorimetry (DSC)

In order to analyze solid complexes by DSC, powdered samples (5–20 mg) are weighed in 40 μL aluminum pans, which are then hermetically sealed. An empty pan is usually employed as reference. Dynamic scans over a wide temperature range (e.g., −80 to 180 °C) are performed to identify the characteristic transition temperature (melting, crystallization, evaporation) of the ligands

we want to encapsulate. Once that temperature has been identified, the start temperature of the run must always be approximate 20 °C below than the ligand transition temperature. It must be taken into account that at temperatures higher than 200–250 °C, most of the CDs and therefore their complexes break down. The scans are conducted under nitrogen gas flow. Measurements should be made at least in duplicate.

Determination of Encapsulation Efficiency

From dynamic thermograms, the melting temperature and the enthalpy of fusion of the pure ligand and of the ligand in the complexes could be obtained. Figure 6 shows the thermograms obtained for pure ligands (TER and CHO) and for their complexes with CDs (TER:HPBCD and CHO:BCD) prepared in a 1:1 molar ratio, compared to those obtained for ligand-CD physical mixtures. The thermograms of the CDs and the pure ligands were also included. Each sample was heated at 10 °C/min from −20 to 110 °C. In all systems, the enthalpy of fusion in the physical mixture was similar to the pure ligand, indicating that no CD complexes were formed by mechanical mixing.

The thermograms obtained for TER:HPBCD did not show any endothermic signal in the melting temperature range of TER, which indicates that the ligand was not free and was completely encapsulated in the CD at the studied molar ratio. However, in the CHO:BCD thermograms, the endothermic signal of the ligand was smaller than that of the pure ligand, indicating that CHO was partially encapsulated in BCD. Therefore, these results suggest that inclusion complexes were formed using the coprecipitation/freeze-drying method.

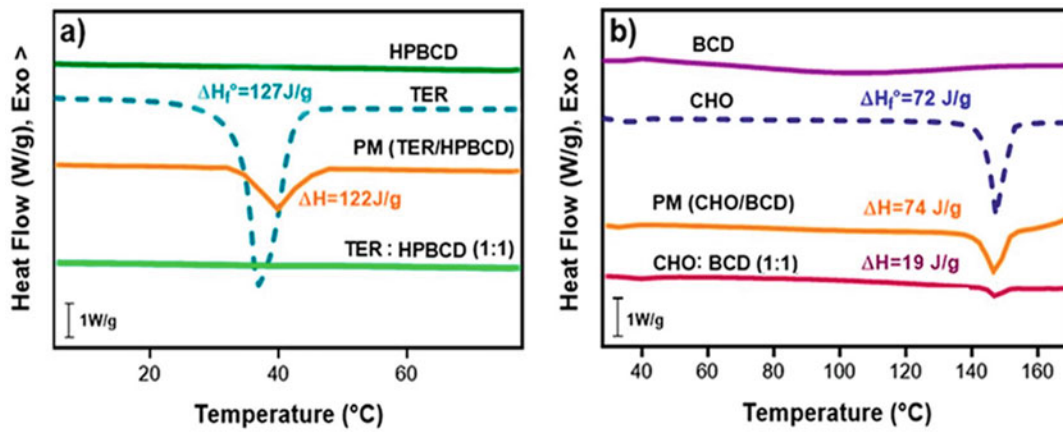

Fig. 6 Thermograms obtained by DSC for (**a**) α-terpineol (TER) and (**b**) cholesterol (CHO) both compared with the thermograms obtained for the ligands combined with 2-hydroxypropyl-beta-cyclodextrin (HPBCD) or beta-cyclodextrin (BCD), respectively, in ligand:CD molar ratios 1:1. The corresponding physical mixture thermogram of each system is also included

Table 4
Optimum stirring time and percentage of encapsulation efficiency (% EE) of each ligand, prepared in ligand:CD molar ratios 1:1 and 1:3, using β-cyclodextrin (BCD) or 2-hydroxypropyl-β-cyclodextrin (HPBCD). Systems were stirred at 25 °C and then freeze-dried as described in Subheading 2.2.1. The standard deviation of the determinations is indicated for $N = 3$. Ligands: cholesterol (CHO), α-terpineol (TER), and myristic acid (MYR)

System	Molar ratio	Optimum stirring time (h)	% EE
CHO:BCD	1:1	24	73 ± 2
CHO:BCD	1:3	24	65 ± 1
TER:BCD	1:1	6	100
TER:BCD	1:3	6	100
TER:HPBCD	1:1	6	100
TER:HPBCD	1:3	6	100
MYR:BCD	1:1	12	35 ± 2
MYR:BCD	1:3	12	45 ± 5
MYR:HPBCD	1:1	12	13 ± 3
MYR:HPBCD	1:3	12	42 ± 5

The encapsulation efficiency (EE%) could be determined as the ratio between the enthalpy of fusion of free ligands in the combined system (corrected considering the mass of ligand in the sample) and the enthalpy of fusion of the pure ligand, measured under identical conditions; according to Equation 4:

$$\text{EE}\ (\%) = \frac{m_0 - m_{\text{L}}}{m_0} \times 100 = \left(1 - \frac{\Delta H_m}{\Delta H_{m_0}}\right) \times 100 \qquad (4)$$

where m_0 is the total mass of the ligand, calculated based on the mass weighed in each DSC pan of the combined systems, m_{L} the nonencapsulated ligand mass, ΔH_{m_0} (J/g ligand) is the enthalpy of fusion of the pure ligand, and ΔH_m (J/g ligand) is the enthalpy of fusion of the ligand in the systems combined with CDs, normalized to the mass ligand ratio in the systems.

Table 4 summarizes the EE% values and the corresponding optimal stirring times we obtained for complexes of CHO, TER, and myristic acid (MYR), prepared in 1:1 and 1:3 ligand:CD molar ratios using the coprecipitation method. The optimal encapsulation conditions for each system were obtained analyzing EE% (Eq. 4) for different stirring times and ligand:CD molar ratios.

From this data, it is observed that the optimal molar ratio for CHO was 1:1. Compared with the other ligands, a longer stirring time was necessary to achieve CHO encapsulation. On the other hand, TER encapsulation seems to be independent of molar ratio and of the type of CD used, and its inclusion was total even with a

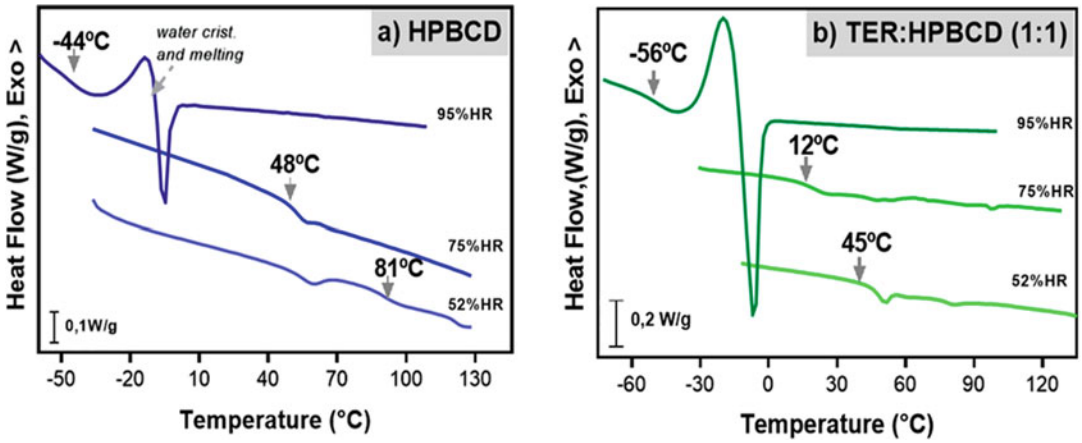

Fig. 7 Thermograms of 2-hydroxypropyl--cyclodextrin (**a**) and α-terpineol:HPBCD complexes (**b**) equilibrated at three relative humidity. Arrows show the onset temperature of the glass transition

shorter stirring time. For MYR, higher EE% was obtained with a 1:3 molar ratio, but encapsulation was partial, showing a lower affinity for HPBCD. This analysis allows us to study host–guest interactions and conclude about how encapsulation is affected by the structure, polarity, and geometry of the compounds.

Determination of Glass Transition Temperature (T_g)

Although many physicochemical properties of different cyclodextrins have been studied, there are not many studies on the influence of the formation of complexes on the glass transition temperature of cyclodextrins. The glass transition temperature is detected by DSC as an endothermic shift in the baseline of dynamic thermograms (obtained as described in Subheading 2.4.1) that occurs due to the change in the specific heat (ΔC_p) of the system at T_g.

Figure 7 shows the thermograms with the step corresponding to the glass transition for HPBCD (a) and for its complexes with TER (b), equilibrated at different relative humidities (% RH). In both systems, the plasticizing effect of water is observed.

For a given water content, the T_g value was lower in the complexes than in the HPBCD, which evidence a certain plasticizing effect of TER. Samples equilibrated at 95% RH (with the higher water content), exhibited exothermic and endothermic peaks corresponding to crystallization and melting of free water in the system. The obtained results show that the encapsulation of the ligand in the CDs involves interactions that promote supramolecular changes in the matrix.

2.4.2 Water Sorption Studies

The water sorption studies are performed by the isopiestic static-gravimetric method. Powdered samples (BCD and CHO:BCD) are distributed into glass vials of 5 mL (around 100 mg/vial) and placed into vacuum desiccators at different relative humidities

provided by different saturated salt solutions: $KCOOCH_3 \cdot 5H_2O$, $MgCl_2 \cdot 6H_2O$, $NaBr \cdot 2H_2O$, NaCl for achieving water activities (a_w) values of 0.22, 0.33, 0.57, 0.75, and 0.84 at 25 °C ± 1 °C, respectively [36]. The water activity of the saturated salt solutions must be confirmed using an a_w meter (AquaLab Series 3, Decagon Devices Inc., Pullman, USA). After reaching the equilibrium condition (difference in weight < 0.0005 g), samples are oven-dried under vacuum (735 mmHg) at 70 °C until constant weight to determine the water content at each relative humidity. The measurements are performed in triplicate, and the average value is informed.

Figure 8a shows the water content of BCD and CHO:BCD complexes at different molar ratios equilibrated at a_w 0.22, 0.33, 0.57, and 0.75 (25 °C).

The complexes show a lower water content than the pure BCD. One of the driving forces behind encapsulation in CDs is the replacement of the water molecules in the cavity by the ligand, so it can be used to verify the encapsulation (Fig. 8b). The presence of cholesterol in the system considerably modified the CD water sorption. The inclusion of the ligand modifies the CD–water interactions, and the water adsorbed by the complexes is less. This study allows us to confirm the encapsulation of the ligand and to analyze some properties of the complexes.

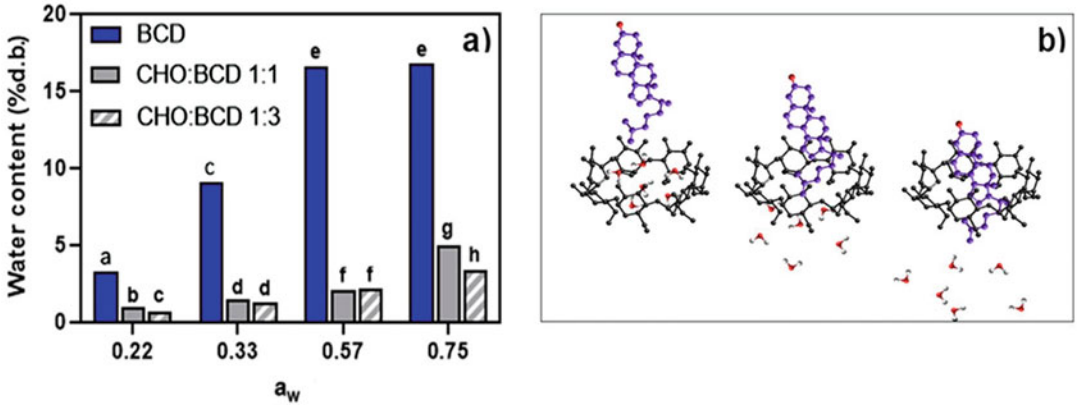

Fig. 8 (**a**) Water content of β-cyclodextrin (BCD) and of the cholesterol complexes (CHO:BCD) with 1:1 and 1:3 ligand:CD molar ratios, as a function of water activity, at 25 °C. Means with the same letter are not significantly different ($p > 0.05$). (**b**) Schematic representation of the displacement of water molecules by cholesterol inclusion in BCD cavity in aqueous solution. Structures of CD (black) and cholesterol (violet) were simulated with the HyperChem Professional V7.5 program. Only water molecules involved in the encapsulation process were represented to simplify the scheme

3 Conclusions

Encapsulation of different compounds in cyclodextrins can be carried out following the methods described in this chapter. Several techniques are described to confirm the formation of these complexes, both in solution and in solid state, and to characterize them for their application as food ingredients.

Through theoretical foundations and practical examples, it is intended to provide simple and clear protocols to encapsulate different types of compounds in cyclodextrins (BCD, HPBCD). We also pretend to contribute to a better understanding of the critical variables of the confinement process and of the interaction mechanisms involved.

4 Notes

1. If solutions are stored at temperatures lower than 4 °C, the CDs precipitate and must be reheated for dissolution. Hence, we suggest preparing only the CD solution volume required for the experiment to avoid solubility and contamination problems.
2. Ligands, such as terpenes and flavonoids that are completely encapsulated in the cavity of the CDs, form complexes at molar ratio 1:1. Other ligands, such as fatty acids, are not entirely encapsulated, and the stable inclusion complex with CD would be obtained with 1:2 or 1:3 ligand:CD molar ratios [22]. We recommend using different molar ratios to verify which is the most suitable to obtain the complexes (Subheading 2.4.1).
3. Stirring time and temperature must be selected according to ligand properties and CD solubility and have to be optimized to achieve a high encapsulation efficiency (Subheading 2.4.1).
4. Some poor water soluble ligands can form emulsions, so a centrifugation step with cooling could be necessary to favor the precipitation of the complexes. For example, in our experiments, a centrifugation of 20 min at 4 °C and 8000 × *g* was necessary to favor the CD:cholesterol complexes precipitation [21].
5. The selection of ligand:CD molar ratio must be based on preliminary calculations of the molecular weight of the ligand and CD characteristics. If ligands are part of a mixture, we recommend selecting the majority compound to calculate this molar ratio. For mixtures of different compounds, we recommend considering the one found in the highest proportion to calculate the ligand:CD molar ratio.

6. As several variables have to be considered, some statistics designs such as multiple response surface to optimize the parameters are recommended.
7. Quinine sulfate could be dissolved in 0.5 M H_2SO_4. Then the standard quinine solutions are measured at an excitation wavelength of 310 nm, to maximize the quantum yield of this molecule. These solutions are very stable at refrigeration temperature.
8. Commonly, statistical parameters "LQ" and "LD" are determined by measuring the standard deviation (StDv0) of the blank system (solvent without the interest compound).
9. It is assumed that the equilibrium between both species is reached after shaking or sonicating the system for a certain time at constant temperature and pressure.
10. The ligand or complex concentration could also be determined by different techniques such as high-performance liquid chromatography or NMR.

Acknowledgments

This work was supported by Universidad de Buenos Aires (UBACyT-20020190200402BA; UBACyT-20020170100557BA); Agencia Nacional de Promoción Científica y Tecnológica [PICT 2017-1744 and 0569; PICT 2018-01822]; and Consejo Nacional de Investigaciones Científicas y Técnicas.

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