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# Evaluation of the properties of the essential oil citronellal nanoencapsulated by cyclodextrins

Carolina Abril- Sánchez, Adrián Matencio, Silvia Navarro-Orcajada, Francisco García-Carmona, José Manuel López-Nicolás*

*Department of Biochemistry and Molecular Biology-A, Faculty of Biology, University of Murcia, Campus de Espinardo, 30071, Murcia, Spain*

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ABSTRACT

In this work we used natural and modified cyclodextrins (β-CD and HP-β-CD) as encapsulating agents to improve citronellal properties. Using fluorimetric techniques, its aggregation behavior was studied for the first time. Its critical micellar concentration was seen to vary with the presence of cyclodextrins, which form 1:1 stoichiometry complexes with citronellal. The encapsulation constants and the scores obtained by Molecular Docking were correlated. Chromatographic (GC–MS) and sensory analysis confirmed that cyclodextrins improve the persistence of the aroma. Finally, the antimicrobial effect of citronellal against *Escherichia coli* and *Bacillus subtilis* in the presence and absence of cyclodextrins was studied. A combinatorial effect of citronellal, HP-β-cyclodextrin and Glucobay® as an antimicrobial mixture was observed. The results of this study not only demonstrated the potential of CD mixtures, but also that the growth caused by CD digestion may sometimes be greater that the antimicrobial effect of the agents used in this study.

## 1. Introduction

Antimicrobial resistance is a problem in our society. Although several authors have focused on seeking new molecules with antimicrobial activity, resistance has recently increased (Høiby et al., 2010; Holmes et al., 2016). Plants are a well-known source of bioactive compounds, one is Citronellal (3,7-dimethyl-6-octenal), a bioactive compound present in a large number of plants of the *Poaceae* family, such as *Cymbopogon nardus* and *Cymbopogon winterianus* (Lenardão et al., 2007). Among its properties, it acts as an effective insect repellent (Sakulku et al., 2009) and antimicrobial (Sánchez-García et al., 2007), and it has an intense aromatic profile. These three qualities make it a candidate for use as an ingredient in gels, vaporizers and other hygiene products.

However, its poor solubility in water makes it difficult to add to hydrophilic matrices. In an attempt to solve this problem in this work we used cyclodextrins as encapsulating agents to facilitate the water solubility of citronellal.

Cyclodextrins (CD) are cyclic oligosaccharides made up of α-(1,4) linked glucose units. The most common CDs are α, β and γ-CD, which contain six, seven and eight glucose units, respectively (Del Valle, 2004; Szente and Szejtli, 2004). CDs have a hydrophobic center, which forms inclusion complexes with a large number of molecules, making them soluble in water. Some molecules with antimicrobial activity are of a hydrophobic nature, for example families of antibiotics such as penicillins, or more common molecules (polyphenols, alcohols, etc.), whose solubility can be increased using CDs (Maffeo et al., 2006; Matencio et al., 2016; Paczkowska et al., 2016). Their "internal cavity" of a hydrophobic nature is a fundamental structural feature of cyclodextrins, which gives them the ability to form complexes with other molecules of a very diverse nature. These molecules should have a size compatible with the inner cavity of the cyclodextrin, allowing the formation of a stable "inclusion complex" (Crini, 2014; Marques, 2010), which is also highly water-soluble. Indeed, the effect of their bioactivity when biomolecules are complexed has been the subject of continuous evaluation (Del Valle, 2004; Doorne et al., 1988; Zhao et al., 2010).

As our research group has previously reported, the encapsulation of bioactive compounds such as fatty acids (Bru et al., 1995; López-Nicolás et al., 1997a), vitamins, stilbenes (Matencio et al., 2017a), etc. in cyclodextrins can modify their water solubility as well as other properties, including their sensory profile and antimicrobial characteristics.

The complexation of citronellal with β-CD is well-known. Its physicochemical characterization of citronellal with β-CD and the mosquito repellency were previously investigated (Songkro et al., 2012). The mosquito repellent effect of the β-CD complex was found to be higher than free citronellal (Pujiastuti et al., 2017). Also, the β-CD complex of citronellal showed antinociceptive effect in animal experiments

* Corresponding author.
*E-mail address:* josemln@um.es (J.M. López-Nicolás).

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exceeding that of the non-complexed terpene (Santos et al., 2016). Finally, the solubilization of citronellal by certain CD derivatives was found to be higher than with β-CD (Ajisaka et al., 2000). Nevertheless, the study with HP-β-CD is not so well documented and its critical micellar concentration (c.m.c.), has not been evaluated. HP-β-CD is generally safer than β-CD (Gould and Scott, 2005), even is an orphan drug for Niemann–Pick disease type C (Matencio et al., 2018). So, this CD would be the first option for medical products.

Furthermore, no author has suggested that the CD uptake as carbon source by bacteria could mask the real effect of any weak antimicrobial agent. Bacteria are a great source of enzymes and some of them could degrade CDs, such us amylases (Saranraj, 2013). In this paper, we also demonstrated this possibility. This is particularly important when dealing with less powerful antimicrobials, such as essential oils (Burt, 2004; Lopez-Romero et al., 2015).

For all of the above reasons, the general objective of this work was to study the process of encapsulation of citronellal in cyclodextrins for use in the development of new products.

Some specific objectives were:

1 To evaluate, for the first time, the aggregation behavior of citronellal in the presence and absence of cyclodextrins.
2 To study the stoichiometry of CD/citronellal complexes.
3 To determine the encapsulation constants of citronellal in two types of cyclodextrins: β-CD and HP-β-CD.
4 To study the possible modification of the sensorial profile of this terpene in the presence of cyclodextrins.
5 To determine the antimicrobial effect of citronellal against *E. coli* and *B. subtilis* complexed with CDs in different conditions.

## 2. Materials and methods

### 2.1. Materials

Citronellal, cyclodextrins, DHPT (1,6-Diphenyl-1,3,5-hexatriene fluorescence), LBbroth and LB Agar were purchased from Sigma-Aldrich (Madrid, Spain). *E. coli strain* BL21 and *B. subtilis* strain 168 were obtained from CECT (number 674 and 461 respectively). Glucobay® was bought in a local pharmacy. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

### 2.2. Methods

#### 2.2.1. Fluorimetric determination of critical micellar concentration (c.m.c.), stoichiometry and complexation constants $K_F$

The complexes formed by HP-β-CD and β-CD with citronellal were studied. The wavelengths used were 430 nm emission and 358 nm excitation of DPHT (López-Nicolás et al., 2006, 1995). A 1 cm quartz cuvette with optical path and a Kontron SFM-25 fluorometer (Zurich, Switzerland) were used.

The concentrations of citronellal used were 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mM + 10% ETOH. The concentrations of HP-β-CD used were 0, 0.25, 0.75, 1 and 2 mM. In the case of β-CD the concentrations were 0, 0.25, 0.5 and 1 mM. Ascending concentrations of citronellal are used to determine the c.m.c. and to be able to observe its increase with respect to the increase of CD concentration.

The buffer used was 0.1 M phosphate at pH 7. All analyses were performed at 25 °C +/- 0.2 °C.

Diphenylhexatriene (DPHT) was from Fluka (Madrid, Spain) and tetrahydrofuran was from Merck (Darmstadt, Germany) and used in cuvette at a concentration of 0.88 μm (López-Nicolás et al., 1997b). The samples were incubated for half an hour at 25 °C in the dark to achieve equilibrium and to avoid photoisomerization of the fluorescent probe.

To determine the stoichiometry of the encapsulation the following expressions were used (Junquera et al., 1992):

For a 1: 1 model (one molecule of citronellal by one of CD) the following expression was used:

$$[\text{c.m.c. }^*]\ t = [\text{c.m.c}_0] + K_F\,[\text{c.m.c}_0]\,[\text{CD}] \quad (1)$$

and for a 1: 2 model (one molecule of citronellal by two of CD):

$$[\text{c.m.c. }^*]\ t = [\text{c.m.c}_0] + K_{F12}\,[\text{c.m.c.}_0]\,[\text{CD}]^2 \quad (2)$$

where c.m.c.* is the observed and $\text{c.m.c.}_0$expression is in the absence of cyclodextrins, $K_{F1}$ is the complexation constants of the encapsulation for a 1:1 model and $K_{F12}$ is the complexation constants of the encapsulation for a 1:2 model.

Three measurements were made in each case, with an ANOVA (GraphPad Software) at a confidence level of $P < 0.05$.Regressions were made using SigmaPlot (Systat Software, version 10.0)

**Table 1**
Values of c.m.c atdifferent HPβ-CD concentrations, pH 7 and 25 °C.

| [HP-β-CD] mM | CMC mM |
|---|---|
| 0 | 1.6 |
| 0.25 | 1.9 |
| 0.75 | 2.2 |
| 1 | 2.5 |
| 2 | 3.2 |

#### 2.2.2. Molecular docking

The molecular structures used in this study were created with Avogadro software (Hanwell et al., 2012) or were obtained from databases. The structure of the β-CD was obtained from crystallized structures with the Protein data Bank (PDB) code 1Z0N. The structure of the HP-β-CD was created by adding the hydroxypropyl radicals to β-CD, while the citronellal structure was obtained using Jmol software.

The Molecular Docking study was performed with Autodock Vina program (Trott and Olson, 2010), in which the cyclodextrins were considered as rigid, the representation was made using the PyMOL application (Molecular Graphics System, version 1.3, Schrödinger, LLC) with default parameters to display hydrogen bonds (Matencio et al., 2018).

#### 2.2.3. GC–MS chromatography

To verify the volatility of citronellal (Milone et al., 2000), two samples were analyzed in the presence and absence of cyclodextrins by GC–MS, the first with a concentration of 1 mM citronellal + 10% ETOH and the second with a concentration of 1 mM + HP-β-CD 5 mM + 10% ETOH. The samples contained in vials were removed to measure the volatility of the citronellal alone and in the presence of cyclodextrins, taking measurements at different times: 0, 30, 60, 90, 120, 240 and 1440 min. Three measurements were made in each case.

The samples were analyzed by GC–MS with an Agilent CP9205 chromatograph with an MSD5977 detector and a Gerstel MPS SPME Injection autosampler, using helium as carrier gas. A Sigma-Aldrich MP5-5 ms rapid apolar column with a stationary phase efficiency of 5% diphenyl/ 95% dimethyl polysiloxane, 30 m long, 0.250 mm in diameter and 0.25 μm thick film-bound, was used, at a flow rate of 1 ml /min and a back pressure of 7.069 psi. The initial temperature was 40 °C increasing by 12 °C /min to 220 °C.

A SPME 50/30 μm DVB/Carboxen/PDMS sample taker was used. For the injector, the sample was incubated at 35 °C and 300 rpm for 3 min, with 10 min extraction and 3 min desorption.

For the MS, Acquisition Mode Scan, a solvent delay of 0 min, MS Source 230 °C, MS Quad 150 °C and a Mass Range of 45–400 were used.

#### 2.2.4. Sensory analysis

To evaluate the aromatic profile of citronellal in the presence and absence of cyclodextrins a sensory session was performed in the tasting

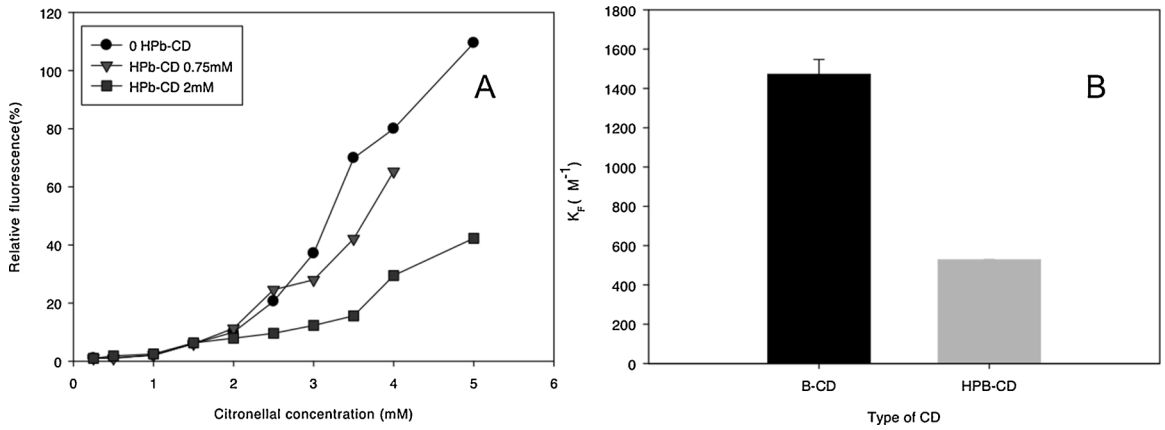


**Fig. 1.** (A) Effect of citronellal concentration on relative fluorescence at different concentrations of HP-β-CD 0 mM, 0.75 mM and 2 mM. (B) Values of the encapsulation constants $K_F$for β-CD and HP-β-CD.

**Table 2**
Values of experiemntal$K_F$ and at pH 7 and 25 °C and predicted Scores.

| CD type | $KM^{-1}$ | SD (+/-) | Score | $R^2$ |
|---|---|---|---|---|
| β-CD | 1472.27 | 75.39 | −4.1 | 0.98 |
| HP-β-CD | 525.82 | 2.85 | −3.4 | 0.99 |

room of the Food Technology Department, of the Faculty of Veterinary Medicine (University of Murcia), with individualized cabins using the published method of (Chitwood et al., 1983).

A group of 20 random individuals smelled the samples at different times (0, 30, 60, 90, 120, 720 and 1440 min) and were asked to identify the intensity of the aroma, In a professional tasting room a panel of 20 individuals smelled a solution of 1 mM citronellal in the presence and absence of 15 mM HP-β-CD, indicating the suggestion of greater or lesser intensity. The intensities ranged from 0 (no odor) to 5 (very intense). The same operation was repeated for the first five time intervals (2 h in total), and then again at 12 h and 24 h.

At a short distance, citronellal has an intense aromatic profile, which would have saturated the olfactory receptors of the panelists and resulted in the same intensity being recorded for the different concentrations. To avoid this, a sample of coffee was intercalated between the different concentrations of citronellal, both alone and with HP-β-CD.

### 2.2.5. Glucobay® preparation

Glucobay® was prepared as follows: one tablet of Glucobay® (containing 50 mg of acarbose) was crushed and dissolved in miliQ water to reach 0.75 mg/mL of acarbose (maximum solubility 1 mg/mL according to Pubchem database). The solution was filtered using a 0.22 µm diameter filter into Eppendorfs tubes and vigorously shaken. The Eppendorf tubes were stored at 4 °C in darkness.

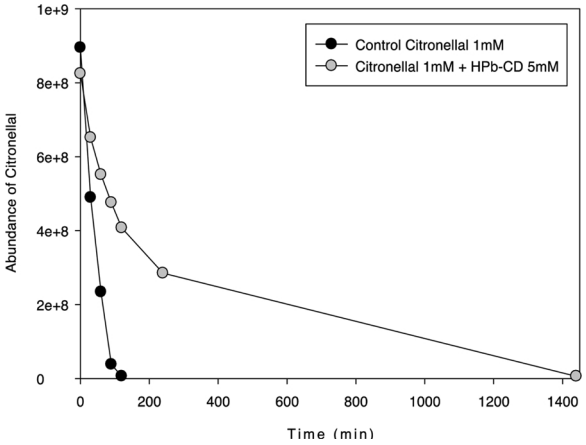


**Fig. 3.** Loss of citronellal abundance with time in the absence and presence of HP-β-CD. 5 mM.

### 2.2.6. Microbiological analysis

Different cultures of *E. coli* and *B. subtilis* at 37 and 30 °C, respectively, were incubated in the presence of different proportions of Glucobay® (v/v) and HP-β-CD. All reagents and materials were previously sterilized. Samples of each culture were taken and measured every 30 min. The growth rate was analyzed from the optical density (O.D.) at 600 nm with a UV-1700 Spectrophotometer (Shimazdu, Japan). All cultures were carried out three times.

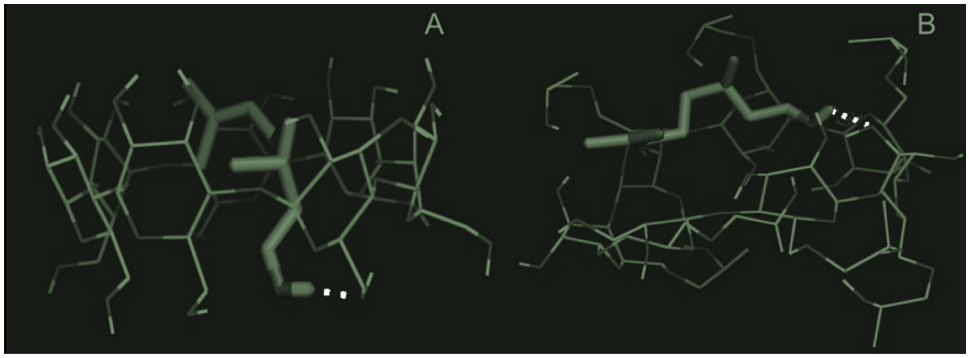


**Fig. 2.** (A) Molecular Docking Pose between β-CD and citronellal, indicated in white, the hydrogen bonds. (B) Molecular Docking Pose between HP-β-CD and citronellal; indicated in white, the hydrogen bonds.

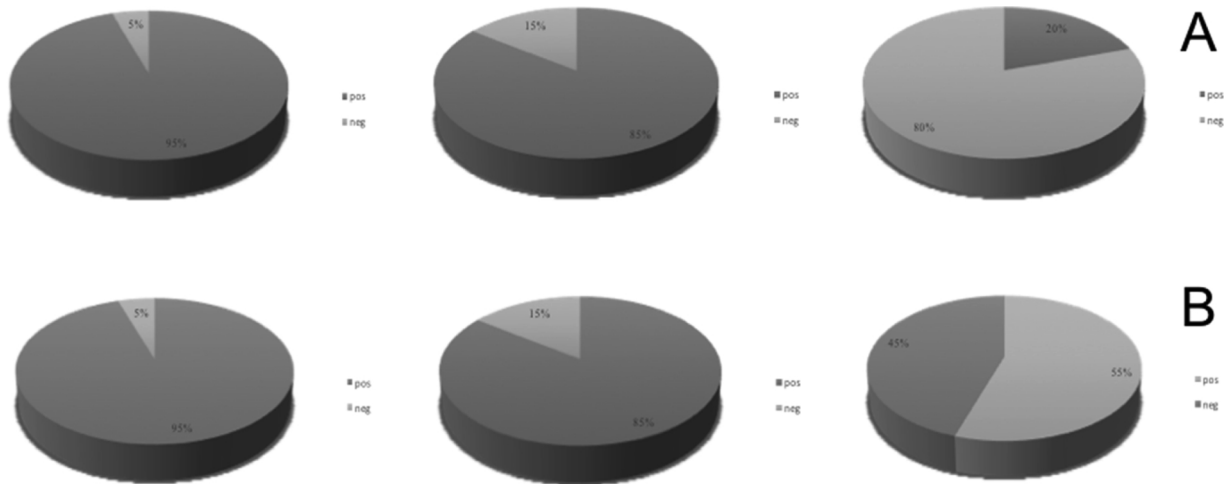


**Fig. 4.** (A) Score of the panel at 0, 1.5 and 24 h for the citronellal sample alone (black, score equal to or greater than 3 out of 5; grey, score less than 3 out of 5). (B) Score of the panel at 0, 1.5 and 24 h for the citronellal sample in the presence of HP-β-CD (black, score equal to or greater than 3 out of 5; grey, score less than 3 out of 5).

*2.2.7. Bioinformatic analysis*

The uniprot database was used to obtain the human (uniprot code P04746), *E. coli* (uniprot code P26612) and *B. subtilis* (uniprot code P00691). MAFFT (Multiple Alignment using Fast Fourier Transform) was carried out using the EBI website (version 7.215) with default parameters.

## 3. Results and discussion

### *3.1. Fluorimetric determination of critical micellar concentration (c.m.c.), stoichiometry and complexation constants $K_F$*

When certain molecules are at low concentrations they come in the form of monomers. However, at a certain concentration, the aqueous phase becomes “saturated” with monomers and colloidal-like aggregates are formed which prevent citronellal solubilization. The exact concentration of a molecule at which it suddenly passes from monomeric to aggregate form is called the critical micellar concentration (c.m.c.). The c.m.c. can be determined by means of a fluorimetric method using a fluorescent probe, diphenylhexatriene, which is incapable of emitting fluorescence in a polar environment but in an apolar environment its fluorescence increase. In other words, when the c.m.c. is exceeded, the probe is incorporated into the aggregate, which is more apolar than the surrounding medium, drastically increasing the fluorescence of the solution. (Table 1 and Fig. 1**A**)

Fig. 1A shows the c.m.c. of citronellal in the absence and presence of cyclodextrins. Without encapsulating agents in the reaction medium, the c.m.c. is low but in the presence of different concentrations of HP-β-CD the c.m.c. increases when the monomers are introduced into the internal cavity of the cyclodextrins and thus hinder the aggregation phenomenon of citronellal.

The same occurs with β-CD, which implies that the solubility of the citronellal increases in the presence of cyclodextrin. This would favor its use in the development of new products of an aqueous nature.

By adjusting the data obtained to Eqs. (1) and (2), both the stoichiometry of the reaction and the encapsulation constants of the citronellal in cyclodextrins can be calculated. For both HP-β-CD and β-CD a 1: 1 stoichiometry (Matencio et al., 2018; Songkro et al., 2012) (one molecule of citronellal per molecule of cyclodextrin) was obtained. The linear dependence of c.m.c.∗ on the β-CD and HP-β-CD concentration ($r > 0.99$) indicated that a 1:1 model is the optimum for the encapsulation of citronellal by β-CD or HP-β-CD. For β-CD the determined complexation constant was 1472.27 ± 75.39 $M^{-1}$ while HP-β-CD 525.82 ± 2.85 $M^{-1}$. These data are in accordance with Kfoury et al. (2015), this fact demonstrates that the c.m.c method could be a good alternative to another techniques. As can be seen, natural β-CD cyclodextrin encapsulated citronellal better than the modified HP-β-CD, but at high concentrations β-CD precipitated, which is why it was used for the study (Fig. 1B).

### *3.2. Molecular docking studies*

In the field of computational chemistry, Molecular Docking predicts the preferred conformation of a given molecule to bind to another, in order to form a stable complex. Knowledge of the preferred orientation is also used to predict the strength of the association using scoring functions that can be interpreted similarly to the Gibbs' free energy (Matencio et al., 2017a). (Table 2)

Table 2 shows the scores obtained for the interaction of citronellal with two types of cyclodextrins. These values reflect the non-covalent interactions of the complex and measure the spontaneity of the process, the results of which suggest that the more negative the score, the greater the encapsulation force. As expected, the encapsulation constants determined by the fluorometric method and the scores calculated by Molecular Docking are correlated since the β-CD /citronellal complex presents a higher constant value and a more negative score than HP-β-CD /citronellal (Fig. 2**A** and B).

Fig. 2A shows how the citronellal only has 2 hydrogen bonds with β-CD, whereas Fig. 2B shows that the citronellal forms 3 hydrogen bonds with the HP-β-CD. However, the larger size produced by β-CD derivatizations, as well as their possible negative interaction with citronellal, could lower the encapsulation efficiency.

By this technique the orientation and interactions between the ligand, in this case the citronellal, and the receptor (cyclodextrins) can be predicted.

### *3.3. Effect of the presence of cyclodextrins on the volatility of citronellal*

One of the main characteristics of citronellal is its strong aroma. However, the intensity of the same disappears quickly as it is a very volatile compound, which explains the search for new systems that increase the permanence in the environment of citronellal when used in vaporizers. As shown in Fig. 3, the abundance of the citronellal analyzed by gas chromatography coupled to a mass detector disappears at 90 min. However, in the presence of a 5 mM HP-β-CD concentration, citronellal can be detected up to 24 h later. (Fig. 3)

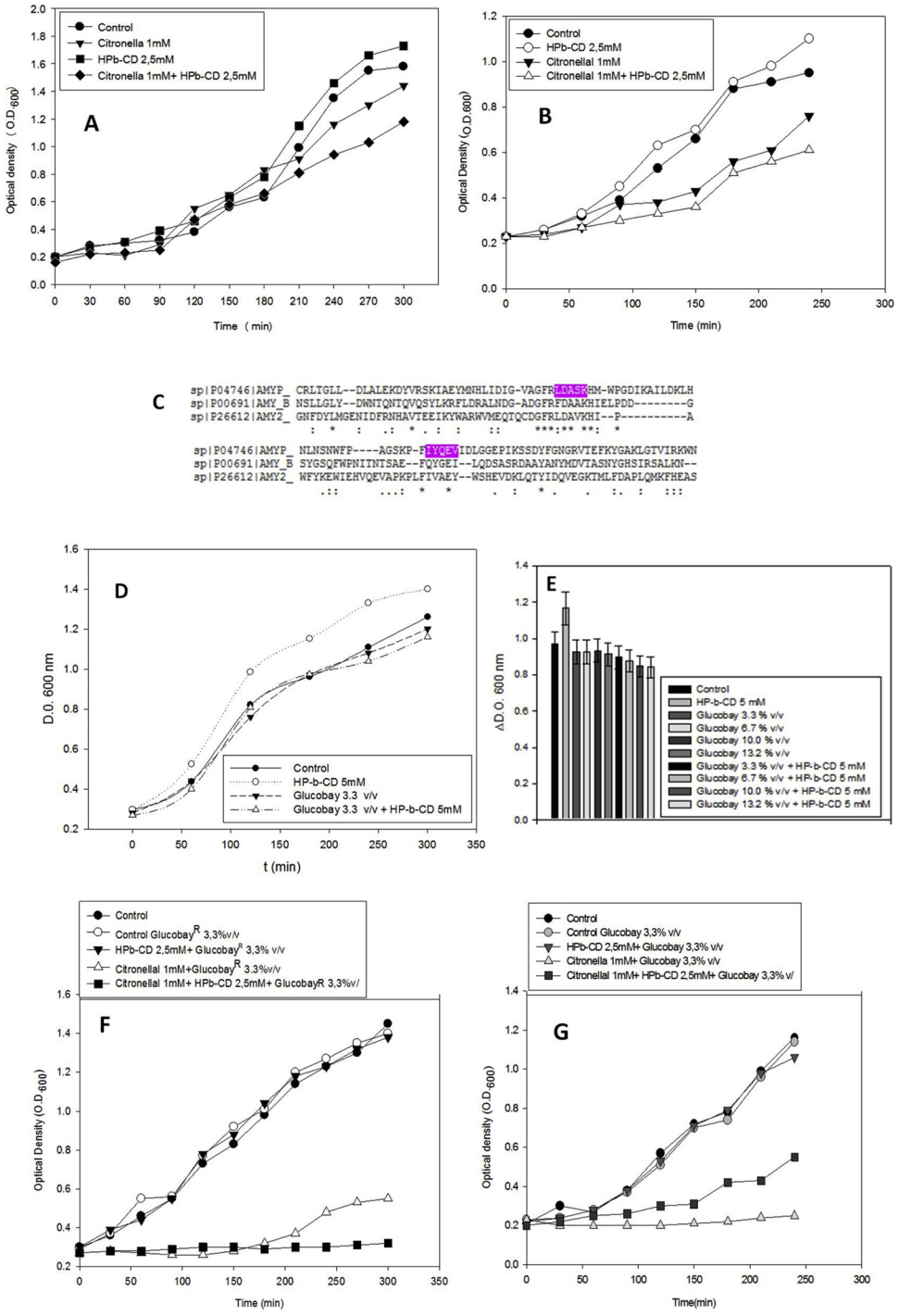


**Fig. 5.** (A) Effect of the presence of citronellal, HP-β-CD and a mix of both on *E. coli* and (B) *B. subtilis* cultures (LB medium at 37 and 30 °C, respectively). (C) MAFFT results for pancreatic human amylase (P04746), *E. coli* cytoplasmatic amylase (P26612) and *B. subtilis* amylase (P00691). The active site of the enzymes is indicated. (D) O.D. of *E. coli* at 600 nm in the presence and absence of HP-β-CD and Glucobay® (Conditions: 37 °C in LB medium). (E) Δ.D.O. of *E. coli* in presence and absence of different proportions of HP-β-CD and Glucobay® (Conditions, 37 °C in LB medium). (F) Effect of the presence of Glucobay® with citronellal, HP-β-CD and a mix of both on *E. coli* and (G) *B. subtilis* cultures (LB medium at 37 and 30 °C, respectively).

### 3.4. Sensory analysis of the cyclodextrin/citronellal complex

Because the general objective of this study is to characterize the process of encapsulation of citronellal in cyclodextrins for use in the development of new products, it is necessary to observe whether the process of encapsulation affects consumer acceptance. For this, a sensory testing was carried out as described in Materials and Methods.

The results were conclusive and correlated with those obtained for the analysis by GC–MS. Both in the presence and absence of cyclodextrins, the aroma intensity decreased with time. However, after two hours, individuals did not perceive the presence of citronellal in the sample that did not include cyclodextrin. When the encapsulating agent was present in the medium, the individuals perceived the aroma 24 h later, as seen in Fig. 4A and B.

### 3.5. Antimicrobial effect of citronellal in presence and absence of CDs

As mentioned in the introduction, citronellal has antimicrobial activity, possibly as a result of making membranes porous (Burt, 2004; Songkro et al., 2012). The next step was to analyze its antimicrobial activity in the presence and absence of CDs. The results (Fig. 5A) showed that in the presence of 2.5 mM HP-β-CD, E. coli O.D. was higher than that of the control culture. The fact that the same results were obtained for *B. subtilis* (Fig. 5B) suggests that CDs are being used as carbon source. However, it has never been suggested that this intake could affect the antimicrobial activity of citronellal. The more

pronounced effect of citronellal on *B. subtilis* growth than on *E. coli*, could be due to their different membrane compositions. Such a possibility could well be affecting the results of research groups testing antimicrobials.

The next step was to prevent CDs digestion. It was thought that *E. coli* and *B. subtilis* could be using a secondary activity of the amylase they contain to digest CDs (Suetsugu et al., 1974; Marshall and Miwa, 1981). A common inhibitor of both activities is acarbose (Kim et al., 1999). Indeed, an easily available drug called Glucobay® is formulated with acarbose as active compound to treat diabetes. Additionally, a MAFFT analysis using a human pancreatic amylase sequence, *B. subtilis* amylase and cytoplasmatic *E. coli* amylase suggested that the active site is well preserved in akk sequences (Fig. 5C). Although the coincidence was not total, it was judged sufficient to merit study. Fig. 5D and E shows the result when the inhibitory effect of different v/v proportions of Glucobay® in *E. coli* cultures was studied. As can be seen, the more Glucobay® was introduced, the greater the inhibitory effect, possibly due to an alteration in the metabolism. However, as the only aim was to block CD degradation, a 3.3% v/v was selected because this caused minimum interference in the growth behavior of the cultures.

To assess whether the mixture of Citronellal, HP-β-Cyclodextrin and Glucobay® is capable of improving the inhibition of *E. coli* and *B. subtilis* growth, cultures were made including all three. As can be seen (Fig. 5F and G), the combination of the three compounds stopped growth in both cases. With CDs present in the culture, Citronellal seems to puncture the membrane (Burt, 2004; Matencio et al., 2017b; Rodríguez-Bonilla et al., 2010). Furthermore, the presence of citronellal and Glucobay® alone also inhibited growth, perhaps because Glucobay® crosses the membrane and affects the metabolism. To sum up, whereas Glucobay® prevents the degradation of CDs, the complex Citronellal/HP-β-CD releases Citronellal slowly into the culture altering the membrane.

## 4. Conclusions

The encapsulation of citronellal in different types of cyclodextrins was characterized. Fluorometric studies determined the aggregation status of citronellal in the absence and in the presence of cyclodextrins, which were seen to increase the solubility of citronellal. Using this technique, the stoichiometry of the cyclodextrin/citronellal complexes was determined to be 1:1. In addition, the encapsulation constants were calculated. Molecular Docking studies showed the type of connections existing in the complex. GC–MS and sensory analysis confirm that encapsulation regulates the volatility of citronellal. In addition, the effect of citronellal as antimicrobial compound in the presence and absence of CDs was studied. In light of the low effect provoked used alone and the increase in *E. coli* and *B. subtilis* growth, we focused our efforts on inhibiting the action of Amylases, the enzymes considered most probably responsible of its degradation, using a solution of the drug Glucobay®.

The results showed that this drug can maintain the growth rate at the same level in the presence and absence of CDs. When the synergetic action of Citronellal, HP-β-Cyclodextrin and Glucobay® as antimicrobial mixture was studied, growth was totally inhibited.

The results of this study not only demonstrate the potential of the mixture in question (improving its retention in solution, the aroma can be made more durable when cyclodextrins form part of the composition), but also confirmed that growth caused by CD digestion may sometimes be greater than the antimicrobial effect of some agents. The above findings make increase the feasibility of including citronellal in lotions or vaporizers and establish a new opportunity to use low antimicrobial capacity agents in hydrophilic solutions.

## Conflict of interest statement

The authors declared that they have no conflicts of interest to this work.

## Acknowledgments

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