LWT - Food Science and Technology 167 (2022) 113811

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Solubility, sorption isotherms and thermodynamic parameters of β-cyclodextrin complexes with *poplar* propolis components: Practical implicances

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ARTICLE INFO

Keywords:
Propolis
Cyclodextrins
Polyphenols
Inclusion complex
Thermodynamic parameters

ABSTRACT

The aim of this work was to establish experimental conditions to improve water solubility of *poplar* propolis components by complexation with β-cyclodextrin (BCD). Water sorption properties, stability constants and thermodynamic parameters of the encapsulation process were studied. Propolis greatly modified BCD sorption isotherms, being water sorption lower in propolis-BCD system. These results are consistent with the displacement of water molecules from the inner cavity of the BCD by propolis components and evidence propolis component-BCD interactions. Results showed a positive linear relationship between phenolic compounds water solubility and the BCD amount in aqueous solution. The solubility increase depends on the polarity and spatial geometry of these components. Phase solubility data indicated the formation of 1:1 M ratio complexes and studies at different temperatures allowed to calculate the stability constants and the thermodynamic parameters of the inclusion process. The negative ΔH (-22 kJ mol^{-1}) and ΔG (-12.8 kJ mol^{-1}) values indicate that the inclusion of propolis components in BCD is an exothermic and spontaneous process, respectively, that is mainly enthalpy driven. The obtained ΔS (-32 J mol^{-1}.K^{-1}) is typical of low energy interactions. Present results could be of interest to develop aqueous propolis formulations avoiding the use of organic solvents and without undesirable tastes or flavors.

1. Introduction

Propolis, a natural resinous material, is of particular interest as a source of bioactive compounds due to its potential beneficial effects on health (Kurek-Górecka et al., 2013; Peixoto, Freitas, Cunha, Oliveira, & Almeida-Aguiar, 2021). Besides its action against microorganisms, propolis possesses many other beneficial biological activities such as antioxidant, anti-inflammatory, antitumour, antimutagenic (Barbarić et al., 2011; Gargouri, Osés, Fernández-Muiño, Sancho, & Kechaou, 2019; Peixoto et al., 2021). Propolis can be added as an antioxidant and antioxidative agent during food storage (Pobiega, Kraśniewska, & Gniewosz, 2018).

More than 150 constituents, including polyphenols, flavonoids, terpenoids, steroids, sugars and amino acids, have been detected in raw propolis (Kumazawa, Ahn, Fujimoto, & Kato, 2010). Some of these phytochemical compounds present in propolis as myricetin, quercetin, and caffeic acid, are being studied for the prevention of coronavirus contagion in humans as potential interferents of cell invasion by SARS-CoV-2 (Berretta, Duarte Silveira, Cóndor Capcha, De Jong, 2020). The application of propolis as an ingredient in the food industry is limited not only by its heterogeneous composition but also by its low water solubility, strong and unpleasant taste and aromatic odor (Pobiega et al., 2018). For medical, dietetic and cosmetic purposes raw propolis is rarely used, but rather a condensed 70–80% ethanolic extract of propolis (EEP) (Gargouri et al., 2019; Kurek-Górecka et al., 2013). There is little data on the production and composition of aqueous solutions of propolis

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https://doi.org/10.1016/j.lwt.2022.113811
Received 29 September 2021; Received in revised form 15 July 2022; Accepted 21 July 2022
Available online 31 July 2022

and their bio-active compounds (Kubiliene et al., 2015).

Cyclodextrins (CDs) are cyclic oligosaccharides commonly composed by 6 to glucopyranose units with a relatively hydrophobic central cavity and hydrophilic outer surface. They are produced using enzymes and the addition of organic solvents usually helps to improve the yield (Li et al., 2021). CDs form complexes with a wide range of guest molecules by including them into the inner hydrophobic cavity, while the hydrophilic CD exterior can modify guest molecule physicochemical properties, such as solubility in water (Astray, Mejuto, & Simal-Gándara, 2020; Fenyvesi, Vikmon, & Szente, 2016). Thus, they could be used to increase aqueous solubility and stability of the included compounds (Crini et al., 2018). CDs–guest complexes are stabilized via noncovalent bonds such as van der Waals, hydrogen bonds, and hydrophobic interactions (Gonzalez Pereira et al., 2021).

β-Cyclodextrin (BCD), composed of 7 glucopyranose units, is listed in the generally regarded as safe (GRAS) list for use as a food additive (Crini et al., 2018; Fenyvesi & Szente, 2021). CDs are widely used in pharmaceuticals, drug delivery systems, cosmetics, and the food and chemical industries. Its applications in food industry as auxiliary agent, for the enhancement of physicochemical properties of food components, solubilization of poorly water-soluble vitamins or additives, taste masking, or removal of undesired components, have been reviewed by several authors (Astray et al., 2020; Astray, Mejuto, Morales, Rial-Otero, & Simal-Gándara, 2010; Fenyvesi & Szente, 2021; Matencio, Navarro-Orcajada, García-Carmona.&; López Nicholas, 2020; Szente & Szejtli, 2004; Tian et al., 2020).

Thermodynamic studies of complex formation via the determination of Gibbs free energy (ΔG), enthalpy (ΔH) and entropy changes (ΔS) are important to understand the molecular inclusion process and the relevant interactions. The estimation of the these parameters could be done using different methods: determining the equilibrium constants at different temperatures and via the van 't Hoff integrated equation, the ΔG, ΔH and ΔS can be calculated. Another method uses the isothermal titration calorimetry (ITC) to evaluate stoichiometry, binding constants and enthalpy change, then the binding free energy and entropy change can be determined and the ΔCp could be estimated from the temperature dependence of the ΔH (dos Santos Silva Araújo, Lazzara, & Chiappisi, 2021).

There has been disagreements in reported enthalpy values calculated using the van 't Hoff approach or ITC measurements for cyclodextrins complexes (Tellinghuisen, 2006). However, Kantonen, Henriksen, and Gilson (2018) argued that binding enthalpies obtained from calorimetry data (such as ITC) and from the van 't Hoff method should be similar, and that differences between them could be due to experimental errors. These authors proposed a modified and integrated form of the van 't Hoff equation, in order to consider the changes in heat capacity (ΔC_p), correcting errors derived from the assumption that the enthalpy is temperature dependent. Similar equations were reported by other authors (Schönbeck & Holm, 2019; Sharma & First, 2009).

The free energy change for the inclusion process can be considered as an enthalpic term (usually associated to electrostatic, dipole-dipole and van der Waals forces) plus an entropic term, corresponding to hydrophobic interactions (Schönbeck & Holm, 2019). The hydrophobic interactions, associated to a positive ΔS value, are commonly explained by the release of water molecules, previously structured around non-polar molecular surfaces (dos Santos Silva Araújo et al., 2021). Connors (1997) stated that, as the ΔH and ΔS values associated to CDs inclusion processs are commonly negative, hydrophobic interactions are commonly thought to not be an important factor for complex formation. However, there could be other contributing factors to hydrophobic effects in CDs complexes formation, such as an enthalpically favored interaction between the non-polar guest molecule and the CD inner cavity (Liu & Guo, 2002). The ΔC_p value for the inclusion process, usually negative, is characteristic of such hydrophobic interactions (Paul, Ghosh, & Mukherjee, 2016). Also, the release of high-energy, highly-structured, bound water molecules from the inner CD cavity could be an enthalpic driving force for the inclusion process (Connors, 1997; dos Santos Silva Araújo et al., 2021).

If ΔG and ΔH values are similar and negative, with a relatively small $T\Delta S$ term, and a negative ΔC_p, the CD inclusion process could be considered enthalpically driven, with significant contribution of water molecules release, and van der Waals, hydrogen bond and hydrophobic interactions (Liu & Guo, 2002; Rekharsky & Inoue, 1998).

The aim of this work was to determine the experimental conditions to enhance water solubility of propolis components by their encapsulation in β-cyclodextrin. The stability constants at different temperatures and the thermodynamic parameters of the encapsulation process were calculated using the van 't Hoff integrated equation assuming both temperature-independent and temperature-dependent enthalpy (Kantonen et al., 2018). Since propolis bioactive compounds often present very low solubilities and bioavailabilities due to their hydrophobic character, encapsulation is an interesting option to enhance the polyphenols and flavonoid compounds' solubility (Guang-Jiao et al., 2018; Popović et al., 2021).

2. Materials and methods

2.1. Materials

Propolis was obtained from colonies of honeybees, *Apis mellifera* L., in Slovenia and the main plant of origin was poplar (*Populus* sp.).

β-Cyclodextrin (BCD) (containing 8 water molecules/molecule of BCD, MW.1135), was purchase from Roquette-Food, France.

Gallic acid and Folin–Ciocalteau reagent, and standards and solvents for HPLC were purchased from Sigma-Aldrich (St. Louis, MO, USA).

All other chemicals were of analytical grade and purchased from Mallinckrodt Chemical Works (St. Louis, MO, USA).

2.2. Preparation of propolis samples

2.2.1. Propolis purification

Propolis was purificated according to Busch et al., 2017. Briefly, 25.0 g of propolis were ground in a mortar and extracted under stirring for 24 h with ethanol, in a propolis/ethanol ratio 30/100 (w/v). Then, it was filtered under vacuum (0.45 μm pore size paper filter). In order to remove all remaining wax, the ethanolic extract was kept at −20 °C for 24 h and centrifuged at −5 °C (4500 rpm, 15 min).

2.2.2. Ethanolic propolis extract (EEP)

The clear supernatant of the purification was evaporated in a rotary evaporator at 50 °C to a final volume of 50 mL. The final concentration of the ethanolic extract (EEP) was 0.115 g/mL of purified propolis.

2.2.3. Propolis powder (EP)

Propolis was grounded to powder in a mortar with liquid nitrogen and immediately used to avoid its agglomeration.

2.3. Preparation of the solid inclusion complexes

Inclusion complexes of BCD with propolis were prepared by the coprecipitation and freeze-drying method (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007). Solutions of BCD (1.85 g/100 ml) were prepared and heated at 50 °C, shaking until complete dissolution of the CD. Dry ethanolic extract (EEP) was dispersed in the BCD aqueous solution (5% w/v concentration) to obtain EEP-BCD systems.

The systems were stirred at a constant rate for 3 h at 50 °C and for 24 h at room temperature (25 °C). The obtained solutions were then stored overnight at 3 °C to promote precipitation of the complexes. The suspensions were filtered (PTFE filters of 0.45 μm average pore diameter), and 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).

2.4. High-performance liquid chromatography (HPLC) with UV detector

HPLC with UV detector was used to identify and determine the major components in poplar propolis extract prepared in ethanol (EEP). The HPLC system used was an Agilent 1100 series with variable wavelength detector (VWD). The separation was achieved with a column Purosphere® STAR RP-18 (5 μm, 150 × 4.6 mm). The mobile phase consisted of 1% formic acid and acetonitrile in gradient. A flow rate of 0.7 ml/min was used. For analysis, UV spectra were recorded at 290 nm at a rate of 0.8 spectrum/s and a resolution of 5 nm (Busch et al., 2017). The EEP samples were dissolved in ethanol (5 mg/ml), filtered with a 0.45-μm sieve (Sartorius) prior to the injection of a volume of 10 μl into the HPLC system. This HPLC method was used to identify the main propolis components, employing as standards methanol solutions of chrysin, pinocembrin, galangin, pinocembrin derivate (pinocembrin-3-methyl-ether) and quercetin, which were selected from bibliographical data (Barbarić et al., 2011; Gargouri et al., 2019).

HPLC was also used to evaluate the solubility of the propolis components in aqueous BCD solutions. Combined systems of BCD and propolis ethanolic extract (EEP-BCD) were prepared as described in section 2.2.1 (stirring at a constant rate for 3 h at 50 °C and for 24 h at 25 °C). These solutions were concentrated by spray drying to obtain stable solid EEP-BCD systems. The conditions of operation of the spray-dryer (Büchi-minispray dryer, B-290) were: temperature inlet 120 °C, outlet 52 °C, aspirator 100%, pump 10%, pressure 80 mbar. The powders obtained by spray drying or the EEP were solubilized in methanol (0.1%w/v), filtered (with a 0.45-μm filter) and injected into the HPLC. The EEP constituents that can be dissolved in the presence or absence of BCD were evaluated by determining the concentration (mg/g initial propolis) of each compound in solution (Kalogeropoulos, Konteles, Mourtzinos et al., 2009).

The percentage of the solubility increase of the i-eth component (% $SI)_i$ was calculated with equation 1

$$\%SI = \frac{\left[\left(C_{fi} - C_{oi}\right)\right]}{C_{oi}} 100 \quad \text{(Eq.1)}$$

where C_{oi} is the concentration of the i-eth component in EEP propolis extract and C_{fi} is the concentration of the i-eth component in the combined solutions of EEP-BCD after the encapsulation and spray drying process.

2.5. Determination of the water sorption isotherms

Sorption isotherms were determined by the standard isopiestic static-gravimetric method (Greenspan, 1977). After freeze-drying, samples of BCD, EEP, PP or their complexes were distributed into 5 ml glass vials (around 200 mg/vial). These vials were stored in vacuum desiccators at 25 (±1 °C) for approximately 3 weeks under different relative water vapor pressures, determined by saturated solutions of selected salts with water activity (a_w) of 0.11 (LiCl), 0.22 ($KCOOCH_3$), 0.33 ($MgCl_2$), 0.43 (K_2CO_3), 0.64 (NaBr), 0.75 (NaCl) and 0.84 (KCl) and 0.97 (K_2SO_4). After they reached equilibrium, water content was gravimetrically determined, by drying the samples in a vacuum oven at 50 °C. Average values from three measurements are reported. Water content was expressed as percentage in dry basis, % d.b. (g of water/100 g of dried matter).

2.6. Folin-Ciocalteu assay

The colorimetric assay based on the reaction of Folin-Ciocalteu reagent is a method widely used for the determination of total phenols in different extracts, in particular in bees products such as propolis or honey (Bankova, 2005; Popović et al., 2021). An aliquot of 50 μl of test solution, 125 μl of Folin–Ciocalteau reagent, 125 μl of 20% solution of Na_2CO_3 and 800 μl of distilled water were mixed. The reaction mixture was centrifuged at 10,000 rpm for 10 min at room temperature. After 30 min the absorbance of the supernatant was measured at 765 nm (Singleton & Rossi, 1965).

Gallic acid (GA) was employed as calibration standard and results of the total phenolic content (TPC) were expressed as gallic acid equivalents (mM GA) or as mg GA/g of propolis extract (Popović et al., 2021). All spectrophotometric data were acquired using a Jasco V-630 UV-VIS spectrophotometer (JASCO Inc., USA).

2.7. Phase solubility studies

The impact of the presence of BCD on the aqueous solubility of propolis extract was measured at different temperatures. Phase solubility studies were carried out according to the method described by Higuchi and Connors (1965). Saturated solution of BCD (15 mM) was prepared, heated at 50 °C and shaken until complete solubilization of the CD. Solutions of 6 and 10 mM of β-cyclodextrin were prepared by dilution of the saturated solution (15 mM).

Dry ethanolic propolis extract (EEP, 50 mg) was dispersed in 10,0 ml of each BCD aqueous solutions (6, 10 and 15 mM). The systems were stirred in the dark at a constant rate for 48 h at 20, 25, 40 and 60 °C. A blank with 10 mL of distilled water was made.

Then, the samples were centrifugated and the solubility was monitored by determining total phenolics content in the supernatant by the Folin-Ciocalteu assay.

The stability constants of the inclusion complexes (K_S), were calculated from the straight-line portion of the phase solubility diagram according to equation (2):

$$K_s = \frac{slope}{S_0(1 - slope)} \quad \text{(Eq.2)}$$

where S_o is the propolis solubility in the absence of BCD and the slope is the slope obtained by linear regression on the straight-line portion of the solubility diagram.

2.8. Theoretical calculations for molecular properties and 3D geometry

The theorical models and properties of the studied molecules were calculated using the free software http://www.molinspiration.com and its Galaxy Visualizer (3D-GV). 3D-GV allows to visualize molecular lipophilicity potential (MLP) on the molecular surface to localize which parts are hydrophobic (encoded by violet and blue colors) and which ones are hydrophilic (orange, yellow and red). MLP was calculated from atomic hydrophobicity contributions, the same that are used to calculate the octanol-water partition coefficient (log P). 3D-molecular geometries were obtained from molecular connectivity information (SMILES) by the Molinspiration 3D structure generator Galaxy, trained using parameters from a dataset optimized by the semiempirical AM1 method.

2.9. Statistical and regression analysis

All experiments were carried out in triplicate. Analysis of variance (ANOVA) was used to determine any significant differences ($p < 0.05$) using GraphPad Prism 6.0 software (San Diego, California, USA). Pearson correlation coefficients and p-values were used to show correlations and their significance. All statistical analyses were carried out at a 95% confidence level. The nonlinear regression for the van 't Hoff plot was performed with Python 3.7 using the Numpy and Matplotlib packages.

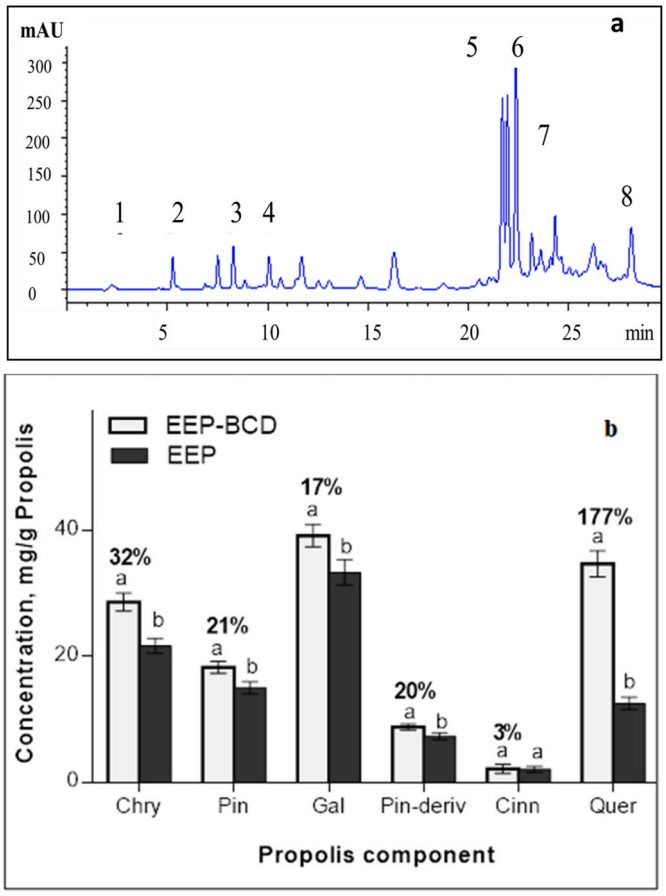


Fig. 1. a) HPLC chomatogram of ethanolic *poplar* propolis extract (EEP): 1- Caffeic acid (tr 3.56); 2- Coumaric acid (tr 4.8); 3- Quercetine (tr 8.24); 4- Cinnamic acid (tr 10.02); 5- Chrysin (tr 21.6); 6- Pinocembrine (tr 21.99); 7- Galangin (tr 22.4); 8- Pinocembrine derivative (tr 28.2). b) Concentration of selected components of propolis (mg/g propolis) determined by HPLC in absence (EEP) and in presence of BCD (EE-BCD). The numbers over bars indicate the % solubility increase of each component. Chry: chrysin, Pin: pinocembrin, Gal: galangin, Pin deriv.: pinocembrin derivate, Cinn: cinnamic acid, Quer: quercetin.

3. Results and discussion

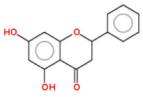
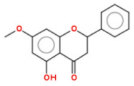
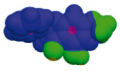
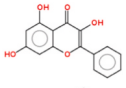
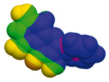
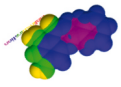
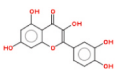
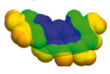
3.1. Composition of poplar propolis and influence of BCD on its aqueous solubility

As propolis extracts contains compounds with different interaction and binding properties with BCD, one interesting approach is to determine the solubility or encapsulation efficiency of each of the main components of propolis (Kalogeropoulos, Konteles, Mourtzinos, et al., 2009). The hypothesis behind this methodology is that each compound is individually encapsulated, and its polarity and size/shape are crutial for the formation of the inclusion complexes (Astray et al., 2010; Zhou et al., 2014).

The poplar (Populus spp.) propolis used in this study was characterized by HPLC/UV detection. Fig. 1a shows the HPLC chromatogram of EEP samples. The main identified propolis compounds, by comparing to standard solutions, were phenolic acids and flavonoids: chrysine (Chry), pinocembrine (Pin), galangin (Gal), pinocembrin derivate (Pin deriv., pinocembrin-3-methyl-ether), cinnamic acid (Cinn), quercetin (Quer). These characteristic compounds were also detected in other poplar propolis from China, Hungary, Bulgaria, Uruguay and Argentina (Barbarić et al., 2011; Kumazawa et al., 2010). BCD efficiency for improving the polyphenols solubility was also determined by HPLC, by relating their concentration in BCD aqueous solution to the initial amount of propolis in the ethanolic extract (Fig. 1b).

The concentration of Chry, Pin, Pin derivative and Gal in the aqueous solution containing BCD increased around 20–30% with respect to their solubilities in the EEP. The cinnamic acid solubility was not affected by the presence of BCD. The concentration of Quer increased nearly three times in BCD solutions, and this increment was greater than the observed for the other compounds. The increment of propolis constituents solubility could be related to BCD complexes formation (Kalogeropoulos, Konteles, Mourtzinos, et al., 2009). Log P, obtained from the ratio of distribution of substrate between water and 1-octanol, is commonly used as a measure of the hydrophobicity of substances. Astray et al. (2010) established a positive correlation between flavors hydrophobicity (log P) and CDs binding constants. Deviations from this correlation could be attributed to the different geometry of the substrates, which in some cases may play a prominent role in the formation of these inclusion complexes (Astray et al., 2010; dos Santos, Buera, & Mazzobre, 2017; Szente & Fenyvesi, 2017).

Table 1
Components of poplar propolis properties. Log P, molecular structure, molecular volume and 3D-MLP (Molecular Lipophilic Potential) calculated with MICP-Molinspiration Calculator Property. Using the Galaxy Visualizer, the molecular lipophilicity potential (MLP) was encoded with violet and blue colors on the molecular surface to see hydrophobic parts and with orange, yellow and red for the hydrophilic parts.

Compound	Type of compound	Molecular structure	3D-MLP	Log P	MW, g.mol^{-1}	Volume
Pinocembrin	Flavonone			2.60	256	222
Pinocembrin -3-Methyl-Ether	Flavonone			3.13	270	240
Galangin	Flavonol			2.65	270	224
Chrysin	Flavone			2.94	254	216
Cinnamic acid	Organic ácid			1.91	148	138
Quercetin	Flavonol			1.68	302	240

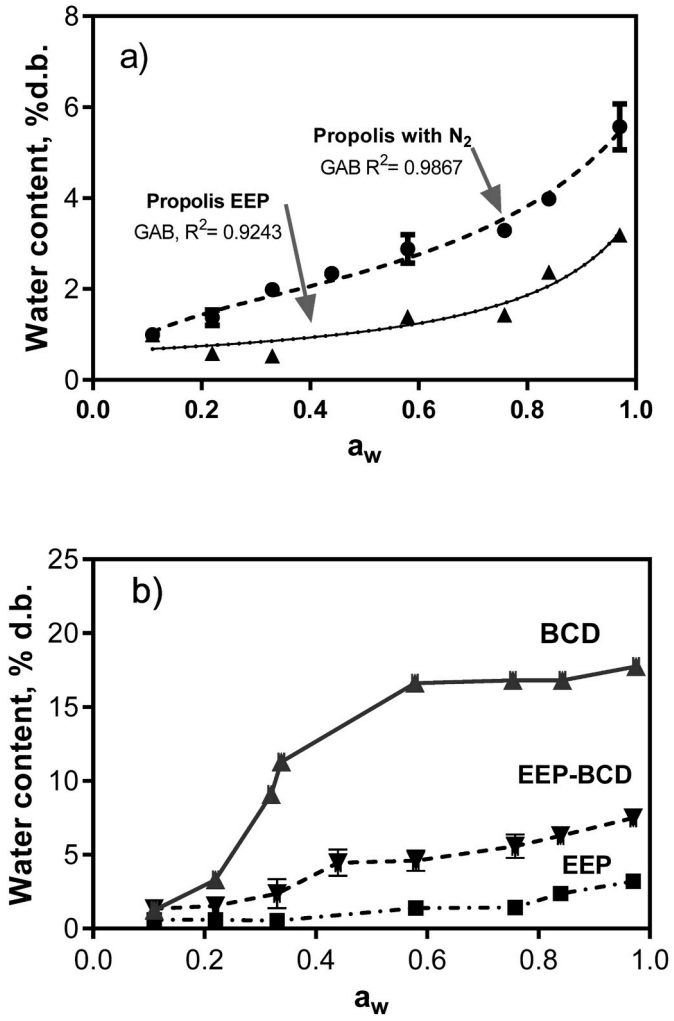


Fig. 2. Water sorption isotherms: (a) propolis powdered in liquid nitrogen, EP (●) and dry ethanolic propolis extract, EEP (▲) fitted with GAB equation. (b) EEP extract (■); EEP-β-cyclodextrin (EEP-BCD) (▼) and BCD, (▲). For some data the error bars (between 4% and 5% of the absolute values) lye below the symbols.

Table 1 shows data of molecular properties (structure, volume, log P, hydrophobic surface area) of propolis components. Chry, Pin, Pin deriv. and Gal are flavonoids of similar polarities and molecular weight (*MW*). The theorical calculations for Pin deriv. were performed on the pinocembrin-3-methyl-ether.

Analysis of 3-dimensional distribution of hydrophobicity on molecular surface is particularly helpful to analyze differences in observed ADME (absorption, distribution, metabolism and excretion) properties of molecules with the same logP, since 3D parameter contains much more information than that resumed in logP, being a single value.

Chry, Pin, Pin deriv. and Gal behave similarly in BCD solutions since their molecular properties and hydrophobic/hydrophilic ligand-BCD interactions are comparable. Cinn in solution was slightly affected by the presence of BCD in comparison with the other flavonoids. The cinnamic acid molecule has a high MLP, comparable to the other polyphenols, except Quer, but it is smaller than the other flavonoids and hence can be most efficiently encapsulated. In agreement with these results, Kalogeropoulos, Konteles, Mourtzinos, et al. (2009) observed that the stability constant (K_s) of Cinn-BCD complex is greater than the K_S of other propolis flavonoids. Thus, there would be more Cinn encapsulated than in solution. Quercetine is more soluble in water than the other flavonoids (Fig. 1b) and also more polar than the other tested compounds (Table 1), which explains its solubility behavior in BCD solutions. The encapsulation of quercetin in CDs has been studied by other authors that also observed an enhanced aqueous solubility of this flavonoid in presence of cyclodextrins (Guang-Jiao et al., 2018; Jullian, Moyano, Yañez, & Olea-Azar, 2007).

3.2. Water sorption isotherms of propolis

The water sorption isotherms of propolis are shown in Fig. 2. Isotherms obtained for propolis ground under liquid nitrogen (PP) and for the dried ethanolic extract (EEP) (Fig. 2a) show the typical sigmoid shape of amorphous food systems. It has to be noted that, due to the hydrophobic characteristics of propolis, the water adsorption is markedly lower than that commonly observed for food matrices. Fig. 2a also shows that the water sorption of propolis extract is influenced by the preparation method of propolis samples; e.g. the propolis powdered with liquid nitrogen adsorbs more water than the ethanolic propolis extract.

The Guggenheim, Anderson, de Boer (GAB) equation (Timmermann, Chirife, & Iglesias, 2001) was employed to describe the water sorption isotherms presented in Fig. 2a through equation (3).

$$X = \frac{a_w\ C\ X_0 k}{[(1 - ka_w)(1 + (1 - C)ka_w)]} \quad \text{(Eq.3)}$$

In this expression, X is the equilibrium material moisture content (g water/100 g dry basis), a_w is the water activity, X_0 is the monolayer water content, and C and k are constants associated with the energetic difference between the water molecules on the monolayer and on the other layers.

The calculated GAB parameters k, C and X_0 were 0.77 ± 0.03, 15 ± 2 and 1.8 ± 0.1 for systems grounded with liquid nitrogen and 0.78 ± 0.05, 7 ± 2 and 0.6 ± 0.1 for the dried ethanolic extract, respectively. R^2 values indicate that these isotherms could be well described by GAB equation (Fig. 2a). The X_0 value, considered the monolayer value, also called the hydration limit (Lechuga-Ballesteros, Danforth, & Zhang, 2002), is very low, which reflects the hydrophobic characteristics of propolis. The difference of X_0 values between the samples grounded with liquid nitrogen and those from the ethanolic extracts could be attributed to the compositional or structural changes upon extraction. The k values are lower than one, as in many food matrices and biological material. The C parameter, which is related to the heat sorption, is higher for the samples grounded under liquid nitrogen.

Considering the high influence of water interactions on complex formation and stability (dos Santos, Buera, & Mazzobre, 2012), the analysis of the water adsorption behaviour was performed for pure BCD and for BCD complexes (EEP-BCD) (Fig. 2b). The BCD isotherm does not show the typical sigmoid shape of amorph food systems: between 33% and 75% RH, the BCD adsorbs more water and reaches a plateau from 52% RH due to the formation of a stable crystalline hydrate. A similar water sorption pattern was described by dos Santos, Buera, and Mazzobre (2011) for natural and branched CDs. The water content of the plateau corresponds to a crystalline hydrated form containing 12 mol of water per mol of BCD, which was stable up to at least 97% RH (dos Santos et al., 2011; 2012; Szejtli, 1998).

The encapsulation of propolis components greatly modified the BCD sorption curves. The water sorption is lower in the EEP-BCD system, which is evidence of propolis-BCD interaction.

In an aqueous solution, the non-polar cyclodextrin cavity is occupied by water molecules which are energetically unfavored (due to polar-non-polar molecular interactions), and therefore can be readily substituted by appropriate guest molecules, which are less polar than water. The cyclodextrin is considered the "host" molecule, and one of the driving force factors responsible for the complex formation is this substitution of the high-enthalpy water molecules by an appropriate "guest" molecule (dos Santos et al., 2017; Szejtli, 1998). This driving force for complexation is not yet completely understood (Astray et al., 2010) but it seems that it is the result of various effects: substitution of

Table 2
Parameters obtained from the linear regression of the experimental data of phase solubility diagrams of ethanolic propolis extract (EEP) with β-cyclodextrin (BCD) at 20, 25, 40 and 60 °C. S_0: water solubility expressed as gallic acid miliequivalents (mM GA). *Ks*: stability constant. Each value is the average of triplicate measurements. Different letters show significant differences for values for the same parameters.

Temperature (°C)	S_0 (mM AG)	K_s (M^{-1})	R^2
20	0.092 ± 0.003^a	199 ± 3^a	0,93
25	0.103 ± 0.002^b	169 ± 4^b	0,91
40	0.275 ± 0.005^c	119 ± 4^c	0,98
60	0.81 ± 0.02^d	65 ± 5^d	0,99

water molecules from the inner cavity, which is energetically favored, the lowering of CD ring strain when the complex is formed, van der Waals forces, hydrogen bond and hydrophobic interactions, which are established when the complex is formed (dos Santos Silva Araújo et al., 2021; Matencio, Navarro-Orcajada, García-Carmona, & López Nicholas, 2020; Rekharsky & Inoue, 1998).

3.3. Phase solubility studies

The estimation of the propolis extract solubility in water, in the presence or absence of BCD, was carried out by performing the Folin-Ciocalteu assay (Kalogeropoulos, Konteles, Troullidou, Mourtzinos, & Karathanos, 2009; Kubiliene et al., 2015).

The total polyphenol content in solution (TPC, expressed as mM GA) was determined after equilibration (stirring during 48 hs) of the combined systems at selected temperatures (20, 25, 40 and 60 °C). TPC increases with increasing temperature and with increasing BCD concentration at a given temperature.

Phase solubility studies of TPC as a function of BCD concentration were performed in the aqueous systems at different temperatures to calculate the stability constants (K_S) and the thermodynamic parameters for the encapsulation of the propolis components of EEP in BCD.

The EEP is composed of numerous components, so the phase solubility diagrams obtained by the Higuchi and Connors method (1965) have a different interpretation from that proposed by these authors. The K_S values and the thermodynamic data obtained from the diagrams are for the whole extracts, not for a particular compound encapsulated in BCD. Parameters obtained from the linear regression of the experimental data of phase solubility diagrams at the different temperatures are shown in Table 2. The solubility of propolis showed a linear correlation ($R^2 > 0.90$) with the BCD concentration at the four studied temperatures. Consequently, according to Higuchi and Connors models, the plots can be considered as an A_L-type diagrams, suggesting the formation of 1:1 complexes between BCD and the polyphenols present in propolis. The water solubility of propolis increased with increasing temperature (Table 2). Kalogeropoulos, Konteles, Mourtzinos, et al. (2009) reported a similar dependence of Greek propolis with BCD concentration. The global result of increasing temperature and BCD concentration is a higher amount of propolis in the aqueous phase.

As EEP dissolution takes place due to the incorporation of its components into the BCD cavity, these results provide an indirect confirmation of inclusion complexes formation between propolis constituents and BCD. Several studies with pure substances evidenced the formation of 1:1 inclusion complexes of BCD with common flavonoids and some propolis polyphenols (Guang-Jiao et al., 2018; Jullian et al., 2007; Tommasini et al., 2004).

It has to be noted that in present Higuchi and Connors approach, K_S represents a global constant for all encapsulated polyphenols present in propolis. Table 2 shows that the solubility of propolis in water (S_o values) were higher for higher temperatures and the obtained K_S values decreased with increasing temperature, as expected for an exothermic process.

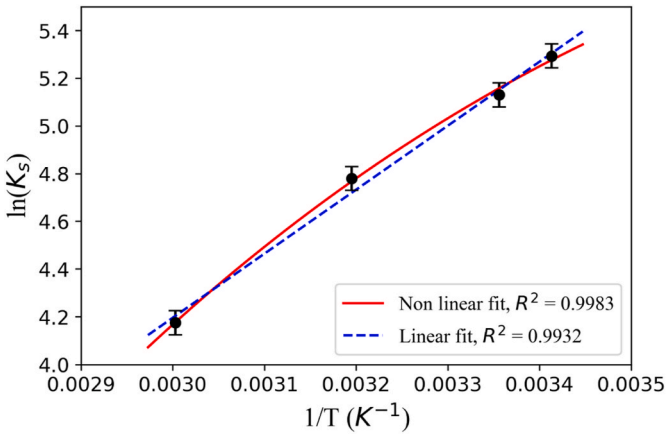


Fig. 3. van 't Hoff plot of the formation of the complex between EEP propolis and BCD fitted by linear (Eq. (4)) and non linear (Eq. (5)) equations. KS: complex stability constant and T: absolute temperature (K).

Table 3
Parameters obtained for linear integrated van 't Hoff (considering $\Delta C_p = 0$, Eq. (4)) and for nonlinear van 't Hoff modified equations (considering a constant $\Delta C_p \neq 0$, Eq. (5)).

Regression	ΔC_p (J. K^{-1}Mol-^{1})	ΔH (KJ/mol)	ΔS (J/mol.K)	ΔG (KJ/mol.
Linear	0	-22 ± 3^a	-32 ± 3^a	$-12,8 \pm 0,5^a$
Nonlinear	$-304.7 \pm 0,5$	-18 ± 2^a	-17 ± 1^b	$-12,7 \pm 0.2^a$

Similar temperature effect on the stability constants were obtained by other authors (Karathanos et al., 2007; Tommasini et al., 2004). The obtained phase solubility data allowed us to calculate the thermodynamic parameters involved in the solubilization of polyphenols presents in propolis by BCD. The integrated form of the van 't Hoff equation (Eq. (4)) was employed for calculating the enthalpy *(ΔH)* and entropy changes *(ΔS),* depending on the variations of the stability constants with temperature (Connors, 1997). This equation assumes that ΔH and ΔS are constant over the studied temperature range.

$$\ln K_s = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad \text{(Eq. 4)}$$

where R is the universal gas constant and T is the absolute temperature.

If enthalpy is not assumed to be constant over the temperature range (i.e., $\Delta Cp \neq 0$), equation 4 does not hold. For cyclodextrin complexes, ΔCp can be assumed to be approximately constant in the studied temperature range (Schönbeck & Holm, 2019), and the van 't Hoff equation can be modified to include the temperature dependence of ΔH and ΔS (Kantonen et al., 2018)

$$\ln K_s = -\frac{\Delta H^0_{T_0}}{R}\frac{1}{T} + \frac{\Delta S^0_{T_0}}{R} - \frac{\Delta C_p}{RT}\left[T - T_0 - \ln\left(\frac{T}{T_0}\right)\right] \quad \text{Eq. (5)}$$

Where $T_0 = 298$ K is a reference temperature, and $\Delta H^0_{T_0}$, $\Delta S^0_{T_0}$ are the standard enthalpy and entropy changes for the inclusion process at T_0. The three thermodynamic parameters of this equation can be obtained from a non linear least squares regression, with the inverse of the temperature as the independent variable, and the natural logarithm of the stability constants at the corresponding temperatures as dependent variable.

Fig. 3 shows the curves of best linear (Eq. (4)) and nonlinear (Eq. (5)) fit to the data. The parameters obtained from both fits are shown in Table 3.

The combined EEP-BCD systems showed a positive correlation between natural logarithm of the stability K_S and the inverse of the absolute temperature (Fig. 3). The fit was better for the non-linear van 't Hoff

Table 4
Thermodynamic values for complex formation of different flavonoids with β-cyclodextrin at 25 °C based on Van't Hoff analysis.

	ΔH (kJ. mol^{-1})	ΔS (J.mol^{-1}. K^{-1})	ΔG_{25} (kJ. mol^{-1})	Method	Solvent	Authors
Propolis	**−22 ± 3**	**−32 ± 3**	**−12.8 ± 0.5**	Phase- solubility	Water	Present work
Pinocembrin	NR	NR	−20.09	Nonlinear least squares curve-fitting method	Water-Ethanol (4v/1v)	Zhou et al. (2014)
Chlorogenic acid	−12.7	−8.1	−14.8		Water	Górnaś, Neunert, Baczyński, and Polewski (2009)
Caffeic acid	−10.7	−10.4	−13.8		Water	Górnaś et al. (2009)
Hesperitin	−35.49	−71.29	−14.25			Tommasini et al. (2004)
Naringenin	−50.14	−119.65	−14.48			Tommasini et al. (2004)
Chrysin	−97,55	−270.0	−17.99	UV-spect. (Hildebrand-Benesi method)	Water - Ethanol	Chakraborty, Basu, Lahiri, and Basak (2010)

equation (Eq. (5)), but the difference was not significative.

The magnitude of ΔCp obtained by the nonlinear fit is similar to other values reported by Schönbeck and Holm (2019) for the inclusion of adamantane in BCD, a non-polar ligand that is totally included inside the CD cavity. Its negative value is associated to hydrophobic interactions between guest molecule and CD.

The difference in ΔH values obtained by both fits is not significative, and the magnitude of ΔH is characteristic of an enthalpically driven CD inclusion process. The ΔS values are negative and statistically different for the two methods, but as the $T\Delta S$ term is small in comparison to the ΔH term, also supports an enthalpically driven inclusion process. Thus, the ΔG value is not significantly different for both methods. These values agree with those reported by Schönbeck and Holm (2019) and other authors for natural CDs: large negative enthalpy ($\Delta H^\circ \approx -25$ to -30 kJ/mol) and relative small entropy changes ($T\Delta S^\circ \approx 0$ to -7 kJ/mol).

The calculated relative thermodynamic parameters through the linear van 't Hoff equation (Eq. (4)) are presented in Table 4, compared with the values obtained by other authors for flavonoids such as pinocembrin (Zhou et al., 2014), hesperidin and naringin (Tommasini et al., 2004) using a similar methodology. The values obtained for the encapsulation process of propolis in BCD are in the same order of magnitude as those found in literature for flavonoids and polyphenols individually encapsulated in BCD. The negative value of the enthalpy change (ΔH) indicates that the interaction process of the ligands with BCD leading to complex fomation is exothermic. 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 (Astray et al., 2010; Rekharsky & Inoue, 1998).

The negative value of ΔG^0 for the global propolis encapsulation process, as for the ligands (Table 4), indicates that the inclusion process in BCD is spontaneous.

Present results confirm the formation of the complexes and support the idea of substitution of high-enthalpy water molecules from the inner cavity by appropriate hydrophobic ligands (dos Santos et al., 2017; Szejtli, 1998). Furthermore, these results suggest that the water molecules inside the cavity could be easily removed by compounds of an adequate size and hydrophobicity to occupy the CD cavity, and thus, form energetically favored inclusion complexes.

From the global viewpoint of supramolecular chemistry, the determination of the thermodynamic parameters is indispensable for a quantitative understanding of the molecular recognition phenomena involving various hosts, guests and solvents (dos Santos Silva Araujo et al., 2021; Rekharsky & Inoue, 1998).

4. Conclusions

The water sorption isotherm of poplar propolis extract is presented for the first time. The presence of BCD greatly increased the amount of water adsorbed by propolis extract. However, the amount of adsorbed water in BCD-propolis system was smaller than that in BCD. These results are consistent with the displacement of water molecules from the inner cavity of the CDs when the propolis components are included.

The analysis of water adsorption behavior of the BCD-propolis complexes becomes of fundamental importance to define the conditions for their appropriate storage in dehydrated conditions.

The limited water solubility of propolis could be overcome by the formation of BCD inclusion complexes. The results of the present study showed a linear relationship between the dissolved phenolic compounds and the amount of β-cyclodextrin at the studied temperatures, and that the inclusion of propolis components is an energetically favored process. The entropy and enthalpy changes have an important role in the comprehension of the interactions between the guest molecule and the solvated hydrophobic cavity of the BCD.

The obtained data could be of interest for the development of aqueous propolis formulations avoiding the use of ethanol or other organic solvents. Furthermore, the use of cyclodextrins would also allow the masking intrinsic undesirable propolis flavors, allowing its use as functional food ingredient.

CRediT authorship contribution statement

Cristina I. dos Santos Ferreira: Investigation, Methodology, Writing – review & editing. **Adriana Pereyra Gonzales:** Formal analysis. **María Florencia Mazzobre:** Project administration, Writing – review & editing. **Natasha Poklar Ulrih:** Conceptualization, Resources. **María del Pilar Buera:** Project administration, Writing – review & editing.

Declaration of competing interest

The authors of this work declare that they have no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge financial support by ANPCyT (Agencia Nacional de Promocion de la Investigacion, el Desarrollo Tecnologico y la Innovacion. Argentina) (PICT 2017-1744 and PICT 2018-01822) and UBA (Universidad de Buenos Aires, Argentina) (UBACYT N° 20020170100557BA; 20020190200402BA, 20020170100459BA and 20020130100443BA). MFM and MPB are members of CONICET. Authors also thank Nicolás Villagran for his contributions in the statistical analyzes and general editing.

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