Molecular Modeling and Physicochemical Properties of Supramolecular Complexes of Limonene with α - and β -Cyclodextrins

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Abstract. This study evaluated three different methods for the formation of an inclusion complex between α - and β -cyclodextrin (α - and β -CD) and limonene (LIM) with the goal of improving the physicochemical properties of limonene. The study samples were prepared through physical mixing (PM), paste complexation (PC), and slurry complexation (SC) methods in the molar ratio of 1:1 (cyclodextrin:limonene). The complexes prepared were evaluated with thermogravimetry/derivate thermogravimetry, infrared spectroscopy, X-ray diffraction, complexation efficiency through gas chromatography/mass spectrometry analyses, molecular modeling, and nuclear magnetic resonance. The results showed that the physical mixing procedure did not produce complexation, but the paste and slurry methods produced inclusion complexes, which demonstrated interactions outside of the cavity of the CDs. However, the paste obtained with β -cyclodextrin did not demonstrate complexation in the gas chromatographic technique because, after extraction, most of the limonene was either surface-adsorbed by β -cyclodextrin or volatilized during the procedure. We conclude that paste complexation and slurry complexation are effective and economic methods to improve the physicochemical character of limonene and could have important applications in pharmacological activities in terms of an increase in solubility.

KEY WORDS: cyclodextrin; inclusion complex; limonene; monoterpene.

INTRODUCTION

Essential oils are widely used in pharmaceuticals, food products, and cosmetics as flavoring agents due to their

biological properties (1). D-limonene (4-isopropenyl-1-methylcyclohexene) (LIM), a monocyclic monoterpene is the major constituent of the essential citrus oils found in fruits such as lemons, oranges, limes, and grapefruits. Its antitumoral, antioxidant (2), antimicrobial (3), and antidiabetic (4) activities have been described in the literature (5,6). However, LIM is a hydrophobic molecule susceptible to oxidative degradation, which reduces its utilization (7). In order to overcome this limitation, cyclodextrins (CDs) have been used to form inclusion complexes with non-soluble molecules such as LIM to improve their solubility (8,9). The structure of CDs comprises hydroxyl groups orientated outwards: with the primary hydroxyl groups of the glucose residues at the narrow edge of the truncated cone and the secondary hydroxyl groups located at the wider edge, resulting in a hydrophilic outer surface exhibiting the form of a truncate. Skeletal carbons and ethereal oxygen groups, providing a lipophilic environment, optimal for entrapping non-polar drugs, structurally compose the central cavity (10,11). The natural CDs include α , β , and γ , which contain

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6, 7, and 8 glucopyranose units, respectively (12). The inclusion phenomenon is characterized by the entrapment of a lipophilic molecule in the somewhat hydrophobic CD central cavity usually via a reversible process (13). The aim of this study was to evaluate the formation of an inclusion complex between α - and β -cyclodextrin (α - and β -CD) and LIM through the physical mixing (PM), paste complexation (PC), and slurry complexation (SC) methods and its characteristics by analytic techniques of molecular modeling, thermogravimetry/derivative thermogravimetry (TG/DTG), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), gas chromatography/mass spectrometry (GC/GCMS) analyses, and nuclear magnetic resonance (NMR).

MATERIAL AND METHODS

Materials

LIM (97%, molecular weight 136.23), α -CD ($\geq 98\%$, molecular weight 972.84), and β -CD ($\geq 97\%$, molecular weight 1134.98) were purchased from Sigma-Aldrich (St. Louis, USA).

Preparation of Binary Mixture of LIM with α - and β -CD

All samples were prepared in a 1:1 molar ratio of LIM to α - or β -CD, based on the method used in other studies involving complexation of monoterpenes (14–16). After processing, the binary mixtures were stored in a glass desiccator at room temperature until being submitted for analysis.

Physical Mixture (PM)

An equimolar mixture of LIM and α - or β -CD was accurately weighed and mixed for 15 min and stored.

Paste Complex (PC)

LIM (136.23 mg) and α -CD (972.84 mg) or β -CD (1134.98 mg) were mixed for 10 min and then 2 mL of water was added until it was a paste. Then, the material was dried (2 days) in a glass desiccator at room temperature and constant weight (fully dried in those conditions) was achieved.

Slurry Complex (SC)

LIM (136.23 mg) and α -CD (972.84 mg) or β -CD (1134.98 mg) were mixed for 10 min and then water (20 mL) was added. The suspension was stirred for 36 h by a magnetic stirring device operating at 400 rpm (Quimis Q 261A21, Brazil). The material was then dried (7 days) in a glass desiccator at room temperature and constant weight (fully dried in those conditions) was achieved.

Characterization of Samples

Thermogravimetry/Derivate Thermogravimetry

Thermogravimetry/derivative thermogravimetry (TG/DTG) curves were obtained with a TGA 60 (Shimadzu) thermobalance in the temperature range of 25–900°C, performed with platinum crucibles containing approximately 3 mg of samples, under dynamic nitrogen atmosphere (50 mL min⁻¹) and heating rate of 10°C min⁻¹. The TG/DTG was calibrated with calcium oxalate monohydrate, according to the ASTM standard.

Fourier Transform Infrared (FT-IR) Spectroscopy

The infrared spectra of samples were obtained in the range of 4000–500 cm⁻¹ in KBr pellets using an IRTracer-100 (Shimadzu) Fourier Transform Infrared Spectrophotometer, at room temperature.

X-ray Diffractometry (XRD)

Powder X-ray diffractometer results were obtained on a Rigaku XRD with a tube of CuK α , in the range of 3–65 θ (2 θ) and 1 s of pass time, carried out with the XRD powder (X-ray diffraction) method.

Complexation Efficiency (CE) Using Gas Chromatography/Mass Spectrometry (GC/GCMS) Analyses

GC analyses were performed using a GC-MS/FID (QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an AOC-20i auto-injector autosampler (Shimadzu). Separations were accomplished using an Rtx®-5MS Restek fused silica capillary column (5%-diphenyl-95-dimethyl polysiloxane) of 30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness, at a constant helium (99.999%) flow rate of 1.2 mL min⁻¹. Injection volume of 0.5 μ L (5 mg mL⁻¹) was employed, with split ratio of 1:10. The oven temperature was programmed from 50°C (isothermal for 1.5 min), with an increase of 4°C min⁻¹ to 200°C, then 10°C min⁻¹ to 250°C, ending with a 5-min isothermal at 250°C.

The FID and MS data were simultaneously acquired using a detector splitting system; the split flow ratio was 4:1 (MS:FID). A 0.62 m $\times$ 0.15-mm i.d. restrictor tube (capillary column) was used to connect the splitter to the MS detector; a 0.74 m $\times$ 0.22-mm i.d. restrictor tube was used to connect the splitter to the FID detector. The MS data (total ion chromatogram, TIC) were acquired in the full scan mode (m/z of 40–350) at a scan rate of 0.3 scan s⁻¹ using the electron ionization (EI) with electron energy of 70 eV. The injector temperature was 250°C and the ion-source temperature was 250°C. The FID temperature was set to 250°C, and the gas supplies for the FID were hydrogen, air, and helium at flow rates of 30, 300, and 30 mL min⁻¹, respectively. Quantification of each constituent was estimated by normalizing the peak area generated in the FID—(%). Compound concentrations were calculated from the GC peak areas and were arranged in order of elution from the GC.

Extraction of Total LIM. Total LIM adsorbed in the α - or β -CD was determined through extraction according to the

method described by Marreto *et al.* (17). Distilled water (8 mL) plus hexane (4 mL) and 0.2 g of the sample were put in a round-bottom flask which was kept in a water bath at 85°C for 20 min, with constant shaking. The organic phase was decanted (3 times) and concentrated to approximately 1 mL. Afterwards, an internal standard (menthol, 2 mg) was added and stored until GC-MS/FID analysis.

Extraction of Surface-Adsorbed LIM. The amount of LIM adsorbed on the surface of α - or β -CD was determined through washing. A sample (3 g) with hexane (20 mL) was shaken for 20 min. The suspension was filtered and the residue was washed with hexane (10 mL). After that, hexane (1 mL) and an internal standard (menthol, 2 mg) were added to the filtrate, which was concentrated and analyzed by means of GC-MS/FID. Thus, the difference between the total LIM (surface-adsorbed LIM and hosted in the cavity) and the surface-adsorbed LIM corresponds to the amount complexed in the α - or β -CD cavity.

Molecular Modeling

The chemical structures of the compounds (LIM and α - and β -CD) were drawn using Marvin 14.9.8.0, 2014, ChemAxon (<http://www.chemaxon.com>). The software Standardizer, JChem 14.9.8.0, 2014, ChemAxon (<http://www.chemaxon.com>) was used to canonize structure, add hydrogens, clean the molecular graph in three dimensions, and finally save it in.sdf format. The process uses a divide-and-conquer approach. The structure is split into small fragments and organized into a building tree using its original connectivity information. Conformers generated for the initial structure (represented by the root node in the building tree) are also optimized. The building process uses a proprietary extended version of the Dreiding force field (18). All geometry optimization and conformational search were performed using Spartan for Windows 10.0 software (19). The geometry of the chemical structure of the compound was initially optimized using MMFF force field (20) and subsequently by performing a new geometry optimization based on the semi-empirical method AM1 (Austin Model 1) (21). We selected the systematic search method, analyzing up to 1000 conformers and selecting up to 10 conformers of the lowest minimum energy using AM1 and Monte-Carlo algorithm. The dihedrals were evaluated by means of rotation in accordance with the standard (default) conditions of the program. The conformers of the lowest minimum energy were selected, and then we performed geometry optimization and vibrational mode calculation using AM1 (21). The docking simulations were performed using the AutoDock 4.2 software. Receptor and ligand preparation was carried out using and VEGA ZZ 3.0.3 (22). Ligand and receptor structures were saved in PDBQT format to be further used for docking calculations. PyRx 0.9 software (23) (X10) was used to aid the steps of job submission and analysis of the results. A three-dimensional grid box with 50, 44, and 50 points for x, y, and z, with a spacing of 0.375 Å, was created. All other parameters were left at the AutoDock 4.2 default settings (22). The results for each calculation were analyzed

to obtain the affinity energy (Kcal/mol) values for each ligand conformation in its respective complex and the probable structure inaccuracies were ignored in the calculations. In order to verify the number of hydrogen bonds, hydrophobic interactions, and docking interpretation, the program Discovery Studio 4.0 was employed.

Nuclear Magnetic Resonance (NMR)

^1H spectra and H–H spatial dipolar correlation 2D NMR were recorded for LIM, α -CD, β -CD, inclusion complexes (prepared through the SC and PC methods), and physical mixtures (PM) dissolved in DMSO- d_6 (0.02 M) using a Bruker AVANCE 400 NMR spectrometer. The spectra have been acquired at 298 K in 5-mm o.b. tubes. Two-dimensional rotating-frame Overhauser effect spectroscopy 2D (ROESY) experiments were performed using the standard pulse sequence found in the Bruker pulse program library, applying a set of mixing times of 500, 300, or 150 ms under spin lock condition. During the acquisition, 256 increments were collected with 32 repetitions and the data matrix measured was processed as a matrix of 2k (F2) by 1k (F1) data points. ^1H chemical shifts were referenced to DMSO (2.50 ppm).

RESULTS

Thermogravimetry/Derivate Thermogravimetry Analysis (TG/DTG)

The TG/DTG curve (Table I and Fig. 1) for LIM showed weight loss of 100% in the range of 30–169°C. The α -CD curve indicated two steps of weight loss after decomposition and elimination of carbonaceous material. The first and second steps in the range of 25–120°C demonstrated weight loss of 10.7%, which corresponds to the elimination of water of α -CD. The third step ($\Delta m = 75.6\%/270\text{--}350^\circ\text{C}$) refers to decomposition followed by carbonization.

According to Fig. 1, the curve of PM was similar to that of α -CD while in the range of 120–278°C, PC, and SC/ α -CD showed higher values of weight loss, indicating a stronger interaction of LIM with CDs by these methods. In addition, between 120 and 278°C, weight loss of oil complexed in CDs occurred, which happens because the CDs are thermally stable in this range and the complexed oil needs a higher temperature to volatilize when compared to PM/ α -CD (Table I). Thus, the values of the second step indicate the efficiency of complexation, once there is no LIM free in this step as noted by Barbosa *et al.* (24). As shown in Table I, the PC/ α -CD and SC/ β -CD methods suggested larger percentages of complexation.

Fourier Transform Infrared (FT-IR) Spectroscopy

The FTIR spectra of pure compounds and their binary systems (1:1 molar ratio) are shown in Fig. 2. The FTIR spectrum of α -CD showed characteristic absorption bands at 3389 and 1637 cm^{-1} corresponding to the –OH stretching vibration. The PM and SC/ α -CD showed spectra similar to that of the α -CD. At 1027 cm^{-1} , the spectrum of β -CD showed the C–O–C stretching band in the range $\sim 1027\text{ cm}^{-1}$. Similar study was developed by Vargacini *et al.* (2015) that

Table I. Weight Loss (%) of LIM, α -CD, β -CD, PM, PC, and SC Obtained with TG/DTG Techniques

Weight loss/%				
	1st step	2nd step	3rd step	4th step
LIM	100 ^a	—	—	—
α -CD	10.7 $\pm$ 1.4 ^b	0.8 $\pm$ 0.1 ^d	75.6 ^e	11.9 ^f
PM	10.1 $\pm$ 1.5 ^c	1.8 $\pm$ 0.6 ^d	72.6 ^e	12.8 ^f
PC	11.7 $\pm$ 0.1 ^c	2.5 $\pm$ 0.5 ^d	72.7 ^e	9.9 ^f
SC	10.6 $\pm$ 0.5 ^c	2.3 $\pm$ 0.5 ^d	74.7 ^e	10.7 ^f
β -CD	11.7 $\pm$ 0.4 ^b	0.3 $\pm$ 0.3 ^d	37.8 ^e	50.5 ^f
PM	12.5 $\pm$ 1.3 ^c	1.4 $\pm$ 0.6 ^d	70.9 ^e	16.9 ^f
PC	10.5 $\pm$ 2.3 ^c	8.0 $\pm$ 1.3 ^d	70.6 ^e	15.4 ^f
SC	9.6 $\pm$ 1.4 ^c	8.3 $\pm$ 1.1 ^d	72.8 ^e	12.4 ^f

LIM limonene, α -CD alpha-cyclodextrin, β -CD beta-cyclodextrin, PM physical mixture, PC paste complex, SC slurry complex

^a Percentage of LIM evaporated to 149°C

^b Percentage of water released up to 120°C

^c Weight loss related to the release of LIM and eliminated water up to 120°C

^d Weight loss probably related to the release of LIM complexed in the range of 120–270°C

^e Thermal decomposition between 270 and 350°C

^f Formation of elementary carbon of samples in the range of 350–900°C

Fig. 1. TG curves of LIM, α -CD (a), β -CD (b), PM, PC, and SC obtained in atmosphere of nitrogen

described C–O–C vibrations with peak at $\sim 1109\text{ cm}^{-1}$ in inclusion complex involving 4,4'-bipyridinium-bis(siloxane) and β -CD. This value peak is attributed to β -CD such as the present study (25). The PC and SC/ β -CD showed intense bands of LIM's alkene group at 937 cm^{-1} suggesting complexation by these methods. Thus, the PM did not form an inclusion complex with no CDs. These results indicated complexation by PC/ α -CD, PC/ β -CD, and SC/ β -CD.

X-ray Powder Diffraction Analysis (XRD)

Figure 3 represents the XRD patterns of α -, β -, PM, PC, and SC. The diffractograms showed the crystallinity of the CDs and similar results for PMs that show low efficiency of complexation. When we observed the PCs, new peaks and a

Fig. 2. FTIR spectra of α -CD (a), β -CD (b), LIM/ α -CD, and LIM/ β -CD samples obtained through physical mixture (PM), paste complex (PC), and slurry complex (SC) methods

Fig. 3. X-ray powder diffractograms of LIM, α -CD (a), β -CD (b), LIM/ α -CD, and LIM/ β -CD (1:1 molar) systems prepared from physical mixture (PM), paste complex (PC), and slurry complex (SC) methods

disappearance of the characteristic diffraction lines of CDs could be seen at 6.5θ , 7.5θ , 12.5θ (PC/ α -CD), 6.0θ , and 9.5θ (PC/ β -CD and SC/ β -CD). The PC and SC methods with β -CD showed the new peaks in relation to β -CD-free state.

Complexation Efficiency (CE) by GC/MS

The formation of inclusion complexes for each complexation method was investigated through GC/MS analysis (Table II). Thus, the subtraction of the total LIM from the surface-adsorbed LIM corresponds to the amount complexed in the CD cavities. In some cases, a higher percentage of total limonene in relation to surface limonene was observed; this occurred because the limonene not complexed was retained on the surface and volatilized on heating as described in methodology. Haloci *et al.* (2014) noted similar findings, where the *Satureja montana* essential oil loss could be attributed to the oil, β -CD, and complex dissipation. The authors reported that are several factors, which may contribute to the loss oil, retention of the oil in the solution after

forming the complex, equilibrium of flavors between the liquid and the complexed state, evaporation of surface oil during the long complexation process, and evaporation during the drying step. The loss of the β -CD and complex powder is mainly assigned to their solubility in water (26). The PC and SC with α -CD such as the SC/ β -CD methods entrapped LIM effectively, suggesting that a small molecular weight compound such as LIM was suitable for the inclusion into CDs. On the other hand, trace amounts of oil surface-adsorbed for the SC/ α -CD were observed, indicating a good interaction ratio for this method. As can be seen in the Table II, the PM procedure showed no significant results.

Molecular Modeling and Nuclear Magnetic Resonance (NMR)

1D and 2D ^1H NMR analyses were used to study inclusion complex formation in solution phase. The differences in the hydrogen chemical shift between LIM, β -CD, and α -CD in the free and complexed states (SC and PC methods) suggest a complexation process by non-covalent interactions and are presented in Tables III and IV. In order to obtain further information on the complex formation, 2D ROESY experiments were carried out. Figure 4 shows the evidence of spatial proximities between aliphatic protons of LIM and protons outside the cavity of β -CD. Intermolecular cross-peaks were observed between protons of LIM (H-4, H-5, H-6b, and H-10) with the H-2 proton of β -CD. The same intermolecular cross-peaks described were observed with LIM- β -CD complexes through the SC and PC methods. For LIM- α -CD complexes (SC and PC methods), intermolecular cross-peaks were observed between the H-4 proton of α -CD and protons of LIM (H-1, H-4, H-5, H-7, and H-10) (Fig. 5). The 2D ROESY results suggest binding outside of the CDs cavity, and no inclusion complexes were formed between LIM and β -CD and α -CD. No intermolecular cross-peaks were observed in the physical mixture for both CDs studied.

The theoretical results obtained through molecular modeling corroborate with the NMR results, indicating that LIM was not totally complexed inside the cavity of CDs. The green circle in the Fig. 6 shows the space in the cavity of CD after interaction with the LIM. This space was observed with the 10 conformers generated by the software.

Furthermore, Fig. 7 shows the spatial proximity of aliphatic protons of LIM and protons outside the cavity of β -CD. The figure shows the proximity of the hydrogen 7 of

Table II. Complexation Efficiency Values of the Physical Mixture (PM), Paste Complex (PC), and Slurry Complex (SC) and α - and β -CD/Limonene Inclusion Complexes

Sample	Extracts (%)		Conc. % difference of extracts from original limonene	
	Surface limonene	Total limonene	Compl. limonene/%	Compl. ratio
PM/ α -CD	0.86	0.20	-0.66	None
PC/ α -CD	25.46	37.65	12.19	1:0.49
SC/ α -CD	0.44	8.41	7.97	1:18, 11
PM/ β -CD	0.90	0.64	-0.26	None
PC/ β -CD	9.19	1.01	-8.18	None
SC/ β -CD	6.51	15.91	9.4	1:1.44

α -CD alpha-cyclodextrin, β -CD beta-cyclodextrin

Table III. Proton Chemical Shift Data (ppm) of Limonene and β -CD in the Free and in the Complex State (SC and PC) in DMSO- d_6 Solution

	δ Free	δ SC	δ PC	$\Delta\delta$ SC	$\Delta\delta$ PC

Limonene					
H-3	5.3705	5.3709	5.3698	0.0004	-0.0007
H-9	4.6853	4.6828	4.6814	-0.0025	-0.0039
H-4 + H-5	2.0135	2.0175	2.0176	0.0040	-0.0041
H-7	1.9601	1.8953	1.8944	-0.0648	-0.0657
H-6a	1.7299	1.7267	1.7207	-0.0032	-0.0092
H-10	1.6909	1.6882	1.6866	-0.0027	-0.0043
H-1	1.6079	1.6062	1.6047	-0.0017	0.0032
H-6b	1.3853	1.3804	1.3784	-0.0049	-0.0069
β -CD					
OH-2	5.7525	5.7523	5.7555	-0.0002	0.0030
OH-3	5.6934	5.6926	5.6948	-0.0008	0.0014
H-1	4.8210	4.8211	4.8211	0.0001	0.0001
OH-6	4.4867	4.4800	4.4906	-0.0067	0.0039
H-3	3.6226	3.6269	3.6233	-0.0043	0.0007
H-5	3.5500	3.5511	3.5503	-0.0011	0.0003
H-2	3.3440	3.3456	3.3434	0.0016	-0.0006
H-4	3.2942	3.2943	3.2944	0.0001	0.0002

β -CD beta-cyclodextrin, PC paste complex, SC slurry complex

limonene (white) and H2 β -CD (green) (1.344 Å), also identified in NMR analysis.

DISCUSSION

CDs are well known in the pharmaceutical field and many studies have shown that they can form inclusion complexes with various molecules, not only in aqueous solution but also in the solid state (9,27). Recently, many supramolecular assemblies based on CDs have attracted great attention since the target guest molecules for CDs are expanding to include low molecular weight compounds such as essential oils and terpenes (9,28).

According to a review study by Mura, the assessment of the formation of drug-CD inclusion complexes and their full characterization is not a simple task and often requires the use of different analytical methods, whose results have to be combined and examined together, since each method explores a particular feature of the inclusion complex. The concomitant use of different techniques can allow a better and more in-depth understanding of host-guest interactions and help in selection of the most appropriate CD to receive a guest molecule (29).

In the present study, we observed that the results in the range of 30–169°C shown in the LIM curve using the TG technique correspond to the volatilization of LIM in the study of Lévic *et al.* (5). Furthermore, Giordano *et al.* reported that a water loss of α -CD may occur in a single step expansion or in two distinct expansion steps that involve water loss and

Table IV. Proton Chemical Shift Data (ppm) of Limonene and α -CD in the Free and in the Complex State (SC and PC) in DMSO- d_6 Solution

	δ Free	δ SC	δ PC	$\Delta\delta$ SC	$\Delta\delta$ PC

Limonene					
H-3	5.3705	5.3714	5.3622	0.0009	-0.0083
H-9	4.6853	4.6834	4.6772	-0.0019	-0.0081
H-4 + H-5	2.0135	2.0186	2.0142	0.0051	0.0007
H-7	1.9601	1.9148	1.8920	-0.0453	-0.0681
H-6a	1.7299	1.7176	1.7199	-0.0123	-0.0100
H-10	1.6909	1.6878	1.6814	-0.0031	-0.0095
H-1	1.6079	1.6056	1.5988	-0.0023	-0.0091
H-6b	1.3853	1.3894	1.3755	-0.0041	-0.0098
α -CD					
OH-2	5.5412	5.5322	5.5403	-0.0090	-0.0009
OH-3	5.4518	5.4473	5.4553	-0.0045	0.0035
H-1	4.5191	4.5118	4.5358	-0.0073	0.0167
OH-6	3.7652	3.7635	3.7560	-0.0017	-0.0092
H-3	3.6392	3.6315	3.6262	-0.0077	-0.0130
H-5	3.5744	3.5732	3.5670	-0.0012	-0.0074
H-2	3.4000	3.3803	3.3742	-0.0197	-0.0009
H-4	3.2700	3.2703	3.2691	0.0012	-0.0258

α -CD alpha-cyclodextrin, PC paste complex, SC slurry complex

structural rearrangements (30). Our results significantly comply with these findings.

The complexation evidence occurred because in the first step (25–120°C) of the TG curves, water and oil adsorbed onto the surface of the CD were eliminated, as has been described by other studies (16,27). So, the content measured in the second step was attributed to the quantity of complexed LIM. The TG technique does not specify the difference between oil and water loss individually because this occurred in a similar temperature range.

Fig. 4. Expansion of a 2D ROESY of limonene- β -CD complex (SC method) in DMSO- d_6

Fig. 5. Expansion of a 2D ROESY of limonene- α -CD complex (PC method) in DMSO- d_6

The FTIR analysis showed the specific signals related to the functional groups of molecules observed in PC/ α -CD and SC/ β -CD mentioned in results section. In the PC/ α -CD spectrum, two large bands were found at 2955–2896 and 1682–1611 cm^{-1} similar to the intense absorption band at 947 cm^{-1} attributed to the alkene group of LIM. The spectrum of β -CD showed absorption bands at 1647 and 3341 cm^{-1} corresponding to the free –OH stretching vibration as described by Dandawate *et al.* (31). The results are in good coincidence with the complex structure determined by PC/ α -CD, PC/ β -CD, and SC/ β -CD methods. There is a lot of evidence in the literature that this technique characterizes inclusion complexes and identifies the presence or absence of the guest in the host such as been described by some authors (25,32).

Furthermore, several articles have described the XRD technique as a useful qualitative method for confirming the formation of inclusion complexes from the analysis of the new solid phase or amorphization of CDs when complexed (33,34). As other techniques have also evidenced, our results demonstrated that the PC and SC methods exhibited better profiles, with new crystalline phase formation, than the PM

Fig. 6. Ten possible interactions of LIM and CDs obtained through molecular modeling. The green space represents the CD cavity

Fig. 7. Theoretical spatial proximities between aliphatic protons of LIM and protons outside the cavity of CDs obtained through molecular modeling in a vacuum

method. Similar results were observed by Abarca *et al.* in a study of complexation 2-nonanone, an essential oil, into β -CD by the co-precipitation method (35). These authors associated the new peaks with changes in the molecular organization of the CD's from cage-type packaging to channel-type packing, which is strong evidence of inclusion complex formation. However, in the PM methods, the diffraction pattern showed the same characteristic as the crystalline β -CD patterns, demonstrating no inclusion.

Additionally, the SC method had a major complexation efficiency (5.52%—SC/ α -CD and 69.41%—SC/ β -CD). However, it did not form a 1:1 inclusion complex, indicating partial complexation with both CDs. This was explained in our previous study, which demonstrated that larger quantities of water, steric factors, chemical structure, and the physical properties of the guest molecule can be influenced in this phenomenon (36). Furthermore, the type of method selected to obtain the samples may be crucial in the complexation efficiency percentage. Hill *et al.* reported a complexation efficiency of around 90% for the complex β -CD:eugenol with a 1:1 ratio, by freeze-drying at low temperatures (37). Additionally, Kamimura *et al.* compared the complexation efficiency of inclusion complexes with a molar ratio of 1:1, based on a complex of carvacrol and hydroxypropyl- β -cyclodextrin, by two different methods: kneading (mixing in mortar) and lyophilization. The results showed higher complexation efficiency for complexes synthesized by lyophilization (83.7%) than for inclusion complexes prepared by the kneading method (78.1%) (38). In this context, other studies with terpenes have assigned the loss of oil to volatilization during the complexation process and drying step (17,35,39).

The 1D and 2D ^1H NMR analyses were used to study the inclusion complex formation in the solution phase. The signals of H-3 and H-5 protons on the inner surface of the CD's changed only slightly, which proves the non-formation of an inclusion complex in the 1:1 molar ratio. The NOE (Nuclear Overhauser Effect) can provide information on internuclear distances and arise between nuclei that share a mutual dipolar coupling (through space interaction) and thus relax each other via the dipole–dipole relaxation mechanism. When performing NOE experiments, one can face a great problem associated with the “zero-crossing” region in the

same conditions, where it will produce a very small NOE signal. To overcome this situation, one option is to perform the NOE measurement in a rotating frame (ROE)—the 2D ROESY experiment. This experiment produces NOE signals, which are always positive. The 2D ROESY results suggest binding outside of the CDs' cavity. The GC results corroborate this effect, showing low complexation efficiency. No intermolecular cross-peaks were observed in the physical mixture for both CDs studied.

The *in silico* data significantly associate with all instrumental results. In fact, the physicochemical characterization of LIM-CDs in the PC and SC methods is improved when compared to the LIM-free or PM method. Furthermore, the theoretical data showed ten possible interactions of LIM with CDs, but none is indicated as showing total complexation in the above-mentioned results. There are just a few differences between α -CD-limonene and β -CD-limonene complexes. The complex formed with α -CD is more stable, with a binding energy of -4.54 kcal. In the complex formed with β -CD, the LIM seems slightly less stable, with -4.05 kcal. The larger cavity β -cyclodextrin complexation allows greater efficiency than α -cyclodextrin; results mainly observed in the second step TG weight loss. However, this is a less intense interaction as observed by docking.

CONCLUSION

This study aimed to elucidate the physicochemical characteristics and interactions between LIM in α - and β -CDs. It concluded that total inclusion complexes were not formed, but interactions outside of the cavity of CDs were observed through the PC and SC methods by the *in silico* data. Moreover, these results corroborated other analyses in this study, showing the improvement in the physicochemical characterization of samples when compared with limonene-free and a satisfactory complexation efficiency showing partial complexation.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declared that they have no conflict of interest.

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