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Characterization of carvacrol beta-cyclodextrin inclusion complexes as delivery systems for antibacterial and antioxidant applications

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ABSTRACT

Carvacrol is a good natural antimicrobial and antioxidant agent; however, its poor aqueous solubility and high volatility limit its application in food systems. Beta-cyclodextrin (BCD) is able to encapsulate hydrophobic molecules improving its aqueous solubility and reducing its volatility. BCD-carvacrol inclusion complexes were prepared using kneading (KN) and freeze drying (FD) methods. Sizes of BCD-carvacrol complexes were 441 ± 12 nm and 899 ± 44 nm and entrapment efficiencies were 83.79 ± 2.89% and 91.31 ± 0.41% for KN and FD BCD-carvacrol complexes, respectively. Polydispersity index was higher ($P < 0.05$) than 0.1 for both methods, indicating a polydisperse system. Differential thermograms and phase solubility study indicated formation of 1:1 stoichiometry inclusion complex. Trolox Equivalent Antioxidant Capacity (TEAC) values ranged from 7491 to 6421 μmol TE/g among treatments, where KN BCD-carvacrol complex showed the lowest ($P < 0.05$) antioxidant activity. Storage stability of BCD-carvacrol complexes proved beneficial to carvacrol encapsulation. Antimicrobial activity against *Escherichia coli* K12 and *Salmonella enterica* serovar Typhimurium LT2 showed that all BCD-carvacrol complexes inhibited bacterial growth at lower ($P < 0.05$) carvacrol concentrations (values ranged from 300 to 350 μg/mL) compared to free carvacrol (≥1000 μg/mL). Results indicate that these BCD-carvacrol complexes could have important applications in food systems due to their storage stability and improved antimicrobial activity.

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1. Introduction

Foodborne pathogen infections and reactive oxygen species (ROS) are two threats which humans, animals and food are continuously exposed to. Foodborne diseases remain a global public health challenge, as some diseases are controlled, others emerge as new threats. The Centers for Disease Control and Prevention (CDC) estimates that each year 48 million people get sick, 128,000 are hospitalized, and 3000 die of foodborne diseases. *Salmonella* and *Escherichia coli* are two of the major pathogens that cause foodborne illnesses (CDC, 2012). There is an urgent need for new effective intervention strategies in the food industry to help prevent foodborne illness. On the other hand, ROS such as hydroxyl radicals, superoxide radicals, singlet oxygen, and hydrogen peroxide radical may lead to oxidative stress, which has been related to aging and many pathological disorders, including cancer, atherosclerosis, inflammation, and neurodegenerative disorders (Yin, Xu, & Porter, 2011). Antioxidants are compounds which slow down or prevent the oxidation of other molecules (Brewer, 2011). They interact with free radicals and prevent the damage by ROS. Thus, the treatment with antioxidants is potentially a way to overcome oxidative stress. Food products also deteriorate when exposed to ROS (Brewer, 2011). Therefore, compounds possessing antimicrobial and antioxidant activities present great potential in food systems applications. In particular, phenolic compounds are known for their antimicrobial and antioxidant properties (Beena & Rawat, 2013).

There has been a growing interest in the use of natural antimicrobials and antioxidants for application in food products. This is mainly due to a consumer preference for natural ingredients combined with concerns about toxic effects of synthetic compounds (Puertas-Mejia, Hillebrand, Stashenko, & Winterhalter, 2002). Carvacrol (5-isopropyl-2-methylphenol) is a phenolic monoterpene constituent of essential oils produced by numerous aromatic plants and spices such as black cumin (*Nigella sativa* L.), marjoram (*Origanum majorana* L.), oregano (*Origanum vulgare* L.),

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and thyme (*Thymus vulgaris* L.) (Silva et al., 2012). Many biological effects have been described for carvacrol, such as pronounced antioxidant effect *in vitro*, inhibitory action against 3-nitrotyrosin and malondialdehyde formation, free radical scavenger, and anti-lipidperoxidative agent (Beena & Rawat, 2013; Silva et al., 2012). When compared to its isomer thymol, carvacrol has shown higher ($P < 0.05$) antioxidant activity by radical scavenging assay (DPPH, 2,2-diphenyl-1-picrylhydrazyl) (Beena & Rawat, 2013). In another study, using an aldehyde-carboxylic acid assay Lee, Umano, Shibamoto, and Lee (2005) demonstrated that carvacrol and thymol (5 mg/L) could inhibit oxidation almost completely for 30 days. Moreover, carvacrol also exhibits antibacterial, antifungal, antiviral, antitumor and anti-inflammatory activities (Beena & Rawat, 2013; Silva et al., 2012).

Carvacrol is Generally Recognized as Safe (GRAS) for consumption, and it is approved by the US Food and Drug Administration for food use and has been included by the Council of Europe in the list of chemical flavorings that may be added to foodstuffs (De Vicenzi, Stammati, De Vicenzi, & Silano, 2004). Carvacrol and thymol have been used as antiseptic in medical practice, agriculture, cosmetics and food industry. However, there are challenges to use carvacrol as antimicrobial and antioxidant in food products: (i) it has an extremely low flavor threshold and can drastically change the sensory properties of the food, (ii) it is highly insoluble in water due to its lipophilic nature and may have limited contact with pathogens in high moisture content foods (Kalemba & Kunicka, 2003); and (iii) it is oxidized, decomposed, or evaporated when exposed to the air, light, or heat (Locci, Lai, Piras, Marongiu, & Lai, 2004). Inclusion of carvacrol in cyclodextrins (CD) is one method to overcome these problems because this technique greatly reduces volatility, oxidation, and heat decomposition (Cabral-Marques, 1994; Szente & Szejtli, 2004).

CDs have a rigid structure with a hydrophilic outer surface and a singular hydrophobic cavity due the absence of hydroxyl groups. Due to their distinctive structure, CDs are able to form inclusion complexes, often a 1:1 interaction, with essential oils and several compounds, enhancing their solubility, chemical stability, and bioavailability (Del Valle, 2004). Beta-cyclodextrin (BCD) is one of the most widely used due to its cavity size that is suitable for common drugs with molecular weights between 200 and 800 g/mol and has been on the GRAS list since 1998, as a flavor carrier and protectant (Szente & Szejtli, 2004; Waleczek, Marques, Hempel, & Schmidit, 2003).

Several methods have been used to prepare cyclodextrin inclusion complexes such as coprecipitation, neutralization, spray-drying, coevaporation, kneading, and freeze-drying (Liu, Lo, Tsai, & Cham, 2010). Among them, kneading method, also known as slurry complexation, is a method that requires small amount of solvent in the preparation and gives a very good yield of inclusion. Consequently, it is conduced to a more easily scaled-up process and lower production costs (Hedges, Shieh, & Sikorski, 1995). Freeze drying is another method that produces a powdered sample in a very good yield of inclusion formation. The low temperature minimizes the loss of extremely volatile guests, being especially useful for heat labile guests (Del Valle, 2004). Many studies have reported carvacrol antimicrobial and antioxidant activities; however, how different encapsulation processes affect these activities when using BCD to form inclusion complexes has not been studied, nor has its physico-chemical properties and storage stability once encapsulated.

Considering the requirements of effectiveness and convenience of the application of natural antimicrobial in food systems, this project aimed to: (1) to prepare carvacrol-BCD inclusion complexes using kneading and freeze-drying methods and characterize their physicochemical properties; (2) to determine the resulting antimicrobial activity against *E. coli* K12 and *S.* Typhimurium LT2, antioxidant activity, and storage stability.

2. Materials and methods

2.1. Materials

Carvacrol 98% was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). β-cyclodextrin (BCD, average MW = 1135.01) was purchased from Alfa Aesar (Heysham, England), and tryptic soy agar (TSA), tryptic soy broth (TSB), peptone water, for bacterial growth and enumeration were purchased from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA), Tween 20 was obtained from VWR (West Chester, PA, USA). HPLC-grade acetonitrile was purchased from EMD Chemicals (Darmstadt, Germany). For the antioxidant activity determination, potassium peroxydisulfate 99% (Alfa Aesar), ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and diammonium salt were obtained from AMRESCO (Solon, OH, USA), and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) from Tokyo Chemical (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Preparation of beta-cyclodextrin inclusion complexes

2.2.1. Freeze-drying

Carvacrol (1.2 g) was dispersed in 500 mL of BCD aqueous solution (16 mmol/L – 9.08 g) using a 1:1 molecular ratio and mixed in a laboratory stirrer for 48 h at room temperature (25 °C) to allow for complex formation and prevent loss of volatiles to the atmosphere (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007). The solution was frozen at −20 °C and lyophilized at −50 °C under 1.09 Pa for 48 h in a Labconco Freeze Dryer-5 (Kansas City, MO, USA). The lyophilized samples were stored in sealed containers inside a desiccator at −20 °C until further use.

2.2.2. Kneading

Carvacrol (1.2 g) and BCD (9.08 g) were initially mixed in a 1:1 molecular ratio in a mortar for 10 min. Then a small amount of ethanol was added to make a homogeneous paste. The paste was further kneaded manually for 45 min. The obtained mass was dried in a desiccator under vacuum for 48 h and stored in a desiccator at −20 °C until further analyses (Manolikar & Sawant, 2003).

2.3. Entrapment efficiency (EE)

The amount of carvacrol entrapped in the inclusion complexes was determined spectrophotometrically (spectrophometer model Genesys 10S UV-Vis, Thermo Scientific, Madison, WI, USA) at 275 nm. For both inclusion complexes, 5 mg of sample was dissolved in 5 mL of 95 g/100 mL acetonitrile and left for 48 h at room temperature after being well mixed to allow enough time for all entrapped carvacrol to be in solution, providing the total carvacrol from inclusion complexes (entrapped in the BCD cavity plus the surface-adsorbed). The carvacrol adsorbed on the surface of the inclusion complexes were determined according to Marreto et al. (2008) by washing 0.5 g of sample with 5 mL of acetonitrile for 20 min with intermittent shaking. Before measurement, the solutions were centrifuged at 3200 × g for 15 min (centrifuge model Clinical 200, VWR International, Darmstadt, Germany) to remove any BCD from the solution, leaving only the active compound. A standard curve of carvacrol was prepared with concentrations ranging from 2.5 to 30 μg/mL, under the same conditions. The EE was calculated according to equation (1) (Gomes, Moreira, & Castell-Perez, 2011):

$$EE = \frac{\text{amount of active compound entrapped}}{\text{initial active compound amount}} \times 100 \quad (1)$$

where amount of active compound entrapped was the difference between total carvacrol amount and surface-adsorbed carvacrol in the inclusion complexes.

2.4. Differential scanning calorimetry

A differential scanning calorimeter (DSC) model Q20 (TA Instruments, New Castle, DE, USA) at Polymer Science Center (Texas A&M University, College Station, TX, USA) was used to study complex formation between carvacrol and BCD. Analysis was performed with a scanning rate of 90 °C/min from 25 °C to 120 °C, maintained at 120 °C for 1 min to ensure even sample heating, then heated to 400 °C at a rate of 10 °C/min under a nitrogen atmosphere. The instrument was calibrated using zinc and indium metals before sample testing. Samples of free carvacrol, and its inclusion complexes were accurately weighed (~2 mg) and placed in aluminum pans (40 μL) with one hole in their lid (Mourtzinos, Kalegeroupoulos, Papadakis, Konstantinou, & Karathanos, 2008).

2.5. Particle size and morphology

A Delsa™ Nano C particle analyzer (Beckman Coulter, Brea, CA, USA) was used to measure the average size and polydispersity indices (PDI) for BCD inclusion complexes. Approximately, 0.07 g of particles were weighed and suspended in 10 mL of distilled water in 1 cm path length plastic cuvettes at scattering angle of 165°, with a pinhole set to 50 μm, and a refractive index of 1.3328 for 120 continuous accumulation times (Hill, Gomes, & Taylor, 2013).

Aqueous suspensions of inclusion complex particles were examined using a FEI Morgagni Transmission Electron Microscope (TEM) (FEI Company, Hillsboro, OR, USA) at the School of Veterinary Medicine and Biomedical Sciences of Texas A&M University. Polyvinyl alcohol (PVA) at 0.01 g/100 mL was added to the aqueous suspensions to charge the particles (Seo, Min, & Choi, 2010). Aqueous suspensions of particles were placed on 0.037 mm copper grids and stained with a 2.0 g/100 mL uranyl acetate aqueous stain (Electron Microscopy Sciences, Hatfield, PA, USA) to provide contrast under magnification. Excess liquid on the mesh was removed with filter paper and the grid was allowed to dry before viewing under 50,000–100,000 times magnification. Observations were performed at 80 kV.

2.6. Phase solubility

Phase solubility experiments were carried out for the inclusion complexes as described by Higuchi and Connors (Higuchi & Connors, 1965). An excess of carvacrol was added to 10 mL aqueous solutions of BCD ranging in concentration from 0 to 15 mmol/L and placed at 10, 25, and 35 ± 0.4 °C in a laboratory shaker (VWR 18L Shaking Water Bath, Radnor, PA, USA) for 48 h at 350 rpm. Then, samples were filtered using 0.45-μm PTFE filters (IC Millex-LH, Millipore, Billerica, MA, USA) and the quantity of carvacrol was measured spectrophotometrically (model Genesys 10S UV-Vis, Thermo Scientific, Madison, WI, USA) at 275 nm. The stability constants, K_c, were calculated according to equation (2):

$$K_c = \frac{\text{slope}}{\text{intercept} \cdot (1 - \text{slope})} \quad (2)$$

The experimental data from the phase solubility can be used to estimate thermodynamic properties of the complex formation using the experimentally determined K_c values and the integrated form of the Van't Hoff equation (3) (Hill et al., 2013):

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

where ΔH is the enthalpy change for the reaction (J/mol), R is the universal gas constant (J/mol K), T is the absolute temperature (K), and ΔS is the entropy change for the reaction (J/mol K). The Gibbs free energy (ΔG) can be calculated using the above-mentioned parameters and equation (4) at standard pressure and temperature (Hill et al., 2013).

$$\Delta G = \Delta H - T \cdot \Delta S \quad (4)$$

2.7. Determination of total antioxidant activity and storage stability

The antioxidant activity of free carvacrol and its inclusion complexes was evaluated according to the modified Trolox Equivalent Antioxidant Capacity (TEAC) method (Re et al., 1999). In this assay, the blue-green ABTS radical cation chromophore ($ABTS^{\bullet+}$) was produced directly by reacting 7 mmol/L ABTS solution and 2.45 mmol/L potassium persulfate and allowing the mixture to stand for 16 h in the dark at room temperature. The $ABTS^{\bullet+}$ solution was diluted with ethanol 50 g/100 mL to an absorbance of 0.70 ± 0.2 at 734 nm.

After addition of 3 mL of diluted $ABTS^{\bullet+}$ solution to 30 μl of free carvacrol and inclusion complexes aqueous solutions or Trolox standard (final concentration 100–2000 μmol/L) in ethanol, the absorbance reading was taken 6 min after initial mixing at 734 nm. Appropriate solvent blanks were run in each assay. TEAC values were calculated according to Murcia et al. (2004). Briefly, a calibration curve was prepared with different concentrations of Trolox. From this curve, the absorbance corresponding to 1000 μmol/L of Trolox was calculated. Then, the carvacrol and the inclusion complexes solutions absorbance measurements were plotted as a function of their concentrations (in g/L). These plots were used to calculate the TEAC, where the absorbance previously calculated was used to find the sample concentration equivalent to 1000 μmol/L of Trolox. The final result, expressed in μmol/g, was obtained following equation (5).

$$TEAC\left(\mu mol/g\right) = \frac{1000\ \mu mol/L\ \text{of trolox}}{\text{sample concentration } (g/L) \text{ equivalent to } 1000 \mu mol/L \text{ of trolox}} \quad (5)$$

In order to study the storage stability of free carvacrol, freeze-dried and kneaded inclusion complexes, samples of each treatment were exposed to light and their antioxidant activity were measured throughout storage. Briefly, samples (200 mg–1 g) were transferred into small transparent containers (45 × 42 mm, polystyrene 1540, Semadeni, Rotronic AG, German). The experiments were carried out under two different conditions: under fluorescent

light (T8 32W, 2800 lumens, ca. 7000 lux) and stored in the dark, both at room temperature for 3 months (Martinez, Penci, Ixtaina, Ribotta, & Maestri, 2013; Zhang et al., 2012). The light source was selected to resemble the illumination conditions commonly used in storage facilities. At different time intervals (0, 1, 2, 7, 15, 30, 45, 60 and 90 days), TEAC analysis was carried out for each treatment.

2.8. Minimum inhibitory and bactericidal concentration

E. coli K12 and *S.* Typhimurium LT2 were obtained from Texas A&M University Food Microbiology Laboratory. These strains were selected for their importance to the food industry and are representatives of pathogenic bacteria commonly occurring in various food products. *E. coli* K12 and *S.* Typhimurium were resuscitated in TSB by identical duplicate transfers and incubated aerobically for 24 h at 35 °C. The bacterial cultures were maintained on TSA slants at 4 °C for no more than 3 months. Transfers from slants were conducted similarly to the resuscitation method to prepare microorganisms for analysis.

The minimum inhibitory concentrations (MICs) for the inclusion complexes and free carvacrol were determined using a broth dilution assay (Brandt et al., 2010). *E. coli* K12 and *S.* Typhimurium cultures were incubated 22 h and then prepared by serial dilution in double-strength TSB (2×TSB) for an initial inoculum of approximately 5.0 log CFU/mL in each sample cuvette. Initial inocula were enumerated via spread plating on TSA and incubated for 24 h at 35 °C. Aliquots of 100-μl of all antimicrobial solutions and solvents blanks were spread plated to ensure sterility.

The MIC analyses were carried out in 3 mL-plastic cuvettes. The antimicrobial inclusion complexes were added to the cuvettes as aqueous suspensions, while the carvacrol was added as aqueous microemulsions containing 1.0 g/100 g ethanol and 0.1 g/100 g tween 20. The concentration of inclusion complexes added to the test cuvettes ranged from 2000 to 8000 μg/mL (equivalent to 200–800 μg/mL of carvacrol concentration based on the entrapment efficiency), while the concentration of free carvacrol ranged from 250 to 1600 μg/mL.

Positive controls were prepared containing inoculum and sterile distilled water, tween 20, free BCD, and ethanol at test concentrations (combined and singly) to guarantee solvents and additives had no effect on optical density values at 630 nm (OD630) or antimicrobial activity. Negative controls were also prepared with antimicrobial solutions and sterile 2× broth to ensure they were not contaminated. Once cuvettes with the solutions and bacteria were prepared, they were covered with parafilm and OD630 was measured (0 h). Then, the cuvettes were incubated at 35 °C and after 24 h another OD630 was measured. Any antimicrobial test cuvettes that showed ≤0.05 change in OD630 were considered "inhibited" by the antimicrobial. The MIC was determined as the lowest concentration of an antimicrobial that inhibited the visible growth of a microorganism after overnight incubation (Andrews, 2001).

All cuvettes that showed inhibition of the test microorganism were then tested for bactericidal capability by spreading 0.1 mL from each cuvette showing inhibition onto TSA plates and incubating for 24 h at 35 °C. If no colonies were observed on the plate surfaces following incubation, the treatment concentration was considered bactericidal. The lowest concentration of antimicrobial demonstrating bactericidal activity across all replicates was considered the minimum bactericidal concentration (MBC) (Hill et al., 2013).

2.9. Statistical analysis

This experiment was based on a completely randomized design with equal replications. For all analyses, determinations were made in triplicate as independent experiments. Data analysis was performed using JMP v. 9 software (SAS Institute, Cary, NC, USA) for size, PDI of inclusion complexes, entrapment efficiency, phase solubility, antioxidant activity, storage stability, and antimicrobial activity. Differences between variables were tested for significance by one-way analysis of variance (ANOVA). Significantly different means ($P < 0.05$) were separated by the Tukey's Honestly Significant Differences (HSD) test. Linear regression and analysis of covariance with 95% confidence interval were used when appropriate.

3. Results

3.1. Entrapment efficiency

The entrapment efficiencies of BCD-carvacrol inclusion complexes prepared by FD and KN methods are listed in Table 1. Both encapsulation methods entrapped carvacrol very effectively, suggesting that a small molecular weight compound such as carvacrol is suitable for inclusion into BCD. Only trace amounts were observed for surface-adsorbed carvacrol for both methods (data not shown), indicating that both methods provided good contact conditions between guest (carvacrol) and host (BCD) to ensure high complexation efficiency. The KN method showed a lower ($P < 0.05$) entrapment efficiency than FD method. This difference could be associated with the loss of carvacrol due to volatilization during the complexation process and drying step that are carried out in an open container at room temperature for the KN method. Similar observations were reported by Marreto et al. (2008) where differences among methods were associated with evaporation of volatile components.

Previous studies have reported high entrapment efficiencies ranging from 90.9% to 91.7% for eugenol, a compound with similar molecular weight to carvacrol, in BCD inclusion complexes prepared by freeze-drying method, similar to that reported in this study (Table 1) (Choi, Soottitantawat, Nuchuchua, Min, & Ruktanonchai, 2009; Hill et al., 2013; Seo et al., 2010).

3.2. Differential scanning calorimetry

The formation of inclusion complexes for each encapsulation method was confirmed by DSC analysis. DSC thermograms of free carvacrol, free BCD, and kneaded and freeze-dried inclusion complexes are shown in Fig. 1. The DSC curve of carvacrol shows a sharp endothermic peak at 238.67 °C which corresponds to its boiling point. The thermograms of carvacrol-BCD inclusion complexes for both methods did not show any sharp endothermic peak in the temperature range of the carvacrol's boiling point, indicating the molecular encapsulation of carvacrol inside the BCD cavity. The elimination of this peak is an indirect proof that an inclusion complex has been formed (Gomes et al., 2011; Karathanos et al., 2007) between carvacrol and BCD by comparing the thermal

Table 1
Entrapment efficiency (EE) values of kneaded (KN) and freeze-dried (FD) beta-cyclodextrin (BCD)-carvacrol inclusion complexes.

Inclusion complex	EE [%]*
FD BCD-carvacrol	91.3[a] (0.4)
KN BCD-carvacrol	83.8[b] (2.9)

*Values given are averages of three replicate samples; standard deviations are displayed in parentheses. Entrapment efficiency values with differing superscript letters indicate significantly different values ($P < 0.05$).

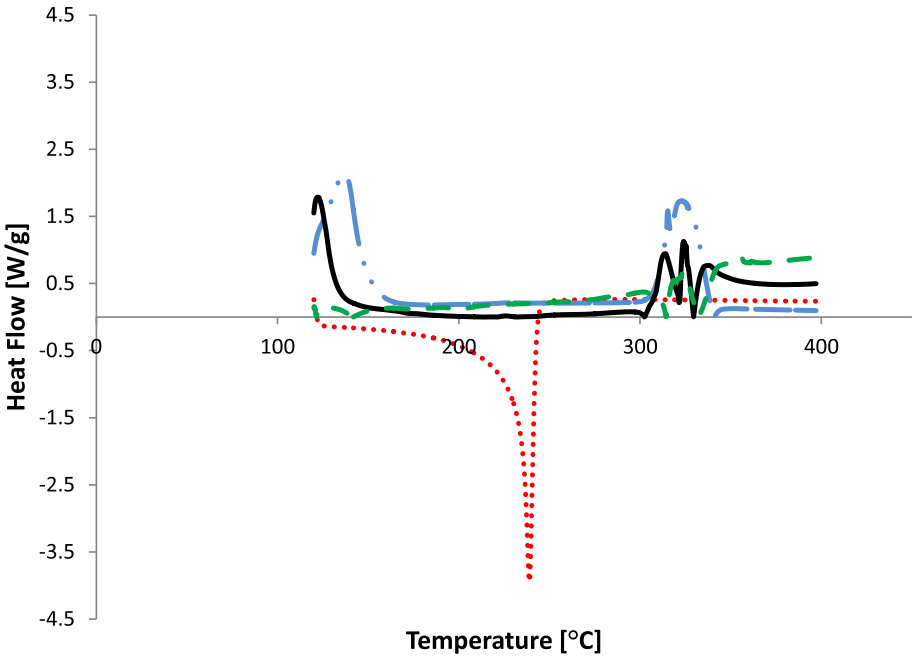

Fig. 1. Differential scanning calorimetry thermograms of carvacrol (·····), free beta-cyclodextrin (— ·), kneaded (——), and freeze-dried (– –) beta-cyclodextrin carvacrol inclusion complexes.

stability of the free carvacrol with the encapsulated form. Exothermics peaks around 320 °C for the BCD and inclusion complexes samples are probably due to melting and thermal decomposition of the BCD itself (Hedges et al., 1995).

3.3. Particle size analysis and morphology

The average inclusion complexes diameters with their respective PDIs for FD and KN methods are shown in Table 2. Average diameters and PDIs were significantly different between encapsulation methods with FD inclusion complexes having the highest ($P < 0.05$) values. The size of FD inclusion complexes was within the size range found by other studies with BCD and essential oils (Hill et al., 2013; Seo et al., 2010). Size and PDI of inclusion complex with BCD was dependent of complexation time. After 8 h of complexation in solution, BCD-eugenol molecular inclusion complex started to precipitate leading to self-aggregation (Chun, You, Lee, Choi, & Min, 2012). Since KN inclusion complexes are formed in a shorter period of time and require less water, self-aggregation was less pronounced, resulting in particles with lower ($P < 0.05$) PDI and particle diameter.

PDI is a measure of the uniformity of particle sizes present in the suspension. Cyclodextrins and their complexes form water soluble aggregates in aqueous solutions and these aggregates are able to solubilize lipophilic water-insoluble drugs through non-inclusion complexation or micelle-like structures (Loftsson, Masson, & Brewster, 2004). PDIs higher than 0.10 (polydisperse systems) (Zigoneanu, Astete, & Sabliov, 2008) could be due to the tendency of the BCD inclusion complex particles to agglomerate (Choi et al., 2009) creating larger particle size than anticipated.

TEM images (Fig. 2) show the size and morphology of BCD-carvacrol complexes for both encapsulation methods. The images reveal evidence of agglomeration for the inclusion complexes where large particles appear to be attracting smaller particles, which explains the PDI values. The size of the inclusion complexes measured by the particle analyzer is consistent with the particle sizes estimated from TEM images. The inclusion complexes showed a spherical shape (Fig. 2 (B and C)) and smooth surfaces as previously reported (Choi et al., 2009; Hill et al., 2013; Salustio, Cabral-Marques, Costa, & Pinto, 2011; Seo et al., 2010). Fig. 2 (B) demonstrates evidence of agglomeration of the inclusion complexes where their irregular form is a consequence of the self-assembly of BCD in water (Seo et al., 2010) due to the lack of significant net charge on the inclusion complex particles, which means there are no repulsive forces to prevent particle agglomeration (Hill et al., 2013).

3.4. Phase solubility

The phase solubility curves of carvacrol with BCD at different temperatures (10, 25 and 35 °C) are presented in Fig. 3. The curves show linear trends with slopes less than one for all temperatures studied. According to Higuchi and Connors (1965), diagrams exhibiting linear relationship are considered as A_L-type (i.e., linear increase of carvacrol solubility with increasing BCD concentration). Type A indicates a complexation reaction where carvacrol solubility increases as the BCD concentration increases, subscript L refers to a 1:1 molecular ratio formation of soluble complexes, as anticipated during preparation. Fig. 3 shows that increasing temperature results in increasing water solubility of carvacrol, as the y-intercepts increase with temperature.

The stability constants, (K_c), of the carvacrol-BCD complexes and their thermodynamic parameters at three different temperatures are shown in Table 3. The decrease of K_c constants with increasing temperature is expected for an exothermic process. Same

Table 2
Polydispersity index and average diameter of kneaded (KN) and freeze-dried (FD) beta-cyclodextrin (BCD)-carvacrol inclusion complexes.

Inclusion complex	Polydispersity index*	Particle diameter [μm]*
FD BCD-carvacrol	0.339[a] (0.009)	0.899[a] (0.044)
KN BCD-carvacrol	0.206[b] (0.029)	0.441[b] (0.012)

*Values given are averages of three replicates; standard deviations are displayed in parentheses. Average values with differing superscript letters (within columns) differ statistically ($P < 0.05$).

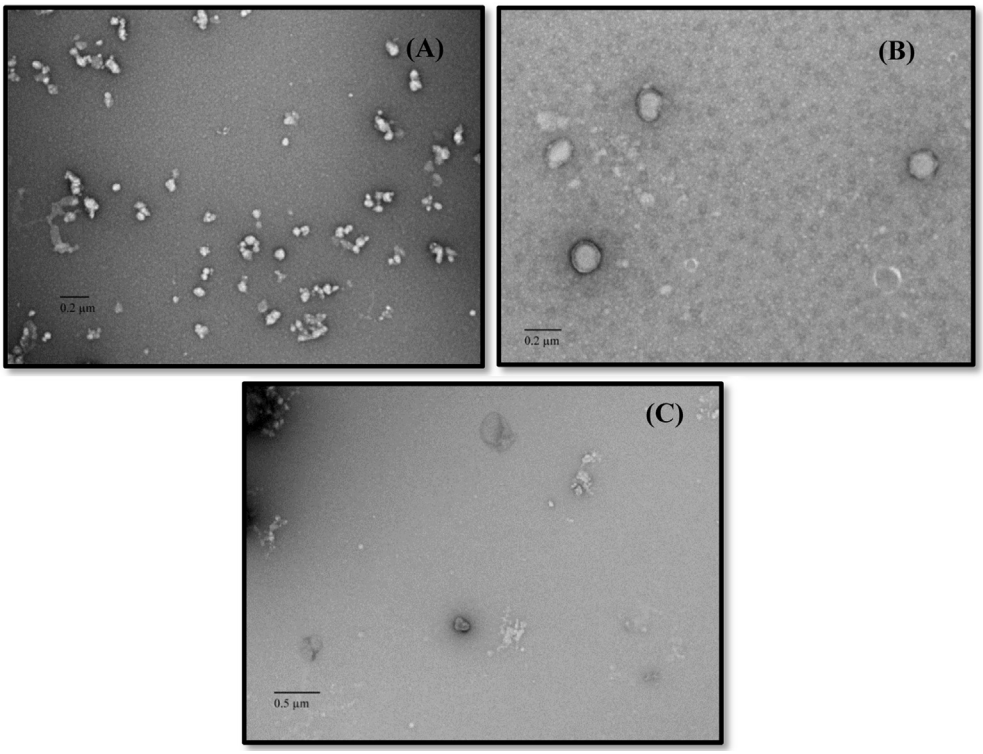

Fig. 2. Transmission electron microscope (TEM) images of beta-cyclodextrin (BCD) inclusion complexes. Images are representative of samples and depict free BCD (A), freeze-dried carvacrol-BCD inclusion complexes (B); kneaded carvacrol-BCD inclusion complexes (C) at 28,000; 36,000; and 18; 000 times magnification at 80 kV, respectively.

temperature effect on the stability constants and water solubilities for different compounds with BCD was reported by Hill et al. (2013); Mourtzinos et al. (2008); Seo et al. (2010); and Tommasini et al. (2004). K_c values for the same temperatures are within the same range to previously reported K_c values for BCD inclusion complexes with terpernoids (Haiyee et al., 2009; Mourtzinos, Salta, Yannakopoulou, Chiou, & Karathanos, 2007; Tommasini et al., 2004). The K_c values are good indicators to estimate the binding strength between the ligand and host (Higuchi & Connors, 1965; Loftsson et al., 2004). A small K_c value indicates a weak interaction with a higher amount of free ligand, while large K_c value indicates that the equilibrium is displaced towards the complex formation. K_c values within the range of 100–1000 L/mol are considered ideal (Mukne & Nagarsenker, 2004).

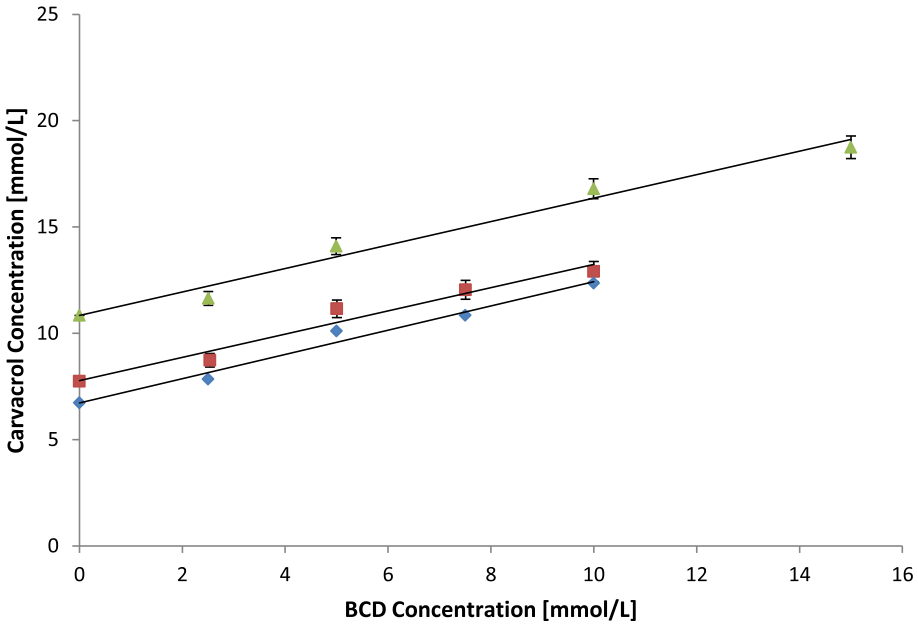

Fig. 3. Phase solubility of carvacrol with beta-cyclodextrin at 10 (◆), 25 (■), and 35 °C (▲). Values given are averages of three replicate samples ± error bars (standard deviations).

Table 3
Stability constants (K_c) as well as the slope and intercept values used to determine them for complex formation between beta-cyclodextrin (BCD) and carvacrol and the thermodynamic properties of complex formation reactions at specified temperatures.

Compound	Temperature (°C)	Intercept	Slope	K_c (L/mol)	[a]R^2	ΔH (J/mol)	ΔS (J/mol·K)	ΔG (J/mol·K)
Carvacrol	10	6.73	0.571	197a	0.98	−15649.44	−11.13	−12331.54
	25	7.78	0.546	155b	0.96	–	–	–
	35	10.84	0.552	113c	0.98	–	–	–

a,b Means within a column which are not followed by a common letter are significantly different ($P < 0.05$).
[a] Coefficient of determination of the linear relationship between BCD concentration vs. carvacrol.

The negative enthalpy (ΔH) value indicates that the complex formation between carvacrol and BCD is an exothermic reaction. According to Mourtzinos et al. (2008), the magnitude of the enthalpy change provides an indication that the complex formation is driven by low-energy interactions such as hydrophobic interactions due to the displacement of water molecules from the cavity of the BCD, increase of van der Waals intermolecular interactions, and hydrogen bonds formation. An explanation for the negative value of entropy (ΔS), which reflects in a more ordered system, can be due to a decrease in translational and rotational degrees of freedom of the complexed molecules in comparison to free ones (Mourtzinos et al., 2008). The negative Gibbs energy indicates that inclusion complex formation is a spontaneous process (Karathanos et al., 2007). Previous studies by Hill et al. (2013); Karathanos et al. (2007); and Mourtzinos et al. (2008) reported similar behavior for guest-cyclodextrin interactions. Moreover, these thermodynamic parameters are within similar range to previously reported for inclusion complex formation of terpenoids with BCD, as observed for eugenol and *t*-cinnamaldehyde (Hill et al., 2013), vanillin (Karathanos et al., 2007), and narigenin and hesperetin (Tommasini et al., 2004). The differences in the thermodynamic properties among compounds can be explained by specific guest–host interactions and differences in the equilibrium between their complexed and free states. The release rate depends on the affinities of the compound for the cyclodextrin cavity and the compound concentration (Ayala-Zavala et al., 2008; Higuchi & Connors, 1965; Loftsson et al., 2004).

3.5. Determination of total antioxidant activity and storage stability

The antioxidant activity of carvacrol and its inclusion complexes by kneading and freeze drying methods are shown in Table 4. This assay compares the capacity of the test compound to quench the $ABTS^+$ radical cation in respect to Trolox as reference antioxidant (Miguel et al., 2009). Free BCD did not show any antioxidant activity of its own when tested at the same concentration range of carvacrol and its inclusion complexes (data not shown). TEAC values ranged from 7491 to 6421 μmol TE/g among treatments (Table 4), where free carvacrol showed the highest ($P < 0.05$) antioxidant capacity, indicating that inclusion of carvacrol with BCD makes it less available to react with the free radical. Similar behavior was reported by Miguel et al. (2009) which was explained by the partial inclusion of compound (deferiprone) moiety in the cyclodextrin cavity. Encapsulation method influenced ($P < 0.05$) antioxidant capacity with FD inclusion complexes showing higher antioxidant capacity than KN inclusion complexes. Since only trace amounts of surface-adsorbed carvacrol was observed for both methods, the difference in antioxidant capacity indicates that complex formation was different between methods (different carvacrol positioning inside BCD cavity) which could be attributed to differences between processing methods (water amount, agitation time and method). Previous studies have reported different structural conformation of carvacrol inside BCD cavity (Locci et al., 2004; Mulinacci, Melani, Vincieri, Mazzi, & Romani, 1996). In this case the hydroxyl group of carvacrol could be less available to react with radical species. Similar TEAC values have been observed in previous studies for carvacrol and its inclusion complexes with BCD (Dorman, Surai, & Deans, 2000; Miguel et al., 2009; Munoz-Acevedo, Kouznetsov, & Stashenko, 2009; Undeger, Basaran, Degen, & Basaran, 2009).

Table 4
Antioxidant activity of carvacrol and its kneaded (KN) and freeze-dried (FD) inclusion complexes with beta-cyclodextrin (BCD).

Compound	TEAC (μmol TE/g carvacrol)*
Free carvacrol	7491[a] (269)
FD BCD-carvacrol	7042[b] (127)
KN BCD-carvacrol	6421[c] (191)

*Values given are averages of three replicates; standard deviations are displayed in parentheses. Average values with differing superscript letters (within columns) differ statistically ($P < 0.05$). TEAC – Trolox Equivalent Antioxidant Capacity.

Storage stability results measured for 3 months at room temperature for carvacrol and its inclusion complexes are shown in Fig. 4. Light exposure did not affect ($P < 0.05$) antioxidant activity of all treatments throughout storage. Although both inclusion complexes have shown lower ($P < 0.05$) antioxidant activity than free carvacrol, they retained this property over time ($P < 0.05$), similarly free carvacrol, when exposed to light. Similar storage stability study evaluating antioxidant activity for carvacrol has not been reported in the literature. However, Marcolino, Zanin, Durrant, Benassi, and Matioli (2011) evaluated the loss of color of bixin and curcumin encapsulated in BCD when stored under natural light and in the dark. Encapsulation with BCD did not protect against color loss of curcumin, but it conferred color protection of bixin when compared to the free compounds. Koontz, Marcy, Barbeau, and Duncan (2003) verified that aqueous solutions of natamycin and its BCD, hydroxypropyl-BCD and γ-CD inclusion complexes were completely degraded after 24 h of exposure to 1000 l× fluorescent lighting at 4 °C. These studies show that the protection by cyclodextrin may depend on the guest compound and the storage conditions. Different types of cyclodextrin may lead to different modes of inclusion for the same molecule; depending on cyclodextrin, the degradation of the drug can be accelerated, retarded, or remain the same (Loftsson et al., 2004). These results indicate that complexation of carvacrol with BCD did not affect negatively ($P < 0.05$) carvacrol stability to light throughout storage.

3.6. Minimum inhibitory and bactericidal concentration

The MIC and MBC of carvacrol and its BCD inclusion complexes for *S.* Typhimurium and *E. coli* K12 are provided in Table 5. The MICs values for the free carvacrol against both bacteria are in agreement with Rivas et al. (2010). Results of the positive controls indicated complete absence of inhibition for both bacteria species (data not shown). Encapsulation with BCD improved water solubility of carvacrol and improved carvacrol's antimicrobial efficacy at lower ($P < 0.05$) concentrations of active compound for both bacteria. The

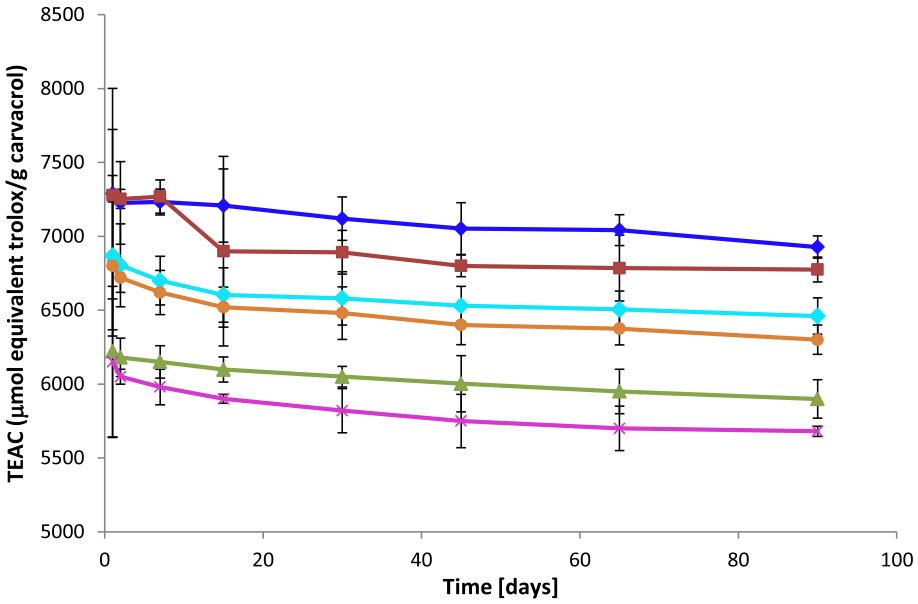

Fig. 4. Storage stability study as a function of antioxidant capacity (Trolox Equivalent Antioxidant Capacity Trolox – TEAC) and light exposure for 3 months at room temperature. Treatments corresponded to carvacrol stored under dark (♦) and light (■); freeze-dried beta-cyclodextrin (BCD) carvacrol complexes stored under dark (♦) and light (●); and kneaded BCD carvacrol complexes stored under dark (▲) and light (×). Values given are averages of three replicate samples ± error bars (standard deviations).

MIC values for BCD encapsulated carvacrol for both bacteria were 350 and 300 μg/mL for FD and KN methods, respectively. These are an improvement in inhibition of 65% and 70% for *E. coli* and 68% and 72.7% for *S.* Typhimurium, respectively. Moreover, bactericidal activity was also improved ($P < 0.05$) for both pathogens reducing carvacrol concentration needed to be effective. Previous studies have reported similar improvement of MIC values for essential oils encapsulated with BCD (Hill et al., 2013; Liang, Yuan, Vriesekoop, & Fei, 2012). The MIC and MBC for FD and KN complexes were significantly different ($P < 0.05$). The MIC for KN particles was lower ($P < 0.05$) for both bacteria species probably due to the increase in the carvacrol-carrier contact surface as a consequence of the more drastic mechanical treatment (Fernandes, Vieira, & Veiga, 2002). Moreover, the MICs and MBCs are different between the two bacteria because each bacterial strain responds differently to the essential oil (Kim, Marshall, & Wei, 1995).

Based on previous studies, Ait-Ouazzou et al. (2013) related hydrophobicity, the presence of a free hydroxyl group, and a delocalized system allowing proton exchange were key factors in the mechanism of inactivation by carvacrol. According to Wang, Li, and Chen (2011), since the primary sites of action of essential oils are at the membrane and inside the cytoplasm of bacteria, the improvement for the antimicrobial activity of the carvacrol is probably because BCD may have enhanced carvacrol access to these regions by increasing carvacrol aqueous solubility. Since it is possible to use less concentration of carvacrol, these results shows that encapsulation with BCD are able to improve delivery of these antimicrobials to the site where they can be active; that is the pathogenic microorganism, providing an exciting potential for the future.

Table 5
Minimum inhibitory and bactericidal concentration (MIC, MBC) against *E. coli* K12 and *S.* Typhimurium for free carvacrol and carvacrol beta-cyclodextrin (BCD) inclusion complexes (freeze-dried (FD) and kneaded (KN)).

Antimicrobial compound	MIC[a] [μg/mL]	MBC[a] [μg/mL]
Escherichia coli K12		
carvacrol	1000	>1000[b]
FD BCD-carvacrol	350	750
KN BCD-carvacrol	300	750
Salmonella Typhimurium		
carvacrol	1100	1100
FD BCD-carvacrol	350	800
KN BCD-carvacrol	300	800

[a] Values are the lowest concentration of carvacrol or carvacrol-BCD inclusion complexes for which a ≤0.05 OD630 change was observed after 24 h incubation at 35 °C in tryptic soy broth. MIC and MBC values are given based on carvacrol concentration.
[b] Values preceded by a higher than (>) means that tested concentrations were not sufficient to determine the MIC or MBC values.

4. Conclusions

Inclusion complex formation with carvacrol for both methods was confirmed by DSC and phase solubility analysis. Moreover, both methods presented high entrapment efficiencies and increased carvacrol water solubility, with FD complexes having higher ($P < 0.05$) entrapment efficiencies than KN complexes, probably because carvacrol was more susceptible to evaporation during the kneading process. Both types of complex inhibited *S.* Typhimurium and *E. coli* K12 at a lower concentration than free carvacrol indicating that encapsulation can enhance the mechanism of antimicrobial action and decrease the concentration of antimicrobial compound needed for inhibition. FD and KN complexes presented lower ($P < 0.05$) antioxidant activity than free carvacrol, indicating that BCD was blocking the hydroxyl group of carvacrol to react with the free radical. Moreover, FD and KN complexes were not degraded by light during storage, providing stable free-flowing powders. Overall, both encapsulation methods showed very positive results and either one could be used for commercial applications. Usually, for large-scale production kneaded method is preferred since it requires less water during the process. The results indicate that these BCD-carvacrol complexes could be useful antimicrobial delivery systems as for application in a variety of food systems where foodborne pathogens could present a risk.

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References

Ait-Ouazzou, A., Espina, L., Gelaw, T. K., de Lamo-Castellvi, S., Pagan, R., & Garcia-Gonzalo, D. (2013). New insights in mechanisms of bacterial inactivation by carvacrol. *Journal of Applied Microbiology, 114*(1), 173–185.

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy, 48*, 5–16.

Ayala-Zavala, J. F., Soto-Valdez, H., Gonzalez-Leon, A., Alvarez-Parrilla, E., Martin-Belloso, O., & Gonzalez-Aguilar, G. A. (2008). Microencapsulation of cinnamon leaf (*Cinnamonum zeylanicum*) and garlic (*Allium sativum*) oils in beta-cyclodextrin. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 60*, 359–368.

Beena, D. K., & Rawat, D. S. (2013). Synthesis and antioxidant activity of thymol and carvacrol based Schiff bases. *Bioorganic & Medicinal Chemistry Letters, 23*, 641–645.

Brandt, A. L., Castillo, A., Harris, K. B., Keeton, J. T., Hardin, M. D., & Taylor, T. M. (2010). Inhibition of *Listeria monocytogenes* by food antimicrobials applied singly and in combination. *Journal of Food Science, 75*(9), 557–563.

Brewer, M. S. (2011). Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety, 10*, 221–247.

Cabral-Marques, H. M. C. (1994). Applications of cyclodextrins. Thermodynamic aspects of cyclodextrin complexes. *Revista Portugues de Farmacia., 44*, 85–96.

CDC. (2012). Center for Disease Control and Prevention. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food. Foodborne diseases active surveillance network, 10 U.S. Site, 1996–2010. *Morbidity and Mortality Weekly Report, 60*(22), 749–755.

Choi, M. J., Soottitantawat, A., Nuchuchua, O., Min, S. G., & Ruktanonchai, U. (2009). Physical and light oxidative properties of eugenol encapsulated by molecular incluison and emulsion diffusion method. *Food Research International, 42*(1), 148–156.

Chun, J.-Y., You, S.-K., Lee, M.-Y., Choi, M. J., & Min, S. G. (2012). Characterization of beta-cyclodextrin self-aggregates for eugenol encapsulation. *International Journal of Food Engineering, 8*(2), 1–19.

De Vicenzi, M., Stammati, A., De Vicenzi, A., & Silano, M. (2004). Constituents of aromatic plants: carvacrol. *Fitoterapia, 75*, 801–804.

Del Valle, E. M. M. (2004). Cyclodextrins and their uses: a review. *Process Biochemistry, 39*, 1033–1046.

Dorman, H. J. D., Surai, P., & Deans, S. G. (2000). In vitro antioxidant activity of a number of plant essential oils and phytoconstituents. *Journal of Essential Oil Research, 12*(2), 241–248.

Fernandes, C. M., Vieira, M. T., & Veiga, F. J. B. (2002). Physicochemical characterization and in vitro dissolution behavior of nicardipine-cyclodextrins inclusion compounds. *European Journal of Pharmaceutical Sciences, 15*, 79–88.

Gomes, C., Moreira, R. G., & Castell-Perez, E. (2011). Microencapsulated antimicrobial compounds as a means to enhance electron beam irradiation treatment for inactivation of pathogens on fresh spinach leaves. *Journal of Food Science, 76*(6), 49–488.

Haiyee, Z. a., Saim, N., Said, M., Illias, R. M., Mustapha, W. A. W., & Hassan, O. (2009). Characterization of cyclodextrin complexes with turmeric oleoresin. *Food Chemistry, 114*, 459–465.

Hedges, A. R., Shieh, W. J., & Sikorski, C. T. (1995). Use of cyclodextrins for encapsulation in the use and treatment of food products. In S. J. Risch, & G. A. Reineccius (Eds.), *Encapsulation and controlled release of food ingredients* (Vol. 590, pp. 60–73). Washignton, DC: American Chemical Society.

Higuchi, L., & Connors, K. A. (1965). Phase solubility techniques. *Advances in Analytical Chemistry Instrumentation, 4*(2), 117–212.

Hill, L. E., Gomes, C., & Taylor, T. M. (2013). Characterization of beta-cyclodextrin inclusion complexes containing essential oils (trans-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. *LWT-Food Science and Technology, 51*, 86–93.

Kalemba, D., & Kunicka, A. (2003). Antibacterial and antifungal properties of essential oils. *Current Medicinal Chemistry, 10*, 813–829.

Karathanos, V. T., Mourtzinos, I., Yannakopoulou, K., & Andrikopoulos, N. K. (2007). Study of the solubility, antioxidant activity and structure of inclusion complex of vanillin with beta-cyclodextrin. *Food Chemistry, 101*(2), 652–658.

Kim, J., Marshall, M. R., & Wei, C. (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. *Journal of Agricultural and Food Chemistry, 43*(11), 2839–2845.

Koontz, J. L., Marcy, J. E., Barbeau, W. E., & Duncan, S. E. (2003). Stability of natamycin and its cyclodextrin inclusion complexes in aqueous solution. *Journal of Agriculture and Food Chemistry, 51*(24), 7111–7114.

Lee, A. J., Umano, K., Shibamoto, T., & Lee, K. G. (2005). Identification of volatile components in basil (*Ocimum basilicum* L.) and thyme leaves (*Thymus vulgaris* L.) and their antioxidant properties. *Food Chemistry, 91*(1), 131–137.

Liang, H., Yuan, Q., Vriesekoop, F., & Fei, L. (2012). Effects of cyclodextrins on the antimicrobial activity of plant-derived essential oil compounds. *Food Chemistry, 135*, 1020–1027.

Liu, H.-K., Lo, Y.-K., Tsai, T.-R., & Cham, T.-M. (2010). Physicochemical characterizations of osthole-hydroxypropyl-beta-cyclodextrin inclusion complexes with high-pressure homogenization. *Journal of Food and Drug Analysis, 18*(6), 391–397.

Locci, E., Lai, S., Piras, A., Marongiu, B., & Lai, A. (2004). 13C-CPMAS and 1H-NMR study of the inclusion complexes of beta-cyclodextrin with carvacrol, thymol, and eugenol prepared in supercritical carbon dioxide. *Chemistry & Biodiversity, 1*, 1354–1366.

Loftsson, T., Masson, M., & Brewster, M. E. (2004). Self-association of cyclodextrins and cyclodextrin complexes. *Journal of Pharmaceutical Sciences, 93*(5), 1091–1099.

Manolikar, M. K., & Sawant, M. R. (2003). Study of solubility of isoproturon by its complexation with beta-cyclodextrin. *Chemosphere, 51*, 811–816.

Marcolino, V. A., Zanin, G. M., Durrant, L. R., Benassi, M. T., & Matioli, G. (2011). Interaction of curcumin and bixin with beta cyclodextrin: complexation methdos, stability, and applications in food. *Journal of Agriculture and Food Chemistry, 59*, 3348–3357.

Marreto, R. N., Almeida, E. E. C. V., Alves, P. B., Niculau, E. S., Nunes, R. S., Matos, C. R. S., et al. (2008). Thermal analysis and gas chromatography coupled mass spectrometry analyses of hydroxypropyl-beta-cyclodextrin inclusion complex containing *Lippia gracilis* essential oil. *Thermochimica Acta, 475*, 53–58.

Martinez, M. L., Penci, M. C., Ixtaina, V., Ribotta, P. D., & Maestri, D. (2013). Effect of natural and synthetic antioxidants on the oxidative stability of walnut oil under different storage conditions. *LWT-Food Science and Technology, 51*, 44–50.

Miguel, M. G., Dandlen, S. A., Figueiredo, A. C., Pedro, L. G., Barroso, J. G., & Marques, M. H. (2009). Comparative evaluation of the antioxidant activities of thymol and carvacrol and the corresponding beta cylodextrin complexes. *Acta Horticulturae, 853*, 363–368.

Mourtzinos, I., Kalegeroupoulos, N., Papadakis, S., Konstantinou, K., & Karathanos, V. T. (2008). Encapsulation of nutraceutical monoterpenes in beta-cyclodextrin and modified starch. *Journal of Food Science, 73*(1), 89–94.

Mourtzinos, I., Salta, F., Yannakopoulou, K., Chiou, A., & Karathanos, V. T. (2007). Encapsulation of olive leaf extract in beta cyclodextrin. *Journal of Agricultural and Food Chemistry, 55*(20), 8088–8094.

Mukne, A. P., & Nagarsenker, M. S. (2004). Traimterene-β-cyclodextrin systems preparation characterization and *in-vivo* evaluation. *American Association of Pharmaceutical Scientists (AAPS) PharmSciTech, 5*(1), E19.

Mulinacci, N., Melani, F., Vincieri, F. F., Mazzi, G., & Romani, A. (1996). ^{1}H-NMR NOE and molecular modelling to characterize thymol and carvacrol β-cyclodextrin complexes. *International Journal of Pharmaceutics, 128*, 81–88.

Munoz-Acevedo, A., Kouznetsov, V. V., & Stashenko, E. E. (2009). Composition and in-vitro antioxidant capacity of essential oils rich in thymol, carvacrol, trans-anethole or estragole. *Salud UIS, 41*(3), 287–294.

Murcia, M. A., Egea, I., Romojaro, F., Parras, P., Jiménez, A. M., & Martínez-Tomé, M. (2004). Antioxidant evaluation in dessert spices compared with common food additives. Influence of irradiation procedure. *Journal of Agricultural and Food Chemistry, 52*, 1872–1881.

Puertas-Mejia, M., Hillebrand, S., Stashenko, E., & Winterhalter, P. (2002). In vitro radical scavenging activity of essential oils of Columbian plants and fractions from oregano (*Origanum vulgare* L.) essential oil. *Flavour and Fragance Journal, 17*, 380–384.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26*(9–10), 1231–1237.

Rivas, L., McDonnell, M. J., Burgess, C. M., O'Brien, M., Navarro-Villa, A., Fanning, S., et al. (2010). Inhibition of verocytotoxigenic *Escherichia coli* in model broth and rumen systems by carvacrol and thymol. *International Journal of Food Microbiology, 139*, 70–78.

Salustio, P. J., Cabral-Marques, H. M., Costa, P. C., & Pinto, J. F. (2011). Comparison of ibuprofen release from minitablets and capsules containing ibuprofen: beta-cyclodextrin complex. *European Journal of Pharmaceutics and Biopharmaceutics, 78*(1), 58–66.

Seo, E., Min, S., & Choi, M. (2010). Release characteristics of freeze-dried eugenol encapsulated with beta-cyclodextrin by molecular inclusion method. *Journal of Microencapsulation, 27*(6), 496–505.

Silva, F., Guimaraes, A., Silva, E., Sousa-Neto, B., Machado, F., Quintans-Junior, L., et al. (2012). Anti-inflammatory and anti-ulcer activities of carvacrol, a monoterpene present in the essential oil of oregano. *Journal of Medicinal Food, 15*(11), 984–991.

Szente, L., & Szejtli, J. (2004). Cyclodextrins as food ingredients. *Trends in Food Science & Technology, 15*, 137–142.

Tommasini, S., Raneri, D., Ficarra, R., Calabro, M. L., Stancanelli, R., & Ficarra, P. (2004). Improvement in solubility and dissolution rate of flavonoids by complexation with beta cyclodextrin. *Journal of Pharmaceutial and Biomedical Analysis, 35*, 379–387.

Undeger, U., Basaran, A., Degen, G. H., & Basaran, N. (2009). Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. *Food and Chemical Toxicology, 47*, 2037–2043.

Waleczek, K. J., Marques, H. M. C., Hempel, B., & Schmidit, P. C. (2003). Phase solubility studies of pure (-)-a-bisabolol and camomile essential oil with beta-cyclodextrin. *European Journal of Pharmaceutics and Biopharmaceutics, 55*, 247–251.

Wang, T., Li, B., Si, H., & Chen, L. (2011). Release characterisitics and antibacterial activity of solid state eugenol/beta-cyclodextrin inclusion complex. *Journal of Inclusion Phenomena and Macrocyclic Chemistry, 71*(1–2), 207–213.

Yin, H., Xu, L., & Porter, N. A. (2011). Free radical lipid peroxidation: mechanisms and analysis. *Chemical Reviews, 111*(10), 5944–5972.

Zhang, Y., Smuts, J. P., Dodbiba, E., Rangarajan, R., Lang, J. C., & Armstrong, D. W. (2012). Degradation study of carnosic acid, carnosol, rosamarinic acid, and rosemary extract (*Rosmarinus officinalis* L.) assessed using HPLC. *Journal of Agricultural and Food Chemistry, 60*, 9305–9314.

Zigoneanu, I. G., Astete, C. E., & Sabliov, C. M. (2008). Nanoparticles with entrapped α-tocopherol: synthesis, characterization, and controlled release. *Nanotechnology, 19*, 105606–105613.