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Original Article

Development and validation of a quantification method for α-humulene and trans-caryophyllene in *Cordia verbenacea* by high performance liquid chromatography

Márcio Vinícius da Silva Gomes [a], J.D. da Silva [a], Ana Ferreira Ribeiro [a,b], Lúcio M. Cabral [a], V.P. de Sousa [a,*]

[a] *Departamento de Fármacos de Medicamentos, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[b] *Faculdade de Farmácia, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

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ABSTRACT

Acheflan® herbal medicine is the first medicine developed and produced in Brazil using *Cordia verbenacea* DC., Boraginaceae, essential oil as a constituent. *C. verbenacea* has anti-inflammatory properties, which have been directly related to α-humulene and *trans*-caryophyllene. Currently, the quantification of α-humulene and *trans*-caryophyllene in *C. verbenacea* has only been described by GC-MS. Although this technique is widely used it cannot be directly applied to finished medicinal products since they contain aqueous constituents. In this context, the objective of this work was to develop a methodology for quantifying *C. verbenacea* in raw materials and in pharmaceutical formulations using liquid chromatography. The Box–Behnken experimental design was used successfully to optimize the analytical method. The method developed in this study was validated and proven to be effective in the proper separation of α-humulene and *trans*-caryophyllene. In addition, the developed method was applied to commercial formulations (cream and aerosol) containing *C. verbenacea* essential oil, which resulted in very satisfactory separation without interference from excipients. *C. verbenacea* oil contains four times higher concentrations of *trans*-caryophyllene than α-humulene. Therefore, we propose that *trans*-caryophyllene can be used as a marker of *C. verbenacea* essential oil. Evaluation of *trans*-caryophyllene content would be especially valuable for applications where the concentrations are very low such as in permeation and release studies of dermatological formulations.

Introduction

Cordia verbenacea DC., Boraginaceae, botanical synonyms of *Varronia curissavica* Jacq. and *Cordia curassavica* (Jacq.) Roem. & Schult., is a perennial plant commonly found throughout the Brazilian coast (Miller and Gottschling, 2007; Figueira et al., 2010; Falkenberg, 2011). Folk medicine describes the anti-inflammatory properties of the *C. verbenacea* extract when administered through either oral or topical routes, which was confirmed by several research studies (Sertié et al., 1991, 2005; Passos et al., 2007; Rogerio et al., 2009; Perini et al., 2015). This observed topical anti-inflammatory effect is directly related to the presence of two sesquiterpenes, α-humulene and *trans*-caryophyllene in the oil (Passos et al., 2007; Fernandes et al., 2007; Medeiros et al., 2007; Rogerio et al., 2009).

The α-humulene (HUM) is a sesquiterpene with known anti-inflammatory activity that acts on the pro-inflammatory cytokine TNF-α IL-1β (Fernandes et al., 2007) and inhibits NF-κB (Medeiros et al., 2007). On the other hand, the *trans*-caryophyllene (CAR) has been shown to have anti-inflammatory activity through its agonist action on the receptor CB2 (Klauke et al., 2014; Cheng et al., 2014; Dutra et al., 2016) and inflammatory mediators such as TNF-α (Fernandes et al., 2007). The two terpenes synergistically reduce the production of PGE_2, as well as the expression of COX-2 and inducible NOS (Fernandes et al., 2007).

In 2004, Anvisa approved Acheflan® an herbal medicine, which was the first medicine developed and produced in Brazil containing the essential oil of *C. verbenacea* (Calixto, 2005). It is formulated as a cream and aerosol, containing 5 mg of oil/g of formulation and standardized in 2.3 and 2.9% α-humulene, however it is not

* Corresponding author.
E-mail: valeria@pharma.ufrj.br (V. de Sousa).

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standardized for *trans*-caryophyllene, the other active oil. The absorption of α-humulene has been supported by an independent study for Chaves and colleagues (Chaves et al., 2008).

Currently, the quantification of HUM and CAR in the essential oil of *C. verbenacea* is only performed by GC-MS (Carvalho et al., 2004; Quispe-Condori et al., 2008; Michielin et al., 2009; Rodrigues et al., 2012). Quantification of HUM and CAR by GC-MS (Carvalho et al., 2004), after isolation by hydrodistillation, indicated that the relative amounts of CAR and HUM, quantified by total ion chromatogram raw data, are 25.3% and 4.6%, respectively. Similar concentrations were reported by Rodrigues et al. (2012), confirming that using hydrodistillation to extract essential oils is highly reproducible.

Despite the fact, that the GC-MS technique is widely described in the literature; it cannot be used to analyze finished medicinal products since they contain aqueous constituents. In this context, the objective of this work was to develop a methodology to quantify *C. verbenacea* raw material and *C. verbenacea*-containing pharmaceutical formulations using liquid chromatography.

Experimental methods

Drug and reagents

The essential oil of *Cordia verbenacea* DC., Boraginaceae, was generously donated by Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA) – Unicamp (São Paulo, Brazil). Fresh leaves and stems of *C. verbenacea* were collected from CPQBA and the species was identified by Kátia Kalago. A voucher specimen (UEC 112744) is deposited at the Biological Institute of Unicamp. The commercial products Acheflan® cream and aerosol were purchased from pharmacies in Rio de Janeiro (Brazil). Acetone (HPLC grade) and phosphoric acid were obtained from Vetec química (Rio de Janeiro, Brazil), acetonitrile (ACN) and methanol (MeOH), both HPLC grade, were obtained from Tedia Brazil (Rio de Janeiro, Brazil). The triethylamine, HUM standard (>98.5%; batch BCBD0607V), CAR standard (>98.5%; batch 1321406V), hydroxypropyl-β-cyclodextrin (HP–β–CD) were purchased from Sigma Aldrich (São Paulo, Brazil), while α-cyclodextrin (α-CD) and β-cyclodextrin (β-CD) were obtained from Wacker Química do Brasil (São Paulo, Brazil).

Apparatus

The analytical quantification of the *C. verbenacea* essential oil was performed using high- performance liquid chromatography (HPLC) from Merck-Hitachi, composed of a pump model L-2130, auto injector model L-2200, oven compartment for column model L-2300, diode array detector (DAD) model L-2455 and software EZChrom Elite. Seven chromatographic columns were tested during the development of the analytical method: Agilent Zorbax CN (250 mm × 4.6 mm; 5 μm), Maxsil RP2 65A (250 mm × 4.6 mm; 5 μm), PerkinElmer® Brownlee Choice PFP Propyl (150 mm × 2.1 mm; 5 μm), Kromasil® Kromasil 300 $5C_4$ (250 mm × 4.6 mm; 5 μm), Gemini C_6-Phenyl 110 Angstroms (150 mm × 4.6 mm; 5 μm), Kromasil® Kromasil 100 $5C_8$ (150 mm × 4.6 mm; 5 μm) and μBondapak C_{18} Waters (300 mm × 3.9 mm; 10 μm).

Chromatographic procedure

Preparation of solutions

The HUM and CAR standard solutions were prepared by precisely weighing 25 mg standard in a 25 ml test flask, then solubilizing them in a mixture of 20% acetone and 80% acetonitrile. The flasks were sonicated until the standards were completely solubilized. The required dilutions were made from these standard stock solutions.

The sample solution of the *C. verbenacea* essential oil was performed by diluting 40 mg oil in a flask containing a 20 ml mixture of 20% acetone and 80% acetonitrile. Then 2.5 ml of the solution was transferred to a 10 ml flask, resulting in a 500 μg/ml solution, which was used as the working solution. Two commercial dosage forms cream and aerosol were evaluated, containing 5 mg of *C. verbenacea* essential oil for each gram of product. Both were diluted in approximately 8 ml of acetone: acetonitrile (1:5) mixture, then quickly closed with parafilm, and sonicated for 15 min. Subsequently, the commercial dosage forms were transferred separately into a 10 ml flask using a 0.45 μm filter syringe, and quiesced with the same solvent mixture.

Experimental design

Different columns were tested during the early steps of method development, along with variations in some of the chromatographic conditions (mobile phase composition, flow rate, column temperature), to determine the factors to be studied during the experimental design. Based on the results obtained in the preliminary experiments, a Box–Behnken (BB) design followed by response surface analysis was used to evaluate the influence of four different factors in six responses and to find the best condition for use in the separation procedure. During these preliminary tests, a four factor three level BB matrix, with six replicates at the center point, was generated, resulting in thirty experimental runs. The factors that were studied were flow rate of the mobile phase (X_1), column temperature (X_2), the proportion of methanol in the mobile phase (X_3) and the concentration of HP-β-CD in the aqueous solution (X_4). The evaluated responses were the resolution between CAR and the adjacent component immediately prior to the peak ($R_{CMP1\text{-}CAR}$) (Y_1), the resolution between CAR and the adjacent component immediately after the peak ($R_{CAR\text{-}CMP2}$) (Y_2) and CAR retention time (RT_{CAR}) (Y_3). The same principles were considered for the HUM peak, resulting in the responses Y_4, Y_5 and Y6 (Table 3).

All the preliminary studies and BB design experiments were performed using the *C. verbenacea* oil at a concentration of 500 μg/ml. The chromatograms were obtained at 210 nm.

Validation of the HPLC method

The optimized analytical method for quantification of HUM and CAR in *C. verbenacea* essential oil was validated according to United States Pharmacopeia (USP) and International Conference on Harmonization (ICH) (ICH, 2005; USP, 2017). To this end, the following parameters were determined: linearity, precision, accuracy, specificity/selectivity, limit of detection and quantification.

The selectivity and specificity analysis of the method for pharmaceutical products were evaluated using the Ezchrom Elite® software. Analysis of the HUM and CAR standard solutions (100 μg/ml), the medicinal products (500 μg/ml) and the placebo were performed. The spectra obtained at the beginning, middle and end of each signal were overlapped for comparison. The purity index was obtained by a *ratiogram*, which calculates the ratio of absorbance to elution time. Evaluations were made to determine whether other substances were present close to the chromatographic signals using the DAD. The identity of the chromatographic signals was confirmed after addition of HUM and CAR standards in the oil.

Linearity was assessed by analyzing five different concentrations of standard solutions of HUM in triplicate with a concentration range ranging from 5 to 25 μg/ml. and standard solutions of CAR

at a concentration range between 64 and 96 μg/ml. All solutions were pre-filtered using a 0.45 μm filter membrane prior to injections. The areas were plotted at their corresponding concentrations and statistical analysis was performed by linear regression analysis using the least squares method in Microsoft Excel® software and $\alpha < 0.05$ was considered significant.

Precision was evaluated at two levels to ensure repeatability and intermediate accuracy. Nine samples were prepared and quantified at 80, 100 and 120% of the working concentration, equivalent to 500 μg/ml *C. verbenacea* oil on different days. Results were expressed as relative standard deviation (% RSD). Accuracy was determined by adding known concentrations of the HUM and CAR standards to the *C. verbenacea* essential oil solution at 80, 100 and 120% of the working concentration. This was used to determine the percentage of recovered standard.

The detection (LD) and quantification (LQ) limits for the chromatographic response were calculated based on the intercept and the slope of the regression equations, $LD = SD \times 3/S$ and $LQ = SD \times 10/S$, where SD is the standard deviation of the intercept of the y-axis of three calibration curves and S is the average of angular coefficients of the respective curves.

Data analysis

Statistical analysis of the results obtained in the preliminary experiments and validation procedure were performed using the software GraphPad Prism®, version 5.0 (GraphPad Software, La Jolla, CA, USA). The software Statistica®, version 10.1 (StatSoft Inc., USA) was used for the generation and analysis of the BB design, as well as for the construction of the response surface graphs.

Results and discussion

Column selection

Copaiba oleoresin contains sesquiterpenes similar, to those found in the essential oil of *C. verbenacea*. Thus, the method developed by Borges et al. (2013) was used as the starting point for the analysis of *C. verbenacea* oil. A CN column was used with a mobile phase consisting of acetonitrile and 9 mM phosphate buffer pH 3.0 with 1.1% triethylamine in a gradient elution with detection at 210 nm. This method was found to be unsuitable for *C. verbenacea* analysis, since the HUM and CAR signals were not well resolved and overlapped with other components in the oil. Additional method variations were also evaluated, as shown in Table 1, resolutions higher than 2.0 were not attained as recommended by the USP (2017). Other stationary phases were evaluated including: dimethylsilane (C_2), propylsilane (C_3), butylsilane (C_4), C_6-phenyl, octylsilane (C_8) and octadecylsilane (C_{18}). The mobile phase (FM) evaluated consisted of water and acetonitrile since they have low cut off values at the wavelength used.

Since sesquiterpenes have low molecular weights and are hydrophobic the C_2 column was first evaluated (Table 1). The C_3 column (propyl) also did not promote the separation of sesquiterpenes present in the *C. verbenacea* essential oil, likely due to the low affinity of the analytes. On the other hand, analytes were retained, however with low resolution between the signals. Optimal mobile phase conditions were tested by varying the mobile phase in the C_3 column first beginning with room temperature acetonitrile: water mixture. Results of the tests indicated individual HUM and CAR signals of low purity, which suggests that other components present in the oil contributed to the signal. Alterations in the mobile phase gradient were also tested (Table 1), as well as variations in flow and temperature, however these adjustments did not improve peak purities. The use of methanol in the FM also did not improve the resolution of the peaks.

The C_4 column is useful in the separation of medium to low molecular weight molecules (between 50 to 500 Da) including mono- and sesquiterpenes, which are the main constituents of the *C. verbenacea* essential oil. The initial composition of the mobile phase was an acetonitrile gradient using 9 mM phosphate buffer pH 5 (1.1% triethylamine). As the buffer and trimethylamine, were removed from the mobile phase, no considerable changes occurred in the retention time and the resolution between HUM and CAR. The retention times of these analytes were greater than 44 min when the gradient contained a greater amount of organic versus aqueous phase. By equalizing the concentrations of these solvents in the mobile phase, the retention times were decreased to approximately 26 min and provided good resolution between the analyte signals. However, an unidentified component (CMP) coeluted with CAR, and was present in all the methods tested using this mobile phase mixture; therefore the resolution of the last peak was directly affected. Alterations in the flow and temperature, as well as changes in the gradient, did not yield better separation between CMP and CAR (Table 1).

As the separation of CMP and CAR was difficult to achieve, other columns were tested. The C_6-Phenyl column, using a mixture of acetonitrile and water as the mobile phase, however did not provide favorable conditions for analyte retention in the column, regardless of the methods tested. This result can be justified by the absence of potential interactions between the phenyl groups in the stationary phase with π electrons of the terpenes in the essential oil of *C. verbenacea*. Therefore, the C_8 column was tested next and showed high affinity for the analytes. Using the following conditions: mobile phase mixture of acetonitrile and water, room temperature and flow rate of 1 ml/min, a good separation between HUM and CAR was obtained. However, both analytes were found to contain components that coeluted. Other methods tested (shown in Table 1) did not result in better analyte separation, therefore this column was ineffective for the proposed goal, since both eluted HUM and CAR exhibited low purity as evidenced by the chromatograph.

The C_{18} column effectively separated HUM and CAR, however retention times were greater than 4 min. Despite this, it was possible to observe other oil components between the analytes, which resulted in coelution for both HUM and CAR (Table 1). Attempts to vary flow and temperature, did not improve the separation of analytes from these unknown components, evidencing the similar physicochemical nature between the analytes and the other components. The chromatograms referring to the best results of each column are shown in Fig. 1.

Development of analytical methodology for Cordia verbenacea *oil*

Of all the columns evaluated, the most promising results were obtained using the C_4 column, which showed total separation between HUM and the other oil components, however the same degree of separation was not observed for CAR. Further tests were performed on column C_4 using a mixture of acetonitrile (57%) and water (43%) and flow rate of 1.2 ml/min at room temperature (25 °C). These conditions more adequately separated HUM and CAR from adjacent components. Despite the similar polarity between methanol and acetonitrile, the latter has a lower analytical selectivity since it forms donor-acceptor electron complexes with the stationary phase or with the analytes, thus suppressing their interactions with the stationary phase. In all the chromatograms obtained using the C_4 column, a small signal very close to CAR was observed. Based on the hypothesis this component may be an isomer of the analyte, we performed tests using cyclodextrins. The literature extensively describes the separation of isomers using cyclodextrins and *C. verbenacea* essential oil is composed mostly

Table 1
Methods applied for the evaluation of the tested columns.

Column	Method	Cromatographic conditions					Cromatographic Parameters			
		t (min)	OP%	AP%	*F*	*T*	HUM t_R	CAR t_R	*R* HUM	*R* CAR
CN	1	0	43.6	56.4	1.2	25	21	22.12		0.65
		14	50.3	49.7						
		22	50.3	49.7						
		22.1	43.6	56.4						
		30	43.6	56.4						
	2	0	43.6	56.4	1.2	25	10.39	10.97		0.611
		4	50.3	49.7						
		22	50.3	49.7						
		23	43.6	56.4						
		31	43.6	56.4						
	3	0	43.6	56.4	1.2	25	13.66	14.68		1.074
		15	43.6	56.4						
		5	50.3	49.7						
		5	50.3	49.7						
		1	43.6	56.4						
		5	43.6	56.4						
	4	0	43.6	56.4	1.2	25	16.01	17.12		0.925
		20	50.3	49.7						
		40	50.3	49.7						
		41	43.6	56.4						
		50	43.6	56.4						
C_2	5	0	10	90	1.2	25	–	–	[b]	[b]
		30	50	50						
	6	0–40[a]	25	75	1.2	25	3.51	3.62	[b]	[b]
	7	0–40[a]	15	85	1.2	25	3.82	3.9	[b]	[b]
	8	0–40[a]	25	75	1.0	25	4.01	4.22	[b]	[b]
	9	0–40[a]	15	85	1.0	25	4.12	4.18	[b]	[b]
C_3	10	0	50	50	1.0	25	9.8	10.2	[b]	[b]
		30	90	10						
	11	0–30[a]	60	40	1.0	25	6.22	6.56	[b]	[b]
	12	0–40[a]	50	50	1.0	25	16.92	18.33	[b]	[b]
C_4	13	0	10	90	1.2	25	44.01	44.79	0.71	0.32
		60	90	10						
	14	0	50	50	1.2	25	26.08	26.73	0.92	0.88
		30	90	10						
	15	0–30[a]	57	43	1.2	25	16.89	18.97	2.8	0.88
	16	0–45[a]	52	48	1.4	40	18.16	20.64	1.54	0.9
	17	0–45[a]	52	48	1.4	25	21.81	24.81	0.92	1.23
	18	0–45[a]	45	55	2.0	40	26.55	31.07	1.63	0.63
	19	0–45[a]	45	55	2.0	25	34.03	40.06	1.79	0.72
	20	0–45[a]	43	57	2.2	40	31.26	36.97	1.81	0.98
	21	0	44	56	2.0	40	19.45	23.72	1.46	0.96
		10	44	56						
		10.1	53	47						
		14	53	47						
		14.1	45	55						
		50	45	55						
C_6-Phenyl	22	0	10	90	1.0	25	[b]	[b]	[b]	[b]
		60	90	10						
	23	0	5	95	1.0	25	[b]	[b]	[b]	[b]
		60	25	75						
	24	0–45[a]	62	38	0.8	25	[b]	[b]	[b]	[b]
C_8	25	0	50	50	1.0	25	25.76	26.73	1.01	0.81
		30	90	10						
	26	0	65	35	1.2	25	17.37	18.76	0.48	0.4
		30	95	5						
	27	0	62	38	1.5	25	17.07	18.57	1.22	1.27
		30	90	10						
C_{18}	28	0	50	50	1.0	25	22.79	23.33	1.29	0.65
		30	90	10						
	29	0–30[a]	70	30	1.0	25	10.21	12.05	0.33	1.49
	30	0–30[a]	60	40	1.0	25	25.13	29.69	0.6	0.95

[a] Isocratic elution.
[b] Resolution not calculated due to high quantity of components coeluted with HUM and CAR.
t, run time (min); OP, organic phase (acetonitrile in all methods); AP, Aqueous phase (9 mM Phosphate buffer pH 5.0 + 1.1% TEA for 1–5 method and MilliQ water for 5–30 method); *F*, flow rate (ml/min); *T*, temperature (°C); *R*, resolution; t_R (min), retention time.

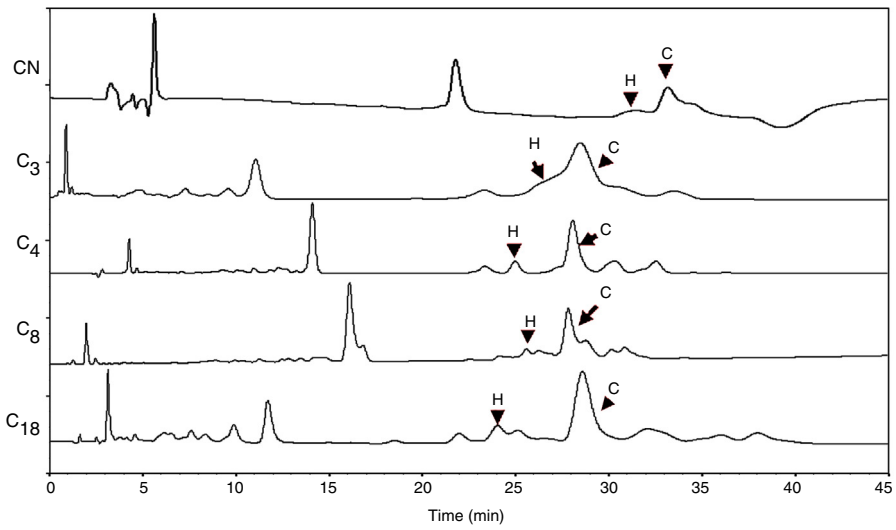

Fig. 1. Representative chromatograms of the methods with the most adequate parameters, among the evaluated columns: CN (method 1), C_3 (method 12), C_4 (method 15), C_8 (method 27) and C_{18} (method 29) at 210 nm. Legend: H. α-humulene; C. *trans*-caryophyllene.

of mono- and sesquiterpenes of very similar molecular formulas (Ajisaka et al., 2000; Lange and Ahkami, 2013; Lima et al., 2016).

Therefore, in an attempt to separate the isomers, α-cyclodextrin (α-CD), β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) were added to the mobile phase and evaluated, using method 15 (Table 1). The first two CD are widely described in the literature due to their ability to form complexes with low molecular weight molecules. β-CD has relatively low solubility in aqueous solvents when used in the mobile phase (Loftsson et al., 2005; Sabadini et al., 2006). Whereas, HP-β-CD was evaluated for its high solubility in water and internal diameter equivalent to β-CD (Lima et al., 2016).

The influence of β-CD was tested at a concentration of 10 mM since it has limited solubility, whereas the established concentration for α-CD and HP-β-CD was 25 mM. The ratio of the mobile phase was utilized as listed in method 15. Molarities greater than 25 mM CD can generate high pressure in chromatographic systems, as described by Ye et al. (2006). The effect of different CD on isomer separation was tested while they were added to the aqueous solvent of the mobile phase. No significant differences were observed when either α-CD or β-CD were included. HP-β-CD, on the other hand, provided better separation of HUM and CAR from the other components in the oil. Thus, HP-β-CD was selected for further analysis at a concentration of 25 mM, and the assays performed are shown in Table 2. The method resulting in the best resolution of HUM and CAR from coeluting components was method 32. Additional oil components could be observed that eluted before and after each of the analytes, which resulted in an additional parameter to be evaluated. Based on method 32, the influence of aqueous phase pH on the selected method was also evaluated, comparing pH 3.0 and 6.0. These results indicated that the aqueous phase at pH 3.0 provided higher resolution between the analytes and the adjacent components compared to the aqueous phase at pH 6.0.

Experimental design

Based on the preliminary experiments, the C_4 column was chosen for use in the experimental design, since it showed the best performance among the columns tested. Moreover, the proportion of the HP-β-CD aqueous solution (pH 3.0) in the mobile phase was fixed to 51%, considering that the range between 50 and 52% resulted in the best resolution. Thus, four factors were selected to be investigated in determining the optimal experimental design (Table 3) and generating a BB design matrix of thirty experiments. The results of the responses obtained for each experimental run performed with the BB matrix are presented in Table 4.

Mathematical models were elaborated for each response and the non-significant coefficients were excluded so that the equations could be recalculated using only significant coefficients. The equations obtained for the HUM resolution responses (Y_4 and Y_5) presented extremely low correlation coefficients ($R^2 < 0.5$), making them unsuitable for prediction purposes. On the other hand, better fitted models were obtained for all the CAR responses, since the equations for Y_1, Y_2 and Y_3 (Equations 1 to 3) presented correlation coefficients of 0.9668, 0.9700 and 0.9452, respectively, without evidence of lack of fit in the statistical analysis (ANOVA) of the regressions. Therefore, only the CAR responses were used for the surface response analysis and optimization of the chromatographic conditions.

$$\begin{aligned} Y_1 = {} & 2.055 - 0.266X_2 - 0.237X_4 + 0.592X_2X_4 - 0.374X_3X_4 \\ & + 0.58X_1^2X_2 - 0.577X_1^2X_4 + 0.712X_2X_3^2 \\ & + 0.278X_2^2X_3 + 0.62X_2^2X_4 + 0.576X_1^2X_2^2 + 1.364X_1^2X_3^2 \\ & - 0.897X_1^2 - 0.471X_2^2 - 0.398X_3^2 - 0.344X_4^2 \end{aligned} \quad (1)$$

$$\begin{aligned} Y_2 = {} & 1.690 + 0.305X_2 + 0.201X_3 - 0.234X_4 + 0.582X_2X_4 \\ & - 0.385X_3X_4 + 0.239X_1^2X_2 - 0.287X_1^2X_3 + 0.302X_1^2X_4 \\ & + 0.462X_2X_3^2 - 0.617X_2^2X_3 - 0.205X_1^2X_3^2 + 0.173X_3^2 \\ & - 0.410X_4^2 \end{aligned} \quad (2)$$

$$Y_3 = 32.692 - 6.696X_1 - 3.714X_2 - 1.905X_4 \quad (3)$$

Surface response (SR) graphs were constructed using mathematical models of CAR responses, to study the relationships between the selected factors and the responses. These models assist in finding the chromatographic conditions that provide the best resolution responses with suitable retention times.

Resolution is an important chromatographic parameter that measures the separation between adjacent peaks in the HPLC chromatogram and is directly affected by the mobile phase flow rate and the column temperature. As can be seen in Fig. 2 (panels A and B), the CAR resolution responses (Y_1 and Y_2) were influenced differently by the flow rate and column temperature. At low temperature values, Y_1 is much more affected by flow rate (Fig. 2A), while Y_2 is

Table 2
Methods with HP-β-CD 25 mM using isocratic elution and C_4 column.

Method	Chromatographic conditions						Chromatographic parameters			
	t (min)	ACN%	HP-β-CD%	MeOH	*F*	*T*	HUM t_R	CAR t_R	*R* HUM	*R* CAR
31	0–45*	40	50	10	1.0	50	24.64	28.84	1.13	1.7
32	0–45*	38	52	10	1.0	50	26.26	31.04	1.72	1.85
33	0–45*	34	50	16	1.0	50	24.97	29.48	1.08	**
34	0–45*	41	51	8	1.0	50	26.65	29.61	1.16	1.64
35	0–45*	42	51	7	1.0	50	25.01	29.33	1.21	1.09

t, run time (min); ACN, acetonitrile; HP-β-CD, hydroxypropyl-β-cyclodextrin; MeOH, methanol; *F*, flow rate (ml/min); *T*, temperature (°C); *R*, resolution; t_R (min), retention time.
* means isocratic elution of the mobile phase
** means resolution not calculated due to high amount of component coeluted with HUM and CAR

Table 3
Variables employed in the Box–Behnken design.

Factors (Independent variables)	Level (−)	Level (0)	Level (+)
X_1: Flow rate (ml/min)	0.8	1.0	1.2
X_2: Column temperature (°C)	30	40	50
X_3: Methanol proportion in the mobile phase (%)	6	8	10
X_4: HP-β-CD concentration (mM)	15	20	25

Responses (Dependent variables)	Abbreviation
Y_1: Resolution between component 1 and caryophyllene	$R_{CMP1\text{-}CAR}$
Y_2: Resolution between caryophyllene and component 2	$R_{CAR\text{-}CMP2}$
Y_3: Retention time of caryophyllene	RT_{CAR}
Y_4: Resolution between component 3 and humulene	$R_{CMP3\text{-}HUM}$
Y_5: Resolution between humulene and component 4	$R_{HUM\text{-}CMP4}$
Y_6: Retention time of humulene	RT_{HUM}

Table 4
Box–Behnken design matrix and corresponding results for the responses.

Trial	Factors				Responses					
	X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	0.8	30	8	20	0.947	1.332	45.32	1.230	1.124	37.87
2	0.8	50	8	20	1.593	2.286	36.26	1.336	0.000	31.15
3	1.0	40	6	15	1.261	1.079	31.93	1.380	0.460	27.31
4	1.0	40	10	15	1.840	2.25	34.53	1.282	0.000	29.29
5	0.8	40	8	15	1.562	1.274	41.24	1.210	0.000	35.19
6	1.2	40	8	15	1.694	1.343	28.16	1.311	0.000	24.00
7	1.0	30	6	20	0.268	1.414	35.37	0.000	1.607	29.66
8	1.0	30	10	20	1.213	0.741	37.44	1.840	0.000	31.10
9	0.8	40	6	20	2.288	1.721	39.46	1.138	0.819	33.53
10	0.8	40	10	20	1.935	1.598	41.78	1.891	1.574	35.26
11	1.0	30	8	15	1.716	1.717	38.43	1.148	1.035	32.26
12	1.0	50	8	15	0.000	1.163	29.93	1.334	0.633	25.79
13[c]	1.0	40	8	20	2.227	1.852	30.02	1.249	0.663	25.27
14[c]	1.0	40	8	20	1.989	1.634	32.71	1.163	1.088	27.70
15[c]	1.0	40	8	20	1.852	1.566	32.41	1.117	1.820	27.44
16	1.2	30	8	20	0.951	1.113	30.05	0.000	0.407	25.03
17	1.2	50	8	20	1.561	2.334	23.95	1.254	0.400	20.54
18	1.0	40	6	25	1.534	1.465	28.79	1.054	1.391	24.43
19	1.0	40	10	25	0.617	1.097	30.30	0.925	0.509	25.55
20	0.8	40	8	25	0.000	1.493	37.58	1.669	1.055	31.79
21	1.2	40	8	25	0.000	1.396	24.72	0.996	0.908	20.94
22	1.0	50	6	20	1.549	3.105	28.25	1.319	0.000	24.29
23	1.0	50	10	20	1.715	2.115	29.63	1.221	0.623	25.35
24	1.2	40	6	20	2.331	1.768	26.49	1.192	0.904	22.50
25	1.2	40	10	20	1.940	1.548	27.92	1.795	1.512	23.55
26	1.0	30	8	25	1.297	0.000	32.97	1.033	2.277	27.48
27	1.0	50	8	25	1.949	1.773	26.99	1.172	0.845	23.21
28[c]	1.0	40	8	20	1.940	1.548	32.67	1.795	1.512	27.67
29[c]	1.0	40	8	20	2.269	1.632	32.63	1.107	1.238	27.61
30[c]	1.0	40	8	20	2.054	1.683	32.83	1.178	1.158	27.79

Letter [c] indicates the replicates at the center point of the design.

less affected at the same condition (Fig. 2B). Midway values of flow rate and column temperature led to the higher values of Y_1, indicating that this is the condition that these factors are most likely improve resolution.

The concentration of HP-β-CD had the greatest influence on the Y_1 and Y_2 responses. At higher temperatures, variations in the HP-β-CD concentration strongly affected Y_1 (resolution between CMP1 and CAR), while at low temperatures the concentration of HP-β-CD weakly influences this response (Fig. 2C). Interestingly, the same factors had the exact opposite influence on Y_2 (resolution between CAR and CMP2) (Fig. 2D), which could be explained by different affinities between each component and the free cyclodextrin

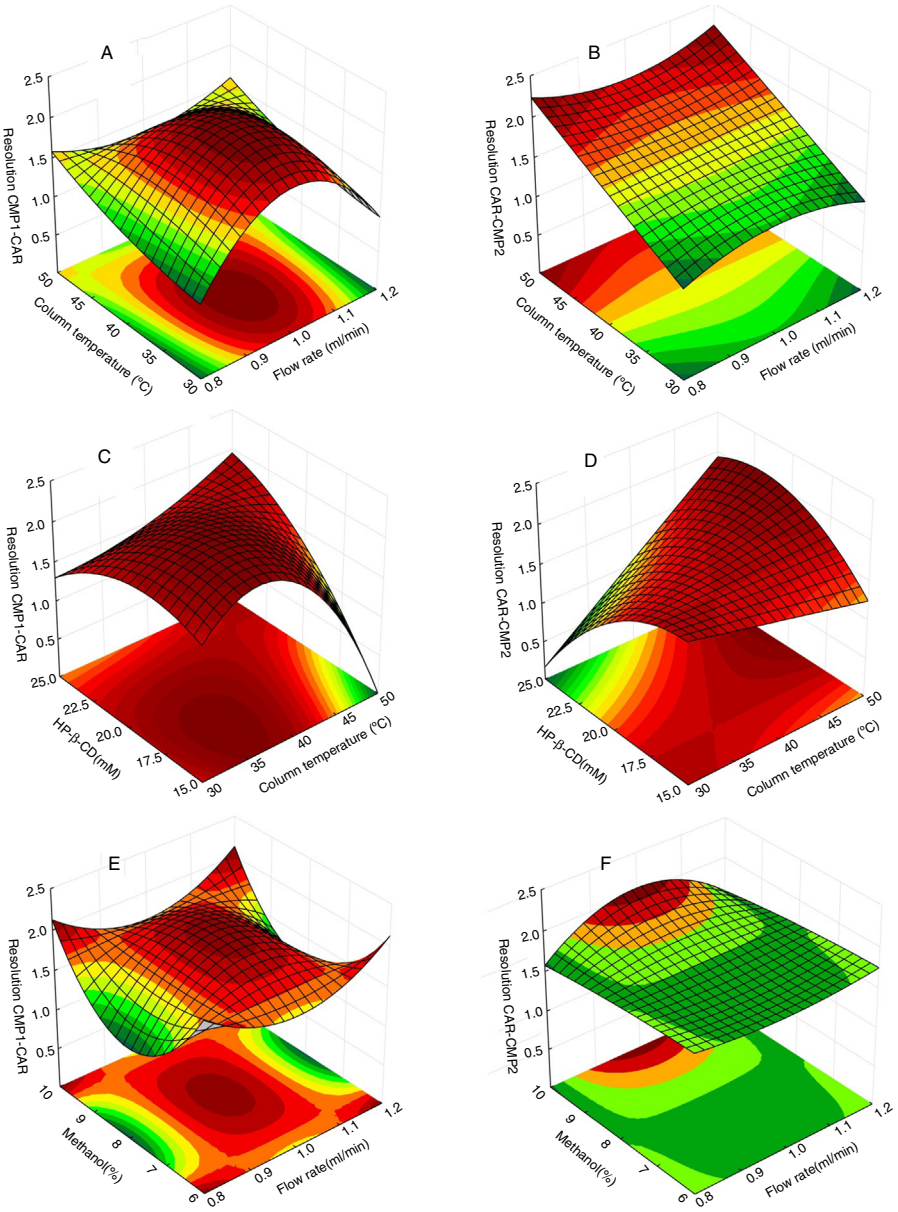

Fig. 2. Response surface graphs showing the effects of flow rate (X_1) and column temperature (X_2) on the responses Y_1 (resolution between CMP1 and CAR) (A) and Y_2 (resolution between CAR and CMP2) (B); the column temperature (X_2) and the HP-β-CD concentration (X_4) on the responses Y_1 (C) and Y_2 (D); and the flow rate (X_1) and proportion of methanol (X_3) on the responses Y_1 (E) and Y_2 (F). CAR. *trans*-caryophyllene; CMP. unidentified component.

in the mobile phase. Therefore, the chromatographic conditions should be equilibrated to avoid hindering one of the responses while improving the other.

Different behaviors were observed for the Y_1 and Y_2 responses with respect to the simultaneous influence of flow rate and the proportion of methanol in the mobile phase. These two factors when combined at mid-range values led to the best resolution results for Y_1. On the other hand, when the proportion of methanol is at mid value, the use of extreme values of flow rate (very high or very low) resulted in very poor resolution values (Fig. 2E). However, the best values for Y_2 could be obtained using higher proportions of methanol combined with mid-range flow rates (Fig. 2F).

According to the linear equation proposed for Y_3, the retention time is negatively affected by the flow rate (X_1), column temperature (X_2) and HP-β-CD concentration (X_4). The proportion of methanol in the mobile phase (X_3), however, did not significantly influence this response (Eq. (3)). In agreement with data in the literature, the combination of high flow rate values and column temperatures considerably reduces retention times; therefore directly affecting the efficiency of the chromatographic analysis. However, this condition could lead to unsuitable resolution results, thus an equilibrated combination between these factors appears crucial for the success of the separation method.

Considering the discussions taken from the RS graphs analysis, it was concluded that using mid-range conditions, where all factors are maintained near their central levels, provides the most ideal combinations. Subsequently, these conditions improve the resolution between CAR and adjacent components, while providing suitable retention times. Therefore, the optimal conditions for the method were determined using a flow rate of 1 ml/min, column temperature of 40 °C, a 20 mM HP-β-CD concentration and 8% methanol, 51% HP-β-CD in aqueous solution (pH 3.0), 41% acetonitrile on a C_4 column. Under these conditions, the CAR responses had predicted resolution values of 2.05 between CMP1 and CAR ($R_{CMP1\text{-}CAR}$), resolution between CAR and CMP2 ($R_{CAR\text{-}CMP2}$) of 1.69 and a retention time for CAR of 32.7 ml/min. A series of runs were

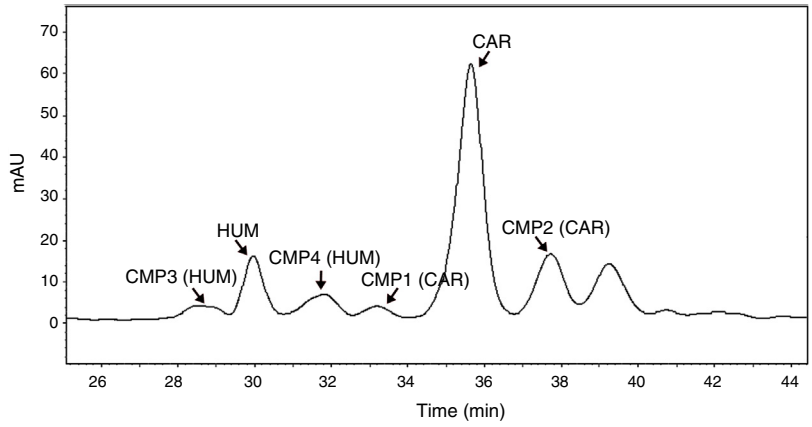

Fig. 3. HPLC Chromatogram obtained for the optimized conditions. HUM. α-humulene; CAR. *trans*-caryophyllene; CMP. unidentified component.

Table 5
Validation parameters for HUM and CAR quantification.

Parameters			HUM		CAR	
Linearity	Range		5–25 μg/ml		64–96 μg/ml	
	Coefficient of determination (R^2)		0.9987		0.9932	
	F regression slope non-zero (F_{critic} = 7.71)		2963.0		591.7	
	Slope [1]/F_{calc} (F_{critic} = 4.26)		[153255.6 ± 1982/0.398]		[180,008.8 ± 1033.7/0.014]	
	Intercept[1]/F_{calc} (F_{critic} = 3.98)		[−87,666.4 ± 3760.3/2.513]		[−1,755,483 ± 47,318/0.214]	
	Concentration (%)	Day	Intraday (DPR%)	Interday (DPR%)	Intraday (DPR%)	Interday (DPR%)
Precision	80	1	0.69	0.57	0.79	1.04
		2	0.59		1.44	
	100	1	0.23	0.17	1.53	1.76
		2	0.05		1.04	
	120	1	0.92	0.59	0.69	0.79
		2	0.07		1.04	
Accuracy	Low		101.83 ± 0.69		98.72 ± 0.79	
	Medium		100.56 ± 0.22		100.74 ± 1.53	
	High		99.33 ± 0.92		98.37 ± 0.69	
Limits	LD (μg/ml)		0.074		0.789	
	LQ (μg/ml)		0.245		2.629	

performed using the optimized conditions that resulted in resolution values for $R_{CMP1\text{-}CAR}$ of 1.81, a $R_{CAR\text{-}CMP2}$ of 1.66 and a CAR retention time of 35.6 min. The representative chromatogram of this analysis is presented on Fig. 3, showing a suitable separation between CAR, HUM and their adjacent components.

Validation of HPLC methodology

The signals related to CAR and HUM of the essential oil *C. verbenacea* were properly identified by comparing the respective UV spectra obtained from the sample to the standard. The specificity was assessed by the purity of the chromatographic signals. The UV scan spectra were superimposed at the beginning, middle and end of the HUM and CAR chromatographic signals. The similarity of the spectra was calculated by the detector software and presented values higher than 0.9999 for both cases, therefore were considered pure. The purity was also confirmed by the ratiogram, indicating a virtually zero absorbance ratio, less than 0.01.

The linearity of the method was evaluated by the correlation coefficients obtained from the standard curve from HUM and CAR, as shown in Table 5. The F test revealed that coefficient (a) was significantly different from zero ($F_{calc} > F_{tab}$), showing that the regression was relevant and the linearity curve was accepted ($p < 0.0001$). The angular coefficient and the intercept showed the F_{calc} was smaller than the F_{tab}, demonstrating that the model is linear and adequate for use at the working range studied with a 95% confidence interval. The R^2 values obtained for HUM and CAR were higher than 0.99 (ICH, 2005; USP, 2017). The method showed good accuracy and precision, with RSD <5% and recovery between 98–102% for HUM and CAR. The LD and LQ were adequate for the application of the developed method.

HUM and CAR quantification in commercial formulations

The *C. verbenacea* essential oil is present in pharmaceutical forms in aerosols and creams at a concentration of 5 mg/g. The labeled concentration of HUM is 2.3–2.9%, equivalent to a mass of 115–145 μg of HUM/g of the product. Using the method developed here, the concentrations were estimated in two different lots of creams and aerosols. In the aerosol, the percentage amounts of HUM in the two lots were 2.85 and 2.87% (142.4 and 143.4 μg of HUM/g of the product) and 10.30 and 10.43% CAR (563.8 and 572.4 μg of CAR/g of the product). In the cream, the percentage amounts of HUM were 2.9% for both of the lots (145.2 and 145.0 μg of HUM/g of the product) and 10.33 and 10.23% of CAR (562.5 and 555.4 μg of CAR/g of the product). In all cases, the determined concentrations of HUM were close to the upper limit described by the manufacturer. The influence of the excipients in the analytical method was also evaluated and no chromatographic signal was observed at the same retention times as HUM and CAR, confirming the applicability of the methodology for the analysis of the Acheflan® herbal medicine.

Conclusion

The method developed in this study has been proven to be effective in properly separating HUM and CAR, which have been previously reported, as the main markers of the essential oil of *C. verbenacea*. This is the first method describing the analysis of this oil by HPLC-DAD. The Box–Behnken experimental design was successfully used in the optimization of the analytical method, resulting in a separation between the HUM and CAR with resolutions close to 1.6, which were adequate to the proposed objectives in the study. The method was successfully validated according to the parameters: linearity, selectivity, precision, accuracy, limit of detection and quantification. The application of the developed method in commercial formulations (cream and aerosol) containing *C. verbenacea* essential oil was also very satisfactory. The content found in the batch analyses closely approximated the labeling information, without interference from the excipients. The concentration of CAR in the oil is about four times higher than that of HUM. For these reasons, we suggest that CAR could be used as a marker of *C. verbenacea* essential oil, especially for applications where its concentrations are very low, such as in permeation and release studies of dermatological formulations.

Authors' contribuition

VPS conceived of the presented idea. LMC helped supervise the project. MVSG carried out all the experiments. JDS contributed to the interpretation of the results. AFR planned the Experimental Design.

Conflicts of interest

The authors declare no conflicts of interest.

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