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Development of electrospun active films of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by the incorporation of cyclodextrin inclusion complexes containing oregano essential oil

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ABSTRACT This paper reports the development of biodegradable active packaging films of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) by the incorporation of alpha- and gamma-cyclodextrins (α-CD and γ-CDs) containing oregano essential oil (OEO). Herein, both the kneading method (KM) and freeze-drying method (FDM) were first explored for the preparation of α-CD:OEO and γ-CD:OEO inclusion complexes at host:guest ratios of 80:20 wt/wt and 85:15 wt/wt, respectively. The results showed that KM was the most efficient method for the encapsulation of OEO in the CDs cavity in terms of simplicity and rapidity, while it was also yielded the inclusion complexes with the highest antimicrobial and antioxidant performance. The α-CD:OEO and γ-CD:OEO inclusion complexes obtained by KM were thereafter incorporated at 10, 15,

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20, 25, and 30 wt% into PHBV fibres by electrospinning and annealed at 160 ºC to produce contact transparent films. It was observed that the optimal concentration of α-CD:OEO and γ-CD:OEO inclusion complexes for homogeneous and continuous film formation was attainad at contents of 15 and 25 wt%, respectively. Higher antimicrobial and antioxidant activities were obtained for the γ-CD:OEO inclusion complexes due to the greater encapsulation efficiency of OEO in γ-CD, resulting in PHBV films with good performance for up to 15 days. This aspect, together with their improved thermal stability and mechanical strength, give interesting applications to these biopolymer films in the design of active-releasing packaging materials to maintain the physical, chemical, and microbiological characteristics of food products.

HIGHLIGHTS

- An effective strategy was designed to obtain active food packaging films of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV).
- The kneading method (KM) was the most efficient for the encapsulation of oregano essential oil (OEO) in alpha- and gamma-cyclodextrins (α-CD and γ-CDs).
- The contents of α-CD:OEO and γ-CD:OEO inclusion complexes were optimal at 15 and 25 wt%, respectively.
- Higher antimicrobial and antioxidant activities were attained for the films incorporating the γ-CD:OEO inclusion complexes due to their greater encapsulation efficiency.
- The newly developed electrospun γ-CD:OEO PHBV films showed high antimicrobial and antioxidant activities for up to 15 days.

Keywords*:* polyhydroxyalakanoates, cyclodextrins, essential oils, antioxidant, antibacterial, active packaging

1. Introduction

Essential oils (EOs) are mixtures of volatile organic compounds obtained from aromatic plants that are well known for their fragrant properties. They are also used in food preservation and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic, and locally anaesthetic remedies (Bakkali, Averbeck, Averbeck, &

Idaomar, 2008; Ribeiro-Santos, Andrade, Melo, & Sanches-Silva, 2017). Their mechanisms of active action, particularly at the antimicrobial level, have been well reported (Owen & Laird, 2018; Sharifi-Rad et al., 2017). The global market of EOs was 226.9 kton/year in 2018 (Research, 2018) while approximately 160 ones are considered as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA, 2016). Therefore, their application is currently growing in the food and beverage, personal care & cosmetics, aromatherapy, and pharmaceutical industries (Bakhtiary, Sayevand, Khaneghah, Haslberger, & Hosseini, 2018; Prakash, Kedia, Mishra, & Dubey, 2015; Prakash, Singh, Kedia, & Dubey, 2012; Raut & Karuppayil, 2014). Among EOs, oregano essential oil (OEO) is one of the most interesting since it is FDA approved and it is also included by the Council of Europe in the list of chemical flavourings that may be added to foodstuffs (De Vincenzi, Stammati, De Vincenzi, & Silano, 2004). In particular, OEO contains a mixture of bioactive related components such as carvacrol and thymol that can be used as antioxidant and antimicrobial agents for active packaging purposes (Kelly J. Figueroa-Lopez, Vicente, Reis, Torres-Giner, & Lagaron, 2019). However, the incorporation of OEO into a food packaging material is a challenging task due to several factors such as potent flavour changes, variations of the sensory perception as a consequence of oxidation, high volatility, chemical instability, low solubility in aqueous systems, etc. (Ju et al., 2019). In particular, OEO can evaporate easily and decompose and oxidize during formulation, processing, and storage due to exposure to heat, pressure, light or oxygen (Beirão-da-Costa et al., 2013; Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013). These inconveniences can be effectively minimized by encapsulation processes in different systems such as films, capsules, liposomes or inclusion complexes (Crini, 2014; Marques, 2010; Sherry, Charcosset, Fessi, & Greige-Gerges, 2013). Encapsulation allows creating a physical barrier between the core and the wall materials to protect OEO from the external medium (moisture, heat, light, etc.) and, thus, it enhances stability and maintains bioactivity (Sagiri, Anis, & Pal, 2016).

Cyclodextrins (CDs) are cyclic oligosaccharide consisting of six, that is, alpha-cyclodextrin (α-CD), seven, that is, beta-cyclodextrin (β-CD) or eight, that is, gamma-cyclodextrin (γ-CD) glucopyranose units modified starch molecules shaped like a hollow truncated cone (Del Valle, 2004). CDs are fairly water soluble, however β-CD shows remarkably lower solubility than α-CD and γ-CD. During crystallization in an aqueous medium, some molecules of water are entrapped into the CD cavity whereas other molecules of water are present as integral parts of the crystal structure, the so-called crystal water. CDs inclusion complexes are formed by the substitution

of the water molecules of the CD cavity by the appropriate guest molecule (Harada, Suzuki, Okada, & Kamachi, 1996; Szejtli, 1998). These inclusion complexes can be used to encapsulate different compounds since CDs cannot only stabilize the compound encapsulated against the degradation mechanisms triggered by environmental conditions, but they also can reduce the sensory changes by masking strong flavours (Marques, 2010; Szejtli, 1998). Furthermore, the CDs can also offer a controlled and sustained release of aromatic substances (Marques, 2010; Wang & Chen, 2005). In addition, the CDs are inert and do not interfere with the biological properties of EOs (Bilia et al., 2014; Del Valle, 2004). They are additionally relatively cost-effective, biodegradable, do not pose a significant safety concern, and encapsulation can be performed both in solution and solid-state (Crini, 2014). Several procedures have been developed to prepare CDs-based inclusion complexes, for instance the kneading method (KM), the co-precipitation, the heating in a sealed container, the freeze-drying method (FDM), the spray drying, and the supercritical fluid technology (Loftsson & Brewster, 1996). The EOs are thermolabile substances sensitive to the effects of light, oxygen, humidity and high temperatures and can be lost activity. It is for this reason that the electrospinning encapsulation technology has been used for the protection, stabilization, solubilization and delivery of the active substances (Gao et al., 2019). In this regard, the electrospinning process is a novel technology that produces ultrathin fibrous mats made of a wide range of polymers and biopolymers with fibre diameters ranging from several nanometres to a few microns (Li & Xia, 2004). This technique is highly suitable for the nanoencapsulation of active and bioactive substances, which is the case of EOs, due to both the high surface-to-volume ratios of the electrospun materials and the high porosity of their mats (Torres-Giner, Pérez-Masiá, & Lagaron, 2016; Torres-Giner, Wilkanowicz, Melendez-Rodriguez, & Lagaron, 2017). Furthermore, it allows processing volatile substances such as EOs because the process is performed at room temperature (Kayaci & Uyar, 2012). The resultant electrospun fibres can be potentially applied in sustainable food packaging applications (Torres-Giner, 2011; Torres-Giner, Busolo, Cherpinski, & Lagaron, 2018) either in the form of coatings or interlayers with bioplastic films (Quiles-Carrillo, Montanes, Lagaron, Balart, & Torres-Giner, 2019a; Torres-Giner, Martinez-Abad, & Lagaron, 2014). Moreover, the electrospun mats can be subjected to a thermal post-treatment, also called annealing, by which they form mechanically strong and transparent films with little porosity due to the fibres coalescence (Cherpinski, Torres-Giner, Cabedo, & Lagaron, 2017). According to the advantages described above, electrospinning has been recently employed to produce multi-functional fibres from different biopolymers (Gao

et al., 2019), such as polyhydroxyalkanoates (PHAs), that are biodegradable microbial polyesters (Zhang, Shishatskaya, Volova, da Silva, & Chen, 2018). Indeed, PHAs are excellent candidates for food packaging applications due to their resistance to water, low oxygen permeability, thermoplastic processability, and good physical and mechanical properties (Dietrich, Dumont, Del Rio, & Orsat, 2019).

In this context, the aim of this research work was first to encapsulate OEO into α- and γ-CDs and the resultant inclusion complexes were incorporated into poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) fibres by electrospinning. The PHBV electrospun mats containing the α-CD:OEO and γ-CD:OEO inclusion complexes were subjected to annealing develop transparent films with improved antioxidant and antimicrobial activity that can be of interest in the design of biodegradable active packaging to extend the shelf life of food products.

2. Experimental

2.1. Materials

OEO with a purity >99% and a density of 0.925–0.955 g/mL was obtained from Gran Velada S.L. (Zaragoza, Spain) and it was processed as received. Wacker Chemie AG (Munich, Germany) supplied the two food-grade cyclodextrin (CDs): α-CD, known as the trademark - CAVAMAX® W6 FOOD with molecular (M_W) of 972.84 g/mol and γ-CD, known as the trademark - CAVAMAX® W8 FOOD with M_W of 1297 g/mol. Their respective empirical formulas are $C_{36}H_{60}O_{30}$ and $C_{48}H_{80}O_{40}$ while further details are gathered in Table 1. Commercial PHBV was ENMAT™ Y1000P, produced by Tianan Biologic Materials (Ningbo, China) and delivered in the form of pellets by Nature Plast (Ifs, France). According to the manufacturer, this biopolymer resin presents a density of 1.23 g/cm^3 and a melt flow index (MFI) of 5–10 g/10 min (190 ºC, 2.16 Kg). The 3HV fraction in the copolyester is 2–3 mol.-%. 2,2,2-trifluoroethanol (TFE), ≥99% purity and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were purchased from Sigma Aldrich S.A. (Madrid, Spain). Ethanol, analytical grade with a purity of 99.8%, was supplied by Sigma-Aldrich. Water Milli-Q® was obtained using a Millipore purification system (resistivity > 18.2 MΩ·cm at 25 ºC).

Table 1

2.2. Preparation of the Inclusion Complexes

2.2.1. Freeze Drying Method (FDM)

The preparation of the inclusion complexes by FDM was carried out according to (Santos, Kamimura, Hill, & Gomes, 2015). The selected weight ratios between host:guest (α-CD:OEO or γ-CD:OEO) were 80:20 wt/wt and 85:15 wt/wt based on the maximum encapsulation efficiency reported by (Petrovi, Stojanovi, & Radulovi, 2010) and (Haloci et al., 2014). To this end, α-CD or γ-CD was dissolved in 2.5 mL of distilled water, then was added OEO and the resultant mixture was magnetically stirred at 250 rpm in a sealed container for 48 h at room temperature (25 ºC) to allow complex formation. Paraffin film and aluminium foil was used to prevent loss of volatiles and to protect the samples from the light. The suspensions were then frozen first at -20 ºC for 24 h and then at -80 ºC for 24 h and finally lyophilized at -50 ºC and 0.1 mbar in a Freeze Dryer (LyoQuest -55 Plus Eco Telstar® Life Science solutions, Hampton, VA, USA) until the water was sublimated (approximately 48 h). The freeze-dried IC was weighed, sealed, and stored at -20 ºC.

2.2.2. Kneading Method (KM)

The preparation of the inclusion complexes by the KM was carried out according to (Santos et al., 2015) and (Hedges, 1998). For this, α-CD or γ-CD was dissolved in 0.25 mL of distilled water, after which it was added OEO and kneaded thoroughly in a mortar and pestle for 18 min until a homogenous blend was obtained. The weight ratios OEO:α-CD or α-CD and γ-CD γ-CD were the same as that used at FDM (that is, 20:80 w/w and 15:85 w/w, respectively). The kneaded inclusion complexes (pasty mass) obtained was dried in a desiccator under vacuum for 48 h at room temperature (25 ºC) and then weighed, sealed, and stored at -20 ºC.

2.3. Characterization of the Inclusion Complexes

2.3.1. Encapsulation Efficiency and Loading Capacity

First of all, 10 mg of each type of inclusion complexes (α-CD:OEO and γ-CD:OEO at 80:20 and 15:85 weight ratios), were dispersed in 10 mL of absolute ethanol and stirred for 30 min in an Eppendorf mixer device (ThermoMixer™ C, Fisher Scientific®, Hampton, VA, USA) at 1000 rpm to allow the entrapped OEO in the CD cavity to be released to the solution for analysis. Then, the solution obtained was sonicated in an ultrasonic bath for 30 min at 37 kHz and 90 W at room temperature and, thereafter, centrifugated for 30 min at 2500 rpm to remove any CD from the solution. This resulted in a solution with a supernatant containing the OEO, which was used for analysis. The OEO content was determined spectrophotometrically (Ultraviolet-Visible

spectrophotometer, UV-VIS 2250, Shimadzu) monitoring the absorbance at wavelength 275 nm. This wavelength absorption belongs to the maximum absorbance wavelength of carvacrol, which is the most representative compound and the major component of OEO (Santos et al., 2015). From this wavelength absorption, the mass of the encapsulated OEO in the absolute ethanol solutions was calculated. A calibration curve of the absorbance versus the concentration of the OEO was previously performed by using OEO solutions of known concentrations dissolved in ethanol.

The encapsulation efficiency (EE, %) and loading capacity (LC, %), for each sample, were calculated according to Equations (1) and (2), respectively (Santos, et al. 2015). Encapsulation efficiency (EE, %) is the encapsulated amount of essential oil expressed as a percentage of the quantity initially used to prepare the solid inclusion complex. The UV-VIS analysis was carried out in triplicate.

$$\text{Encapsulation Efficiency (\%)} = \frac{\text{Total amount of encapsualted essential oil (mg)}}{\text{Initial amount of OEO to be encapsulated (mg)}} \times 100\ (\%) \qquad (1)$$

$$\text{Loading Capacity (\%)} = \frac{\text{Total amount of encapsualted essential oil (mg)}}{\text{Total amount of inclusion complexes (mg)}} \times 100\ (\%) \qquad (2)$$

2.3.2. Morphological Characterization of the Inclusion Complexes

The morphology of the empty CDs and CD:OEO inclusion complexes were examined using a scanning electron microscope (SEM, FEI Quanta 650 FEG, Thermo Fisher Scientific®, Germany). The samples were fixed on aluminium stubs with a double-stick conductive carbon substrate and sputter-coated with gold for 63 s at a working pressure of 1.4 E^{-3} mbar before the SEM measurements to prevent the build-up of a negative electric charge in the specimen, which would induce "imaging artefacts" and to enhance resolution. Observations were carried out with voltage acceleration of 10 kV and15 kV at spot 3. Transmission Electron Microscopy (TEM) was also used. Droplets of 0.1 % (w/v) and 1 % (w/v) aqueous suspensions (empty "*as-received*" α-CD and γ-CD; α-CD:OEO and γ-CD:OEO inclusion complexes) were placed on a copper grid and air-dried overnight. For negative staining, one drop of UranyLess 22409 was used. Observations were carried out with voltage acceleration of 200 kV at α_3, spot 1 and magnification: 30KX-100KX (JEOL JEM 2100, Izasa Scientific®, Carnaxide, Portugal).

The X-ray diffractograms of empty α-CD and γ-CD; α-CD:OEO and γ-CD:OEO inclusion complexes were obtained by wide-angle X- ray diffraction (WAXD) using an X-ray diffractometer (PANalitycal X'pert MPD-PRO (PANalytical, Model: X PERT PRO MRD) Bragg-Brentano θ-θ geometry using CuKα radiation at 45kV and 40 mA. The 2θ scan range was 5° - 80° with a step size of 0.01° and a time/step of 0.5 s.

2.4. Preparation of Electrospun Films

2.4.1. Preparation of Solutions

A PHBV solution for electrospinning was prepared by dissolving 10 % of biopolymer in TFE (wt/vol) at room temperature. The γ-CD:OEO and α-CD:OEO inclusion complexes were incorporated into the PHBV solution at 10, 15, 20, 25, and 30 wt% in relation to the biopolymer. PHBV solutions with γ-CD and α-CD (25 and 15 wt%, respectively), without OEO, were also prepared as control samples.

2.4.2. Electrospinning

The PHBV solutions containing γ-CD:OEO and α-CD:OEO were electrospun using a high-throughput electrospinning/electrospraying pilot line Fluidnatek® LE 500 manufactured and commercialized by Bioinicia S.L. (Valencia, Spain). The solutions were processed under a constant flow using a 24 emitter multi-nozzle injector, scanning vertically onto a flat slightly negatively charged collector. A voltage difference of 18 kV, a flow-rate of 6 mL/h per single emitter, and a tip-to-collector distance of 20 cm were used as these were the most optimal conditions (Melendez-Rodriguez et al., 2019).

2.4.3. Annealing

A thermal pots-treatment below the biopolymer's melting temperature (T_m) was thereafter applied to the electrospun mats in a 4122-model press from Carver, Inc. (Wabash, IN, USA). The mat samples were placed in the hot plates of the press at 160 ºC and closed, for 10 seconds, without pressure. These conditions were selected based on our previous study (Melendez-Rodriguez et al., 2019). The resultant samples had an average thickness of approximately 80 µm.

2.4.4. Characterization of the Electrospun Films

2.4.4.1. Film Thickness

Before testing, the thickness of all films was measured using a digital micrometer (S00014, Mitutoyo, Corp., Kawasaki, Japan) with ± 0.001 mm accuracy. Measurements were performed and averaged in five different points, two in each end and one in the middle.

2.4.4.2. Morphology

The particle shape and size (diameter) distributions of γ-CD and α-CD, the PHBV electrospun fibres and their films containing the γ-CD:OEO and α-CD:OEO inclusion complexes were examined by SEM in a Hitachi S-4800 (Tokyo, Japan) and TEM in Hitachi HT7700 (Tokyo, Japan). For cross-section observations by SEM, the films were previously cryo-fractured by immersion of the sample in liquid nitrogen. The SEM micrographs were taken at an accelerating voltage of 10 kV and a working distance of 8 – 10 mm, the samples were previously sputtered with a gold-palladium mixture for 3 min under vacuum. The size distribution of the particles and average fibres diameter was determined via ImageJ software using at least 20 SEM images.

2.4.4.3. Transparency

The light transmission of the films was determined in specimens of 50 mm x 30 mm by quantifying the absorption of light at wavelengths between 200 and 700 nm, using a UV–Vis spectrophotometer VIS3000 from Dinko, Instruments (Barcelona, Spain). The transparency value (T) was calculated using Equation 3 (K. Figueroa-Lopez, Andrade-Mahecha, & Torres-Vargas, 2018):

$$T = \frac{A_{600}}{L} \quad (3)$$

Where A_{600} is the absorbance at 600 nm and L is the film thickness (mm).

2.4.4.4. Thermal Analysis

Thermogravimetric analysis (TGA) of the γ-CD, α-CD, films containing γ-CD:OEO and α-CD:OEO was performed under nitrogen atmosphere in a Thermobalance TG-STDA Mettler Toledo model TGA/STDA851e/LF/1600 analyser. TGA curves were obtained after conditioning the samples in the sensor for 5 min at 30 °C. The samples were then heated from 25 °C to 700 °C at a heating rate of 10 °C/min. The first

derivatives of the thermogravimetry (DTG) curves, expressing the weight loss rate as the function of time, were obtained using TA analysis software. All tests were carried out in triplicate.

2.4.4.5. Mechanical Tests

Tensile tests were performed according to the ASTM Standard D 638 on an Instron Testing Machine (Model 4469; Instron Corp; Canton, MA, USA). The film samples were dumbbell-shaped. The cross-head speed was fixed at 10 mm/min. At least six samples were tested for each material, and the average values of the mechanical parameters and standard deviations were reported. Tensile modulus (E), tensile strength at break (σ_b), and elongation at break (ε_b) were calculated from the stress–strain curves, estimated from the force–distance data.

2.5. Antimicrobial Activity

Staphylococcus aureus CECT240 (ATCC 6538p) and *Escherichia coli* CECT434 (ATCC 25922) strains were obtained from the Spanish Type Culture Collection (CECT: Valencia, Spain) and stored in phosphate-buffered saline (PBS) with 10 wt% tryptic soy broth (TSB, Conda Laboratories, Madrid, Spain) and 10 wt% glycerol at −80 ºC. Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37 ºC for 24 h. A 100 µL aliquot from the culture was again transferred to TSB and grown at 37 ºC to the mid-exponential phase of growth. The approximate count of 5 x 10^5 colony-forming unit (CFU)/mL of culture having absorbance value of 0.20 as determined by optical density at 600 nm (UV–Vis spectrophotometer VIS3000 from Dinko, Instruments, Barcelona, Spain).

The minimum inhibitory concentration (MIC) and bactericide (MIB) values of the γ-CD:OEO and α-CD:OEO inclusion complexes against food-borne bacteria was tested following the plate micro-dilution protocol based on our previous work (Kelly J. Figueroa-Lopez et al., 2019). For this, 96-well plates with an alpha numeric coordination system (columns 12 and rows A-H) were used, where 10 µL of the tested samples were introduced in the wells with 90 µL of the bacteria medium. In the wells corresponding to A, B, C, E, F, and G columns different concentrations of CD/OEO (0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 µg/mL), were tested, in triplicate, from rows 1 to 10. Columns D and H were used as control of CD:OEO in TSB without bacteria. Row 11 was taken as a positive control, that is, only TSB, and row 12 was used as a negative control, that is, *S. aureus* and *E. coli* in TSB. The plates were incubated at 37 °C for 24 h. Thereafter, 10 µL of resazurin, a

metabolic indicator, was added to each well and incubated again at 37 °C for 2 h. Upon obtaining the resazurin change, the wells were read through the colour difference. The MIC value was determined as the lowest concentration of γ-CD:OEO and α-CD:OEO presenting growth inhibition.

The antimicrobial performance of the electrospun PHBV films containing γ-CD:OEO and α-CD:OEO was evaluated by using a modification of the Japanese Industrial Standard (JIS) Z2801 (ISO 22196:2007) (Kelly Johana Figueroa-Lopez, Castro-Mayorga , Andrade-Mahecha, Cabedo, & Lagaron, 2018). A microorganism suspension of Staphylococcus aureus (*S. aureus*) and Escherichia coli (*E. coli*) was applied onto the test films containing the γ-CD:OEO and α-CD:OEO inclusion complexes and the films without CD:OEO (negative control) sizing 1.5 cm x 1.5 cm that were placed in either open bottles. After incubation at 24 °C and at a relative humidity (RH) of at least 95 % for 24 h, bacteria were recovered with PBS, 10-fold serially diluted and incubated at 37 °C for 24 h in order to quantify the number of viable bacteria by conventional plate count. The antimicrobial activity was evaluated from 1 (initial day), 8, and 15 days. The value of the antimicrobial activity (R) was calculated using Equation 4:

$$R = \left[Log\left(\frac{B}{A}\right) - Log\left(\frac{C}{A}\right)\right] = Log\left(\frac{B}{C}\right) \quad (4)$$

Where A is the average of the number of viable bacteria on the control sample immediately after inoculation, B is the average of the number of viable bacteria on the control sample after 24 h, and C is the average of the number of viable bacteria on the test sample after 24 h. Three replicate experiments were performed for each sample and the antibacterial activity was evaluated with the following assessment: Nonsignificant (R<0.5), slight (R≥0.5 and<1), significant (R≥1 and<3), and strong (R≥3) (Sergio Torres-Giner, Torres, Ferrándiz, Fombuena, & Balart, 2017).

2.6. Antioxidant Activity

The 2,2,1-diphenyl-1-picrylhydrazyl (DPPH) inhibition assay was used to evaluate the free radical scavenging activity of the neat OEO, γ-CD:OEO, α-CD:OEO, and the electrospun PHBV films containing γ-CD:OEO and α-CD:OEO. Samples were weighed in triplicate in cap vials, and then an aliquot of the DPPH solution (0.05 g/L in methanol) was added to each one. Vials without samples were also prepared as controls. All the samples were prepared and immediately stored at room temperature for 2 h in darkness. After this, the absorbance of the solution was measured at 517 nm in the UV 4000 spectrophotometer from Dinko Instruments.

Results were expressed as the percentage of inhibition to DPPH following Equation 5 (Busolo & Lagaron, 2015) and μg equivalent of Trolox per gram of sample, employing a previously prepared calibration curve of Trolox.

$$Inhibition\ DPPH\ (\%) = \frac{A_{Control} - (A_{sample} - A_{blank})}{A_{control}} * 100 \quad (5)$$

Where $A_{control}, A_{blank},$ and A_{sample} are the absorbance values of the DPPH solution, methanol with the test sample, and the test sample, respectively.

2.7. Statistical Analysis

The results of the encapsulation efficiency and loading capacity of the CD:OEO inclusion complexes, mechanical tests, and antioxidant activity assays were evaluated by analysis of variance (ANOVA) and a multiple comparison test (Tukey) with 95% significance level ($p < 0.05$). For this purpose, we used the software OriginPro8 (OriginLab Corporation, USA).

3. Results and Discussions

3.1. Encapsulation Efficiency of the CD:OEO Inclusion Complexes

The EE and LC of the α-CD:OEO and γ-CD:OEO inclusion complexes prepared by FDM and KM are presented in Table 2. Although the results showed that both α-CD and γ-CD are efficient wall materials for encapsulation of OEO, the preparation method and the weight ratio (CDs:OEO) are also key factors to obtain an optimal EE. Indeed, FDM yielded lower encapsulation efficiency (from 36.03 % to 96.7 %) than KM (from 71.2 % to 98.5 %). This is in accordance with the results described by Ozdemir et al. (2018), who studied the encapsulation of black paper oleoresin in β-CD with encapsulation efficiencies from 90.2 % to 79.3 % for KM and FDM, respectively. The higher encapsulation efficiency obtained by KM compared to FDM can be related to the high shear rate applied (Ozdemir et al., 2018) and the use of a low amount of water during the IC formation. In an aqueous solution, the CD cavity is slightly polar and occupied by water molecules, and can therefore be readily replaced by appropriate guest molecules, that are less polar than water (Ponce Cevallos, Buera, & Elizalde, 2010). It is also worthy of mentioning that some differences in the EE values could be associated with evaporation of volatile components during the preparation process studies (Ozdemir et al., 2018; Santos et al.,

2015). In conclusion, the preparation parameters of the α-CD:OEO and γ-CD:OEO inclusion complexes, that is, weight ratio host-guest, nature of cyclodextrin, use of co-solvent and its quantity, mixing time, and shear rate applied could affect the properties of the obtained complexes such as the encapsulation yield. Thus, based on the results obtained in terms of EE, KM revealed to be the most efficient method for the encapsulation of OEO in the CD cavity, which also added value in terms of simplicity, rapidity, and the desired characteristics of the final product.

Table 2

3.2. Morphology of the CD:OEO Inclusion Complexes

Figure 1 gathers the SEM micrographs of the α-CD:OEO and γ-CD:OEO inclusion complexes. It can observed that the size and shape of the α-CD:OEO and γ-CD:OEO inclusion complexes (Figs. 1b, 1d, 1e, 1f, 1g, 1h) are completely different from the empty α- and γ- CDs (Figs. 1a, 1c, 1i, 1j). The shape of empty "*as-received*" α- and γ- CDs appear uneven and the size ranged from 24 up to 254 µm. Large particle sizes were observed suggesting that CDs piled up forming large aggregates. The relatively larger size of empty "*as-received*" α- and γ- CD can be attributed to the agglomeration of empty "*as-received*" α- and γ- CD particles via hydrogen bonding. In the absence of a guest molecule, empty "*as-received*" α- and γ- CD tended to cluster due to lack of significant net charge on the particles, that is, no repulsive forces were produced to prevent agglomeration (Hill, Gomes, & Taylor, 2013). This is also consistent with the observation of smaller particles attraction and adherence to the larger particles (see in detail in Figs. 1a, $1a_1$, $1a_2$ and 1c, $1c_1$, $1c_2$). Similar observations were reported by Santos et al. (2015). Contrarily, this behavior was not observed in the formed α-CD:OEO and γ-CD:OEO inclusion complexes. In particular, the reduction of the particle size in the α-CD:OEO and γ-CD:OEO inclusion complexes indicated a conformational change of empty α-CD and γ-CD that obstructed their agglomeration (Guimarães et al., 2015; Seo, Min, & Choi, 2010). Indeed, compared with empty "*as-received"* α- and γ- CD (particle size from 24 up to 254 µm; similar to that found by Gauret et. al. (2018)), their inclusion complexes showed a remarkable decrease in particle size, range from ~5 µm up to the nanometric level (~100 nm) and with well-defined lamella shaped (tetragonal crystals). In both types of inclusion complexes were observed lamella-like sheets and microrods (Figs. 1b and 1d).

Figs. 1a, 1b, 1c, 1d

In addition, the microrods from inclusion complexes had a very high aspect ratio (see Figs 1e and 1f). Observation over a large number of SEM images suggests that these long microrods stack together to produce the lamella-like sheets.

Figs. 1e and 1f

The morphological similarity of both α-CD:OEO and γ-CD:OEO inclusion complexes can be explained by considering the solubility of the used CDs (see previous Table 1). In this regard, Saokham et al. (2018), and the referenced cited within examined the solubility of α and γ differences compared with β-CD. Briefly, γ-CD, which is the largest of the three, is the most soluble (23.20 mg/100 ml H_2O) while β-CD, which is in the intermediate, is the least soluble in water (1.8 g/100 ml H_2O). These differences in the solubility of the CDs are related to the way the CD glucose units are geometrically aligned with each other. It has been proposed that in the β-CD molecule, all the 7 glucose units lie in the same plane. Hence, in this arrangement, all the glucose primary hydroxyl groups at the CD narrower end can form hydrogen bonds with each other. At the same time, all the secondary hydroxyl groups at the wider CD opening form hydrogen bonds with each other. The hydrogen bonding below and above the ring leads to secondary belts which increases the rigidity of the β-CD and therefore causes low solubility. As opposite, α- and γ- CD which do not have secondary belts therefore their structures are flexible, are hence very soluble due to the availability of free hydroxyl (–OH) groups. During trituration of cyclodextrins in an aqueous medium, a few water molecules could entrap into the cyclodextrin cavity, whereas other molecules of water are present as integral parts of the crystal structure (crystal water). According to Rusa et al. (2002), Szeijtli (1998), and Das et al. (2015) the CD inclusion complexes are formed by the substitution of included water from cyclodextrin cavity by the appropriate guest molecule.

Using the above reasoning, the threading of OEO can occur faster in α-CD and γ-CD molecules than in β-CD. Hence, the morphological similarity of both types of inclusion complexes (α-CD:OEO and γ-CD:OEO) studied in this work could be explained using the above reasoning. Moreover, the particle size distribution appears quite homogenous and have rather smooth and parallel surfaces (Figs. 1b, 1d, 1e, 1f, 1g, 1h). They all have sharp edges, as expected from crystalline structures. The growth of the crystals is definitely preferential in 2D (lamella-shape). Figs. 1g and 1h show other details: the two arrows point out the thickness, around a few hundreds of nm, of a platelet with a size of 100 nm - ~ 5µm. Smaller platelets (under 500 nm) appear to have the same thickness. These results show that the morphological characteristic of inclusion complexes are indeed

different from empty "*as-received*" α-CD and γ- CD; empty "*kneaded for 18 minutes at R.T.*" α-CD and γ- CD, and empty "*kneaded for 18 minutes with 0.25 mL distilled water at R.T.*" α-CD and γ- CD. This morphological difference between the empty CDs and the inclusion complexes obtained is in agreement with the observations reported by Guimaraes et al. (2015) and the references cited within.

Figs. 1g, 1h, 1i, and 1j

To elucidate if the morphology, in terms of particle size and well-defined shape, of empty "*as-received*" α- and γ-CD was changed due to the encapsulation of EO (i.e., low particle size and well-definite lamella shape), was performed SEM also on empty "*kneaded for18 minutes at R.T.*" α- and γ- CD, and empty "*kneaded for 18 minutes at R.T. with 0.25 ml distilled water (the same quantity used at the preparation of inclusion complex)*" α- and γ- CD. The results showed that compared with empty "*as-received*" α- and γ- CD there is not significant morphological modification after their kneaded for 18 minutes at R.T. (Figs. $1a_1$ and $1c_1$) as well as after their kneaded for 18 minutes at R.T. with 0.25 ml distilled water (Figs. $1a_2$ and $1c_3$). The presence of aggregates of size from ~2 up to 242 µm with undefined shape was revealed, except for empty "*kneaded 18 minutes at R.T. with 0.25 ml distilled water*" **γ**-CD which showed a defined shape, that is, prisms shape structure (Fig. $1c_2$). Similar observations on "the agglomeration of the free cyclodextrin" were revealed by Rakmai et al. (2017). It indicates the hydrogen bonding of the cyclodextrin molecules (empty) interact with each other in water producing the cluster of CD. In addition, Shan et al. (2016) have demonstrated that CDs particle agglomeration might be induced also by the moisture content.

The α-CD:OEO and γ-CD:OEO inclusion complexes morphology in the aqueous suspension was also observed using TEM, and Fig. 2 shows clear lamella shapes with diameters from 0.1 to ~1 µm.

In detail, at 0.1 % (w/v) were detected single lamellas with diameters of 350 nm over a large number of grid holes (Fig. 2a), whereas in Figs. $2b_{1\text{-}3}$ were displayed several representative TEM micrographs for 1 % (w/v), lamellas with diameters 170 - 519 nm. In addition, some of these lamellas fused together as indicated by the white arrow, indicating that the lamellas do not simply interact through their surfaces but are able to merge completely in aggregate. Thus, TEM measurements corroborated the results that were obtained from SEM, that is, a well-defined lamella-shape structures of the inclusion complexes. In contrast, empty γ-CD (1 % w/v, aqueous suspension vortex 10 minutes at 2500 rpm, R.T) presented aggregates made up of a larger number of small spherical particles with diameters between 5 nm and 325 nm (Figs. $2c_{1,2}$). These spherical particles seem

to interact and form rod-like shape structure with diameters between 406 nm and 1786 nm (inset of Figs. $2c_{3,4}$ diameter: 790.86 nm). Empty α-CD and α-CD:OEO inclusion complexes revealed similar morphologies (data not shown). These results support the hypothesis that the spherical particles are not indefinitely stable, thus tend to form a larger structure. These observations are in agreement with those revealed by several research groups such as Harada et al. (1990), (1992), (1993), and Ceccato et al. (1997) who reported the formation of the so-called "molecular tube" a rod-like rigid molecule with an empty hydrophobic cavity that can behave as a host for ions or small organic molecules. Furthermore, Bonini et al. (2006) reported evidence of β-cyclodextrin self-aggregation in water. It was showed also that the concentration plays a critical key on their morphology so that polydisperse spherical objects with diameters of about 100 nm were present at low concentration, whereas micrometre planar aggregates are predominated at higher concentrations.

Fig. 2

Finally, WAXD studies were conducted to confirm the formation of CD:OEO inclusion complexes. According to Marques (2010) and references cited herein, X-ray powder diffraction is the most useful method for the detection of inclusion complexes formation, especially in the case of the guest in the form of liquid molecules (e.g., oils and volatiles), because the liquid guest molecules produce no diffraction patterns and any changes in the diffractogram reflects the formation of a new crystal lattice. Figs. 3 shows the WAXD patterns of empty "*as-received*" α-CD and γ-CD compared with their inclusion complexes. Empty "*as-received*" α-CD and γ-CD differed from each other in their diffraction patterns. The WAXD pattern of empty "*as-received*" α-CD and γ-CD revealed several diffraction peaks which are indicative of their crystalline nature, but, according to Rusa et al. (2002) for α-CD there are three salient peaks associated with its crystal structure occurring at 2θ = 12.1°, 14.5°, and 21.8° (Fig. 3a). In the diffractograms of both CD:OEO inclusion complexes, some of these characteristic diffraction peaks disappear.

For the α-CD:OEO inclusion complex, a new intense diffraction peak appeared at 2θ = 19.6°, which was not observed in empty "*as-received*" α-CD. According to Rusa et al. (2002), the peak at 2θ ~20° in the WAXD of α-CD inclusion complexes is characteristic for the channel structure of α-CD when including long guest molecules and polymers in particular. In the γ-CD:OEO inclusion complex, a new sharp diffraction peak at 2θ = 7.6° (Fig. 3b) was revealed. This peak was not observed in empty "*as-received*" γ-CD. According to Harada

et al. (1996), and Rusa et al. (2002) this peak has been suggested as an indicator for γ-CD inclusion complex channel structures. Hence, this behavior can be attributed to an interaction between CD and OEO showing the presence of a new solid phase.

Fig. 3

3.3. Morphology of the Electrospun CD:OEO Inclusion Complexes-Containing PHBV Films

The SEM micrographs of the electrospun fibres of the neat PHBV and the fibres containing the γ-CD:OEO and α-CD:OEO inclusion complexes are shown in Fig. 4. The mean fibre diameters obtained from the SEM images are gathered in Table 3. The diameters of the electrospun fibres of neat PHBV were 0.89 ± 0.30 µm, being very similar to those reported in our previous research work (Melendez-Rodriguez et al., 2019). As shown in Figs. 4a-e, the electrospun PHBV fibres containing different concentrations of γ-CD:OEO inclusion complexes, that is, 10, 15, 20, 25, and 30 wt%, presented mean diameters ranging between 0.87–0.91 µm. Up to contents of 25 wt% γ-CD:OEO inclusion complexes, the electrospinning process yielded regular and continuous fibres of PHBV. In the case of the PHBV fibres mat containing 30 wt% γ-CD:OEO IC, the fibrilar morphology was affected, losing the homogeneity and continuity due to the high concentration of CD. Figs. 4f-j show the SEM micrographs of the electrospun fibers of PHBV containing different concentrations of α-CD:OEO inclusion complexes, that is, 10, 15, 20, 25, and 30 wt%. The electrospun fibres containing 10 and 15 wt% α-CD:OEO showed mean diameters of approximately 0.89 and 0.90 µm, respectively, being homogeneous and with smooth surfaces. The diameter of the fibers containing 20 wt% α-CD:OEO inclusion complexes increased, presenting a mean value of 1.03 ± 0.25 µm, and beaded regions due to potential CD agglomerations. It can be observed that the electrospun PHBV fibers with 25 and 30 wt% α-CD:OEO inclusion complexes, respectively shown in Figs. 4i and 4j, presented mean diameters of 1.17 and 1.22 µm. This slight increase in the fiber diameters can be related to the relatively high amount of CDs incorporated that aggregated during electrospinning and resulted in destabilization of the electrified jet. These results are in agreement with the previous reports of (Topuz & Uyar, 2019), concluding that at low hydroxypropyl-β-CD/laponite concentrations, the fibers do not present any significant change in diameter and shape while, at high concentrations, the diameter of the nanocomposite nanofibers decreases and aggregates are also formed. Furthermore, changes in the solution properties such as viscosity or conductivity may cause variations in the electrospun morphologies. For instance, (Aytac, Ipek, Durgun, Tekinay, & Uyar, 2017) determined that the diameters of nanofibers containing methylated-β-

CD/linalool were lower than those of hydroxypropyl-β-CD/linalool due to the lower viscosity and higher conductivity of the aqueous solution. Therefore, the most optimal fibrillary morphologies were attained for PHBV containing 25 wt% γ-CD:OEO and 15 wt% α-CD:OEO inclusion complexes.

Fig. 4

Table 3

The electrospun fibres mats were thereafter subjected to annealing in order to obtain a continuous film (Figueroa-Lopez et al., 2019; Melendez-Rodriguez et al., 2019). The surface and cross-section areas of the PHBV films containing **γ**-CD:OEO and ***α***-CD:OEO inclusion complexes were observed by SEM images. As shown in Fig. 5, the surface of the electrospun PHBV films containing 10, 15, 20, and 25 wt% ***γ***-CD:OEO inclusion complexes were homogeneous and continuous, showing mean thicknesses of 61 ± 1.1, 63 ± 0.98, 70 ± 0.94, and 72 ± 0.72 µm, respectively (see Table 3). This is in agreement with the electrospun fibre morphologies described above (see Fig. 4) that showed proper fibre formation until 25 wt%. Moreover, the film containing 30 wt% showed a surface with some cracks due to the high concentration of ***γ***-CD:OEO inclusion complexes that difficulted the formation of a continuous film with a higher thickness (~ 77 ± 0.68 µm). The film thicknesses also increased with the concentration of **γ**-CD:OEO inclusion complexes. Based on these results, the best concentration to attain uniform and homogenous films of PHBV was 25 wt% **γ**-CD:OEO inclusion complex.

Fig. 5

Fig. 6 showed the surface and cross-section of the films containing α-CD:OEO inclusion complexes. The thicknesses of the films containing 10 and 15 wt% of α-CD:OEO inclusion complexes were 73 ± 0.99 and 75 ± 0.77 µm, respectively. These films also showed a homogeneous surface. The film thicknesses increased with the amount added of α-CD:OEO inclusion complexes, reaching values of 81 ± 0.91, 83 ± 0.86, and 85 ± 0.69 µm for 20, 25, and 30 wt% of α-CD:OEO, respectively (see Table 3). Increasing the concentration from 20 wt% also affected the surface and generated cracks with different sizes. This phenomenon has been ascribed to the weak interfacial bond between the CDs and the biopolyester matrix (Ashori, Jonoobi, Ayrilmis, Shahreki, & Fashapoyeh, 2019). In this context, (Melendez-Rodriguez et al., 2019) also found that at high concentrations of silica nanoparticles with eugenol, that is, 15 and 20 wt%, the electrospun films showed greater porosity and

also some plastic deformation, which was attributed to a plasticization generated by the released oil and a possible migration during the annealing process. In this case, the best concentration to get uniform and homogenous films was 15 wt% of α-CD:OEO inclusion complexes.

Fig. 6

3.4. Visual Aspect of the Electrospun CD:OEO Inclusion Complexes-Containing PHBV Films

The visual aspect of the electrospun PHBV films containing different concentrations of γ-CD:OEO and α-CD:OEO inclusion complexes was observed to ascertain their contact transparency. In Fig. 7 it can be observed that the contact transparency was high but some differences among the samples were also seen. The neat PHBV film had a transparency value of 4.78 ± 0.08 and opacity of 0.037 ± 0.001. When 10 wt% of γ-CD:OEO inclusion complex was incorporated, slight changes were observed with respect to the neat PHBV´s transparency whereas opacity values without significant differences were obtained. The transparency value and opacity of the films containing 15, 20, and 25 wt% γ-CD:OEO inclusion complexes increased significantly respect to neat PHBV and 10 wt%. When 30 wt% of γ-CD:OEO inclusion complex was incorporated, the transparency and opacity values were higher respect to the others samples (9.23 ± 0.59 and 0.065 ± 0.004, respectively). Also, the films containing α-CD:OEO inclusion complexes presented high changes in the transparency and opacity respect to the γ-CD:OEO inclusion complexes. In particular, for the film sample containing 10 wt% α-CD:OEO inclusion complex, a transparency value of 6.36 ± 0.63 and opacity of 0.047 ± 0.005 was obtained. For the films containing 15 and 20 wt% of α-CD:OEO inclusion complexes, the values were similar while those based on 25 and 30 wt% of α-CD:OEO inclusion complexes presented the highest values. For both inclusion complexes, the increment of the concentration caused a light scattering that produced lower transparency and higher opacity. This phenomenon can be important in the design of food packaging materials due to some food products are sensible to the ultraviolet-visible (UV-Vis) light, which can trigger different enzymatic and oxidative reactions (Figueroa-Lopez et al., 2018).

Fig. 7

3.5.Thermal Stability of the Electrospun CD:OEO Inclusion Complexes-Containing PHBV Films

The TGA curves for the neat PHBV, γ-CD, α-CD, inclusion complexes of γ-CD:OEO and α-CD:OEO, and the PHBV films containing the inclusion complexes are shown in Fig. 8. The values of mass loss at 5% ($T_{5\%}$), mass at 160 ºC (%), which corresponds to the annealing temperature applied to the electrospun mats to produce

the films (see section 2.4.4.4), degradation temperature (T_{deg}), weight loss at T_{deg} (%), and residual mass (%) at 700 °C are gathered in Table 4. In our previous study, the TGA curve for the neat OEO showed a low thermal stability. In particular, it presented a mass loss at 160 ºC around of 40.3 %, having its T_{deg} value at 178.4 °C and the mass loss at T_{deg} was 74.16%, corresponding to the volatilization and/or degradation of principal volatile compounds such as carvacrol, thymol, and pinene (Figueroa-Lopez et al., 2019). This value is also similar to the T_{deg} of 168 ºC reported by (Guimarães et al., 2015). The mass losses at 160 ºC for the empty CDs were 8.86 % (γ-CD) and 9.42 % (α-CD), while the T_{deg} values were 323.12 ºC with a mass loss of 83.01 % for γ-CD and 326.43 ºC with a mass loss of 86.29 % for α-CD. As other authors have indicated (Campos et al., 2018), the thermal degradation of powdered molecules can be affected by different factors such as chemical structure, crystallinity, crystal size, and morphology. Thus, the thermal stability of CDs depends on the size of the crystal, showing greater thermal stability the larger crystals (Giordano, Novak, & Moyano, 2001; Nakanishi et al., 1997). When OEO was encapsulated into CDs, the inclusion complexes enhanced the thermal stability due to the interactions between the guest molecule and the cavity of the cyclodextrins achieving a protection of the volatiles compounds (Kayaci & Uyar, 2012). The mass loss for γ-CD:OEO inclusion complexes at 160 ºC was nearly 16.9%, having a T_{deg} of 326.28 ºC with a mass loss of 86.11 % at T_{deg}. In the case of α-CD:OEO inclusion complexes, the mass loss at 160 ºC was 9.69% and T_{deg} was 330.50 ºC with a mass loss at T_{deg} of approximately 85.70%. The inclusion complexes showed two mass losses, one below 100 °C corresponding to the loss of water from the cavity and another above 280 °C, which is attributed to the main thermal degradation of CDs (Aytac et al., 2017). In this regard, (Shin, Kathuria, & Lee, 2019) reported similar results for triacetyl (TA) encapsulated in β-CD, obtaining a T_{deg} of 293.99 °C for β-CD and for the inclusion complex of TA-β-CD its T_{deg} was 340.62 ºC. The thermal degradation of the PHBV films containing inclusion complexes increased slightly the value of T_{deg} respect to the neat PHBV. The mass loss values at 160 ºC for all films containing CDs and the inclusion complexes were similar, showing values between 1.21 and 1.71 %. The slight differences can be ascribed to the size and load capacity of the two tested CDs. The value of T_{deg} for the PHBV with 25 wt% γ-CD:OEO was 322.52 ºC with a mass loss of 96.12 %, while T_{deg} for the PHBV with 25 wt% γ-CD without OEO was slightly lower (320.11 ºC). Furthermore, the PHBV film containing 15 wt% α-CD:OEO showed a T_{deg} around 313.70 ºC with a mass loss of 96.98 % and the films with 15 wt% α-CD without OEO presented a T_{deg} of 309.27 ºC and mass loss around 96.28 %. Then, one can conclude that the thermal stability of OEO was

improved in the electrospun PHBV films. In this regard, (Yildiz, Celebioglu, Kilic, Durgun, & Uyar, 2018) reported an improvement of the thermal stability of CD:menthol inclusion complex in aqueous solutions nanofibers. Other studies have also suggested that the incorporation of substances such as powder, nanoparticles or EOs into electrospun biopolymer films increased their maximum decomposition temperature (Melendez-Rodriguez et al., 2019; Quiles-Carrillo, Montanes, Lagaron, Balart, & Torres-Giner, 2019b; Zainuddin, Kamrul Hasan, Loeven, & Hosur, 2019).

Fig. 8

Table 4

3.6. Mechanical Properties of the Electrospun CD:OEO Inclusion Complexes-Containing PHBV Films

The mechanical properties of the electrospun PHBV films containing the inclusion complexes are shown in Table 5. The neat PHBV film presented an E value of 1252 MPa, a σ_b value of 18.1 MPa, and a ε_b value of 2.4%, being very similar to the values reported in our previous work (Melendez-Rodriguez et al., 2019). The elastic modulus increased when CDs were included in the PHBV matrix. The E value for the PHBV film with 25% wt γ-CD was 1692 MPa and, for the PHBV film with 15% wt of α-CD, the E value was 1594 MPa. Likewise, the E values were higher in the PHBV films containing CDs with OEO compared with the films with CDs without OEO, showing an E value of 1472 MPa for the PHBV with 25% wt γ-CD:OEO and an E value of 1698 MPa for the PHBV with 25% wt α-CD:OEO. These significant increases of elasticity of PHBV films were induced by the presence of powder particles, that is, CDs, which potentially generated low interfacial interactions between the hydrophilic compounds of γ-CD and α-CD and the hydrophobic PHBV matrix and OEO, producing a reduction in ductility and consequently an increment in mechanical resistance (Zainuddin et al., 2019). Indeed, the values of σ_b decreased in the PHBV containing CDs, with values between 9.04 MPa and 9.83 MPa, while the ε_b values of PHBV films also decreased from 2.4% to 0.78 % due to presence of CDs and OEO. As reported earlier by (Shin et al., 2019), the addition of β-CD containing allyl isothiocyanate (AITC) reduced the tensile strength and elongation by 84% and 96%, respectively, of LDPE films obtained by extrusion. In another work, (Melendez-Rodriguez et al., 2019) reported an improvement of the elastic modulus and tensile strength of electrospun PHBV films when mesoporous silica nanoparticles containing eugenol were incorporated. The PHBV films here-prepared with the inclusion complexes are slightly less deformable and

therefore have greater elasticity than films produced using other commercial biopolymers, which facilitates the development of materials for the design of packaging to protect food (Quiles-Carrillo et al., 2019b).

Table 5

3.7. Antimicrobial activity of the Electrospun CD:OEO Inclusion Complexes-Containing PHBV Films

Table 6 showed the MIC and MBC values of γ-CD:OEO, and α-CD:OEO against *S. aureus* and *E. coli*. In our previous studies (Figueroa-Lopez et al., 2019), it was reported that the MIC and MBC values for pure OEO against *S. aureus* was 0.312 µL/mL and for *E. coli* was 0.625 µL/mL. Results showed that the encapsulation of OEO in CDs increased the antibacterial activity. The MIC and MBC values of γ-CD:OEO against *S. aureus* was 0.039 µg/mL and against *E. coli* was 0.078 µg/mL. In the case of α-CD:OEO, the MIC ad MBC values were 0.078 µg/mL, against *S. aureus*, and 0.156 µg/mL, against *E. coli*, so that these values agree with those reported by (Liang, Yuan, Vriesekoop, & Lv, 2012) for α-CD:carvacrol (MIC = 0.125 µg/mL). The higher antibacterial activity of the γ-CD:OEO inclusion complex can be attributed to its larger cavity size (see previous Table 1, γ-CD inner diameter is 9.5 Å while α-CD inner diameter is 5.7 Å) and improved encapsulation efficiency and loading capacity (see Table 2) compared with the α-CD:OEO inclusion complex. For both inclusion complexes, *S. aureus* was more sensitive than *E. coli*. In this regard, inclusion complexes have been reported to elevate the aqueous solubility of encapsulated hosts resulting in improved antimicrobial efficiency of EOs and their components at lower concentration (Das et al., 2019). (M. Zhang et al., 2018) evaluated the antimicrobial activity of γ-CD:alamethicin complex against *L. monocytogenes*, showing that the use of CD increased the solubility of alamethicin in aqueous medium thereby allowing more alamethicin to interact with the cell membranes resulting in a higher antimicrobial activity.

Table 6

Fig. 9 shows the antibacterial activity results of the PHBV films containing 25 wt% of γ-CD:OEO and 15 wt% of α-CD:OE inclusion complexes in both open and closed systems for up to 15 days. The films used as control, that is, samples without the inclusion complexes, presented an *E. coli* and *S. aureus* growth in the range between 4.16 x 10^6 and 6.05 x 10^6 CFU/mL. As shown in Fig. 9a, the reduction versus *S. aureus* and *E. coli* for the films containing 25 wt% of γ-CD:OEO inclusion complex was strong ($R \geq 3$), reaching a reduction for up to 3.63 and 3.28 Log_{10} (CFU/mL), respectively, after 15 days of evaluation. The PHBV films containing 15 wt% of α-CD:OEO at day 1 presented a significant inhibition ($R \geq 1$ and <3) and at days 3, 8, and 15 the inhibition was

strong, showing a reduction of up to 3.15 Log_{10} (CFU/mL) against *S. aureus*. The inhibition achieved for *E. coli* was significant, obtaining a reduction of 2.64 Log_{10} (CFU/mL) at day 15. These results correlate well with the antimicrobial properties included in Table 6 and Fig. 9 that show that the antibacterial activity of the γ-CD:OEO inclusion complexes was higher than the α-CD:OEO inclusion complexes.

The reduction of the *S. aureus* and *E. coli* using PHBV films containing 25 wt% γ-CD:OEO inclusion complexes and 15 wt% α-CD:OEO inclusion complexes in a closed system for up to 15 days of analysis are showed in Fig. 9b. All values in the closed system showed slightly higher values of reduction compared with the open system due to the release of the volatile compounds that were accumulated in the headspace. The film containing 25 wt% γ-CD:OEO inclusion complexes presented a strong activity against both bacteria during the 15 days of the study ($R \geq 3$). The antimicrobial activity for γ-CD:OEO inclusion complexes was higher than α-CD:OEO inclusion complexes, which is in accordance to the characteristics of the γ-type of CD due to its higher solubility and bigger pore size (Szejtli, 1998). Moreover, this is produced by the CD inclusion complexes mechanism that increases the solubility and, therefore, provides an efficient release of the hydrophobic agent in bacterial medium (Liang et al., 2012). Likewise, (Celebioglu, Umu, Tekinay, & Uyar, 2014) observed that films containing HPβ-CD:triclosan and HPγ-CD:triclosan inclusion complexes showed better antibacterial activity against both bacteria compared to the film with uncomplexed pure triclosan. Furthermore, the inhibition of *S. aureus* was slightly higher compared to *E. coli* due to the cellular wall differences between Gram-negative (G-) and Gram-positive (G+) bacteria (Rakmai, Cheirsilp, Mejuto, Torrado-Agrasar, & Simal-Gándara, 2017).

Fig. 9

3.8. Antioxidant Activity of the Electrospun CD:OEO Inclusion Complexes-Containing PHBV Films

The inhibition percentage (%) of DPPH and concentration (eq. Trolox/g sample) of DPPH for the pure OEO, γ-CD:OEO inclusion complexes, α-CD:OEO inclusion complexes, and the electrospun PHBV films containing γ-CD:OEO and α-CD:OEO inclusion complexes are shown in Fig. 10 and Table 7. These systems were also evaluated in an open and closed system for 15 days. Neat OEO presented a high percentage of inhibition (91.96%) attributed to its main active compounds (carvacrol, thymol, p-cymene, and γ-terpinene) (Figueroa-Lopez et al., 2019). The DPPH inhibition for γ-CD:OEO inclusion complexes was 82.51 % and for α-CD:OEO inclusion complexes it was 76.32 %. Therefore, OEO decreased the percentage of inhibition when it was encapsulated in CDs, which can be related to the encapsulation efficiency and loading capacity of the inclusion

636 complexes reported above in Table 2. The higher antioxidant activity attained with γ-CD:OEO inclusion
637 complexes can be related to its greater encapsulation efficiency when compared with α-CD:OEO inclusion
638 complexes. As indicated by (Lu, Cheng, Hu, Zhang, & Zou, 2009), the antioxidant activity of resveratrol in free
639 form showed little difference with that of resveratrol in complex form at the same concentration. The antioxidant
640 activity of biodegradable films is generally proportional to the amount of bioactive compounds added whereas
641 the thermal process to obtain the films can also highly affect bioactivity since most bioactive compounds are
642 sensitive to temperatures above 80 °C (Jouki, Yazdi, Mortazavi, & Koocheki, 2014). The electrospun films
643 containing OEO, that is, PHBV with 10 wt% OEO, showed a low inhibition of DPPH (24.54 %) with respect
644 to the films containing the inclusion complexes, which were 53.16% for PHBV with 25 wt% of γ-CD:OEO
645 inclusion complexes and 45.34% for PHBV with 15 wt% of α-CD:OEO inclusion complexes, at day 1 of
646 evaluation. From day 3, all the PHBV films started to show lower antioxidant activity. In the closed system,
647 the films presented a slightly higher DPPH inhibition than the films of the open system due to the release of
648 OEO volatile compounds to the simulated packaging headspace. For the last day of evaluation, that is, day 15,
649 the PHBV with 10 wt% of OEO films showed an inhibition of DPPH in the open and closed system of 14.90–
650 15.24 % (15.75 - 16.47 µg eq Trolox/g sample), respectively. The PHBV film containing 15 wt% of α-CD:OEO
651 inclusion complexes presented an inhibition of 36.11–37.24 % (38.42 - 39.26 µg eq Trolox/g sample) while the
652 PHBV film with 25 wt% of γ-CD:OEO inclusion complexes presented the highest antioxidant activity with a
653 DPPH inhibition of 45.26–47.02 % (48.17 - 49.95 µg eq Trolox/g sample). These results demonstrate that the
654 here-prepared inclusion complexes can successfully protect the volatile compounds responsible for the active
655 properties of OEO, a thermolabile substance, in a similar way that observed in the antimicrobial test. These
656 results also agree with the research work of (Aytac et al., 2017) where the antioxidant activity of electrospun
657 fibres of PLA containing β-CD:gallic acid was slightly superior to the fibres of PLA containing neat gallic acid,
658 being this effect attributed to the solubility of gallic acid in alcohols and the position of gallic acid in the cavity
659 of β-CD. Likewise, (Kaolaor, Phunpee, Ruktanonchai, & Suwantong, 2019) determined a high antioxidant
660 activity of β-CD:curcumin in poly(vinyl alcohol) (PVOH) blend films, which was attributed to the complexity
661 of curcumin in the cavity of β-CD. In conclusion, the electrospun films of PHBV incoporating 25 wt% of γ-
662 CD:OEO inclusion complex managed to maintain a high antioxidant activity for a longer period, which

indicates that this film can be used in the design of active packaging to maintain the physical, chemical, and microbiological characteristics of the food products (Robertson, 2005).

Fig. 10

Table 7

4.Conclusions

Herein, KM and FDM were explored for the encapsulation of OEO in two cyclodextrins types (α-CD and γ-CD). The results of this study showed that the encapsulation efficiency was influenced by the encapsulation method and the cyclodextrin type. Although both methods showed high encapsulation efficiencies, KM revealed to be the most efficient method for encapsulating OEO in the CD cavities, and it also offers some other advantages in terms of rapidity (KM: 18 minutes versus FDM > 48 h for the formation of inclusion complex), and the desired characteristics of the final product since the γ-CD:OEO (80:20 wt/wt) showed EE and LC values of 98.5% and 19.6%, respectively. The α-CD:OEO and γ-CD:OEO inclusion complexes presented high antimicrobial and antioxidant activities, which allowed their incorporation into PHBV fibres by electrospinning and subsequent annealing for film formation. The best concentration of α-CD:OEO and γ-CD:OEO inclusion complexes for homogeneous and continuous film formation were observed at 15 wt% α-CD:OEO and 25 wt% γ-CD:OEO inclusion complexes. The films showed high contact transparency whereas the mechanical properties were improved by the addition of the α-CD:OEO and γ-CD:OEO inclusion complexes in the PHBV matrix. The antimicrobial and antioxidant activities for the γ-CD:OEO inclusion complexes were higher than for the α-CD:OEO inclusion complexes, which is in accordance to the higher solubility of OEO in the γ-type of CD and its bigger pore size. The antimicrobial and antioxidant activity of the bioactive films were successfully maintained for up to 15 days due to the high protection offerered by the encapsulation system. In the light of the aforementioned findings, the here-developed electrospun CD:OEO inclusion complexes-containing PHBV films show a great deal of potential to be used in biodegradable active packaging applications to extend the shelf life of foodstuff.

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