López-Miranda Santiago (Orcid ID: 0000-0002-3820-5875)

Modified cyclodextrin type and dehydration methods exert a significant effect on the antimicrobial activity of encapsulated carvacrol and thymol

Running Tittle: Antimicrobial activity of carvacrol and thymol cyclodextrin complexes

Santiago López-Miranda[1]*, Daniel Berdejo[2], Elisa Pagán[2], Diego García-Gonzalo[2] and Rafael Pagán[2]

[1] Department of Food Technology and Nutrition Molecular Recognition and Encapsulation (REM) Group. UCAM Universidad Católica de Murcia. Avenida de los Jerónimos, 135, 30107, Guadalupe, Murcia, Spain.

[2] Departamento de Producción Animal y Ciencia de los Alimentos. Facultad de Veterinaria. Instituto Agroalimentario de Aragón-IA2 (Universidad de Zaragoza-CITA). C/Miguel Servet, 177, 50013, Zaragoza, Spain.

*Corresponding author:

Tel: +34 968 278756

e-mail: slmiranda@ucam.edu

Department of Food Technology and Nutrition. Molecular Recognition and Encapsulation (REM) Group. UCAM Universidad Católica de Murcia. Avenida de los Jerónimos, 135, 30107, Guadalupe, Murcia, Spain.

Keywords

Carvacrol, thymol, cyclodextrin, antimicrobial, spray-drying, freeze-drying.

ABSTRACT

BACKGROUND

The antimicrobial activity of essential oils (EOs) and their constituents has led to increasing interest in using them as natural preservative agents. However, their high sensitivity to light and oxygen, their volatility, and their low aqueous solubility are all obstacles to their application in the food, cosmetic, or pharmaceutical industries. Encapsulation in cyclodextrins (CDs) is a solution for the application of EOs.

RESULTS

The complexation of carvacrol and thymol with hydroxypropyl (HP)-α-, HP-β-, and HP-γ-CDs, the behavior of the solid complexes prepared by freeze-drying and spray-drying methods, and the antibacterial activity of solid complexes was studied. K_c values of HP-α- and HP-γ-CD complexes with carvacrol (118.4 and 365.7 M^{-1}) and thymol (112.5 and 239.7 M^{-1}) were far lower than those observed for HP-β-CD complexes with carvacrol (2268.2 M^{-1}) and thymol (881.6 M^{-1}). The lower stability of HP-α- and HP-γ-CD complexes increased the release of compounds, thereby affecting the antimicrobial activity of carvacrol and thymol to a lesser extent than complexation with HP-β-CD, normally used in the encapsulation of carvacrol and thymol. HP-β-CD encapsulation of carvacrol and thymol drastically reduced their antimicrobial activity. The freeze-drying method barely affected the antimicrobial activity of carvacrol and thymol after encapsulation, while spray-drying could be considered for the production of solid complexes in combination with the appropriate CD.

CONCLUSIONS

It was thus demonstrated that HP-α- and HP-γ-CD are a very suitable alternative for the encapsulation of carvacrol and thymol with the purpose of preserving their bacteriostatic and bactericidal activities.

INTRODUCTION

In recent years, the food industry has profoundly innovated in terms of food preservation processes and food safety. Essential oils (EOs), as well as their constituents, have been shown to be a powerful and natural antimicrobial agent against a large number of pathogens. [1] These antibacterial agents are extracted from natural sources, and are generally regarded as healthy, environmentally friendly, and safe,[2] which makes them attractive to consumers.[3] However, food industrial application of these compounds faces challenges that need to be solved, such as their limited aqueous solubility as well as their high volatility and instability against external agents such as light and oxygen, all of which render difficult a controlled release of the compounds and reduce their antimicrobial efficacy.[4]

One of the techniques most widely used to address these limitations has been encapsulation, comprising a wide and varied range of procedures such as freeze-drying, spray-drying, coacervation, gelation, precipitation, and nanoemulsion,[4,5] as well as a wide range of encapsulating agents such as maltodextrins, gums, starch, chitosan, protein and zeins.[4,5,6,7]

Among all the encapsulation alternatives for EOs and their components, the use of molecular encapsulation in cyclodextrins (CDs) stands out. CDs are cyclic oligomers that are widely used and recommended for industrial use as protectors for light-sensitive, oxygen-sensitive and heat-sensitive compounds, solubilizers of dyes and vitamins, flavor

stabilizers, suppressants of flavors and unpleasant flavors, and regulators of controlled release of food additives and drugs.[8]

Several authors have previously studied the effect of encapsulation in CDs on the solubility, stability, controlled release and bioactive properties, particularly the antimicrobial activity, of different EOs such as clove and oregano, [9] coriander, [10] thyme, [11] cinnamon,[3] basil and tarragon,[12] *Mentha* x *villosa* Hudson,[13] guava leaf,[14] and pepper,[15] as well as some of their main components with high antimicrobial potential such as carvacrol and thymol.[16,17,18,19,20] However, despite a wide range of existing literature, there is no consensus whether the encapsulation of these compounds in CDs positively or negatively affects their biological properties, especially their antimicrobial activity.[21] Although encapsulation in CDs increases the aqueous solubility of EOs and terpenes, the stability of the complex can affect the degree of release of the active compounds as well as their biological activity.

In addition, the majority of previous studies almost exclusively focused on the use of β- and hydroxypropyl (HP)-β-CD, with almost no references to the encapsulation of EOs or their components with modified HP-α- or HP-γ-CDs. In a recent review of the characterization of volatile compounds encapsulated in CDs, Kfoury et al.[22] provided more than 300 values of complexation constants with a series of CDs, but not with HP-α- and HP-γ-CD. Rakmai et al.[23] report 26 technological applications of complexed EOs in CDs, whereby 25 of them are focused on encapsulation with β- or HP-β-CD. On the other hand, Lima et al.[24] published almost 150 references, of which only two consider the complexation of terpenes in HP-γ-CD and none in HP-α-CD. This preference for β- and HP-β-CDs is due to the greater compatibility of their hydrophobic cavity with most terpenes and oils with a reduced molecular weight (200-800 g/mol),[25] which provides complexes with high stability. However, complexes with high stability can reduce

compound release from CDs and limit their functional properties. It is therefore of great interest to study the ability to modulate the biological activity of antimicrobial compounds by complexing with other CDs, especially with modified ones such us HP-α- and HP-γ-CDs, in order to obtain complexes with lower stability constants than those provided by the most widely used β- and HP-β-CDs.

On the other hand, solid state CD complexes are another key aspect for food application, because solid state improves handling, stability, allows for standardized dosage of active compounds. The most frequently used methods for obtaining solid complexes of EOs are those that do not require high temperatures, such as freeze-drying, kneading, and precipitation.[13,15,16,17,25] However, the use of the spray-drying method, despite being an easy system to perform on an industrial scale,[5] is less widespread in the dehydration of EO complexes with CDs due to its high process temperature. Therefore, it is key to advance in the study of interactions between CD type and solid complex dehydration methods in order to modulate the biological activity of the encapsulated compounds for industrial applications.

The aims of this study are, on the one hand, to study the effect that the stability of CD complexes has on the antimicrobial activity of carvacrol and thymol, introducing, in addition to the well-known HP-β-CDs, the modified HP-α-CD and HP-γ-CD, the behavior of which has not been previously described in the literature. On the other hand, the effect of the method of preparing solid complexes, via spray-drying as well as via freeze-drying, was determined for the physical and antimicrobial properties of those complexes.

MATERIAL AND METHODS

Reagents and standards

Carvacrol (98 % purity) and thymol (99 % purity) were purchased from TCI Europe N.V. (Zwijndrecht, Belgium). The HP-α-, HP-β- and HP-γ-CDs were purchased from Winplus International Limited (Ningbo, China). HPLC reagents acetonitrile and water were purchased from JT Baker (Deventer, The Netherlands). Other chemical reagents used were of analytical grade.

Complexation and phase solubility diagrams

The complexation of carvacrol and thymol in CDs was carried out by phase solubility diagrams according to the method described by Higuchi and Connors with some modifications.[26] Excess amounts of carvacrol and thymol were added to 50 mL of aqueous solutions in concentrations increasing from 0 to 50 mmol L^{-1} for HP-α-, HP-β- and HP-γ-CDs, and were continuously stirred during 24 h at 20 ºC in the dark. After 24 h, solutions were filtered using 0.45 μm cellulose acetate membrane filters (Chromafil Macherey-Nagel, Düren, Germany) for HPLC assays. Phase solubility diagrams were made in triplicate.

The complexation constant K_c between carvacrol and thymol and each type of CD was calculated from the slope of the phase solubility diagram and the solubility of the compound aqueous solution (S_0) by using the equation (1):

$$K_c(L\ mol^{-1}) = \frac{slope}{S_0 \cdot (1 - slope)} \quad (1)$$

Complexation efficiency (CE) is the ratio between the dissolved complex and the concentration of free CDs. It is independent of S_0, and was calculated from the slope of the phase solubility profiles by using the equation (2):

$$CE\ (\%) = \frac{Slope}{(1-slope)} x100 \quad (2)$$

The molar ratio drug:cyclodextrin (D:C) was calculated using the CE value with the equation (3):

$$D:C = 1:\left(1 + \frac{1}{CE}\right) \quad (3)$$

The solubilization potential (S_t/S_0) was calculated as the relation between compound solubility at 50 mM CD concentration (S_t) and compound aqueous solubility (S_0).

Solid complexes by freeze-drying and spray-drying

Solid complexes were obtained by dehydrating 100 mL volume with a molar proportion of 50:25 (CD:Compound) for HP-α-CD and HP-γ-CD, and 50:40 (CD:Compound) for HP-β-CD. These molar proportions were selected according to the solubilization potential of the different CDs in order to ensure that all of the active compound was complexed. Solutions were continuously stirred during 24 h at 20 ºC in the dark. After 24 h, the solutions containing the complexes were either freeze- or spray-dried.

Solutions of carvacrol and thymol complexes were freeze-dried in a Christ Alpha 1-2 LD Plus (Martin Christ, Osterode am Harz, Germany) freeze dryer at -48 ºC during three days. Solutions of carvacrol and thymol complexes were spray-dried in a Buchi B-290 device (Flawil, Switzerland). The spray drier configuration was: inlet air temperature 170 ºC, outlet air temperature 68 ºC, inlet air flow 35 $m^3\ h^{-1}$, pump flow 5 mL min^{-1}, and compressed air caudal 360 L h^{-1}. The recovered freeze- and spray-dried solid complexes were stored in an airtight glass container for posterior analysis and characterization. For carvacrol and thymol HPLC quantification, solid complexes were dissolved in distilled

water and filtered using 0.45 μm cellulose acetate membrane filters (Chromafil Macherey-Nagel, Düren, Germany).

The dehydration yield (DY) was calculated using the equation:

$$\text{DY}\ (\%) = \frac{\text{solid complexes obtained (g)}}{\text{total solids in solution (g)}} \cdot 100 \quad (4)$$

The encapsulation efficiency (EE) was calculated using the equation:

$$\text{EE}\ (\%) = \frac{\text{total compound encapsulated in solid complex (mg)}}{\text{initial total compound in solution (mg)}} \cdot 100 \quad (5)$$

The drug load (DL) was calculated using the equation:

$$\text{DL}\ (\text{mg}\ g^{-1}) = \frac{\text{total compound encapsulated in solid complex (mg)}}{\text{total solid complexes (g)}} \quad (6)$$

Wettability is defined as the time taken by a solid to completely submerge in a humid medium. The analysis was carried out by gently dropping 500 mg of powder on the surface of 100 mL of distilled water at 20 ºC.

Encapsulation efficiency, drug load, and wettability were measured in triplicate for each sample for purposes of statistical analysis.

Carvacrol and thymol determination by HPLC

Carvacrol and thymol were quantified by HPLC analysis using an HPLC Agilent Technologies Model 1200 (Agilent, Santa Clara, California, USA) equipped with a diode array detector set at 280 nm, injecting 20 μL of filtered complexes. Separations were carried out on an endcapped (5 μm) HPLC Cartridge 250-4 LiChospher 100 RP-18. The column temperature was set to 30 ºC, and the flow rate was 0.7 mL min^{-1}. The mobile phase was water with 0.5 % of acetic acid (A) *versus* acetonitrile (B), for a total running time of 7 min and a constant proportion of 20 % (A) 80 % (B). Time retentions were 4.3 min for carvacrol and 4.1 min for thymol. The data were processed by Agilent ChemStation software.

Particle size and shape

Shape and structural characteristics of the solid complexes were studied by Field Emission Scanning Electron Microscope (FESEM) images. Uncoated samples were examined using a MERLIN™ VP COMPACT SEM microscope (Carl Zeiss Microscopy, Oberkochen, Germany). Images detailing morphology were taken using an SE2 detector under an accelerating voltage of 1 kV.

Antibacterial activity

Bacteria and growth conditions

Escherichia coli O157:H7 Sakai/*stx1A*/*stx2A* was kindly provided by Kyu-Tae Chang.[27] FPR3757 strain of methicillin-resistant *Staphylococcus aureus* USA300 was provided by Prof. Kolter (Harvard Medical School, Boston, MA, USA). This strain was isolated from an outbreak in the United States.[28]

Throughout this investigation, both strains were kept at -80 °C in cryovials with glycerol. To prepare the broth subcultures, one single colony from a tryptone soya agar plate supplemented with 0.6 % yeast extract (Oxoid, Basingstoke, Hampshire, England; TSAYE) was inoculated in a test tube with 5 mL of sterile tryptone soya broth with 0.6 % yeast extract added (Oxoid; TSBYE). The inoculated tubes were incubated overnight in aerobic conditions at 37 °C (Selecta Incudigit, Barcelona, Spain) to obtain bacterial subcultures. 250 mL Erlenmeyer flasks containing 50 mL of TSBYE were inoculated with those subcultures to a final concentration of 10^5 colony forming units (CFU) mL^{-1}. To reach the stationary growth phase (2 x 10^9 CFU mL^{-1} approximately), bacterial cultures were incubated for 24 h under agitation (130 rpm) at 37 °C (Selecta Rotabit).

Disk diffusion test

A modified disk diffusion test was applied in order to screen the antimicrobial activity of carvacrol and thymol encapsulated in HP-α-, HP-β- and HP-γ-CDs against *E. coli* O157:H7 Sakai and *S. aureus* USA300.[29] Filter paper disks (Whatman No. 1; diameter: 6 mm) containing 600 μg of encapsulated active compound were applied on TSAYE plates that had been previously inoculated with a diluted culture (0.5 McFarland) and spread on the surface. For a period of 24 h, the plates were incubated at the appropriate temperature (37 ºC), and the resulting zone of partial inhibition was observed.

Minimum inhibitory and bactericidal concentration (MIC and MBC)

The MIC and MBC of carvacrol and thymol, free and encapsulated in HP-α, HP-β and HP-γ-CD, were evaluated against *E. coli* O157:H7 Sakai and *S. aureus* USA300 with the purpose of studying the encapsulation effect on the antimicrobial properties of those two compounds following a protocol adapted from CLSI (2015). Test tubes containing 5 mL of Mueller Hinton broth (Oxoid; MHB) with a diluted (10^5 CFU mL^{-1}) stationary phase culture were inoculated with CD-encapsulated thymol and carvacrol at different concentrations (from 150 to 1,000 mg L^{-1} at intervals of 50 mg L^{-1}). Subsequently, test tubes were incubated at 37 ºC for 24 h in a shaking thermostatic incubator (Bunsen, mod. BTG, Madrid, Spain) at 130 rpm. Positive controls containing MHB with microbial cultures at 10^5 CFU mL^{-1} without carvacrol and thymol, as well as negative controls containing MHB with encapsulated carvacrol and thymol without bacteria were also prepared. MIC was determined as the lowest concentration of carvacrol or thymol in the presence of which bacteria showed no visible growth. Additionally, test tubes were aliquoted and spread onto Mueller Hinton agar plates (Oxoid, MHA), and, after incubation (37 ºC / 24 h), survivors were counted with an improved image analyzer

automatic counter (Protos; Analytical Measuring Systems, Cambridge, United Kingdom). The MBC was set as the minimum concentration of the compound that inactivated more than 3 $\log_{10}$ cycles (< 10^2 UFC mL^{-1}).

RESULTS AND DISCUSSION

Complexation of carvacrol and thymol in HP-α-, HP-β- and HP-γ-CD

The phase diagrams of carvacrol and thymol encapsulated in HP-α-, HP-β- and HP-γ-CD are shown in Figure 1. The phase solubility diagrams showed an A_L type profile, for both compounds and for all CDs, indicating that water-soluble complexes had been formed. In all cases studied, the slope of the solubility diagrams was lower than 1, indicating that the stoichiometry of the complexes was 1:1, whereby each molecule of carvacrol or thymol enters into one molecule of CD.[26] The same A_L diagram was previously observed for carvacrol and thymol encapsulated in HP-β-CDs by Kamimura et al. and Kfoury et al.,[16,18] thereby suggesting that this 1:1 stoichiometry is common for these compounds.[30] To the best of our knowledge, these are the first published results on the subject of carvacrol and thymol encapsulation with HP-α- and HP-γ-CDs.

From the information plotted in phase diagrams, different parameters that characterize the complexation of these compounds in CDs were calculated. Table 1 shows complexation constant (K_c), complexation efficiency (CE), molar ratio (D:C), and solubilization potential (S_t/S_0) of HP-α-, HP-β-, and HP-γ-CD complexes.

The complexation constant (K_c) was calculated by using linear regression analysis from the phase solubility diagrams according to equation (1). One can observe that the highest values of K_c were obtained for HP-β-CD. The K_c values for carvacrol and thymol encapsulated in HP-β-CD were 2268.2 L mol^{-1} and 881.6 L mol^{-1} respectively. These values are similar to those obtained by Kfoury et al.[18] K_c values of carvacrol and thymol

complexes with HP-α- and HP-γ-CD were significantly lower ($p < 0.05$) than those obtained for HP-β-CD. K_c values for HP-α-CD were 118.5 and 112.5 L mol^{-1}, and for HP-γ-CD they were 365.7 and 237.7 L mol^{-1} for carvacrol and thymol, respectively. K_c values lay, in general, within the range of 200 to 2000 L mol^{-1}, which are usual values for aromatic compounds.[30] No K_c values for HP-α- and HP-γ-CD have been described in previous literature, neither for these compounds nor for others of a similar nature. In fact, the vast majority of the available literature almost exclusively focuses on studying the interactions of different molecules with β- and HP-β-CD, which have been cited as the most suitable for the encapsulation of compounds of an aromatic nature.[18,20,22,23]

The stability of a CD complex depends on an adequate inclusion of the host molecule inside the hydrophobic cavity of the CD molecule. Based on the classification proposed by Carrier et al.,[31] the stability of carvacrol and thymol complexes with HP-α- and HP-γ-CDs could be classified as very weak (0-500 L mol^{-1}), while that of complexation with HP-β-CD would be between weak (500-1000 L mol^{-1}) and moderate (1000-5000 L mol^{-1}). The K_c values obtained in this study suggest that the size of HP-α-CD could be too small for carvacrol and thymol to be able to complete their encapsulation within the hydrophobic cavity. On the other hand, the cavity size of HP-γ-CD could be too large, thereby causing carvacrol and thymol molecules to fail to establish a sufficient amount of stable interactions with hydrophobic cavity atoms. In previous studies carried out with these and similar molecules, it has been observed that the K_c complexation constants with native CDs have been generally higher for β-CD than for α- and γ-CD.[12,18,22] Obtaining complexes with a different stability by using different CDs could represent an advantage in controlling and modulating the release of biological active compounds.

K_c indicates complexation strength or complex stability, and it is normally used to compare the affinity of any guest molecule with different CD types. However, in order to analyze the solubilizing effect of CDs, complexation efficiency (CE) is more suitable because it is independent of S_0. CE represents the ratio between complex and free CD concentration, and, for 1:1 complexes, it can be calculated from the slope of the phase solubility diagram with equation (2).[32] In addition, CE can be used to calculate the molar ratio (D:CD), which indicates the ratio between the number of CD-forming complexes and the total number of CDs in solution (equation 3).

The obtained values of CE and molar ratio are shown in Table 1. The highest CE value was obtained for the carvacrol-HP-β-CD complex (1769.2 %) followed by the thymol-HP-β-CD complex (537.8 %). These values are similar to those obtained by other authors for the same compounds and CDs.[18] Significantly lower values ($p < 0.05$) were observed for carvacrol and thymol complexes with HP-γ-CD (285.2 % and 146.2 %, respectively). The lowest complexation efficacy values were displayed by carvacrol and thymol complexes with HP-α-CD (92.4 % and 68.6 %, respectively). In general, the CE values obtained in this study were higher than those reported by other authors for different types of compounds and CDs.[32] CE values close to or even greater than 100 % mean that there was a higher proportion of CD-forming soluble complexes than of free CDs, which indicates that CDs were being used effectively to solubilize the guest molecule by forming soluble complexes. Almost all of the dissolved HP-β- and HP-γ-CD molecules were complexed with a carvacrol molecule, as shown by the 1:1.1 and 1:1.4 molar ratio, respectively (Table 1). The behaviour of thymol complexes was similar, with a molar ratio of 1:1.2 and 1:1.7 for HP-β- and HP-γ-CD, respectively. HP-α-CD complexes displayed a lower molar ratio than HP-β- and HP-γ-CD, indicating that one of each two

molecules of HP-α-CD in solution were forming soluble complexes (1:2.1 and 1:2.5 for carvacrol and thymol, respectively).

The solubilization potential (S_t/S_0) was comparable in carvacrol and thymol, although slightly higher values were obtained for thymol than for carvacrol, mainly due to the lower S_0 value of thymol (Table 1), as suggested by previous studies.[18] At a CD concentration of 50 mmol L^{-1}, the solubility of carvacrol and thymol increased more than 7-fold with HP-β-CD and almost 6-fold with HP-γ-CD, which indicates the high solubilization potential not only of HP-β-CD but also of HP-γ-CD. HP-α-CD has shown a significantly ($p < 0.05$) lower solubilization potential, increasing the aqueous solubility 4.07 and 4.30-fold for carvacrol and thymol, respectively.

Characterization of solid complexes

Table 2 shows the characterization parameters of the solid complexes of carvacrol and thymol with HP-α-, HP-β-, and HP-γ-CD dehydrated by freeze- or spray-drying. Dehydration yield (DY, grams of solid complex recovered with respect to the initial grams of soluble solids, equation 4) was higher for freeze-drying than for spray-drying. On average, freeze-drying obtained a recovery yield of 93 % in contrast with 80 % for spray-drying. Despite the lower values for spray-drying relative to freeze-drying, the spray-drying DY of 80 % was over 50 %, which is the minimum recommended recovery rate for adequate dehydration process efficiency.[33] This fact indicates that conditions of temperature, inlet air flow, and atomization level were adequate for obtaining solid complexes by spray-drying. On the other hand, a slightly increasing trend was observed in the dehydration yield from HP-α- < HP-β- < HP-γ-CD. This increasing trend could be due to the fact that the preparation of solid complexes was carried out with the same initial CD concentration of 50 mmol L^{-1}, which means that the initial amount of soluble solids

was increasing from HP-α- < HP-β- < HP-γ-CD, thereby resulting in a more efficient recovery of dehydrated complexes.[34]

Encapsulation efficiency (EE, equation 5) represents the amount of active matter that has been recovered after the dehydration process, indicating whether significant compound losses occur during the dehydration process. For all the solid complexes obtained, EE was significantly higher ($p < 0.05$) for freeze-drying than for spray-drying. On average, the encapsulation efficiency for freeze-drying was 93.5 %. These values were similar to those obtained by Kfoury et al.[30] for thymol encapsulated in HP-β-CD, and slightly higher than those provided by Kamimura et al.[16] for carvacrol likewise encapsulated in HP-β-CD, as well as by Hill et al.[3] for aromatic compounds that were similar although encapsulated in other CDs. The low temperature conditions of the freeze-drying process were key in limiting aromatic compound losses during the process. On the other hand, the EE of spray-drying ranged from 64.9 to 88.8 % for carvacrol, and from 70.2 to 79.8 % for thymol.

Regarding the effect of CD type on EE, no statistically significant differences ($p > 0.05$) were observed among freeze-dryied dehydrated complexes, except between thymol complexes with HP-α-CD and with HP-β-CD. When complexes were dehydrated by spray-drying, EE was higher for HP-α-CD and lower for HP-γ-CD, with HP-β-CD in an intermediate position. A high concentration of soluble solids, as occurred with HP-γ-CD, could increase the solution's viscosity prior to being sprayed, which can negatively affect the retention of encapsulated compounds.[35] Kfoury et al. also observed a reduction in EE for native CDs in the α-CD > β-CD > γ-CD trend.[12]

In relation to the amount of compound that can be encapsulated per gram of complex (drug load, DL, equation 6), the freeze-drying process showed higher values than spray-drying, with statistically significant differences ($p < 0.05$) for all solid

complexes studied. Regarding the effect of CD type, HP-β-CD had the highest DL value for both carvacrol and thymol, and for both the freeze-drying and the spray-drying method. From a toxicological and economic point of view, increasing DL can be advantageous for the bulk formulation and bioavailability of active material.[36] However, it should be noted that when complexes were prepared with HP-α-CD and spray-dried, the lower compound:CD molar ratio and the adequate EE enabled HP-α-CD to have a DL close to that of HP-β-CD, with no statistical differences ($p > 0.05$) between these CDs for carvacrol solid complexes.

One of the physical characteristics of solid complexes that was most affected by the dehydration method was wettability (Table 2). This parameter is affected by different factors such as particle size, water content, and crystalline structure. All solid complexes dehydrated by freeze-drying had a wettability time under 1 s, without any observable difference between CDs or compounds. However, spray-dried complexes had a much higher wettability time. The difference in wettability behavior between dehydration methods is probably related to the solubilization capacity associated with the different crystal and particle structures that result from using a specific procedure. Figure 2 shows the FESEM images of the particles obtained by freeze-drying (Figure 2 A, B) and spray-drying (Figure 2 C, D) for the solid complexes of carvacrol with HP-β-CD. These images were representative of the other solid complexes obtained, regardless of CD type or encapsulated compound. One can observe that the particles of the freeze-dried solid complexes were composed of crystals with angular, sharp shapes and straight edges. Similar shapes were obtained by Tao et al.[25] for thymol and β-CD complexes. Conversely, solid complexes dehydrated by the spray-drying method showed the typical wrinkled globular structure of particles obtained through this procedure.[37] The capacity of water to interact with the crystalline structure of freeze-dried complexes could be closely related

to the short wettability times, which cause an instantaneous dissolution of the lyophilized solid complexes in water. On the other hand, the use of high temperatures in the spray-drying process allows for a semi-permeable membrane to be formed on the surface of the particles, which could slow down the powder's wettability.[4]

Antimicrobial activity of solid complexes

Antimicrobial activity of the solid complexes was initially monitored by using the inhibition halo technique, for *E. coli* O157:H7 Sakai as representative of a Gram-negative bacterium, and for *S. aureus* USA300 as representative of Gram-positive types. This technique has been previously used to evaluate the antimicrobial activity of EOs encapsulated in CDs.[9,10] In view of their high EE and DL, freeze-dried solid complexes were selected to carry out a preliminary evaluation of the antimicrobial activity of carvacrol and thymol complexed in modified CDs. Table 3 shows the results of the inhibition halos of freeze-dried solid complexes of carvacrol and thymol encapsulated with HP-α-, HP-β-, and HP-γ-CD against *E. coli* O157:H7 Sakai and against *S. aureus* USA300. The use of this basic technique revealed the relevance of CD type for the antimicrobial activity of carvacrol and thymol. As shown in Table 3, inhibition halos for both *E. coli* O157:H7 Sakai and *S. aureus* USA300 were significantly higher ($p < 0.05$) for both HP-α-CD and HP-γ-CD than those for HP-β-CD. In order to analyze the interactions between the microorganisms and the complexes in further detail, we carried out MIC and MBC determinations.

Table 4 shows the MIC and MBC values for *E. coli* O157:H7 Sakai and for *S. aureus* USA300 of carvacrol and thymol free and encapsulated in HP-α-, HP-β-, and HP-γ-CD. MIC values of free compounds ranged from 150 to 200 mg L^{-1}, and MBC values from 200 to 250 mg L^{-1}. These values are similar to those previously reported in the

literature,[38,39,40,41] but lower than other previous research works.[16,17,20] When carvacrol and thymol were encapsulated, antimicrobial activity was reduced to a greater or lesser extent as a function of the type of CD used for encapsulation. For freeze-dried complexes, carvacrol and thymol encapsulation in HP-α-CD and HP-γ-CD maintained the antimicrobial activity against *E. coli* O157:H7 Sakai and *S. aureus* USA300 very close to that exhibited by the free compounds, increasing MIC and MBC values only by 50 mg L^{-1} for most cases studied. However, when both compounds were encapsulated in HP-β-CD, antimicrobial activity was clearly reduced. The freeze-dried complexes presented MIC values four and two times higher than free carvacrol and thymol, respectively. Regarding bactericidal activity, the MBC values of HP-β-CD freeze-dried complexes were four and three times higher than those of free compounds, exceeding, in some cases, the maximum tested concentration of 1,000 mg L^{-1}.

Many authors have previously studied the effect of CD encapsulation on the biological properties, mainly the antimicrobial and antioxidant activity, of EOs and/or their components. However, conclusive results cannot be reached. In contrast with our results, Kamimura et al.[16] and Santos et al.[17] observed a significant increase in the antimicrobial activity of carvacrol encapsulated in HP-β-CD and β-CD against *E. coli* K12 and *S.* Typhimurium LT2 with respect to the free compound, although encapsulation conversely reduced antioxidant capacity. Rodríguez et al.[20] also concluded that encapsulation in HP-β-CD improved the antimicrobial capacity of carvacrol and thymol against *E. coli* CECT 943 and *S. aureus* CECT 239. Similar conclusions have likewise been reached by other authors.[3,14] On the other hand, Anaya-Castro et al.[9] observed that encapsulation of mexican oregano EO in β-CD did not significantly affect its antimicrobial activity, and Dima et al.[10] reported a decrease in the antimicrobial and antioxidant capacity of coriander EO encapsulated in β-CD with respect to free EO.

Regarding other biological processes such as antifungal activity, Del Toro-Sánchez et al.[11] observed that the encapsulation of thyme EO in β-CD reduced its antifungal capacity, and Kfoury et al.[42] determined that antifungal activity of phenylpropanoids was reduced when encapsulated in HP-β-CDs. This disparity in the results of previous studies has already been indicated by Kfoury et al., who concluded that the effect of encapsulation in CDs on the biological activity of aromatic compounds can present effects that do not always go in the same direction.[21] One of the reasons that could explain this disparity in results may be related to the use of different methodologies for the characterization of antimicrobial activity of free compounds, which can lead to a wide range of MIC and MBC values reported. Based on previous work, the methodology used in this study only applied vigorous shaking as a method to ensure sufficient dispersion of the terpene in the culture medium.[43] Using this methodology, values of MIC and MBC obtained in this study were in consensus with those reported in previous studies.[38,39,40,41] However, when additional actions were carried out to disperse the terpenes in the culture medium, such as the use of surfactants, the values of MIC and MBC of the terpenes in free state were higher.[16,17,20] As a result, these authors observed a significant increase in the antimicrobial activity of encapsulated compunds, while those who reported lower MIC and MBC values for free compounds, as concluded in this study, affirmed that efficiency was maintained or decreased.[9,10]

Taken into account the method used for antimicrobial activity determination in this study, this research demonstrated that encapsulation in CDs reduces, to a greater or lesser extent, the antimicrobial capacity of carvacrol and thymol, proving that the stability of the complexes, defined as the K_c values and dependent on the type of CD, can modulate these compounds' antimicrobial activity. The highest K_c value, which was determined for the carvacrol-HP-β-CD complex (2268.1 L mol^{-1}, Table 1), indicates a greater stability

of carvacrol-HP-β-CD but a lower amount of release of the antimicrobial compound. As a consequence, the antimicrobial activity of such compounds would decrease, as demonstrated by the highest MIC and MBC value obtained by the freeze-dried HP-β-CD complexes. Furthermore, it was determined that encapsulation of carvacrol and thymol in HP-α-CD and HP-γ-CD, not previously studied in the literature, provided a lower K_c value than HP-β-CD (Table 1), thereby allowing a more efficient release of the compounds while scarcely affecting their antibacterial activity.

Regarding the effect of the type of dehydration method used to obtain solid complexes, it was observed that spray-drying reduced the antimicrobial activity of carvacrol and thymol in relation to the antimicrobial activity of freeze-dried complexes. The MIC and MBC values (Table 4) of HP-α-CD complexes were, on average, 160 % and 147 % higher for spray-dried than for freeze-dried complexes, respectively. For carvacrol-HP-β-CD complexes, the MIC values increased from 700 mg L^{-1} (freeze-dried) to over 1,000 mg L^{-1} (spray-dried) for both bacteria, whereas for thymol-HP-β-CD complexes the MIC values were, on average, 95 % higher for spray-dried than for freeze-dried complexes. The reduction in antimicrobial activity of spray-dried complexes may be related to the high temperatures to which such complexes are subjected during the dehydration process, thereby negatively affecting the antimicrobial capacity of the antibacterial compounds,[44] as well as to the lower ease of water dissolution of spray-dried complexes as shown by their degree of wettability (Table 2), which could hamper the interaction of antimicrobial compounds with bacteria. However, HP-γ-CDs have been shown to better protect the antimicrobial capacity of carvacrol and thymol after spray-drying than HP-α-CD or HP-β-CD. In the case of HP-γ-CD spray-dried complexes, the loss of antimicrobial activity resulted in an increase of MIC values lying between 0 and 50 mg/L for carvacrol against *E. coli* O157:H7 Sakai and against *S. aureus* USA300,

respectively, and corresponding to 100 mg L^{-1} for thymol against both microorganisms. On the one hand, the K_c values of 365.7 and 239.7 L mol^{-1} for carvacrol and thymol with HP-γ-CD (Table 1) may be high enough to protect the compounds during the dehydration process, but, on the other hand, these K_c values were still sufficiently low to allow for fast release of the compound when it was rehydrated. In addition, the solubilization potential values (S_t/S_0) of HP-γ-CD were significantly higher than those of HP-α-CD, allowing for better water dissolution of antimicrobial compounds and thus improving their antimicrobial capacity.

CONCLUSION

In conclusion, this research shows that modified HP-α-CD and HP-γ-CD represent a very suitable alternative for carvacrol and thymol encapsulation, since they obtain complexes with a lower K_c value and release the compounds more readily, thus leading to bacteriostatic and bactericidal activities significantly higher than the complexes with HP-β-CD that are normally used for the encapsulation of these compounds. In this regard, it was observed that the HP-β-CD encapsulation of carvacrol and thymol drastically reduces their antimicrobial activity. Therefore, in order to consider the use of HP-β-CD for the encapsulation of this type of compound, it would be necessary to study mechanisms that activate the release of the host molecule from the inner cavity of the CD: procedures such as combined treatments with changes in pH and/or temperature. On the other hand, freeze-drying is the preferable alternative to obtain solid complexes with the purpose of maintaining the antimicrobial activity of carvacrol and thymol after encapsulation, although the spray-dry method, mainly combined with HP-γ-CD, allows for the obtention of solid complexes that maintain an antimicrobial activity of an efficacy comparable to that displayed by compounds in a free state.

ACKNOWLEDGEMENTS

This study was financially supported by MINECO (Spanish Ministry of Economy and Competitiveness, Project No. PGC2018-093789-B-I00), by the European Social Fund, and by the Aragonese Office of Science, Technology and University Research.

CONFLICT OF INTEREST

The authors declare that this research was carried out in the absence of commercial or financial relationships that could be constructed as a possible conflict of interest.

REFERENCES

1. Rivera J, Crandall PG, O'Bryan CA, and Ricke SC, Essential oils as antimicrobials in food systems – A review. *Food Control* **54**:111-119 (2015). https://doi.org/10.1016/j.foodcont.2014.12.040
2. Ju J, Chen X, Xie Y, Yu H, Gou Y, Cheng Y, Qian H and Yao W, Application of essential oils as a sustained release preparation in food packaging. *Trends Food Sci Technol* **92**:22-32 (2019). https://doi.org/10.1016/j.tifs.2019.08.005
3. Hill LE, Gomes C, and Taylor TM, Characterization of beta-cyclodextrin inclusion complexes containing oils (*trans*-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. *LWT–Food Sci Technol* **51**:86-93 (2013). https://doi.org/10.1016/j.lwt.2012.11.011
4. Alvarenga D, de Barros RV and Vilela S, Microencapsulation of Essential oils using spray drying technology, in *Microencapsulation and Microspheres for Food Applications*, ed. by Sagis LMC. Academic Press, Cambridge (USA) pp. 235-231 (2015). https://doi.org/10.1016/B978-0-12-800350-3.00013-3

5. El Asbahani A, Miladi K, Badri W, Sala M, Aït Addi EH, Casabianca H, El Mousadik A, Hartmann D, Jilale A, Renaud FNR and Elaissari A, Essential oils: From extraction to encapsulation. *Int J Pharm* **483**:220-243 (2015). https://doi.org/10.1016/j.ijpharm.2014.12.069

6. Hasheminejad N, Khodaiyan, F, and Safari M, Improving the antifungal activity of clove essential oil encapsulated by chitosan nanoparticles. *Food Chem* **275**:113-122 (2019). https://doi.org/10.1016/j.foodchem.2018.09.085

7. Merino N, Berdejo D, Bento R, Salman H, Lanz M, Maggi F, Sánchez-Gómez S, García-Gonzalo D and Pagán R, Antimicrobial efficacy of *Thymbra capitata* (L.) Cav. essential oil loaded in self-assembled zein nanoparticles in combination with heat. *Ind Crop Prod* **113**:98-104 (2019). https://doi.org/10.1016/j.indcrop.2019.03.003

8. Astray G, Gonzalez-Barreiro C, Mejuto JC, Rial-Otero R and Simal-Gándara J, A review on the use of cyclodextrins in foods. *Food Hydrocolloids* **23**:1631-1640 (2009). https://doi.org/10.1016/j.foodhyd.2009.01.001

9. Anaya-Castro MA, Ayala-Zavala JF, Muñoz-Castellanos L, Hernández-Ochoa L, Peydecastaing J and Durrieu V, β-Cylodextrin inclusion complexes containing clove (*Eugenia caryophyllata*) and mexicano regano (*Lippia berlandieri*) essential ois: Preparation, physochemical and antimicrobial characterization. *Food Packaging Shelf* **14**:96-101 (2017). https://doi.org/10.1016/j.fpsl.2017.09.002

10. Dima C, Cotarlet M, Tiberius B, Hahrim G, Alexe P and Dima S, Encapsulation of coriander essential oil in beta-cyclodextrin: antioxidant and antimicrobial properties evaluation. *Rom Biotech Lett* **19**:9128-9140 (2014). http://e-repository.org/rbl/vol.19/iss.2/4.pdf

11. Del Toro-Sánchez CL, Ayala-Zavala JF, Machi L, Santacruz H, Villegas-Ochoa MA, Álvarez-Parrilla E and González-Aguilar GA, Controlled release of antifungical

volatiles of thyme essential oil from β-cyclodextrin capsules. *J Incl Phenom Macrocycl Chem* **67**:431-441 (2010). https://link.springer.com/article/10.1007/s10847-009-9726-3

12. Kfourly M, Auezova L, Ruellan S, Greige-Gerges H and Fourmentin S, Complexation of estragole as pure compound and as main component of basil and tarragon essential oils with cyclodextrins. *Carbohydr Polym* **118**:156-164 (2015). https://doi.org/10.1016/j.carbpol.2014.10.073
13. Martins AP, Craveiro AA, Machado MIL, Raffin FN, Moura TF, Novák CS and Éhen Z, Preparation and characterization of *Mentha x Villosa* Hudons oil-β-cyclodextrin complex. *J Therm Anal* **88**:363-371 (2007). https://link.springer.com/article/10.1007/s10973-005-7407-z
14. Rakmai J, Cheirsilp B, Mejuto JC, Simal-Gándara J and Torrado-Agrasar A, Antioxidant and antimicrobial properties of encapsulated guava leaf oil in hydroxypropyl-beta-cyclodextrin. *Ind Crop Prod* **111**:219-225 (2018).
15. Suprani C, Gonçalves S, Diirr L, Oliveira JC, Fontes P, Moreira D, Yoshida MI, Checon JC, Fernandes D and Campos P, β-Cyclodextrin inclusion complexes with essential oils: Obtention, characterization, antimicrobial activity and potential application for food preservative sachets. *Food Res Int* **119**:499-509 (2019). https://doi.org/10.1016/j.foodres.2019.01.016
16. Kamimura JA, Santos EH, Hill LE and Gomes CL, Antimicrobial and antioxidant activities of carvacrol microencapsulated in hydroxypropyl-beta-cyclodextrin. *LWT–Food Sci Technol* **57**:701-709 (2014). https://doi.org/10.1016/j.lwt.2014.02.014
17. Santos EH, Kamimura JA, Hill LE and Gomes CL, Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant

applications. *LWT-Food Sci Technol* **60**:583-592 (2015). https://doi.org/10.1016/j.lwt.2014.08.046

18. Kfoury M, Landy D, Ruellan S, Auezova L, Greige-Gerges H and Fourmentin S, Determination of formation constants and structural characterization of cyclodextrin inclusion complexes with two phenolic isomers: carvacrol and thymol. *J Org Chem* **12**:29-42 (2016). https://www.beilstein-journals.org/bjoc/articles/12/5
19. Rodríguez-López MI, Mercader-Ros MT, López-Miranda S, Pellicer JA, Pérez-Garrido A, Pérez-Sánchez H, Núñez-Delicado E and Gabaldón JA, Thorough characterization and stability of HP-***β***-cyclodextrin thymol inclusion complexes prepared by microwave technology: A required approach to a successful application in food. J Sci Food Agr **99**: 1322-1333 (2018). https://doi.org/10.1002/jsfa.9307
20. Rodríguez-López, MA, Mercader-Ros MT, Pellicer JA, Gómez-López V, Martínez-Romero D, Núñez-Delicado E, and Gabaldón JA, Evaluation of monoterpene-cyclodextrin complexes as bacterial growth effective hurdles. *Food Control* **108**:106814 (2020). https://doi.org/10.1016/j.foodcont.2019.106814
21. Kfourly M, Auezova L, Greige-Gerges H and Fourmentin S, Cyclodextrin for Essential Oils Applications. I Cyclodextrin Application, in *Cyclodextrin Applications in Medicine, Food, Environment and Liquid Crystals*, ed. by Fourmentin S, Crini S and Lichtfouse E. Springer, Berlin, pp. 81-123 (2018). https://doi.org/10.1007/978-3-319-76162-6_4
22. Kfoury M, Landy D and Fourmentin, S, Characterization of cyclodextrin/volatile inclusion complexes: a review. *Molecules* **23**:1204 (2018). https://doi.org/10.3390/molecules23051204
23. Rakmai J, Cheirsilp B, Cid A, Torrado-Agrasar A, Mejuto JC and Simal-Gandara J, Encapsulation of Essential oils by cyclodextrins: characterization and evaluation, in

Cyclodextrin: a versatile ingredient, ed. by Aroa P and Dhingra N. IntechOpen, London, pp. 236-290 (2018). http://dx.doi.org/10.5772/intechopen.73589

24. Lima P, Lucchese A, Araújo-Filho H, Menezes P, Araújo A, Quintans-Júnior L and Quintans J, Inclusion of terpenes in cyclodextrins: preparation, characterization and pharmacological approaches. *Carbohydr Polym* **151**:965-987 (2016). https://doi.org/10.1016/j.carbpol.2016.06.040
25. Tao F, Hill LE, Peng Y and Gomes CL, Synthesis and characterization of β-cyclodextrin inclusion complexes of thymol and thyme oil for antimicrobial delivery applications. *LWT-Food Sci Technol* **59**:247-255 (2014). https://doi.org/10.1016/j.lwt.2014.05.037
26. Higuchi T and Connors KA, Phase solubility techniques. Advances in Analytical *Chem Instrum* **4**:56-63 (1965).
27. Kim SH, Lee SR, Kim KS, Ko A, Kim E, Kim YH and Chang KT, Shiga toxin A subunit mutant of *Escherichia coli* O157:H7 releases outer membrane vesicles containing the B-pentameric complex. *FEMS Immunol Med Microbiol* **58**:412–420 (2010). https://doi.org/10.1111/j.1574-695X.2010.00654.x
28. Kreiswirth B, Kornblum J, Arbeit RD, Eisner W, Maslow JN, McGeer A, Low DE and Novick RP, Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* **259**:227-230 (1993). https://doi.org/10.1126/science.8093647.
29. Meena MR and Sethi V, Antimicrobial activity of essential oils from spices. *J Food Sci Technol - Mysore* **31**:68-70 (1994).
30. Kfoury M, Auezova L, Fourmentin S and Greige-Gerges H, Investigation of monoterpenes complexation with hydroxipropyl-β-cyclodextrin. *J Incl Phenom*

Macrocycl Chem **80**:51-60 (2014). https://link.springer.com/article/10.1007/s10847-014-0385-7

31. Carrier RL, Miller LA and Ahmed I, The utility of cyclodextrins for enhancing oral bioavailability. *J Control Release* **123**:78-99 (2007). https://doi.org/10.1016/j.jconrel.2007.07.018

32. Loftsson T, Hreinsdóttir D and Másson M, The complexation efficiency. *J Incl Phenom Macrocycl Chem* **57**:545-552 (2007). https://link.springer.com/article/10.1007/s10847-006-9247-2

33. Fang Z and Bhandari B, Effect of spray drying and storage on the stability of bay berry polyphenols. *Food Chem* **129**:1139-1147 (2011). https://doi.org/10.1016/j.foodchem.2011.05.093

34. Tontul I and Topuz A, Spray-drying of fruit and vegetable juices: Effect of drying conditions on the product yield and physical properties. *Trends Food Sci Technol* **63**:91-102 (2017). https://doi.org/10.1016/j.tifs.2017.03.009

35. De Barros RV, Borges SV and Botrel DA, Gum Arabic / starch / maltodextrin / inulin as wall materials on the microencapsulation of rosemary essential oil. *Carbohydr Polym* **101**:524-532 (2014). https://doi.org/10.1016/j.carbpol.2013.09.083

36. Loftsson T, Hreinsdottir D and Masson M, Evaluation of cyclodextrin solubilization of drugs. *Int J Pharm* **302**:18-28 (2005). https://doi.org/10.1016/j.ijpharm.2005.05.042

37. Wang S, Shi Y and Han L, Development and evaluation of microencapsulated peony seed oil prepared by spray drying: oxidative stability and its release behavior during in-vitro digestion. *J Food Eng* **231:**1-9 (2018). https://doi.org/10.1016/j.jfoodeng.2018.03.007

38. Ait-Ouazzou A, Cherrat L, Espina L, Lorán S, Rota C and Pagán R, The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innov Food Sci Emerg Technol* **12**:320-329 (2011). https://doi.org/10.1016/j.ifset.2011.04.004

39. Espina L, Pagán R, López D, García-Gonzalo D, Individual constituents from essential oils inhibit biofilm mass production by multi-drug resistant *Staphylococcus aureus*. *Molecules* **20**:11357-11372 (2015). https://doi.org/10.3390/molecules200611357

40. Chueca B, Berdejo D, Gomes-Neto NJ, Pagán R and García-Gonzalo D, Emergence of hyper-resistant *Escherichia coli* MG1655 derivative strains after applying sub-inhibitory doses of individual constituents of essential oils. *Front. Microbiol*. March 2016 (**7**):273 (2016). https://doi.org/10.3389/fmicb.2016.00273

41. García-Salinas S, Elizondo-Castillo H, Arruebo M, Mendoza G and Irusta S, Evaluation of the antimicrobial activity and cytotoxicity of different components of natural origin present in essential oils. *Molecules* **23**:1-18 (2018). https://doi.org/10.3390/molecules23061399

42. Kfoury M, Sahraoui A, Bourbon N, Laruelle F, Fontaine J, Auezova L, Greige-Gerges H and Fourmentin S, Solubility, photostability and antifungal activity of phenylpropanoids encapsulated in cyclodextrins. *Food Chem* **196**:518-525 (2016). https://doi.org/10.1016/j.foodchem.2015.09.078

43. Friedman M, Henika PR, Mandrell RE, Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Prot* **65**:1545-1560 (2002). https://doi.org/10.4315/0362-028X-65.10.1545

44. Dos Santos R, Da Silva RA, Corso MP, and Canan, C. Essential oils microencapsulated obtained by spray drying: a review, *J Essent Oil Res* **31**:457-473 (2019). https://doi.org/10.1080/10412905.2019.1612788

Table 1. Aqueous solubility (S_0), complexation constant (K_c), complexation efficiency (CE), Molar Ratio (D:C), and solubility ratio (S_t/S_0) at 50 mmol L^{-1} of CDs for carvacrol and thymol for HP-α-CD, HP-β-CD, and HP-γ-CD.

Compound	CD	S_0 (mmol L^{-1})	K_c (L mol^{-1})	CE (%)	Molar Ratio (D:C)	S_t/S_0
Carvacrol	HP-α-CD	7.80±0.19	118.4±4.8[a]	92.4±3.8[a]	1:2.1±0.04[c]	4.07±0.14[a]
	HP-β-CD		2268.2±184.2[c]	1769.2±143.7[c]	1:1.1±0.00[a]	7.05±0.18[c]
	HP-γ-CD		365.7±13.7[b]	285.2±10.7[b]	1:1.4±0.01[b]	5.73±016[b]
Thymol	HP-α-CD	6.10±0.08	112.5±3.1[a]	68.6±1.9[a]	1:2.5±0.04[c]	4.30±0.12[a]
	HP-β-CD		881.6±6.6[c]	537.8±4.0[c]	1:1.2±0.00[a]	7.85±0.01[c]
	HP-γ-CD		239.7±2.0[b]	146.2±1.2[b]	1:1.7±0.01[b]	5.85±0.00[b]

Values represent means of triplicate determination (±Standard deviation).

In each column, statistical difference between means of HP-α, HP-β and HP-γ-CD for each compound is shown ($p < 0.05$) (a-c).

Table 2. Dehydration yield (DY, %), encapsulation efficiency (EE, %), drug load (DL, mg g^{-1}), and wettability (s) of solid complexes by freeze-drying (FD) and spray-drying (SD) for carvacrol and thymol encapsulated with HP-α-CD, HP-β-CD, and HP-γ-CD.

		DY (%)		EE (%)			DL (mg g^{-1})			Wettability (s)		
Compound	CD	FD	SD	FD	SD	S.S	FD	SD	S.S	FD	SD	S.S
Carvacrol	HP-α-CD	92.3	77.6	96.5±0.2^{a}	88.8±3.2^{b}	**	59.0±0.1^{b}	53.6±1.9^{b}	**	<1^{a}	22.3±1.5^{a}	**
	HP-β-CD	89.8	79.5	98.1±1.7^{a}	81.6±7.7^{b}	*	72.5±1.2^{c}	59.4±5.6^{b}	*	<1^{a}	53.3±5.5^{a}	**
	HP-γ-CD	94.1	81.9	95.7±1.7^{a}	64.9±3.5^{a}	**	40.9±0.7^{a}	28.1±1.5^{a}	**	<1^{a}	145.3±27^{b}	**
Thymol	HP-α-CD	90.0	77.5	88.3±1.6^{a}	79.8±2.0^{c}	**	54.8±1.0^{b}	49.2±1.2^{b}	**	<1^{a}	24.3±4.5^{a}	**
	HP-β-CD	92.3	79.6	99.2±0.1^{b}	75.2±0.5^{b}	**	73.07±0.1^{c}	54.5±0.4^{c}	**	<1^{a}	67.3±4.0^{b}	**
	HP-γ-CD	97.7	85.0	94.3±5.7^{ab}	70.2±1.0^{a}	**	39.5±2.4^{a}	29.9±0.4^{a}	**	<1^{a}	156.7±28.4^{c}	**

Values represent means of triplicate determination (±Standard deviation)

In each column, the statistical difference between means of HP-α, HP-β, and HP-γ-CD for each compound is shown ($p < 0.05$) (a-c).

Statistical significance (S. S.) between freezedrying (FD) and spray-drying (SD): (**) $p < 0.01$; (*) $p < 0,05$; (ns) not significant

Table 3. Inhibition halos (mm) for carvacrol and thymol against *Escherichia coli* O157:H7 Sakai and *Staphylococcus aureus* USA300 encapsulated in HP-α-, HP-β, and HP-γ-CD and dehydrated by freeze-drying; disk diameter 6.0 mm.

		Inhibition halo (mm)	
Compound	Encapsulation	*Escherichia coli*	*Staphylococcus aureus*
Carvacrol	HP-α-CD	12.7±0.4[b]	12.0±0.6[c]
	HP-β-CD	6.4±0.4[a]	7.1±0.5[a]
	HP-γ-CD	11.8±0.4[b]	10.4±0.1[b]
Thymol	HP-α-CD	13.7±0.3[c]	13.8±0.8[c]
	HP-β-CD	8.9±0.5[a]	8.2±0.1[a]
	HP-γ-CD	11.3±0.1[b]	11.8±0.5[b]

Values represent means of triplicate determination (±Standard deviation)

In each column, the statistical difference between means of HP-α, HP-β, and HP-γ-CD for each compound is shown ($p < 0.05$) (a-c).

Table 4. Minimum inhibitory concentration (MIC; mg/L) and minimum bactericidal concentration (MBC; mg L^{-1}) of carvacrol and thymol, free or encapsulated in HP-α-CD, HP-β-CD, and HP-γ-CD using the freeze-dry (FD) and spray-dry (SD) methods, against *Escherichia coli* O157:H7 Sakai and *Staphylococcus aureus* USA300.

		MIC (mg L^{-1})				MBC (mg L^{-1})			
		Escherichia coli		*Staphylococcus aureus*		*Escherichia coli*		*Staphylococcus aureus*	
Compound	Encapsulation	FD	SD	FD	SD	FD	SD	FD	SD
Carvacrol	Free	150		200		200		250	
	HP-α-CD	200	600	250	600	250	900	450	900
	HP-β-CD	700	>1000	700	>1000	>1000	>1000	>1000	>1000
	HP-γ-CD	250	250	250	300	250	500	450	600
Thymol	Free	150		150		250		250	
	HP-α-CD	200	500	200	500	250	500	350	800
	HP-β-CD	350	750	400	700	650	800	900	>1000
	HP-γ-CD	200	300	200	300	300	350	450	600

Figure 1. Phase solubility diagrams of carvacrol (A) and thymol (B) with HP-α-CD (■), HP-β-CD (▲), and HP-γ-CD (●) in aqueous solution.

Figure 2. Field Emission Scanning Electron Microscope (FESEM) images of freeze-dried (A and B) and spray-dried (C and D) complexes of carvacrol in HP-β-CDs