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Effect of cyclodextrins and Mexican oregano (*Lippia graveolens* Kunth) chemotypes on the microencapsulation of essential oil

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ABSTRACT

Lippia graveolens (Kunth) essential oil (EO) contains the bioactive compounds carvacrol and thymol in different concentrations according to their chemotype. We evaluated the effect of the essential oil chemical composition, and different cyclodextrins (CD), β- and γ, on the microencapsulation process and its efficiency. Complex formation of *L. graveolens* EO with β-CD and γ-CD was determined by Scanning Electron Microscopy, X-Ray Diffraction, Thermogravimetric Analysis, and Fourier Transform Infrared Spectroscopy. Carvacrol and thymol concentrations in the γ-CD complexes were higher than in the β-CD complexes, as demonstrated by Gas Chromatography analysis. After 14 days of storage, carvacrol concentration in the β-CD complex remained similar to the initial values; while in the γ-CD complex the concentration was significantly reduced. In the case of thymol concentrations, no significant losses were observed after storage in the complexes obtained with β-CD or γ-CD. Our results confirmed that the microencapsulation of *L. graveolens* EO with CDs retained the bioactive compounds, carvacrol and thymol. Based on the amount of bioactive terpenes in these complexes and their relative stability, the best combinations to be used as delivery systems were the complexes formed between chemotypes thymol- γ-cyclodextrin and carvacrol-β-cyclodextrin.

1. Introduction

Essential oils (EOs) are natural products obtained from aromatic plants and are mainly composed of terpenoids, especially monoterpenes and sesquiterpenes. EOs are generally characterized by two or three major components which are assumed to be responsible for their biological activity (Majeed et al., 2015).

Lippia graveolens (Kunth), known as Mexican oregano, is an aromatic plant of high economic importance for its medicinal and culinary uses (Calvo-Irabien, 2018). Mexican oregano EO shows considerable phytochemical variation. Three different chemotypes have been identified for *L. graveolens* EO according to the ratios of: carvacrol, thymol, and the three sesquiterpenes, β-caryophyllene, α-humulene and caryophyllene oxide (Calvo-Irabién et al., 2014).

Mexican oregano EO presents antimicrobial and acaricidal properties and these properties have been attributed mainly to the presence of carvacrol and thymol. Several works have shown that thymol and carvacrol can control two important pathogens in beekeeping: varroa mites (Brasesco et al., 2017; El-Zemity et al., 2006; Suntres et al., 2015) and the microsporidian *Nosema ceranae* (Costa et al., 2010). The potential of utilizing EOs for pest control is compelling as they are widely accepted and perceived by consumers as natural and safe (Isman et al., 2011). Additionally because EOs are, in fact, complex mixtures of molecules, it has been claimed that the development of resistance in mirco-organisms is improbable (Brasesco et al., 2017; Girardi et al., 2018). However, the use of EOs can present disadvantages from a technological viewpoint, due to their high volatilization and low water solubility, which can limit their applications. In this sense, the encapsulation of EOs with cyclodextrins (CDs) is one of the most efficient strategies to improve their physicochemical

Abbreviations: EOs, essential oils; CDs, cyclodextrins; BCD, β-cyclodextrin; GCD, γ-cyclodextrin; BCD-C, β-cyclodextrin-EO Carvacrol chemotype; BCD-T, β-cyclodextrin-EO Thymol chemotype; BCD-S, β-cyclodextrin-EO Sesquiterpene chemotype; GCD-C, γ-cyclodextrin-EO Carvacrol chemotype; GCD-T, γ-cyclodextrin-EO Thymol chemotype; GCD-S, γ-cyclodextrin-EO Sesquiterpene chemotype; PMs, physical mixture of cyclodextrins and essential oil

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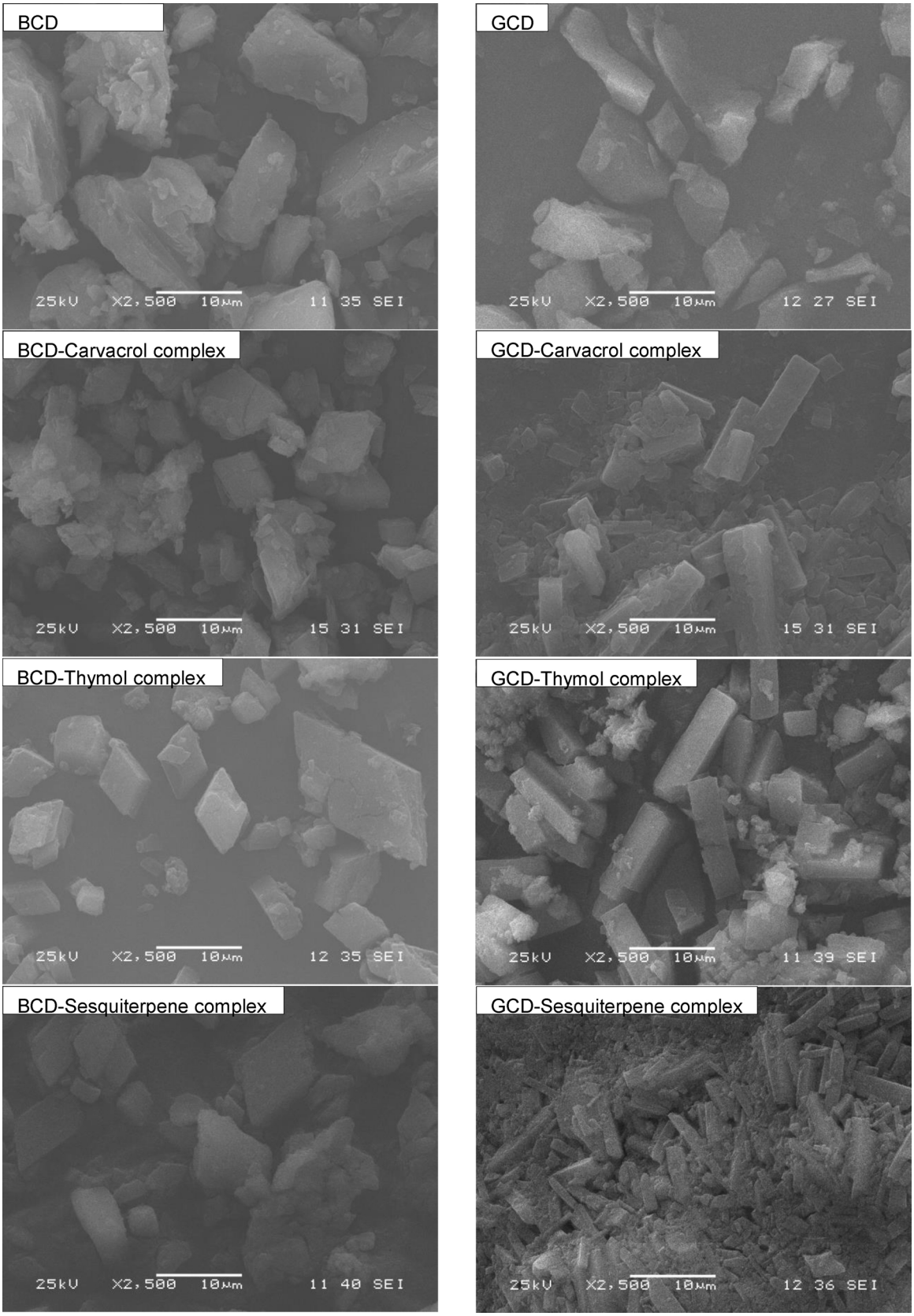

Fig. 1. Morphology of cyclodextrin-*L. graveolens* essential oil inclusion complexes. Photomicrographs (magnification x 2500) of β-cyclodextrin (BCD), γ-cyclodextrin (GCD) and the inclusion complexes obtained for the three chemotypes, Carvacrol, Thymol and Sesquiterpene of *L. graveolens* essential oil. The bar represents 10 µm.

Table 1
Size (μm) of pure β and γ-cyclodextrins, (BCD and GCD) and the complexes cyclodextrin-essential oil (CD-EO) obtained for the three chemotypes: Carvacrol (C), Thymol (T) and Sesquiterpene (S) of *L. graveolens*. Values are means ± SD, n = 6.

BCD	BCD-C	BCD-T	BCD-S	GCD	GCD-C	GCD-T	GCD-S
16.88 ± 3.50	8.30 ± 1.69	10.16 ± 3.55	8.92 ± 1.85	17.08 ± 4.97	11.40 ± 5.41	13.22 ± 3.21	6.97 ± 1.07

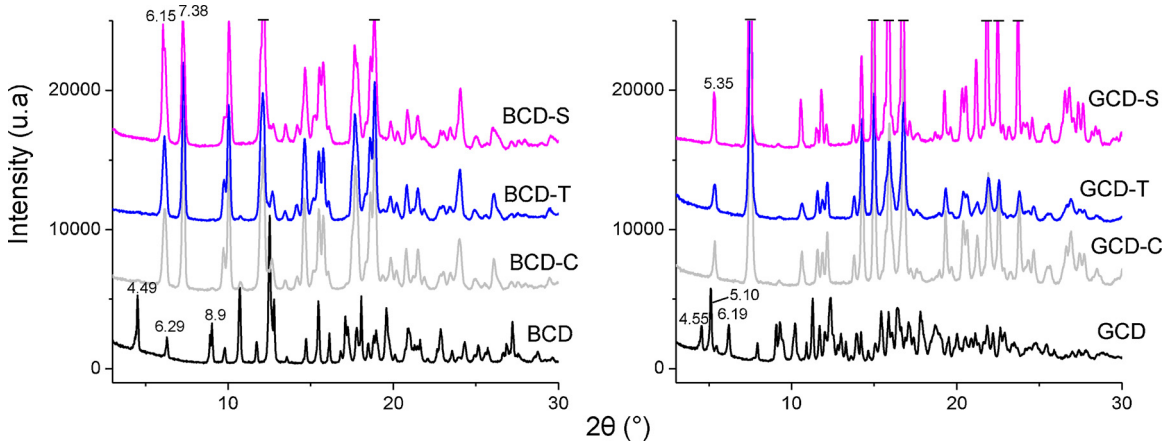

Fig. 2. Powder X-ray diffraction patterns of β-cyclodextrin (BCD), γ-cyclodextrin (GCD) and their complexes with Carvacrol (C), Thymol (T) and Sesquiterpene (S) chemotypes of *L. graveolens* essential oil.

properties (Rakmai et al., 2018). Furthermore, inclusion with CDs converts liquid EOs into a water-soluble powder which is easy-to-handle. Studies have clearly demonstrated the benefits of EO encapsulation (Girardi et al., 2018; Pinto et al., 2016) and highlight that complexation may serve as a stable delivery system for Mexican oregano EO, and its components. It was reported that encapsulation with β-cyclodextrin (BCD) can enhance the antimicrobial action of carvacrol (Kamimura et al., 2014; Santos et al., 2015) and thymol (Tao et al., 2014). Furthermore, LeBlanc et al. (2008) prepared BCD complexes of thymol, carvacrol and *Origanum vulgare* (L.) EO and gave this formula to honeybees. The study found significant levels of thymol and carvacrol in honeybee hemolymph and gut, without toxicity to the bees suggesting that this system could be a promising tool for honeybee health and integrated disease management. Hernández-Hernández et al. (2014) showed that microencapsulation improves the stability and antimicrobial activity of both *L. graveolens* and *O. vulgare* (L.) essential oils. Likewise, Arana-Sánchez et al. (2010) showed that microencapsulation with BCD preserved antimicrobial and antioxidant activity of *L. graveolens* EO, and emphasized that EO composition affected the microencapsulation efficiency.

This study evaluated the effect of different CDs and chemotypes in the microencapsulation of *L. graveolens* EO, taking into consideration the phytochemical variation reported for Mexican oregano EO. Three chemotypes of *L. graveolens* with differing thymol-to-carvacrol ratios, and two types of CD: β-cyclodextrin and γ-cyclodextrin were utilized for this study. This research focused on: (i) characterizing the inclusion complexes formed by using different analytical techniques, (ii) determining the terpene content of those complexes, focusing on the components with documented anti-varroa and anti-*nosema* activity (thymol, carvacrol), and (iii) evaluating the stability of those complexes. To our knowledge, this is the first report on microencapsulation of *L. graveolens* EO using γ-cyclodextrin and the sesquiterpene chemotype of this oil. The results of this study will determine suitable CD combinations for specific *L. graveolens* EO chemotypes in order to determine an efficient delivery system, with potential application for controlling pests in honeybees.

2. Materials and methods

2.1. Essential oils extraction

EOs from the leaves of *Lippia graveolens* Kunth (Verbenaceae) were obtained by hydrodistillation in a Clevenger-type apparatus as previously reported (Calvo-Irabién et al., 2014). As described by Calvo-Irabién et al. (2014), three different chemotypes were identified, hereon designated as: type C, with EO mainly composed of carvacrol (~60% of the chromatogram area), type T, mainly composed of thymol (> 60%) and type S, mainly composed of the sesquiterpenes, β-caryophyllene, α-humulene and caryophyllene oxide (> 40%) and with concentrations of carvacrol and thymol less than 0.3% (Fig. 5a–c). The estimated density for the different EOs was 0.983 g/mL for carvacrol, 0.935 g/mL for thymol and 0.940 g/mL for sesquiterpene chemotypes.

2.2. Preparation of cyclodextrin (CD) complexes

β-cyclodextrin (BCD) (CAVAMAX® W7) and γ-cyclodextrin (GCD) (CAVAMAX® W8) food grade from Wacker Chemical Corporation were used for the inclusion complex formation. This was achieved by a slight modification to the LeBlanc et al. (2008) co-precipitation method. Briefly, 1.25 g of CD were dissolved in 50 mL of deionized water (18 M-Ohm) at 40 °C under constant stirring. Then, the EO (0.75 g) was dissolved in 80 mL of ethanol and slowly added to the CD solution (1 mL/min). This mixture was stirred an additional 60 min and cooled to room temperature. To accelerate precipitation, the mixture was left at −20 °C overnight. The following day the precipitate was separated by centrifugation (6000 rpm, 20 min, 4 °C) and allowed to air-dry overnight in a desiccator vacuum. Six types of complexes were obtained based on the cyclodextrin and chemotypes utilized and will hereon be denoted as: β-cyclodextrin-EO Carvacrol chemotype (BCD-C); β-cyclodextrin-EO Thymol chemotype (BCD-T), β-cyclodextrin-EO Sesquiterpene chemotype (BCD-S); and γ-cyclodextrin-EO Carvacrol chemotype (GCD-C); γ-cyclodextrin-EO Thymol chemotype (GCD-T), γ-cyclodextrin-EO Sesquiterpene chemotype (GCD-S). The total complex yield (expressed as percentage) was calculated as: obtained complex precipitate (g)/Initial EO + CD (g).

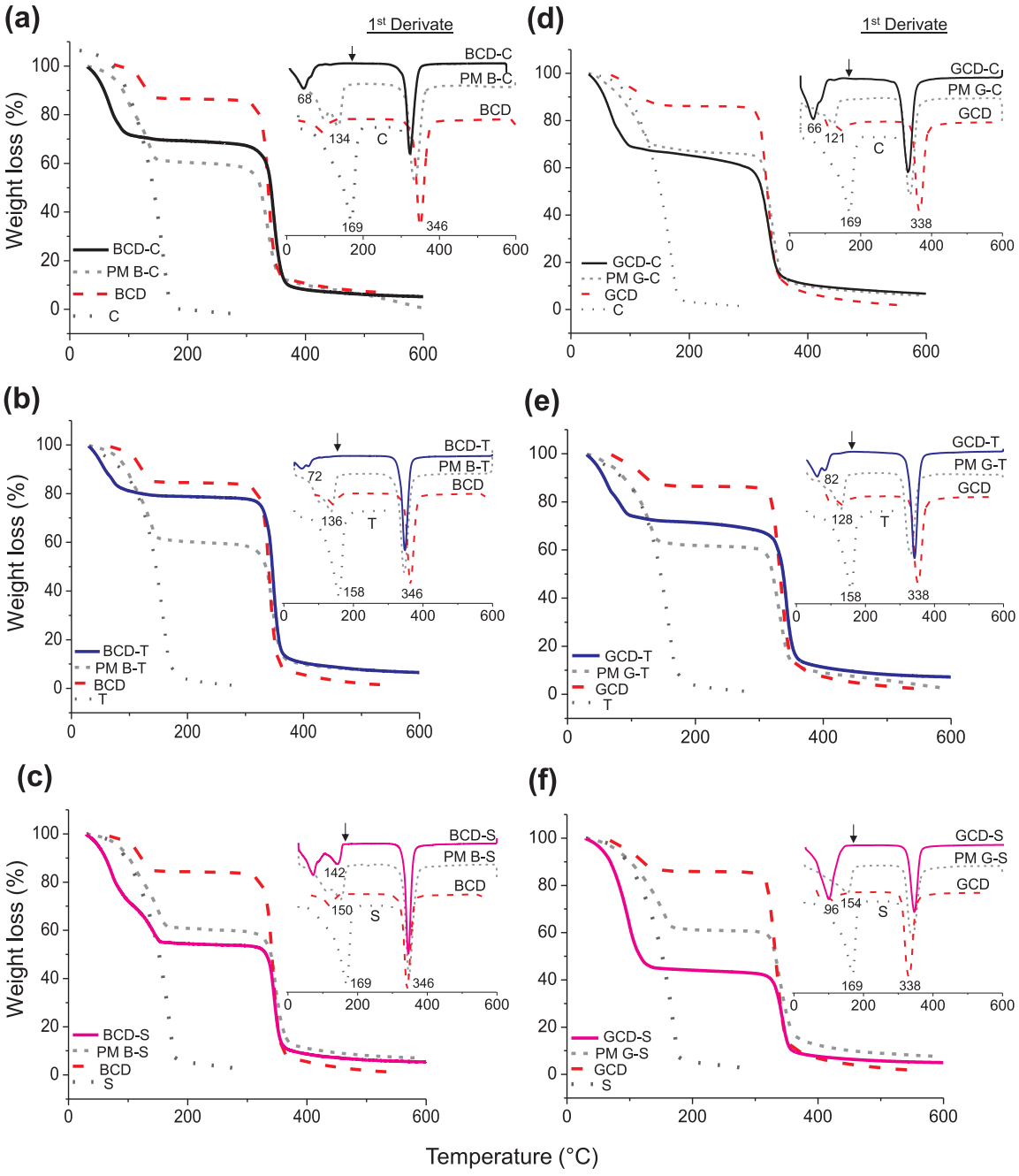

Fig. 3. Thermogravimetric (TG) and their first derivate (DTG) curves of cyclodextrin-*L. graveolens* essential oil complexes for the three chemotypes, Carvacrol (C), Thymol (T) and Sesquiterpene (S); the physical mixtures of cyclodextrin with essential oil (PM); pure cyclodextrins (BCD and GCD) and the *L. graveolens* essential oil. (a), (b), (c): show data for complexes with β-cyclodextrin and (d), (e), (f) for complexes with γ-cyclodextrin.

2.3. Characterization of CD-EO complexes

2.3.1. Scanning electron microscopy (SEM)

SEM images provided the morphology and approximate size of the crystals formed by the inclusion complexes. These properties were determined by using a JEOL 6360 LV (Tokyo, Japan). Samples were gold coated and observed at an accelerating voltage of 25 kV, spot size 31, aperture 1. The dimensions of crystals were determined using ISIS JEOL SEM software. Measurements from six crystals were taken for each CD-EO complex type.

2.3.2. X ray diffraction (XRD)

Crystallinity was confirmed by X-ray diffraction (XRD) using a Siemens D5000 diffractometer (Siemens, Germany) with CuK α radiation (λ = 1.5416 Å) in the 2θ range of 5° to 60° with a step count of 3 s and a step size of 0.02° (2θ).

2.3.3. Thermogravimetric analysis (TGA)

For TGA, 6 mg of the EOs, CDs, CD-EO complexes and CD-EO physical mixtures (PMs) were heated from 30 °C to 600 °C at 10 °C/min in a nitrogen atmosphere using a TGA 7 from Perkin Elmer (Norwalk, CT). Decomposition temperatures (Td) were obtained using the first derivate of the TGA curves. Physical mixtures (PMs) of CD-EO were prepared immediately before the TG characterization, with the same proportions used for the CD-EO complexes.

2.3.4. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the samples were obtained by using a Nicolet

Table 2
Percentage of weight lost during thermogravimetric analysis for *L. graveolens* essential oil, cyclodextrins, physical mixture and cyclodextrin-essential oil complexes.

	Weight loss (%)							
	1st step (30–120 °C)		2nd step (120–280 °C)		3rd step (280–400 °C)		4th step (400–600 °C)	
Essential oil chemotype								
Carvacrol	22.13 [a]		75.27 [b]		–		–	
Thymol	24.21 [a]		72.09 [b]		–		–	
Sesquiterpene	22.07 [a]		73.93 [b]		–		–	
	BCD	GCD	BCD	GCD	BCD	GCD	BCD	GCD
Pure CD	13.40 [c]	11.37 [c]	–	–	71.13 [f]	71.16 [f]	4.75 [g]	6.66 [g]
PM- C	23.97 [d]	23.64 [d]	16.98 [e]	10.29 [e]	49.08 [f]	56.31 [f]	9.35 [g]	3.92 [g]
PM −T	22.21 [d]	23.52 [d]	18.58 [e]	15.24 [e]	49.26 [f]	52.05 [f]	3.41 [g]	6.88 [g]
PM −S	18.01 [d]	16.30 [d]	22.18 [e]	23.11 [e]	48.76 [f]	48.35 [f]	4.09 [g]	4.89 [g]
CD-C	30.70 [d]	34.91 [d]	2.93 [e]	6.00 [e]	58.5 [f]	49.51 [f]	3.17 [g]	3.45 [g]
CD-T	24.38 [d]	28.14 [d]	1.96 [e]	3.82 [e]	63.32 [f]	57.81 [f]	3.64 [g]	3.45 [g]
CD-S	31.66 [d]	51.42 [d]	14.07 [e]	3.93 [e]	45.36 [f]	37.52 [f]	3.19 [g]	2.38 [g]

C: carvacrol, T: thymol and S: sesquiterpene chemotypes, respectively. PM: physical mixture. BCD and GCD: β and γ-cyclodextrins, respectively. Weight loss due to: [a] initial essential oil evaporation; [b] final evaporation of essential oil up to 206 °C (C), 200 °C (T) or 216 °C (S); [c] water release; [d] initial evaporation of the essential oil + water release; [e] external essential oil release; [f] thermal decomposition of CD and evaporation of encapsulated essential oil; [g] carbonization of CD.

8700 FT-IR spectrometer (Thermo Scientific, Madison, WI) equipped with an attenuated total reflectance (ATR) accessory using germanium crystal. Scans in the 4000 and 650 cm^{-1} spectral range were performed and the spectra were registered after averaging 100 scans with a resolution of 2 cm^{-1}.

2.4. Extraction of essential oil from CD-EO complexes and estimation of the inclusion efficiency

The amount of EO in the inclusion complex particles was determined with the hexane extraction method (Petrović et al., 2010). For each type of inclusion complex, 0.3 g of the sample were mixed with 10 mL of distilled water and 5 mL of hexane. The mixture was heated at 85 °C for 20 min. Then, the organic phase containing the volatile compounds was separated from the aqueous phase by decantation. In order to ensure that all the entrapped EO was extracted from the CD-EO complexes, the aqueous phase was extracted three times with hexane using the previously described hexane extraction method. The final hexane extract was dried with anhydrous sodium sulphate; then the hexane was evaporated under a nitrogen flow. The recovered EO was weighed and then stored at 4 °C for Gas Chromatography (GC) analysis. The EO extracted corresponds to the included molecules in the CD cavity plus the essential oil adsorbed at the surface of CD. The efficiency of the inclusion, as a percentage, was calculated using the ratio: initial g of EO/g of recovered EO after hexane extraction.

2.5. Gas chromatography with flame ionization detector (GC-FID)

Composition of the original EO and the EO extracted from the complexes was determined by GC-FID using a Varian 430 gas chromatograph equipped with FID detector (Varian B.V. Inc. Walnut Creek, CA, USA). Separation was carried out on a 60-m × 0.25 mm ID × 0.25 µm film thickness non-polar capillary column Varian DB5 (5%-phenyl-methylpolysiloxane) with N_2 as the carrier gas (1.5 mL/min). Injection volume was 1 µL using a split ratio of 1:40. The temperatures of the injector and detector were 250 °C and 280 °C, respectively. The initial oven temperature was increased from 60 °C (1 min) up to 150 °C at a rate of 4 °C/min and held for 2 min. It was then increased to 250 (4 °C/min) (maintained for 5 min) and finally increased at a rate of 10 °C/min up to 275 °C (for 5 min), resulting in a total analysis time of 58 min. The concentration of each component of the EO was quantified by proper calibration curves of commercial standards of p-cymene, eucalyptol, γ-terpinene, thymol, carvacrol, β-caryophyllene, α-humulene and caryophyllene oxide (Sigma-Aldrich). The chemical composition of the original EO was compared with that of the EO extracted from the complexes. The inclusion of each terpene in the complex was expressed as a complexation ratio: terpene concentration (mg/mL) in the extracted EO/terpene concentration (mg/mL) in the original EO. Additionally, in order to have a standardized estimate for the amount of each type of terpene present in the CD-EO complexes (µg terpene/g complex), the terpene concentration was calculated according to the following equation: ((mg terpene*mg extracted EO/g complex)/mg EO corresponding to 1 mL)*1000.

2.6. Stability of CD-EO complexes

In order to evaluate the release of the encapsulated oils under storage conditions, vials with 0.3 g of each complex were stored in an oven. Oven conditions were 36 °C and an ambient relative humidity of 36%. Samples were stored for a period of 14 days. At day 7 and 14, the EO from these samples was extracted from the CD-EO complexes using the previously mentioned hexane extraction method. Identification and quantification of the components present in the recovered EOs were evaluated by GC-FID using the chromatographic conditions previously described. The temporal dynamic (days 0, 7 and 14) of the terpene content in the complexes was described and the complex stability was evaluated by comparing terpene content (µg) per gram of each complex at day 0 and at day 14. A *t*-test was used to determine if there was a statistically significant difference between the two-time points.

3. Results and discussion

3.1. Yields of CD-EO complexes

BCD-C, BCD-T, BCD-S, GCD-C and GCD-T formed crystalline solids. However, GCD-S exhibited a semi-solid appearance (rubber-like). Total complex yields varied depending on the chemotype-CD combination resulting in yields of 77.12%, 87.73% and 70.64% for BCD-C, T and S complexes respectively. For GCD, values were 79.89%, 61.98% and 112.62%, for the C, T, and S chemotypes, respectively. The relatively high total yield of the GCD-S complex was probably due to its rubber-like consistency; with the laboratory protocol utilized the mixture was not completely dry.

3.2. Scanning electron microscopy (SEM)

SEM images showed that pure BCD and GCD appear as crystalline particles of varied sizes without a defined shape (Fig. 1). The average particle sizes were 16.88 µm for BCD and 17.08 µm for GCD (Table 1). BCD-C, BCD-T and BCD-S complexes showed the typical monoclinic

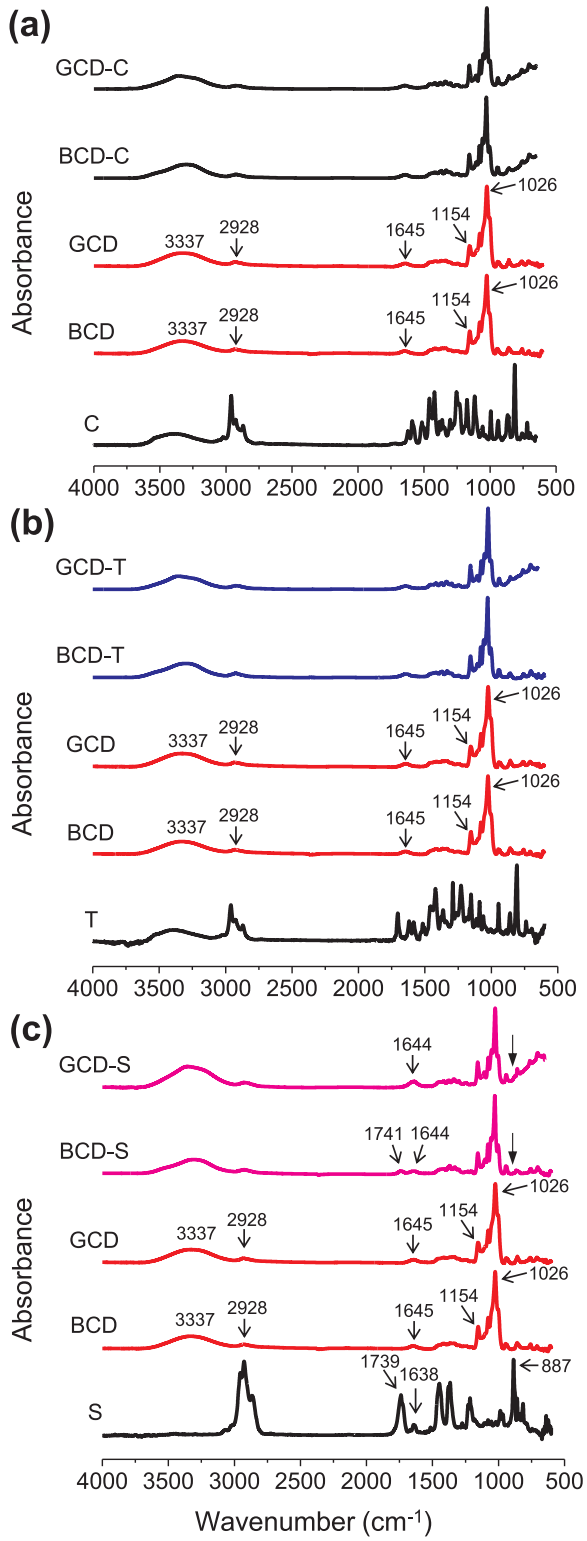

Fig. 4. FTIR spectra of *L. graveolens* essential oil for chemotypes Carvacrol (a), Thymol (b) and Sesquiterpene (c); and their corresponding inclusion complexes. Additionally, FTIR spectra for pure β-cyclodextrin (BCD) and γ-cyclodextrin (GCD) are shown.

crystalline structures that have been reported for the inclusion of thymol and carvacrol in this type of CD (Bethanis et al., 2013). The average size varied between 8 and 10 μm (Table 1). In contrast, GCD-based complexes showed triclinic crystals where the average size depended on the essential oil composition, i.e. an average size of 11.4 and 13.22 μm for GCD-C and GCD-T respectively and smaller 6 μm crystals for GCD-S complexes (Fig. 1 and Table 1).

The physical appearance and size of the CD-EO complexes formed were different from the original CDs, as shown by the SEM microphotographs. In general, crystals in the CD-EO complexes were smaller, nearly half their original size, than pure CD crystals. The change observed in the size and shape in the CD-EO complexes was indicative of the presence of a new solid phase, which clearly indicates cyclodextrin-Mexican oregano EO complex formation. These results are in agreement with previous studies showing that the morphology of inclusion complexes was different from the original morphology of pure CDs and it is considered as an evidence of the formation of the complex (Guimarães et al., 2015; Hădărugă et al., 2012; Menezes et al., 2012). Haiyee et al. (2009) prepared BCD and GCD complexes with turmeric oleoresin using the same co-precipitation method and reported drastic changes in particle shapes and morphologies of the inclusion complex products. Likewise, Hădărugă et al. (2012) studied the complexes of BCD and EOs obtained by the co-precipitation method and reported crystal complexes with smaller rhomboidal crystals rather than the hexagonal crystals of the pristine BCD.

3.3. X-ray diffraction (XRD) of complexes

XRD is a useful method for the detection of CD complexation. The appearance of new diffraction peaks, as well as a shift in the characteristics of peaks of the host molecule, and their relative intensity have all been reported as evidence of the formation of inclusion complexes (Mura, 2015). The XRD pattern of both BCD and GCD are evidence of a clear crystalline nature because their peaks are intense and sharp (Fig. 2, Rotich et al., 2003). The diffraction patterns of inclusion complexes with BCD showed differences from the pattern of pure BCD. The peaks at $2\theta = 4.49°$, $2\theta = 6.29°$ and $2\theta = 8.9°$ of BCD disappeared and new intense and sharp peaks appeared at $2\theta = 6.15°$ and $2\theta = 7.38°$ in the BCD-C, BCD-T and BCD-S complexes (Fig. 2). The change in peaks could be associated with a new molecular organization of the BCD evidencing the complex formation and defining the monoclinic crystalline structure in the complexes. Similar results were described by Abarca et al. (2016) for the system BCD-2-nonanone and by Fernandes et al. (2004) for *Lippia sidoides* oil-BCD complex. Likewise, GCD showed reflection at $2\theta = 4.55°$, 5.10° and 6.19°, all of which were not observed in the diffraction patterns of GCD-C, GCD-T, GCD-S complexes. In addition, the patterns of the CD-EO complexes presented an intense peak at $2\theta = 5.35°$ which was observed in the pure GCD, suggesting a reduction of d-spacing of the crystalline structure (Fig. 2). Martínez and Gómez (2007) described similar changes in the XRD characterization of the GCD-Isotactic polypropylene complexes. Overall, the changes in the CD-EO complex diffraction patterns support the formation of an inclusion complex.

3.4. Characterization by thermogravimetric analysis (TGA)

Thermogravimetric (TG) curves and their first derivate (DTG) can also be used as indicators of CD-EO complex formation (Fernandes et al., 2004; Guimarães et al., 2015; Menezes et al., 2012; Mura, 2015). Thermograms of the pure EO from the three chemotypes showed that 96–97% of the EO weight was lost between 200 and 216 °C, corresponding to EO volatilization (Fig. 3 and Table 2). TGs of pure CDs were characterized by two separate stages of weight loss. The first stage is related to the CD dehydration occurring at temperatures between 30 and 120 °C. In this temperature range 13.40% and 11.37% of mass loss was observed for BCD and GCD, respectively. The second stage, between 280 and 400 °C is ascribed to the decomposition of the CDs and represents the majority of the weight lost. During continuous heating, the sample is carbonized and between 400 and 600 °C, elemental carbon was slowly released (Fig. 3, Table 2). The TGs of the physical mixtures (PMs) of CDs and EOs showed a pattern overlapping the

Table 3
Inclusion efficiency (%) and amount of *L. graveolens* essential oil (EO) in the cyclodextrin complexes (mg of EO/g of complex). Values are means ± SD, n = 2.

	β-cyclodextrin Essential oil chemotype			γ-cyclodextrin Essential oil chemotype		
	Carvacrol	Thymol	Sesquiterpene	Carvacrol	Thymol	Sesquiterpene
Content of EO (mg of EO/g of complex)	28.5 ± 1.5	51.7 ± 8.2	244.3 ± 10.3	51.4 ± 2.1	89.8 ± 0.4	62.4 ± 3.7
Inclusion efficiency (%) (g original EO/g recovered EO after inclusion)	7.4 ± 0.4	13.4 ± 2.1	63.4 ± 2.7	13.4 ± 0.6	23.4 ± 0.2	16.1 ± 1.0

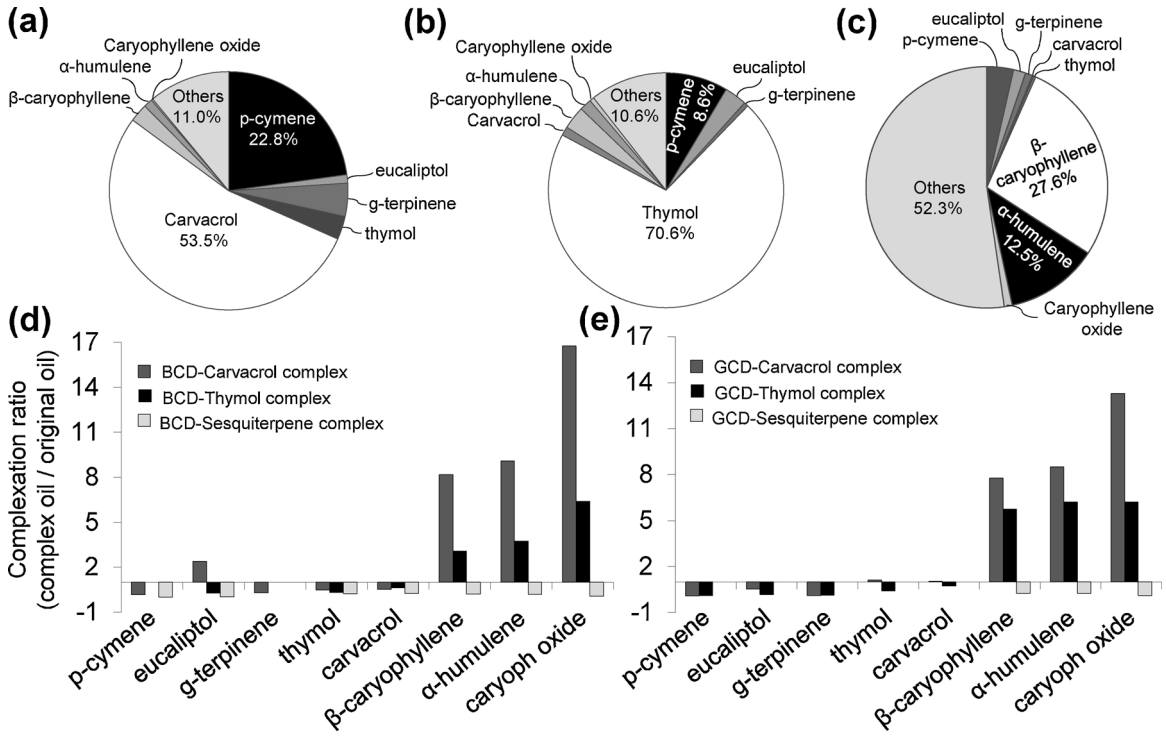

Fig. 5. Changes in the chemical composition of *Lippia graveolens* essential oil after inclusion in β and γ cyclodextrins. The original essential oil composition (percentage values of total chromatogram area) for the three chemotypes Carvacrol (a), Thymol (b) and Sesquiterpene (c) are shown. Complexation ratios for the major essential oil components in β and γ cyclodextrin complexes (d and e, respectively), are shown. Complexation ratio values = 1 represents no change in terpene concentration due to complex formation; < 1 decreased terpene concentration, > 1 increased terpene concentration (see text for details).

characteristic events observed for the individual curves of the pure EOs and CDs, which suggests that no interaction took place between the EO and the CD in the physical mixture. In the PMs, the evaporation of the EO (assumed as surface oil) and the remaining water content in the CD completes at 180 °C; this behavior was independent of the EO chemotype (Fig. 3).

On the other hand, the thermal behavior of the different CD-EO complexes was remarkably different from all the previously described curves. There is no evidence of the presence of peaks corresponding to the EO in the DTG curves of the CD-EO complexes (arrows in Fig. 3). These findings strongly indicate the formation of inclusion complexes in both types of CDs, independent of the EO composition. In the case of the BCD-S complex (Fig. 3c) the peak observed at 142 °C suggests that a portion of the EO was not totally incorporated and that surface EO evaporated; a similar pattern to that observed in the BCD-S DTG curve of the PM sample. Between 120 °C and 280 °C, the percentage of weight lost for the CD-EO complexes was two to ten times lower than the loss observed for their corresponding physical mixture (PM) samples (Table 2). The greater weight loss observed in the PM samples can be attributed to the final release of surface oil; which did not occur with the CD-EO complexes because the EO was strongly encapsulated. The complexes BCD-C and BCD-T demonstrated the smallest percentage of weight lost within the temperature range 120–280 °C, suggesting that these complexes exhibited the highest EO encapsulation. Finally, the encapsulated oil was released in the third temperature range (280–400 °C) as indicated by the increased percentage of weight lost; which is most clearly shown in the BCD-C and BCD-T complexes, when compared with their respective PM samples (Table 2). TG/DTG curves of the CD-EO complexes showed similar curves to those described for complexes of BCD and essential oil components, such as, geraniol (Menezes et al., 2012), carvacrol (Guimarães et al., 2015), *Lippia gracilis* EO (Marreto et al., 2008) and *Lippia sidoides* EO (Fernandes et al., 2004).

3.5. Fourier transform infrared spectroscopy (FTIR)

Specific types of complexation may be demonstrated by the FTIR spectra where the intensity and relative location of the bands observed are altered by their guest molecule. However, its application is limited when the mass of the guest molecule does not exceed 5–15% of the mass of the complex because the alterations are usually masked by the CD spectrum (Marques, 2010). The spectrum of both β and γ cyclodextrins showed absorption bands at 3337 cm^{-1} (symmetrical and asymmetrical stretching of the −OH groups), 2928 cm^{-1} (C−H stretching), 1645 cm^{-1} (H−O-H bending), 1154 cm^{-1} and 1026 cm^{-1} (asymmetric and symmetric stretching of the C−O−C; Fig. 4). Comparable results have been reported (Haiyee et al., 2009; Menezes et al., 2012). Small changes in the spectra of BCD-C, GCD-C, BCD-T and GCD-

Table 4
Concentration of major terpenes in the original *L. graveolens* essential oil and in the oil extracted from the cyclodextrin-essential oil complexes. Concentrations are expressed as mg terpene/mL EO and μg terpene/g CD-EO complex (see text for details).

Original oil		Oil extracted from BCD-EO complex		Oil extracted from GCD-EO complex	
Carvacrol chemotype	mg/mL	mg/mL	μg terpene/g complex	mg/mL	μg terpene/g complex
p-cymene	9.20	1.61	46.58	0.86	44.95
eucalyptol	0.59	1.41	40.80	0.31	16.20
γ-terpinene	1.98	0.57	16.49	0.22	11.50
thymol	2.05	0.99	28.64	2.30	120.23
carvacrol	34.25	17.95	519.35	35.71	1866.67
β-caryophyllene	2.33	19.04	550.89	18.10	946.14
α-humulene	1.15	10.47	302.93	9.77	510.71
caryophyllene oxide	0.29	4.86	140.62	3.85	201.25
Thymol chemotype					
p-cymene	2.47	ND	ND	0.27	26.13
eucalyptol	1.14	0.30	16.81	0.20	19.54
γ-terpinene	0.19	ND	ND	0.03	2.64
thymol	32.71	9.98	552.16	13.39	1286.04
carvacrol	0.56	0.35	19.36	0.41	39.76
β-caryophyllene	2.11	6.49	359.07	12.12	1164.49
α-humulene	1.12	4.20	232.37	6.96	668.18
caryophyllene oxide	0.57	3.65	201.94	3.54	339.73
Sesquiterpene chemotype					
p-cymene	4.54	0.06	15.33	ND	–
eucalyptol	2.71	0.07	19.23	ND	–
γ-terpinene	1.34	ND	ND	ND	–
thymol	0.36	0.09	22.87	ND	–
carvacrol	0.56	0.14	36.39	ND	–
β-caryophyllene	81.66	16.85	4379.21	20.24	1343.59
α-humulene	37.92	7.52	1954.40	9.31	618.03
caryophyllene oxide	4.34	0.31	80.57	0.52	34.52

ND = non detected.

T complexes were observed, presumably due to the low amount of EOs and overlap with the peaks of the CDs (Fig. 4a, b). Conversely, the spectrum of the S chemotype presents an intense peak at 1739 cm^{-1} and another peak at 1638 cm^{-1}, corresponding to C=O and C=C stretching. These two peaks changed their intensities and were observed at 1741 cm^{-1} and 1644 cm^{-1} in the spectrum of BCD-S, corresponding to C=C stretching. In addition, the very intense peak at 887 cm^{-1} observed in the S spectrum, which has been previously reported as the characteristic absorption band of β-caryophyllene associated to an out-of-plane deformation vibration of =CH (Liu et al., 2013), was not detected in the BCD-S complex (arrows in Fig. 4c). For GCD-S complex, the peak at 1741 cm^{-1} was not detected, while the intense peak at 887 cm^{-1} characteristic of β-caryophyllene was not present (arrows in Fig. 4c). Liu et al. (2013) considered that the absence of the characteristic peak of β-caryophyllene in the spectrum of the BCD inclusion complex was related to the formation of intra-molecular hydrogen bonds between β-caryophyllene and β cyclodextrin. Therefore, FTIR only showed evidence of complex formation for β and γ cyclodextrins with Sesquiterpene EO chemotype.

In summary, according to the physicochemical tests performed, we have demonstrated with evidence from various analytical techniques, that the EO of different Mexican oregano chemotypes formed complexes with both β and γ cyclodextrins.

3.6. Efficiency of inclusion and composition of the EOs extracted from the CD-EO complexes

The formation of an inclusion complex between a guest molecule and CDs depends on a variety of physicochemical parameters, such as: the size, the chemical structure and the physical properties of the guest molecules; as well as the type of interaction between the guest molecule and the CD; the type of cyclodextrin used; and finally the method of preparation (Abarca et al., 2016; Del Valle, 2004).

Inclusion efficiency values for the different CD-EO complexes that resulted ranged between 7.4% and 63.4%. The highest percentage of inclusion efficiency was obtained for the BCD-S complex and the lowest value for BCD-C complex. Carvacrol and Thymol chemotypes presented a higher percentage of inclusion in GCD than in BCD; whereas the Sesquiterpene chemotype showed a higher value of inclusion in BCD (Table 3). These differences could be attributed to the distinct chemical structure and physicochemical properties of the major terpene guest molecules present in the EO (e.g. carvacrol, thymol and β-caryophyllene), as well as to the type of interactions established between the guest molecules and the specific cyclodextrin (Abarca et al., 2016; Del Valle, 2004).

Ciobanu et al. (2013) determined the inclusion efficiency as a function of the stability constant for CD-monoterpenes complexes and suggested that monoterpenes (α-pinene, β-pinene, camphene, eucalyptol, limonene, linalool, p-cymene, myrcene, menthone, menthol, trans-anethole, pulegone and camphor) had superior complexation efficiency in β-cyclodextrin than with γ-cyclodextrin. In contrast, our results showed that the oils rich in monoterpenes (Carvacrol and Thymol chemotypes) presented higher inclusion efficiency in GCD than in BCD. This could be explained by the higher water solubility of GCD compared to BCD (Marques, 2010). For the type of monoterpenes present in our EOs, water solubility might be one of the most important factors for complex formation with the co-precipitation method used. For the Sesquiterpene chemotype, composed primarily of β-caryophyllene and α-humulene, our results suggest that BCD has an intrinsic ability to form inclusion complexes with β-caryophyllene as previously reported (Liu et al., 2013; Quintans-Júnior et al., 2016) and could explain the higher complexation efficiency observed in BCD-S compared with GCD-S (Table 3). Furthermore, there are additional factors, such as the retention of oil in the solution after complexation,

Table 5
Final content (μg/g complex) and percentage of major terpenes thymol, carvacrol and β-caryophyllene retained in the *L. graveolens* essential oil-cyclodextrin complexes after 14 day storage.[a]

	carvacrol		thymol		β-caryophyllene	
Complexes	μg/g complex	% retained	μg/g complex	% retained	μg/g complex	% retained
BCD-C	392.5 ± 120.0	75.6	17.07 ± 1.0*	59.3	264.8 ± 14.0*	48.1
BCD-T	7.7 ± 2.3*	40.2	374.9 ± 92.7	67.9	213.0 ± 63.4	59.3
BCD-S	0.6 ± 0.3*	1.7	3.3 ± 0.6*	14.6	943.1 ± 209.9*	21.5
GCD-C	650.3 ± 229.0*	34.8	42.4 ± 14.6*	35.3	382.7 ± 151.0*	40.4
GCD-T	12.0 ± 5.9*	30.2	685.3 ± 283.0	53.3	597.2 ± 130.9	51.3
GCD-S	absent-	–	absent-	–	784.0 ± 22.9*	58.4

C, T and S: Carvacrol, Thymol and Sesquiterpenes chemotypes from *L. graveolens* essential oil. BCD: β-cyclodextrin. GCD: γ-cyclodextrin.
[a] Complexes were stored at 36 °C and 36% ambient relative humidity. Values are means ± SD, n = 2.
* Significantly lower than day 0 ($P < 0.05$). Initial content of the major terpenes in the essential oil is shown in Table 4.

the evaporation of oil during the long complexation process and the evaporation during drying, which may contribute to the loss of EO (or individual components) and consequently, affect the inclusion efficiency (Lima et al., 2016).

Results from the GC-FID analysis confirmed that the major components of the original *L. graveolens* EO were carvacrol (53.5%) for the C chemotype; thymol (70.6%) for the T chemotype and β-caryophyllene (27.6%) and α-humulene (12.5%) for the S chemotype (Fig. 5a–c). These results coincide with previously reported phytochemical variation in the EO composition of Mexican oregano (Calvo-Irabién et al., 2014).

The analysis of the major terpenes present in the essential oils from this study demonstrated different behaviors during CD-EO complexation depending on the EO composition. The concentration of carvacrol in the EO recovered from the BCD-C complex was lower than the concentration in the original Carvacrol EO (Table 4). This terpene presented a complexation ratio of 1:0.52 (Fig. 5d). This means that approximately 52% of the carvacrol present in the original EO was included in the BCD complex. In the case of the EO recovered from the GCD-C complex, the carvacrol concentration was similar to that of the original EO, with a 1:1 complexation ratio (Fig. 5e). Consequently, the combination GCD-C exhibited the maximum amount of carvacrol per gram in complexation (Table 4).

With respect to the complexes formed with the Thymol chemotype of Mexican oregano EO, as expected, the EO extracted from both BCD-T and GCD-T complexes presented thymol as the major compound. The quantity and concentration of thymol in the GCD-T complex was higher than that of the BCD-T complex (Table 4), with complexation ratios of 1:0.41 and 1:0.31 respectively (Fig. 5d, e). Fernandes et al. (2004) and Tao et al. (2014) studied BCD inclusion complexes of pure thymol and of EOs rich in thymol (*Lippia sidoides* and *Thymus vulgaris*, respectively); they reported that the inclusion efficiency in BCD of thymol was higher as a pure compound versus the EO. The reason for this phenomenon could be related to the presence of other compounds in the EO competing for inclusion reactions with the CD cavity. Different molecules have different equilibria in solution that drive them to form inclusion complexes or remain in solution (Marreto et al., 2008; Tao et al., 2014). This could, in part, explain why the CD-T complexes did not present a 1:1 complexation ratio for thymol.

According to our results we can state that carvacrol and thymol, two monoterpenes with documented biological activity, and the major components of the Carvacrol and Thymol chemotypes in *L. graveolens* EO, are more efficiently incorporated into γ than into β-cyclodextrin.

In the EOs extracted from the complexes formed between the chemotype Sesquiterpene and cyclodextrins (BCD-S and GCD-S), β-caryophyllene and α-humulene remained as predominant components. However, both components were present in lower concentrations when compared with the original oil (Table 4). The complexation ratio for these two sesquiterpenes, in both BCD and GCD complexes, were similar (β-caryophyllene: 1:0.21 and 1:0.25; α-humulene: 1:0.20 and 1:0.25 for BCD-S and GCD-S respectively, Fig. 5d, e). In addition, the monoterpenes (p-cymene, eucalyptol, γ-terpinene, thymol and carvacrol) present in the original Sesquiterpene chemotype oil were not found in the oil extracted from the GCD-S complex. Therefore, the major components of this chemotype were better preserved in the BCD-S complexes (Table 4).

During the complexation process, the major terpenes present in the studied EOs showed different affinities for the different CD cavities. Independently of the chemotype, the sesquiterpenes had a greater affinity for CDs than the monoterpenes. β-caryophyllene, α-humulene and caryophyllene oxide showed complexation ratios 9–17 times higher when compared to the other EO components (Fig. 5d, e). These results suggest, as previously mentioned, that CDs have an intrinsic ability to form inclusion complexes with these sesquiterpenes, as has been reported for β-caryophyllene in BCD complexes (Liu et al., 2013; Quintans-Júnior et al., 2016).

The CDs can preferentially encapsulate geometrically compatible hydrophobic compounds (Hădărugă et al., 2012). Once inside the CD cavity, the guest molecule makes conformational adjustments to take maximum advantage of the van der Waals forces (Del Valle, 2004). Thus, the complexation depends mainly on the polarity and geometric configuration between the guest molecule and the cavity. The diameters of the central cavity are 0.6–0.8 nm for BCD and 0.8–1.0 nm for GCD (Marques, 2010). When considering the hydrophobic/hydrophilic balance as the logarithm of the 1-octanol/water partition coefficient (log P), the hydrophobicity values (in ascending order) of the main terpenes in the EOs in this study were: thymol (3.33) < carvacrol (3.49) < caryophyllene oxide (4.43) < β-caryophyllene (6.30) < α-humulene (6.59). These values clearly demonstrate that the sesquiterpenes are more hydrophobic than the monoterpenes, carvacrol and thymol, which could explain the greater affinity of sesquiterpenes for the apolar cavity of CDs. In addition, sesquiterpenes are bigger molecules than monoterpenes, which may allow them to have more points of interaction with the CD cavity. Consequently, during complex formation, and due to the preference of β− and γ− cyclodextrins for the sesquiterpenes β-caryophyllene, α-humulene and caryophyllene oxide, the EO extracted from the CD-EO complexes was considerably enriched in these components when compared with the corresponding original EO (Table 4).

3.7. Stability of CD-EO complexes after 14 days of storage

There were no significant differences in the carvacrol content of the BCD-C complex between day 0 and day 14. In fact, 75.6% of the initial content of carvacrol remained in the BCD-C complex after the storage period (Table 5). Likewise, the BCD-T complex showed no significant losses during the period evaluated suggesting a robust inclusion. The percentage of thymol retained after storage was 67.9%. In the case of β-caryophyllene content, the BCD-S complex decreased drastically under the storage conditions tested and only the 21.5% of the initial content β-caryophyllene remained in the complex (Table 5). We observed a significant loss in the carvacrol content of the GCD-C complex during the storage period. Specifically, the percentage of retained carvacrol in this complex was 34.8% (Table 5). The GCD-T complex withstood storage conditions better, with no differences in the content of thymol observed. After the 14 day storage period 53.3% of the initial thymol content remained in the complex (Table 5). Finally, for GCD-S complex a significant decrease in β-caryophyllene content was observed on day 14 with respect to the initial values (Table 5).

During the formation process of CD inclusion complexes, water must migrate from the interior of the CD cavity to be replaced by the guest molecule. Once the complexes have formed, the addition of water can cause the system to fail. Thus, interactions with water determine the stability of the complex (Ponce Cevallos et al., 2010). Our results have shown the relevance of selecting appropriated storage conditions for terpenes with biological activity, like carvacrol and thymol, in Mexican oregano EO encapsulated in CDs. In this sense, the storage conditions, in terms of relative humidity and temperature, are critical in determining the release of encapsulated volatile compounds in CD-EO complexes and for predicting the functional shelf life of an EO-based product.

Finally, the resulting concentration (μg terpene/g CD-EO complex) of terpenes in the CD-EO complexes is mainly determined by the affinities of the host molecules and the CD and also, by the stability of the complex over time. The bioactive molecules carvacrol, thymol and β-caryophyllene showed the highest final concentration values in the complexes: GCD-Carvacrol chemotype; GCD-Thymol chemotype and BCD-Sesquiterpene chemotype, respectively (Table 4). In brief, our study has shown that the CD-EO complexes with the highest final content of carvacrol, thymol and β-caryophyllene resulted when the EOs of their corresponding chemotypes were used. This suggests that a higher concentration of the terpene of interest in the EO facilitates a

higher final concentration once the CD-EO complex is formed. The monoterpenes, carvacrol and thymol, showed a stronger affinity in complexes with γ-cyclodextrin (especially carvacrol); while β-caryophyllene showed the strongest affinity with β-cyclodextrin. Regarding the stability of CD-EO complexes, both monoterpenes were more stable (i.e. higher% of retention) within the β-cyclodextrin complexes. While β-caryophyllene was more stable in the BCD-T and GCD-S complexes (Table 5)

4. Conclusions

Encapsulation of *L. graveolens* essential oil, with different chemical compositions, on β and γ- cyclodextrins was successfully achieved by the co-precipitation method. Evidence of inclusion complexation was demonstrated by contrasting analytical techniques (SEM, XRD, TGA and FTIR). Furthermore, the ability of both cyclodextrins to bind and retain bioactive components present in the essential oils was dependent on essential oil composition and the type of cyclodextrin.

According to the quantity and stability of the bioactive terpenes, carvacrol and thymol, the best complex combinations were formed between γ-cyclodextrin-Thymol and β-cyclodextrin-Carvacrol chemotypes. The results of this work support the application of the *L. graveolens* essential oil-cyclodextrin complexes as a delivery system with potential in the management of honeybee pests. The evaluation of the effectiveness of the microencapsulated *L. graveolens* EO-cyclodextrin complexes on honeybee pests and their residual effects are being further explored as part of an ongoing research project.

Conflicts of interest

None.

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