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Characterization of hydroxytyrosol- β -cyclodextrin complexes in solution and in the solid state, a potential bioactive ingredient

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ABSTRACT

This study focused for the first time on characterizing the inclusion complexes between β -cyclodextrin (β -CD) and hydroxytyrosol (HT) in the solid state. In solution, HT and β -CD are able to form a 1:1 inclusion complex with an association constant of $33.2 \pm 3.7 \text{ M}^{-1}$. In the solid state, the inclusion complexes prepared by freeze-drying and spray-drying of an equimolar mixture of both partners were characterized and compared by ^{13}C NMR and SEM. After dissolution, their free radical-scavenging ability was also determined by UV-visible spectroscopy. The results show that β -CD and drying processes have no effect on the efficiency of HT to reduce the DPPH radical. The solid state ^{13}C NMR data provided information on the spatial proximity between β -CD and HT and suggest the formation of inclusion complexes for both drying processes compared to the physical mix. However, the morphology of the solids obtained was significantly different, as spherical particles were formed by spray-drying while freeze-drying only provided irregular shapes.

1. Introduction

Phenolic compounds are the main class of secondary metabolites acting at low concentration in plant defenses against ultraviolet damage or predators (Beart, Lilley, & Haslam, 1985; Lattanzio, Kroon, Quideau, & Treutter, 2008). Abundant in fruits and vegetables, phenolic compounds are considered as the major (non-essential) micronutrients in the human diet (Faridi Esfanjani & Jafari, 2016; Tsao, 2010). Their daily consumption provides protection against cardiovascular disease, obesity and diabetes but also against neurodegenerative disorders such as Alzheimer's (de Mello Andrade & Fasolo, 2014). The specific benefits of the phenolic compounds typical of olive oil have led the European Food Safety Agency to issue a health claim about the relation between the dietary consumption of hydroxytyrosol (HT) from olive oil and protection of blood lipids against oxidative damage (Bulotta et al., 2014; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011). The antioxidant activity of phenolic compounds is closely related to their hydroxylation degree (Servili et al., 2013). Thus, the catechol unit of HT is responsible for its general electron-donating capacity. This property underlies the ability of HT to scavenge the reactive

oxygen species (ROS) involved in oxidative stress and also possibly to modulate the expression of genes sensitive to redox regulation (Kitsati, Mantzaris, & Galaris, 2016). Unfortunately, the catechol group is also responsible for the relatively sensitivity of HT to oxidation during food storage or processing.

To answer the current food industrial demand, olive biophenols, in particular HT, could be used as natural functional food additives or ingredients. However, adequate formulation may be required to increase their chemical stability and preserve their bioactivity. The utilization of encapsulated phenolic compounds, instead of free compounds, can overcome the drawbacks of their instability, but also alleviate unpleasant tastes or flavors, enhance their aqueous solubility and potentially control their release, improve their bioavailability and half-life *in vivo* and *in vitro* (Munin & Edwards-Lévy, 2011).

Being recognized as safe for food applications, cyclodextrins (CDs) have been extensively studied as hosts for encapsulation (Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gándara, 2009; Ratnasooriya & Rupasinghe, 2012). CDs are natural cyclic oligosaccharides consisting of glucopyranose subunits bound through α -(1, 4) links. CDs are obtained from the degradation of starch, for example

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by *Bacillus macerans* (Astray et al., 2009; Szejtli, 1988). Their hydrophobic cavity can accommodate a variety of guest compounds to form inclusion complexes, while their hydrophilic outer surface provides aqueous solubility. Examples of phenolic compounds forming inclusion complexes with CDs in aqueous solution are rosmarinic acid (Aksamija, Polidori, Plasson, Dangles, & Tomao, 2016), ferulic acid and gallic acid (da Rosa et al., 2013; Olga, Styliani, & Ioannis, 2015), resveratrol (Lu, Cheng, Hu, Zhang, & Zou, 2009; Lucas-Abellán, Mercader-Ros, Zafrilla, Gabaldón, & Núñez-Delgado, 2011) and phenol-rich plant extracts (Kalogeropoulos, Yannakopoulou, Gioxari, Chiou, & Makris, 2010; Mantegna et al., 2012; Rajha et al., 2015; Ratnasooriya & Rupasinghe, 2012).

With its 7 D-Glc subunits, β -CD has been chosen because of its internal diameter of about 6.6 Å, which is consistent with the molecular size of HT (Uekama, Hirayama, & Irie, 1998). A few works have investigated the β -CD - HT binding in aqueous solution. López-García et al. determined the corresponding binding constant using NMR and studied the protection afforded by β -CD to HT (López-García, López, Maya, & Fernández-Bolaños, 2010). Rescifina et al. investigated the inclusion complexes of olive phenols with β -CD by NMR and molecular dynamics (Rescifina, Chiacchio, Iannazzo, Piperno, & Romeo, 2010). Formation of the HT - β -CD complex in solution does not guarantee its persistence in the solid state. So far, this topic has not been addressed in the literature.

In this work, the encapsulation of HT into β -CD in aqueous solution and in the solid state was investigated. In solution, the complex was characterized by its stoichiometry and stability constant obtained by UV-visible spectroscopy. Then, HT + β -CD powders were prepared by spray- or freeze-drying and the encapsulation yield and efficiency were assessed. The powders were characterized by UV-visible spectroscopy according to the DPPH test. Finally, the shape and size of the inclusion complexes in the solid state were determined by SEM.

2. Materials and methods

2.1. Materials

Hydroxytyrosol was purchased from Extrasynthèse (Genay, France). β -CD was kindly given by Roquette Frères (Lesterm, France). 2,2-diphenyl-1-picrylhydrazyl was supplied from Sigma-Aldrich Co (St Louis, USA) methanol was purchased from Sigma-Aldrich Chimie (Fontenay sous Bois, France), and water was supplied by Fisher Scientific (Leics, UK). Folin-Ciocalteu reagent was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

2.2. Spectrophotometric analysis

UV-visible analyses were recorded using an Agilent 8453 spectrophotometer (Waldbronn, Germany) equipped with a magnetically stirred quartz cell (optical pathlength = 1 cm). The stoichiometry and stability constant of the HT - β -CD complex were calculated from the changes in absorbance monitored at 292 nm.

2.3. Preparation of the complexes in the solid state

An equimolar aqueous solution of HT and β -CD (final concentration of each = 5 mM) was prepared and filtered through a 0.45 μ m syringe filter (VWR International, North America, USA) before the drying processes.

2.3.1. Spray-drying conditions

The Nano Spray Dryer B-90 (Büchi, Switzerland) was operated with the long version of the drying chamber (height = 150 cm, diameter = 55 cm) with the following conditions: gas inlet temperature = 100 °C (spray head temperature = 85 °C), drying gas (air) flow rate = 100 L/min, feed rate = 0.5 mL/min, inside

pressure = 35 mbar, spray rate = 100%, spray mesh corresponding to 4 μ m size holes. The dried powder was collected from the cylindrical particle-collecting electrode using a particle scraper.

2.3.2. Freeze-drying conditions

The freeze-drying was performed using an ALPHA 1-4 LD plus (Christ Martin, Germany). The frozen samples were slowly dried at -50 °C under 0.06 mbar.

2.3.3. Preparation of the physical mix

Accurately weighed equimolar amounts of HT and β -CD (0.1 mmol each) were mixed using a mortar and pestle during 10 min.

2.4. Analysis of the inclusion complex in aqueous solution

2.4.1. Stoichiometry determination

For the calculation of the stoichiometry, equimolar freshly prepared aqueous solutions of β -CD and HT were mixed in various proportions in volumetric flasks (total volume = 10 mL). Thus, the mole fraction r of HT was varied from 0.1 to 0.9, while the sum of the total host and guest concentrations was held constant at 0.7 mM. The absorbance change δ was then measured and plotted against r (Job's plot).

2.4.2. Association constant determination

For the determination of the binding constant K , 0.5 mM aqueous solutions of HT containing β -CD in increasing concentration (0-10 mM) were prepared. The absorbance A was measured at 292 nm. The absorbance data were fitted with the Benesi-Hildebrand double reciprocal plot, as follows:

$$\frac{1}{\Delta A} = \frac{1}{\Delta \epsilon CK [CD]} + \frac{1}{\Delta \epsilon C}$$

Where:

$$K = \frac{[HT - CD]}{[HT][CD]}$$

C = total HT concentration, $\Delta A = A - A_0$, A_0 = absorbance of HT alone = $\epsilon_{HT}C$, $\Delta \epsilon = \epsilon_{HT-CD} - \epsilon_{HT}$

2.4.3. Hydrogen abstraction by DPPH

To 2 mL of a freshly prepared 0.06 mM solution of DPPH in MeOH - H₂O (1:1, v/v) was added 0.05 mL of a freshly prepared 0.6 mM solution of HT or HT + β -CD. After 60 min, the spectra were recorded for the determination of the stoichiometry (number of DPPH radicals reduced per HT molecule) and radical scavenging activity (RSA), as follows:

$$n = \frac{A_0 - AF}{\epsilon_{DPPHC}}$$

Where A_0 = initial absorbance at 515 nm (100% DPPH), AF = final absorbance at 515 nm, C = HT concentration.

2.5. Analysis of solid inclusion complexes

2.5.1. Solid recovery

The solid recovery (SR) of the spray-drying and freeze-drying processes was calculated as follow:

$$\% SR = \frac{m(\text{recovered solid})}{m(\text{initial solid})} \times 100$$

2.5.2. Moisture content and water activity

The moisture content in the samples was determined by oven-drying the powders at 102 °C to a constant weight. Samples were cooled to room temperature in a desiccator prior to weighting. The weight loss of the powders was expressed as a percentage of the initial weight.

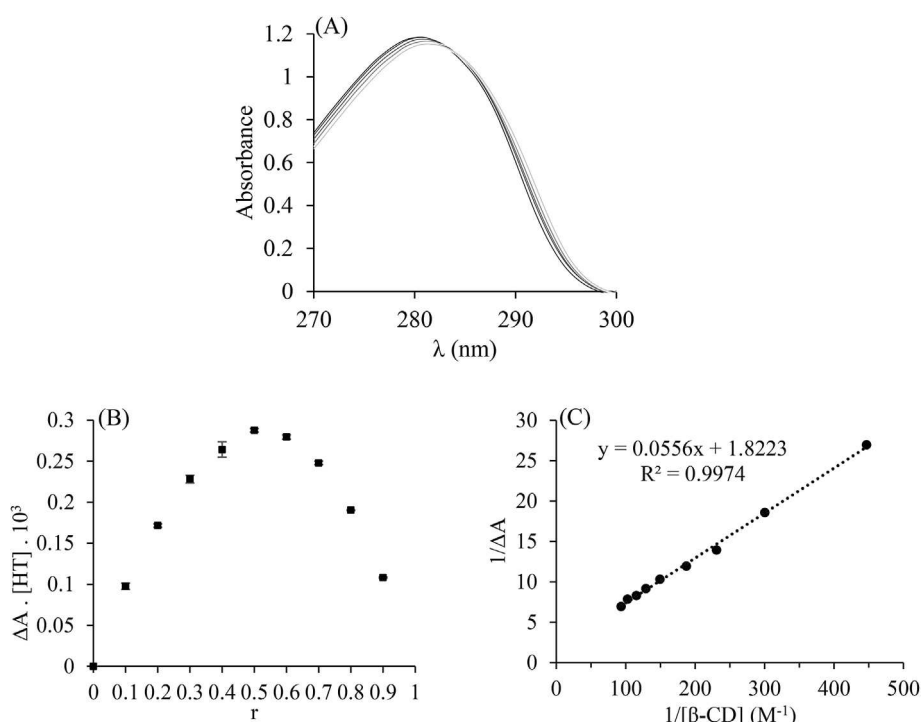


Fig. 1. HT - β -CD binding in aqueous solution. (A) Absorption spectra of HT in the presence of increasing amounts of β -CD. (B) Job's plot for the determination of the stoichiometry. (C) Benesi-Hildebrand plot for the determination of the binding constant determination.

The water activity (a_w) of the samples was assessed at 25 °C using the Aqualab TDL water activity meter (Decagon Devices, Inc., USA) at 25 °C. Four standard salt solutions (Decagon) of known a_w were used for calibration.

2.5.3. Encapsulation efficiency

The encapsulation efficiency (EE) of the spray-drying and freeze-drying processes was estimated as described by da Rosa et al. (2013) with a few modifications: samples of HT + β -CD powder (50 mg) were dissolved in 2 mL MeOH, sonicated for 5 min and centrifuged for 10 min at 3000 rpm. Then, the HT concentration was evaluated by UV-visible spectroscopy. The encapsulation efficiency was finally calculated:

$$\% EE = \frac{TPf}{TPi} \times 100$$

Where TPf is the HT content in the methanolic fraction and TPi is the initial HT content.

2.5.4. Solid nuclear magnetic resonance

The solid-state NMR spectra were obtained on a Bruker AvanceHD-400 MHz NMR spectrometer operating at a ^{13}C resonance frequency of 106 MHz and using a commercial Bruker double-channel probe. About 50 mg of samples were placed in zirconium dioxide rotors of 4-mm outer diameter and spun at a Magic Angle Spinning rate of 10 kHz. The CP technique (Schaefer & Stejskal, 1976) was applied with a ramped ^1H -pulse starting at 100% power and decreasing to 50% during the contact time (2 ms) in order to circumvent Hartmann-Hahn mismatches (Cook, Langford, Yamdagni, & Preston, 1996; Peersen, Wu, Kustanovich, & Smith, 1993).

To improve the resolution, a dipolar decoupling GT8 pulse sequence was applied during the acquisition time (Gerbaud, Ziarelli, & Caldarelli, 2003). To obtain a high signal-to-noise ratio in the ^{13}C CP/MAS experiments, 1K scans were accumulated using a delay of 2.5 s. The proton relaxation time in the rotating frame (^1H T1 ρ) was measured by recording the ^{13}C signal as a function of the ^1H spin-locking time in the

range 0.1–15 ms (12 values) before the CP period in ^{13}C CP/MAS experiments. A frequency field of 65 kHz was used for the spin-lock field B1. The recycle delay was 10 s, the number of transients was 1024 and a contact time of only 0.4 ms was selected to minimize ^1H spin-diffusion. The carbon spin-lattice relaxation time in the rotating frame (^{13}C T1 ρ) was measured using a standard CP pulse sequence with 0.4 ms contact time, proton spin-locking interruption from 0.1 to 20 ms (16 values) over the cross-polarization period, and 10 s recycle delay.

In addition, proton-detected heteronuclear 2D ^1H - ^{13}C HETCOR experiments were carried out to identify correlations that are in close spatial proximity (Schmidt-Rohr, Clauss, & Spiess, 1992). The 2D ^1H - ^{13}C HETCOR experiment was performed with a short contact time (0.3 ms) to reduce spin diffusion effects and with a homonuclear ^1H - ^1H FSLG (Frequency Switch Lee-Goldburg, Lee & Goldberg, 1965) decoupling during the t1 evolution period, to improve the resolution in the second dimension. The recycle delay was set at 3s, the number of scans at 1024 and the number of t1 increment in the second dimension at 64. The chemical shifts were referenced to tetramethylsilane and calibrated with glycine carbonyl signal set at 176.5 ppm.

2.5.5. Scanning electron microscopy (SEM) and size distribution

The outer structures of spray-dried and freeze-dried HT + β -CD particles were investigated by SEM using a Philips XL30 FEG at a 1 kV voltage. The microstructure was not coated and observed at different extensions. The spray-dried particle diameters were estimated by image analysis. The Image J software (an open source image processing program) was used and a minimum of 100 particles were measured using a calibrated micrometric scale. To ensure a good particle size distribution, size classes were selected each 0.2 μm from 0 to 3.4 μm .

3. Results and discussion

3.1. Stoichiometry and association constants determination

β -CD does not absorb the UV light but induces small changes in the UV spectrum of HT ($\lambda_{\text{max}} = 280 \text{ nm}$) (i.e. bathochromic and

Table 1

Solid recovery (SR), encapsulation efficiency (EE), moisture content and water activity (a_w) of spray- and freeze-drying processes.

	Spray-drying	Freeze-drying
SR (%)	53.0	91.0
EE (%)	84.4 ± 3.2	89.6 ± 3.7
% moisture	7.0 ± 0.1	6.1 ± 0.1
a_w	0.42 ± 0.01	0.32 ± 0.02

hyperchromic shifts) that were ascribed to encapsulation (Fig. 1A). These absorbance changes were used for the calculation of the stoichiometry and binding constant of the β -CD - HT complex. For the stoichiometry, the method of continuous variation, known as Job's plot, was selected. The ΔA [HT] product was plotted against the mole fraction of HT (r) ranging from 0.1 to 0.9 (Fig. 1B). A symmetrical curve with a maximum at $r = 0.5$ evidenced the formation of a complex having a 1:1 stoichiometry in accordance with literature data (López-García et al., 2010). Fig. 1C presents the linear Benesi-Hildebrand plot for the determination of the binding constant, which is deduced from the intercept/slope ratio: $K = 33.2 (\pm 3.7) \text{ M}^{-1}$. Using NMR, López-García et al. obtained a close value of $43 (\pm 1) \text{ M}^{-1}$ (López-García et al., 2010).

3.2. Solid recovery and encapsulation efficiency

Spray- and freeze-drying processes were used to recover the HT - β -CD complexes as powders from aqueous solutions. Spray-drying is a fast thermal process whereas freeze-drying is a cold process requiring much more time. As shown in Table 1, the latter provided better yields. Indeed, solid recovery after spray-drying was almost twice lower because only part of the powder was sent to the electrostatic receiver and actually recovered, while the rest remained on the top of the walls of the drying chamber and thus was lost. For instance, Wilkowska et al. prepared blueberry juice polyphenols encapsulated in β -CD by freeze-drying with a 82% solid recovery while Nunes et al. using spray-drying only obtained a 51% recovery yield of encapsulated lycopene (Nunes & Mercadante, 2007; Wilkowska, Ambroziak, Czyżowska, & Adamiec, 2016).

The moisture content represents the total amount of water in powders while water activity is a measure of free water, which must be kept low enough ($a_w < 0.60$) to avoid microbial growth and spoilage. The moisture content obtained was 6.1 ± 0.1 by freeze-drying and 7.0 ± 0.1 by spray-drying with a_w values of 0.32 ± 0.02 and 0.42 ± 0.01 , respectively. These low values guarantee a good microbial stability for both powders.

Moreover, the low temperature of the freeze-drying process may be more suitable for the preservation of thermally sensitive compounds such as HT. To our knowledge, no study has reported the encapsulation efficiency of HT into β -CD. However, our results are similar to literature data for other phenolic compounds such as gallic acid. Using hydroxypropyl- β -CD, Olga et al. reached a 89.2% EE by spray-drying while Rosa et al. obtained a slightly lower EE (80%) by freeze-drying (da Rosa et al., 2013; Olga et al., 2015).

3.3. Hydrogen abstraction by DPPH

Phenolic compounds can act as hydrogen or electron donors to radicals and other oxidizing species, thereby preventing the oxidation of important biomolecules such as polyunsaturated lipids (Dangles, 2012). As a first approach, the free radical-scavenging ability of phenolic compounds can be assessed by monitoring the reduction of the stable colored DPPH radical in MeOH. As β -CD is insoluble in MeOH, a 1:1 MeOH - water mixture was used (Ferreira et al., 2013).

Table 2 shows the radical-scavenging percentage values and the

Table 2

DPPH assay: radical scavenging activity and stoichiometry of HT and HT + β -CD powders.

	% RSA ^a	n
HT (control)	83.3 (± 1.0)	3.21 (± 0.09)
Equimolar HT + β -CD mixture	86.0 (± 3.7)	3.30 (± 0.16)
Spray-dried HT + β -CD powder	83.0 (± 3.2)	3.25 (± 0.04)
Freeze-dried HT + β -CD powder	85.8 (± 3.7)	3.27 (± 0.01)

^a RSA: radical scavenging activity.

stoichiometry n of HT samples ($n =$ number of DPPH radicals reduced per HT molecule). Freeze-dried and spray-dried HT + β -CD samples were compared with HT and a 1:1 HT + β -CD physical mix. The % RSA and n values were essentially the same for all samples. So, both encapsulation processes do not affect HT integrity and preserve its H-donating capacity. These results were in accordance with Kfoury et al. who showed that encapsulation into CDs did not change the antioxidant capacity of eugenol, caffeic acid or ferulic acid (Kfoury, Landy, Auezova, Greige-Gerges, & Fourmentin, 2014).

Finally, the stoichiometry values of the different samples in MeOH - H₂O (1:1) are slightly higher than for HT in MeOH: $n \approx 2.5$ (Roche; Dufour, Mora & Dangles, 2005), which could point to an easier regeneration of the catechol nucleus from the *o*-quinone, possibly after tautomerization into a *p*-quinone methide followed by water addition (Dangles, 2012).

3.4. Solid state NMR

The influence of mobility in the relative intensity of the host and guest signals can be probed in the kHz frequency ranges by measuring the ¹H and ¹³C spin-locking (¹H T1 ρ and ¹³C T1 ρ) relaxation times. ¹H T1 ρ and ¹³C T1 ρ were chosen to probe spin diffusion and mobility, respectively. Indeed, motion is the driving mechanism for ¹³C T1 ρ , because it is less influenced by spin diffusion than ¹H T1 ρ . Indeed, the spin diffusion rate increases with the magnetogyric ratio and natural isotopic abundance and is therefore higher for ¹H than for ¹³C. HT and β -CD relaxation times were measured using the signals recorded at ca. 37 ppm and 102 ppm, respectively (see Table 3).

In the case of the HT + β -CD physical mix, different ¹H T1 ρ values were measured for β -CD and HT. This is consistent with 2 independent spin systems (no spin diffusion) and thus suggests that HT and β -CD do not interact. For both spray-dried and freeze-dried samples, similar ¹H T1 ρ were measured for HT and β -CD, which clearly suggests a common spin system for both compounds. In other words, host - guest ¹H - ¹H distances are now small enough to allow spin diffusion in agreement with the formation of an inclusion complex. ¹³C T1 ρ values are lower for HT than for β -CD in all samples with no clear influence of encapsulation. This is consistent with less restricted molecular motion for HT than for β -CD.

To confirm the spatial proximity between HT and β -CD in the complex, a 2D ¹H-¹³C HETCOR experiment was performed on the three samples: the physical mixture of HT and β -CD and the freeze-dried or spray-dried HT/ β -CD complexes (Fig. 2). HETCOR experiments rely on dipolar couplings, which are strongly dependent on nuclear

Table 3

Spin-diffusion (¹H T1 ρ) and mobility (¹³C T1 ρ) for HT + β -CD solid samples.

Samples	¹ H T1 ρ *	(ms)	¹³ C T1 ρ *	(ms)
Physical mix	β -CD	4.5	β -CD	19.5
	HT	2.5	HT	2.6
Spray-dried sample	β -CD	4.5	β -CD	14.5
	HT	4.5	HT	3.5
Freeze-dried sample	β -CD	5.4	β -CD	17.2
	HT	5.5	HT	9.2

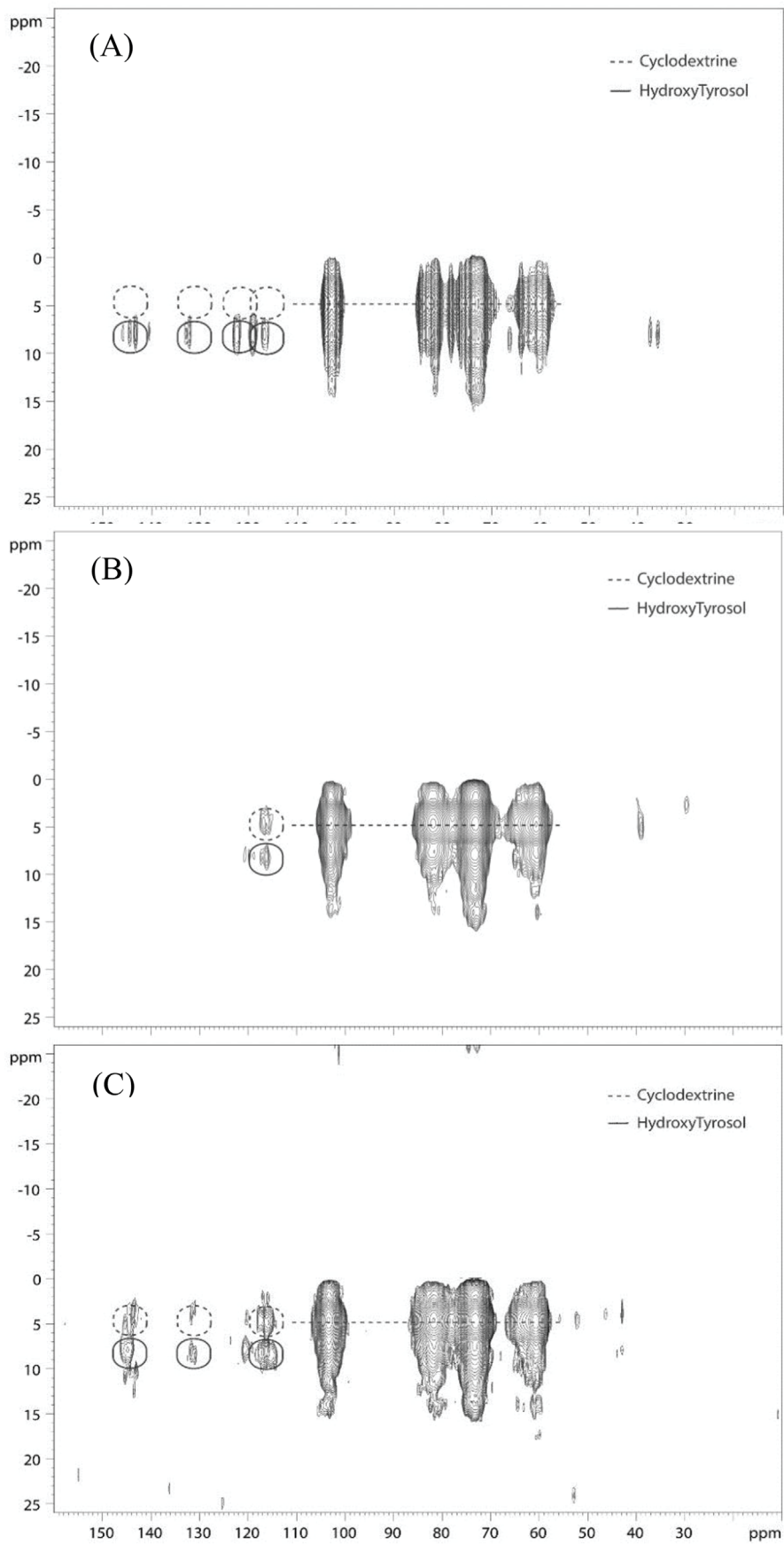


Fig. 2. 2D ^1H - ^{13}C HETCOR NMR spectra of HT + β -CD physical mixture (A), freeze-dried (B) and spray-dried (C) HT + β -CD complexes.

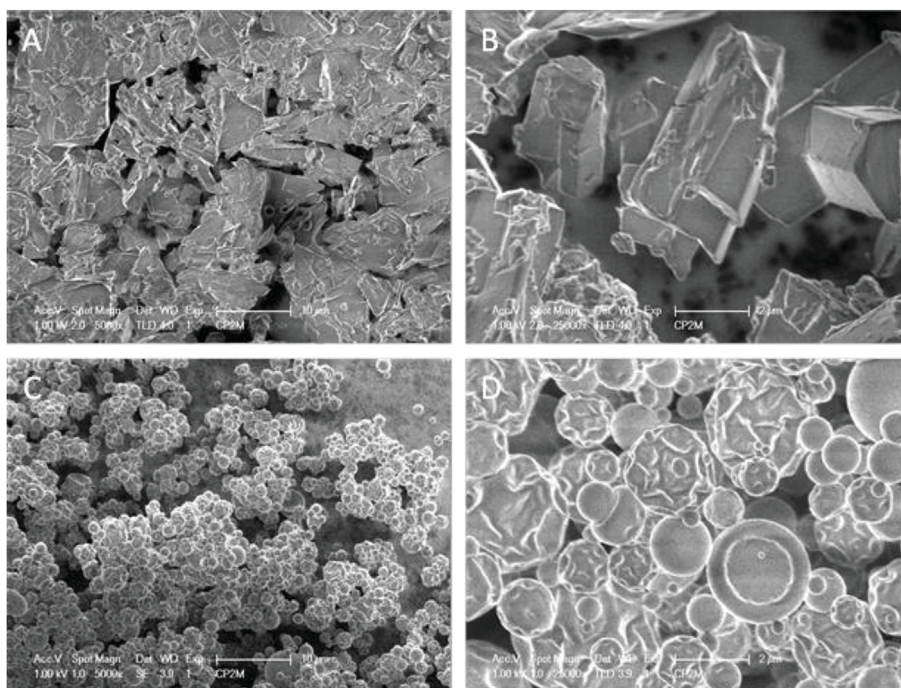


Fig. 3. Scanning electron micrographs at 1 kV of freeze-dried (A, B) and spray-dried (C, D) HT + β -CD powders. Magnification: x5000 (A, C), x25000 (B, D).

spatial proximity at the sub-nanometer scale. In other words, the observation of correlation peaks between pairs of nuclei indicates intimate contact between these nuclei. For the HT + β -CD physical mixture, only independent HT or β -CD signals were observed (Fig. 2A). In contrast, for the HT/ β -CD complexes, correlations (evidenced by dotted circles) between the HT carbons and the CD protons were detected (Fig. 2B and C), thus confirming the close spatial proximity between HT and CD in the complexes.

Malapert et al. (Malapert, Tomao, Dangles, & Reboul, 2018) studied the bioaccessibility and uptake by Caco-2 cells of HT incorporated into a meal either as a pure standard or as a spray-dried β -CD complex. β -CD was shown to act as a protective agent for HT in the oral compartment but did not alter HT bioavailability.

3.5. Scanning electron microscopy (SEM) and size distribution

The micrographs obtained by SEM showed two kinds of external structures according to the selected drying process. Freeze-dried particles (Fig. 3A and B) display irregular structures compared to spray-dried particles (Fig. 3C and D), which have spherical and indented shapes. The wrinkled surface of the spray-dried particles could be attributed to capillary forces and fast dehydration during the process (Foerster, Gengenbach, Woo, & Selomulya, 2016; Yang et al., 2015).

For industrial development, the particle size is an important criteria, influencing the solubility of the inclusion complex (Khadka et al., 2014). The diameter distribution of the spray-dried particles was estimated using the calibrated scale on micrographs and the Image J software (Fig. 4).

The particle diameters ranged from 0.4 to 3.4 μ m, in agreement with previous data on the same spray-dryer (Bürki, Jeon, Arpagaus, & Betz, 2011; Pérez-Masiá et al., 2015). More than 15% of particles had diameters between 1.4 and 1.6 μ m, defining the first population mode. Moreover, particles with diameters distributed around 2.2–2.4 μ m represented ca. 10% of the population. The size polydispersity is a frequent consequence of spray-drying (Huntington, 2004; Yao et al., 2016).

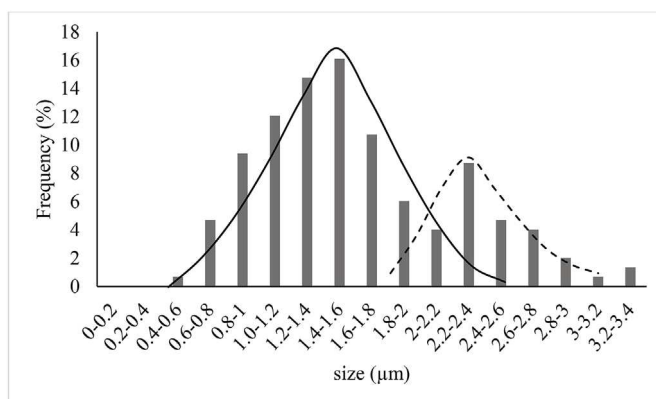


Fig. 4. Diameter distribution in number for the spray-dried HT - β -CD complex determined by image analysis of SEM photographs.

4. Conclusion

Spectroscopic analyses provided evidence that HT and β -CD form a weak inclusion complex of 1:1 stoichiometry in aqueous solution. Based on the K value, the percentage of complex in solution from an equimolar (5 mM) mixture of both partners is lower than 20%. However, spray-drying or freeze-drying of such solutions allowed the simple preparation of HT + β -CD powders with HT encapsulated inside the macrocycle as evidenced by solid state ^{13}C CP/MAS NMR. Such preparations could express extended shelf-life for HT upon storage in the solid state. On the other hand, dissolution of the powders in MeOH – H₂O (1:1) show that the radical-scavenging activity of HT (and thus its chemical integrity) is fully preserved during the drying processes and that in solution HT is immediately released from its complex to express its bioactivity. SEM analysis indicated that freeze-dried particles were irregular in shape, whereas spray-dried particles displayed a spherical and smooth surface. In conclusion, hydroxytyrosol can form inclusion complexes with β -CD in aqueous solution and in the solid state, which could foster its development as a bioactive ingredient.

Declarations of interest

None.

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