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HPLC analysis of β-caryophyllene in copaiba oleoresin: Development, validation and applicability

Análise por CLAE de β-cariofileno em óleo-resina de copaíba: Desenvolvimento, validação e aplicabilidade

Análisis por HPLC de β-cariofileno en aceite-resina de copaiba: Desarrollo, validación y aplicabilidad

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Abstract
Copaiba oil (CO) is an oleoresin containing resinous acids, comprising mainly of diterpenes, and volatile compounds, comprising of sesquiterpenes. CO has been used for many years as a therapeutic agent and cosmetic, being the β-caryophyllene (CAR) one of the main sesquiterpene markers found in CO samples. During the last years, some analytical methods have been developed for analysis of sesquiterpenes like CAR from CO. However, these methods are based on gas chromatography, and requiring additional steps, such as derivatization or extraction of the essential fraction of the CO for sesquiterpenes analysis. Liquid chromatography methodologies have been proposed only for analysis of diterpenes. Therefore, the aim of this study was to develop a high-performance liquid chromatography (HPLC) assay for CAR analysis in CO samples (*Copaifera reticulata* Ducke) and in emulsion systems containing CO. The HPLC system suitability was determined through the capacity factor, repeatability, relative retention, resolution, tailing factor, theoretical plate number and the height of the theoretical plate; where the method developed showed the efficiency for separation of CO compounds. The method was validated displaying specificity, linearity, precision, accuracy, and robustness. Moreover, it showed to be of utmost importance to analyze CO in emulsion systems, displaying versatile and applicability.
Keywords: *Copaifer*a spp.; Beta-caryophyllene; HPLC; Emulsion; Sesquiterpenes analysis.

Resumo
O óleo de copaíba (CO) é um óleo-resina contendo ácidos resinosos, compostos principalmente por diterpenos, e compostos voláteis, constituído por sesquiterpenos. O CO é utilizado há muitos anos como agente terapêutico e

cosmético, sendo o β-cariofileno (CAR) um dos principais marcadores sesquiterpênicos. Nos últimos anos, alguns métodos analíticos foram desenvolvidos para análise de sesquiterpenos como o CAR a partir de amostras de CO. No entanto, esses métodos são baseados em cromatografia gasosa e requerem etapas adicionais, como derivatização ou extração da fração volátil do CO para análise dos sesquiterpenos. Metodologias de cromatografia líquida têm sido propostas apenas para análise dos diterpenos. Portanto, o objetivo deste estudo foi desenvolver um método de cromatografia líquida de alta eficiência (HPLC) para análise de CAR em amostras de CO (*Copaifera reticulata* Ducke) e em sistemas emulsivos contendo CO. A adequação do sistema de HPLC foi determinada através do fator de capacidade, repetibilidade, fator de separação, resolução, fator de simetria, número de pratos teóricos e altura dos pratos teóricos; onde o método desenvolvido mostrou eficiência para separação de compostos presentes no CO. O método foi validado apresentando especificidade, linearidade, precisão, exatidão e robustez. Além disso, mostrou-se de suma importância analisar o CO em sistemas emulsivos, apresentando versatilidade e aplicabilidade.
Palavras-chave: *Copaifera* spp.; Beta-cariofileno; CLAE; Emulsão; Análise de sesquiterpeno.

Resumen

El aceite de copaiba (CO) es una oleorresina que contiene ácidos resinosos, compuestos principalmente por diterpenos, y compuestos volátiles, compuestos por sesquiterpenos. El CO se ha utilizado durante muchos años como agente terapéutico y cosmético, siendo el β-cariofileno (CAR) uno de los principales marcadores de sesquiterpenos encontrados en muestras de CO. Durante los últimos años se han desarrollado algunos métodos analíticos para el análisis de sesquiterpenos como CAR a partir de CO. Sin embargo, estos métodos se basan en la cromatografía de gases y requieren pasos adicionales, como la derivatización o extracción de la fracción esencial del CO para el análisis de sesquiterpenos. Las metodologías de cromatografía líquida se han propuesto solo para el análisis de diterpenos. Por lo tanto, el objetivo de este estudio fue desarrollar un ensayo de cromatografía líquida de alta resolución (HPLC) para el análisis de CAR en muestras de CO (*Copaifera reticulata* Ducke) y en sistemas emulsionantes que contienen CO. La idoneidad del sistema de HPLC se determinó mediante el factor de capacidad, la repetibilidad, retención relativa, resolución, factor de simetría, número de plato teórico y altura del plato teórico; donde el método desarrollado mostró la eficiencia para la separación de compuestos de CO. El método fue validado mostrando especificidad, linealidad, precisión, exactitud y robustez. Además, demostró ser de suma importancia analizar CO en sistemas emulsionantes, mostrando versatilidad y aplicabilidad.
Palabras clave: *Copaifera* spp.; Beta-cariofileno; HPLC; Emulsión; Análisis de sesquiterpenos.

1. Introduction

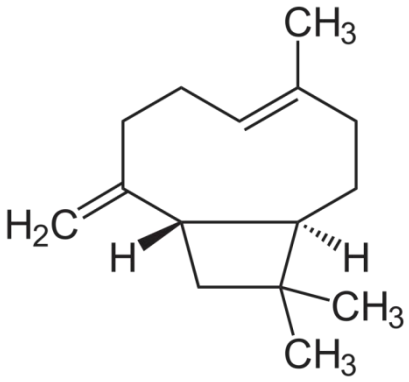
Copaiba oleoresin (CO) is a natural transparent liquid displaying coloration ranging from yellow to brown (Veiga Junior & Pinto, 2002), obtained through perforations or incisions in the trunks of copaiba trees (*Copaifera* spp.), which are commonly found in Latin America and West of Africa (da Trindade et al., 2018; Pieri et al., 2009; Romero, 2007). There are more than 70 species of *Copaifera* around the world, and Brazil is the country with the greater number of them (26 species and eight varieties) (da Trindade et al., 2018).

Many applications of CO have been reported, such as a clean fuel, in cosmetics and perfumes, and as therapeutic agent in many diseases (Veiga Junior & Pinto, 2002). The first applications of CO as therapeutic agent come from sixteenth century and, since that, some CO biological activities have been described, as wound healing, anti-inflammatory, antimicrobial, anti-Leishmania, anti-asthmatic and antitumor (Bardají et al., 2016; dos Santos et al., 2012; Lima & Lima, 2012; Veiga Junior & Pinto, 2002).

Chemically, the CO is composed of a resinous portion consisting of diterpenes acids, and a volatile portion composed mainly of sesquiterpenes (Cascon & Gilbert, 2000). Among the main terpenes found in CO, there are the hardwickiic acid, copalic acid, β-bisabolene, α-humulene and β-caryophyllene (CAR) (Pieri et al., 2009).

The CAR is a sesquiterpene (Figure 1) that can display anti-inflammatory, antibacterial and antifungal properties, being one of the main compounds responsible for the therapeutic activities of CO (Pieri et al., 2009).

Figure 1. Chemical structure of β-caryophyllene.



Source: Authors.

The development of analytical methods can be carried out for verifying the quality and amount to the compounds present in formulations containing bioactives from natural origin, for example (Borges et al., 2013). Due to CAR is one of the main constituents of the volatile portion of CO, some quantitative analytical methods based on gas chromatography (GC) have been proposed for analysis of this sesquiterpene (Sousa et al., 2011; Veiga Junior & Pinto, 2002). Generally, these GC methodologies show the need for additional steps during the sample preparation, such as derivatization or extraction of the essential/volatile part of the CO (Borges et al., 2013). This can result in a greater time and higher costs for analysis.

Therefore, with the aim to develop more accessible quantitative methods with better practicality in the preparation of samples, High Performance Liquid Chromatography (HPLC) and Ultra-High Performance Liquid Chromatography (UHPLC) methods have been developed for sample analysis of CO (Carneiro et al., 2018; da Silva et al., 2017). However, these methods are based on the analysis of diterpenes, being necessary to carry out the isolation of these compounds from the CO samples and the determination of its purity (Carneiro et al., 2018; da Silva et al., 2017).

The liquid chromatography method consists of a separation technique where different substances in a liquid mobile phase can interact with a stationary phase (Lozano-Sánchez et al., 2018). Depending on the intensity/force of interactions, the separation of compounds occurs. HPLC is a separation method frequently used to analyze qualitatively and quantitatively natural and finished products containing organic compounds (Sarker & Nahar, 2015). Therefore, the aim of this work was to develop and validate an HPLC method for the analysis of the sesquiterpene CAR from CO and emulsion systems. The validation, suitability and applicability of the methodology were carried out and discussed, confirming that the developed method can provide reliable data.

2. Methodology

2.1 Chemicals and reagents

Acetonitrile (HPLC grade) was purchased from Honeywell Riedel-de-Haën (Indianapolis, IN, USA) and β-caryophyllene standard (purity ≥ 80%) was acquired from Sigma-Aldrich (Sao Paulo, SP, Brazil). Acetic acid and polyethylene glycol 400 analytical grades were obtained from Synth (Sao Paulo, SP, Brazil). Soluplus® was kindly donated by BASF (Ludwigshafen am Rhein, Germany) and the copaiba oleoresin (*Copaifera reticulata* Ducke; SISGEN authorization nº AE28797) was purchased from Ariapuana-Guariba Agroextractive Association (Apuai, AM, Brazil).

2.2 Apparatus and analytical conditions

The chromatographic analyses were performed on a high-performance liquid chromatographer, compact model Prominence-i LC-2030C (Shimadzu, Tokyo, Japan), equipped with automatic sample injections, with an oven (model FCV-14AH, part of compact model Prominence-I LC-2030C HPLC system, Shimadzu, Tokyo, Japan) to control the column temperature and a photodiode array detector (Tungsten lamp for PDA, ASSY, part n° S22857110-41; and High-Speed Cell for PDA, part n° S228-45618-54 part of compact model Prominence-I LC-2030C HPLC system, Shimadzu, Tokyo, Japan) for analyte detection. Moreover, the temperature of analysis was 22 °C, the injection sample volume was de 20 µL and the wavelength for detection was λ = 210 nm. The chromatographic column was a Supelcosil LC-18 (25 cm x 4.6 mm, 5 µm; Supelco, Saint Louis, MO, USA) with a guard column Gemini C18 (4 x 3 mm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile (Phase A) and ultra-purified water with acetic acid 0.1% ($v.v^{-1}$) (Phase B), with a flow rate of 1.0 $mL.min^{-1}$ and analysis time of 25 min. The gradient elution is described in Table 1.

The software *LabSolutions* version *LCSolution Lite* (Shimadzu, Tokyo, Japan) was used to obtain the chromatograms and others parameters.

Table 1 - Gradient elution system utilized in the HPLC method. Phase A was acetonitrile and Phase B was composed of ultra-purified water with acetic acid 1% ($v.v^{-1}$).

Analysis time (min)	Phase A (%)	Phase B (%)
0 – 2	60	40
2 – 4	65	35
4 – 6	70	30
6 – 8	75	25
8 – 10	80	20
10 – 12	100	0
12 - 25	70	30

Source: Authors.

2.3 Preparation of standard and sample stock solutions

The CAR standard stock solution was prepared by adding 12.5 mg of CAR in a volumetric flask, to make up to 25 mL with acetonitrile, obtaining a solution with 500 $µg.mL^{-1}$ of CAR.

The samples analyzed were CO (*Copaifera reticulata* Ducke) and emulsion systems containing CO: an emulsion (EM) containing CO, the surfactant Soluplus® and ultra-purified water; and a self-emulsifying drug delivery system (SEDDS) with CO, the surfactant Soluplus® and the polyethylene glycol 400 as a co-surfactant.

The CO sample stock solution was prepared by adding 75 mg of CO in a 25 mL volumetric flask, and the final volume was adjusted with acetonitrile, obtaining a solution with 3 $mg.mL^{-1}$ of CO. The sample solutions of emulsion systems were prepared adding 15 mg of each formulation in a volumetric flask, completing the volume (10 mL) with acetonitrile, obtaining solutions with 1.5 $mg.mL^{-1}$ of each formulation.

2.4 System suitability

For the determination of the system suitability parameters, it was utilized the guide for Validation of Chromatographic Methods from the Food and Drug Administration (FDA) (FDA, 1994). A CAR solution (90 $µg.mL^{-1}$) was utilized to determine

the system suitability. This solution was prepared by adding 900 µL of CAR standard stock solution in a 5 mL volumetric flask and completing the volume with acetonitrile. The CO, EM and SEDDS solutions were from their respective sample stock solution, previously described. The standard and sample solutions were filtered through 0.45 µm (13 mm) PTFE membrane (Filtrilo, PR, Brazil) before be packed in vials and were maintained on the HPLC rack at temperature controlled of 5 °C until the injection and analysis.

2.4.1 Capacity factor (k')

The capacity factor (*k'*) is a measure of time retention about the peak of interest in relation with the void time. Thus, to calculate the capacity factor was used the equation Equation 1:

$$k' = \frac{t_{r1} - t_0}{t_0} \quad (1)$$

Where t_{r1} is the retention time of CAR and t_0 is the retention time of void volume or non-retained components.

At least five replicates of CAR solutions, CO solutions and emulsion system solutions were analyzed.

2.4.2 Repeatability

The repeatability was performed in relation to CAR retention time in the method developed, expressed as relative standard deviation with less than 1%, in at least five replicates.

2.4.3 Relative retention (α)

The relative retention (α) was determined in relation to the two peaks using the Equation 2:

$$\alpha = \frac{k'_1}{k'_2} \quad (2)$$

Where k'_1 is the capacity factor of CAR and k'_2 is the capacity factor of the closest peak.

At least five replicates of CO solutions and emulsion system solutions were used.

2.4.4 Resolution (Rs)

Resolution (*Rs*) is utilized for the determination of how well the peaks are separated. So, it was calculated using the equation Equation 3:

$$Rs = \frac{t_{r2} - t_{r1}}{1/2 \times (W_1 + W_2)} \quad (3)$$

Where t_{r1} and t_{r2} are the retention time of CAR and the peak closest, respectively; W_1 and W_2 are the width of CAR's peak and the closest peak, respectively.

At least five replicate samples of CO solutions and emulsion system solutions were analyzed.

2.4.5 Tailing factor (T)

Tailing factor (T) was calculated according to the equation Equation 4:

$$T = \frac{W_1}{2 \times f} \quad (4)$$

Where W_1 is the width of CAR's peak and *f* is the distance between the peak maximum and the peak front at W_1.

At least five replicate samples of CO solutions and emulsion system solutions were analyzed.

2.4.6 Number of theoretical plates (N)

The equation Equation 5 was utilized for determination of the number of theoretical plates:

$$N = 16 \times \left(\frac{t_{r1}}{W_1}\right)^2 \quad (5)$$

2.4.7 Height of the theoretical plate (H)

The determination of height of the theoretical plate (*H*) was accomplished according to the Equation 6:

$$H = \frac{L}{N} \quad (6)$$

Where *L* is the column length and *N* is the theoretical plate number.

At least five replicate samples of CAR solutions, CO solutions and emulsion system solutions were analyzed.

2.5 Validation

The validation of chromatographic method was performed according to the guidelines of the Validation of Analytical Procedures Q2 (R1) of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, 2005) and Resolution RDC nº 166 from the Brazilian Health Regulatory Agency (ANVISA, 2017).

2.5.1 Specificity

The specificity of the method was evaluated by using the CAR standard stock solution 500 µg.mL^{-1} and the stock solution of samples (3 mg.mL^{-1} of CO; 1.5 mg.mL^{-1} of EM and SEDDS), as previously described. The solutions were filtered through 0.45 µm (13 mm) PTFE membrane before be packed in vials and were maintained on the HPLC rack at 5 °C until the injection and analysis. The specificity was determined by analyzing the CAR peak in different samples. In addition, to confirm the retention time and the selectivity method, 12.5 mg of CAR standard was added to the solution of 3 mg.mL^{-1} of CO.

2.5.2 Linearity

Linearity was determined by the graphic representation (analysis curve) of the areas as function of the different CAR standard concentrations (20 – 100 µg.mL^{-1}) obtained from standard stock solution. The solutions were filtered and maintained on equipment rack at 5 °C until injection. Statistical analysis was performed using the Analysis of Variance (ANOVA) to evaluate the significance of linear regression and the analysis of lack of fit. At least five replicate samples were evaluated.

2.5.3 Limits of detection and quantitation

The limits of detection (LD) and quantitation (LQ) were determined according to Equations 7 and 8, respectively:

$$LD = \frac{3.3 \times \sigma}{S} \quad (7)$$

$$LQ = \frac{10 \times \sigma}{S} \quad (8)$$

Where σ is the standard deviation and *S* is the slope of analysis curve.

At least five replicate samples were analyzed.

2.5.4 Precision

The evaluation of precision was performed by determination of repeatability and intermediate precision. For repeatability, three replicate samples of CAR standard solutions (50, 60 and 70 μg.mL^{-1}) were prepared on the same day. For the intermediate precision, three replicate samples of CAR standard solutions (50, 60 and 70 μg.mL^{-1}) were prepared during different days and between different analysts. The solutions were filtered and maintained on equipment rack at 5 °C until injection. The results were evaluated by ANOVA statistical analysis.

2.5.5 Accuracy

The accuracy was determined as the percent recovery, as shown in Equation 9, between the concentration determined experimentally and the theoretical concentration of three replicate samples of CAR standard concentration (50, 60 and 70 μg.mL^{-1}). The solutions were filtered and maintained on equipment rack at 5 °C until injection.

$$\text{Recovery } (\%) = \frac{\text{Experimental concentration}}{\text{Theoretical concentration}} \times 100 \quad (9)$$

2.5.6 Robustness

To determine the robustness of the method, variations of the following parameters were performed: wavelengths (λ = 205, 208, 209, 210, 211, 212 and 214 nm), flow (0.9, 1.0 and 1.1 mL.min^{-1}) and oven temperature (20, 22 and 24 °C). The analyses were performed using three replicate samples of CAR standard solution (50, 60 and 70 μg.mL^{-1}). The solutions were filtered and maintained on equipment rack at 5 °C until injection. The ANOVA was done to evaluate the results.

2.6 Analysis of CAR in CO samples

During the analysis of CAR in CO samples, the amount of 1.6 mg of CO was added in a 10 mL volumetric flask and the volume was made up with acetonitrile, obtaining a solution with 160 μg.mL^{-1} of CO. The solution was filtered and maintained on equipment rack at 5 °C until injection. The analysis of CAR was performed using the method developed with the chromatographic conditions previously described and determined using the analytical curve. The analysis was performed in at least six replicate samples.

2.7 Applicability of method

The CAR content in emulsion systems was determined using the analytical curve and the trapping efficiency (TE) was determined according to Equation 10. The sample stock solutions of EM and SEDDS previously described were filtered and

maintained on equipment rack at 5 °C until injection.

$$\text{TE}\ (\%) = \frac{\text{Experimental CAR content}}{\text{Theoretical CAR content}} \times 100 \quad (10)$$

3. Results and Discussion

3.1 HPLC method

The HPLC analysis consists of passing a mobile phase, containing the dispersed analyte, by a stationary phase, being both (mobile and stationary phase) immiscible each other (Lozano-Sánchez et al., 2018). Depending on the some parameters of analyte, such as chemical structure, ionic charge and molecular weight, it should be chosen the liquid chromatography most appropriate to occur the separation of compounds (Lozano-Sánchez et al., 2018). Analyzing the CO composition, the majority compounds is terpenoids (diterpenes and sesquiterpenes), which are substances consist of nonpolar pentacarbonated isoprene units (PubChem, 2022; Simões et al., 2007). Due it, the HPLC method selected was the reversed-phase liquid chromatography, where the mobile phase is polar while the stationary phase is no polar or less polar, being commonly used for separation of a wide variety of natural compounds (Lozano-Sánchez et al., 2018; Poole & Lenca, 2017). Therefore, in this work a reversed-phase liquid chromatography was used for separation of CO compounds, being the polar mobile phase composed of acetonitrile and water with acetic acid, and a column C18 (reversed phase) was utilized as stationary phase.

3.2 Determination of system suitability

The analysis of the peaks and the separation of the CO compounds in HPLC system can be determined by the system suitability (FDA, 1994). This parameter can indicate the method's ability to generate reliable data, being this reliance confirmed by the validation of developed method. Table 2 displays the parameters calculated for system suitability using the CAR standard and the samples CO, EM and SEDDS.

The evaluation of capacity factor (*k*') indicates the retention capacity of the analyte in the system (Moldoveanu & David, 2013), being strongly linked to the physical properties of the stationary phase (Serban C. et al., 2017). Therefore, low values capacity factor (*k*' < 2) indicate insufficient analyte retention, which may not lead to good separation (Moldoveanu & David, 2013). Likewise, very high values capacity factor (*k*' > 10) indicate a strong retention of the analyte, resulting in longer retention times and, consequently, longer analyzes and with wide peaks (Moldoveanu & David, 2013). Thus, analyzing the results in the Table 2, it is possible to observe that, in all samples, the system showed suitable values of k', displaying no significant difference between them ($p = 0.975$). These results indicate a good capacity factor of CAR, either of in the standard or in different samples (CO, EM and SEDDS).

Table 2 - Suitability parameters of system for the HPLC method: capacity factor (k'); repeatability; relative retention (α); resolution (Rs); tailing factor (T); number of theoretical plates (N); height of the theoretical plate (H).

Parameters	**CAR**	**CO**	**EM**	**SEDDS**
k' (dimensionless)	2.87 ± 0.01	2.88 ± 0.08	2.87 ± 0.02	2.87 ± 0.02
Repeatability (%)[a]	0.12	0.29	0.05	0.03
α (dimensionless)	-	1.13 ± 0.003	1.13 ± 0.0004	1.13 ± 0.0004
Rs (dimensionless)	-	0.88 ± 0,02	1.74 ± 0.04	1.78 ± 0.05
T (dimensionless)	1.37 ± 0,02	1.30 ± 0.02	0.91 ± 0.02	0.89 ± 0.03
N (dimensionless)	8755.17 ± 240.49	987.79 ± 91.36	4535.18 ± 265.96	4853.63 ± 421.00
H (mm)	0.03 ± 0.001	0.25 ± 0.02	0.06 ± 0.003	0.01 ± 0.0004

[a]Results expressed as relative standard deviation (RSD, %). Source: Authors.

The retention time is one of the main factors used to identify the compound, being mainly dependent of the flow and composition of the mobile phase and stationary phase (Ornaf & Dong, 2005). CAR is one of the main terpenes in CO (Borges et al., 2013; Souza et al., 2011). Therefore, the repeatability was determined in relation to CAR retention time (around 14 min) in the developed method. According to FDA's Chromatographic Methods Validation guide, the HPLC system must show relative standard deviation (RSD) with less than 1% in at least five replicates for the system to have good repeatability. According to the Table 2, it was possible to observe that the CAR standard and the samples (CO, EM and SEDDS) displayed suitable repeatability (RSD < 1%) in relation the CAR retention time.

The tailing factor (T) indicates whether the peak is symmetrical in the method developed. Ideally, the peaks should be symmetrical, with no peak fronting and peak tailing (Ornaf & Dong, 2005). The peak fronting is usually resulting to strong adsorption and/or interaction of the compound with stationary phase, while the peak tailing are caused by column overloading, chemical reactions or isomerization of compounds during the analysis (Ornaf & Dong, 2005). Therefore, FDA's Chromatographic Methods Validation guide indicates T values ≤ 2.0 as being ideal (FDA, 1994). All samples (CO, EM and SEDDS) and the standard CAR showed the symmetry of the CAR peak within the interval defined (Table 2).

The column efficiency can be measured through the theoretical plate number (N) and the height equivalent to these theoretical plates (H) (Ornaf & Dong, 2005). These parameters are efficiency column quantity measures, being related to mainly the diameter of silica particles in column packing, as well as the extent of mobile phase flow caused by column length (Ornaf & Dong, 2005). Thus, the higher theoretical plate number and lower height equivalent to these theoretical plates, better the efficiency of separation of compounds (Ornaf & Dong, 2005).

It was also possible to observe that the CAR standard and the emulsion systems (EM and SEDDS) displayed theoretical plate number higher than 2000 (Table 2), as usually have been found in HPLC systems (FDA, 1994). However, only in the CO samples showed a decrease of the theoretical plate number, indicating a tendency to a lower separation compound efficiency in that sample. The same was observed for at height of theoretical plates. CAR standard and the emulsion system displayed the lower heights, while the CO sample, as consequence of theoretical plate number decrease, showed higher of theoretical plate values (Table 2).

A complete separation occurs due to the different retention time of the compounds, caused by the different interactions with the mobile phase and stationary phase (Ornaf & Dong, 2005). Thus, the relative retention (α) of the HPLC system is calculated as the elution difference between the compounds closer next to each other, being considered a good peaks separation if relative retention values are greater than 1.0 (Ornaf & Dong, 2005). Therefore, according to relative retention (α)

(Table 2), the HPLC system showed good peak separation ($\alpha > 1$), in all samples (CO, EM and SEDDS), indicating that the method developed could efficiently separate the compounds, even when analyze the CAR indifferent samples.

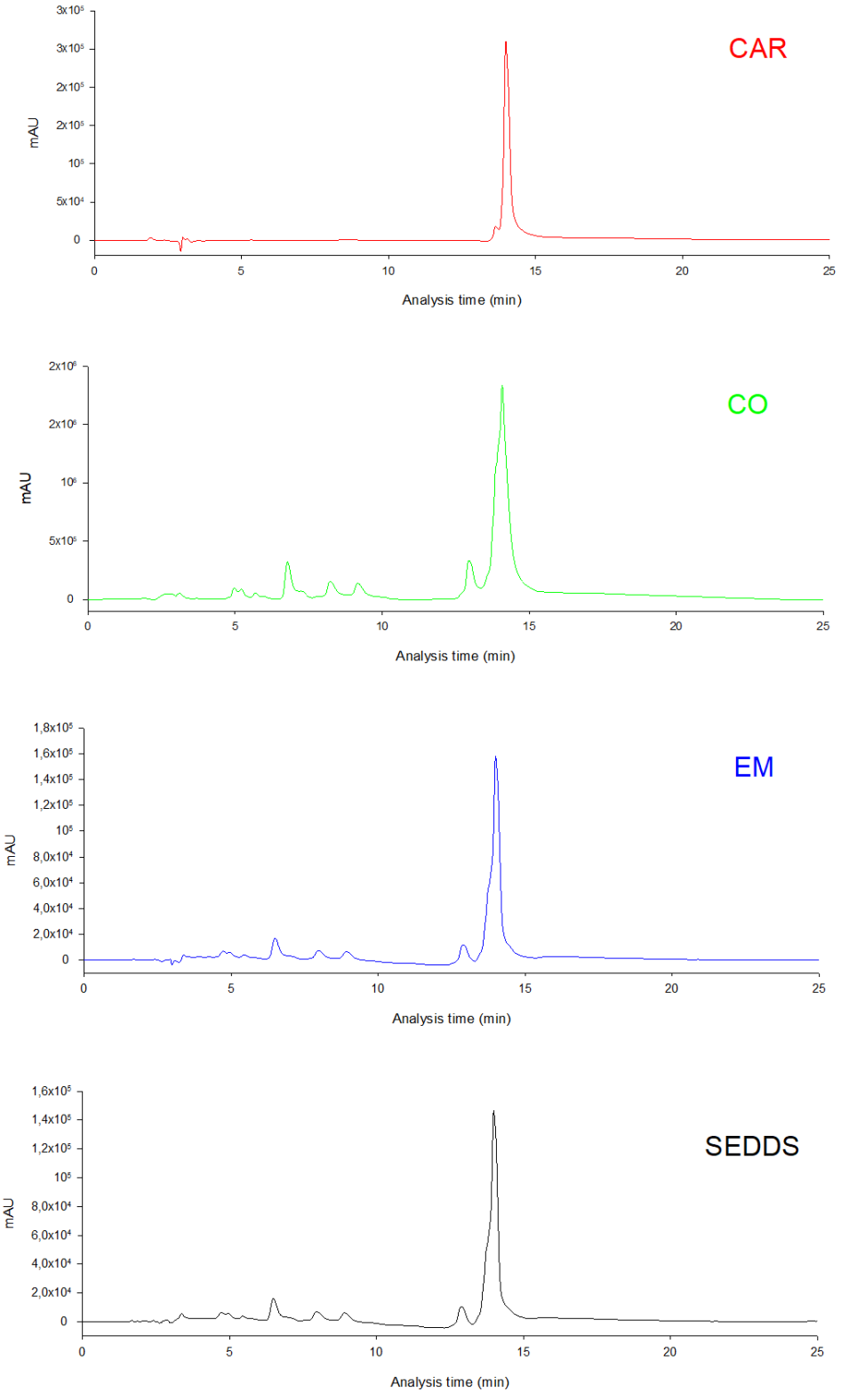
Resolution is another parameter involving the peak separation efficiency. It represents the degree of separation between two adjacent peaks in the HPLC system, being considered a satisfactory separation when the resolution is greater than 1.5 or sometimes greater than 1.0, depending on the analysis (Moldoveanu & David, 2013; Ornaf & Dong, 2005). The emulsion systems (EM and SEDDS) showed good separation of adjacent peaks, without significant difference between them ($p = 0.530$), while the CO sample demonstrated a lower resolution. These results are according to the theoretical plate number and its height of these theoretical plates previously discussed, since these parameters are related to system separation capacity. Therefore, the developed method showed a system with good compound separation capacity in relation to emulsion systems, and it is an appropriate method for determination of CAR in formulations containing CO.

3.3 Validation of HPLC method

When an analytical method is developed, its validation must determine if the methodology can generate reliable and adequate results (ANVISA, 2017). Thus, for the validation of HPLC method, the specificity, linearity, detection and quantitation limits, precision, accuracy and robustness have been were evaluated.

The specificity demonstrates the ability of method developed to identify or quantify the compound of interest in the presence of impurities, diluents and others components that can be in the sample (ANVISA, 2017; ICH, 2005). Despite the wavelength utilized ($\lambda = 210$ nm) is in an absorption region common for many substances, it is very utilized for chromatographic analysis of compounds like CAR (Bardají et al., 2016; Borges et al., 2013; da Trindade et al., 2018; Romero, 2007; Souza et al., 2011). The specificity of the proposed method is displayed in Figure 2.

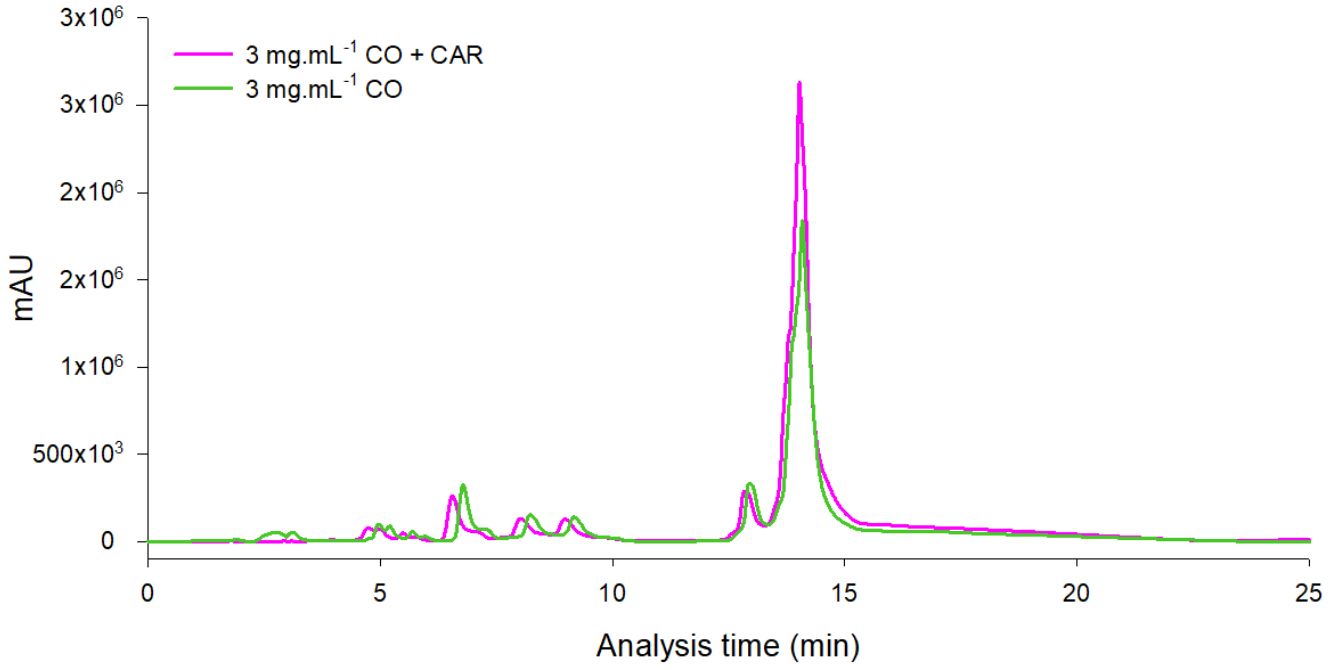
Figure 2. High-performance liquid chromatography chromatograms at 210 nm showing the specificity of the proposed method: (CAR) β-caryophyllene; (CO) copaiba oil-resin; (EM) emulsion system; (SEDDS) self-emulsifying drug delivery system.



Source: Authors.

CAR standard showed retention time of 14 ± 0.03 min, having the peaks similar in one sample to another. The method showed the capacity to separate and identify the component CAR, even in different samples, with different structure and components. Moreover, to confirm the retention time and selectivity of the method, CAR standard was added to the CO samples, resulting in an increase of the CAR peak area in relation those of CO samples without CAR addition (Figure 3).

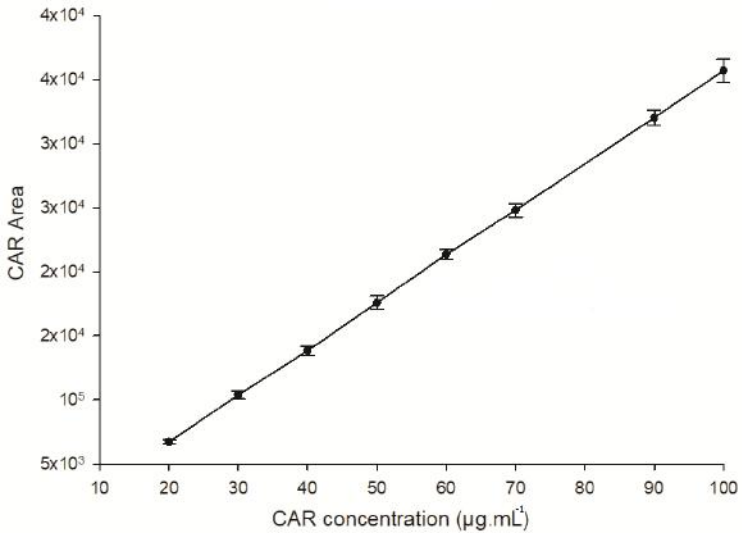
Figure 3. High-performance liquid chromatography chromatograms at 210 nm of copaiba oil-resin (CO) and copaiba oil-resin (CO) added with β-caryophyllene internal standard (CAR).



Source: Authors.

The linearity of analytical methods is demonstrated through the method capacity to obtain results directly proportional to the concentration of analyte in the sample (ICH, 2005). Thus, a graphical representation of the responses in accordance of concentration (analytical curve) was done (Figure 4), obtaining the linear equation y = 36222x - 53443. For this, the interval between the upper and lower concentrations (range) has been determined as from 20 to 100 µg.mL^{-1}.

Figure 4. Analytical curve of β-caryophyllene standard obtained by high-performance liquid chromatography at wavelength (λ) 210 nm.



Source: Authors.

The determination (r^2) and correlation coefficient (r) of regression equation was 0.9975 and 0.9987, respectively, showed a good fit of equation. Proving this was the results of analysis of variance (ANOVA) that have indicated a significant regression (F_{value} has been higher than F_{tab}), while the lack of fit has demonstrated not significant (F_{value} has been lower than F_{tab}), as showed in the Table 3. Therefore, the regression equation has showed to be suitable in representing the CAR concentrations as function of area. Consequently, it could be used to quantify the CAR in different samples.

Table 3 - ANOVA results for linearity: significance of regression and lack of fit analysis.

Parameter	DOF	SQ	MS	F_{value}	F_{tab}
Regression	1	$3.641e^{+13}$	$3.641e^{+13}$	15406.009	4.098
Residual	38	89803751324	2363256614		
Lack of fit	6	2036693084	339448847.4	0.124	2.399
Pure error	32	87767058240	2742720570		

DOF = Degree of freedom; SQ = Sum of square; MS = Mean square. Source: Authors.

The determination of LD and LQ indicates the sensitivity of the method developed. LD is the lowest amount of analyte that can be detected, while the LQ is the lowest amount of analyte that can be quantified (ANVISA, 2017; ICH, 2005). This determination can be performed through analysis of curve parameters (ANVISA, 2017). The values of CAR LD and LQ were 1.681 µg.mL^{-1} and 5.095 µg.mL^{-1}, respectively.

The precision of an analytical method evaluate the proximity among the results, being expressed by the repeatability and intermediate precision (ICH, 2005). The repeatability indicates the method precision under same operating conditions by a short period of time (same day, for example), while the intermediate precision indicates the method precision by some variations, as different days and different analysts (ANVISA, 2017; ICH, 2005). The results showed low relative standard deviation (less than 5%). Also, the statistical analysis have indicated a not significant difference ($p = 0.627$) between the precision intra-day (analysis have done in the same day), inter-day (analysis have done in different days) and inter-analysts

(analysis have done by different analyst), indicating that even when there are some variations in the analysis, the results do not have difference.

The accuracy is determined through the agreement between the results obtained by the analytical method in relation to a value accepted as reference (ICH, 2005). The determination of method accuracy was determined by recovery considering the relation of experimental CAR concentration to theoretical CAR concentration (Table 4). The recovery values were acceptable, since the results were within the range from 80 to 120% (ICH, 2005), and the method developed has shown to be accurate.

Table 4 - Recovery results for method accuracy analysis.

CAR concentration ($\mu g.mL^{-1}$)	**Experimental concentration ($\mu g.mL^{-1}$)**	**Theoretical concentration ($\mu g.mL^{-1}$)**	**Recovery (%)**
50	53.0784 ± 0.4624	50.1760 ± 0.3168	106.5391 ± 1.1124
60	63.4325 ± 0.4035	60.2112 ± 0.3801	106.1073 ± 1.6413
70	73.5786 ± 0.7810	70.2464 ± 0.4435	105.4878 ± 0.8205

Source: Authors.

The robustness of an analytical method is the method capacity to withstand small variations of some parameters (ICH, 2005). Thus, the chromatography parameters chosen were flow, oven temperature and wavelength for detection.

In relation to the wavelength, the method developed was not robust as can be observed in the Table 5 with a significant difference ($p < 0.05$) between the results. Due to $\lambda = 210$ is a region common to several substances, small variations of this parameter cause difference in the CAR analysis. Changing the flow of the method developed, a significant difference ($p < 0.05$) was also observed between the results (Table 5). The HPLC flow is an important factor in the separation during the analysis. It can occur wide peak with a decrease flow and an incomplete separation in larger flow. This way, the analytical methodology has not shown robustness about the flow. The method developed has been robust only about the oven temperature, where was not observed significant difference ($p > 0.05$) between the results, changing the temperature from 20 to 24 °C. This parameter is important to the CAR and others CO compounds due to be volatiles substances. This way, the method developed has shown robustness about the oven temperature, being detached in relation others HPLC methods to CAR analysis (Borges et al., 2013; Shah et al., 2020) that did not report robustness about this parameter during their studies.

Table 5 - Determination of CAR concentrations relative standard deviation and p-value between variations of wavelengths (λ = 205, 208, 209, 210, 211, 212 and 215), flow (0.9, 1.0 and 1.1 mL.min^{-1}) and oven temperature (20, 22 and 24 °C).

Wavelengths λ = 205, 210 and 215 nm			
CAR (µg.mL^{-1})	CAR concentration (µg/mL)	Relative standard deviation (%)	P-value
50	50.3380 ± 12.4175	24.67	6.6 x 10^{-9}
60	58.9346 ± 14.6609	24.88	3.1 x 10^{-9}
70	68.9220 ± 17.2723	25.06	3.2 x 10^{-10}
Wavelengths λ = 208, 210 and 212 nm			
CAR (µg.mL^{-1})	CAR concentration (µg/mL)	Relative standard deviation (%)	P-value
50	51.0799 ± 5.1172	10.02	2.4 x 10^{-6}
60	59.6987 ± 6.0663	10.16	1.1 x 10^{-7}
70	69.7344 ± 7.0842	10.16	1.4 x 10^{-7}
Wavelengths λ = 209, 210 and 211 nm			
CAR (µg.mL^{-1})	CAR concentration (µg/mL)	Relative standard deviation (%)	P-value
50	50.5392± 2.4348	4.82	0.00015
60	59.8843 ± 2.6665	4.45	1.1 x 10^{-8}
70	69.7349 ±3.3173	4.76	1.2 x 10^{-5}
Flow 0.9, 1.0 and 1.1 mL.min^{-1}			
CAR (µg.mL^{-1})	CAR concentration (µg/mL)	Relative standard deviation (%)	P-value
50	42.5620 ± 9.8346	23.11	0.0583
60	53.3443 ± 7.3368	13.75	0.0493
70	63.2617 ± 6.1063	9.65	0.0055
Oven temperature 20, 22 and 24 °C			
CAR (µg.mL^{-1})	CAR concentration (µg/mL)	Relative standard deviation (%)	P-value
50	50.1297 ± 2.3002	4.59	0.1201
60	59.6770 ± 2.4571	4.12	0.1982
70	69.5941 ± 2.2234	3.20	0.1132

Source: Authors.

3.4 Applicability of HPLC method

The method applicability was shown analyzing the CAR content in different samples as CO (*Copaifera reticulata* Ducke) and emulsion formulations containing CO (EM and SEDDS). Analyzing the CAR amount in CO samples, the CAR concentration has found was 42.43 ± 0.97%. Furthermore, as it can be observed in the chromatograms of Figure 2, the CAR peak is the one that contains the greatest intensity and area. So, it is possible to infer that CAR is the major compound found in the CO samples used in this study.

Usually, CO is rich in sesquiterpenes and frequently is reported that the CAR is one of major components (da Trindade et al., 2018). In relation to *Copaifera reticulata* Ducke, Veiga Junior and collaborators (Veiga Junior et al., 2007) found 40.9% of CAR content in their CO sample. Herrero-Jáuregui and colleagues also reported CO samples of *Copaifera reticulata* with 43.4 % of CAR content (Herrero-Jáuregui et al., 2011). Guimarães-Santos and researchers evaluated CO samples of *Copaifera reticulata* Ducke with 37.3% of CAR content (Guimarães-Santos et al., 2012).

Although the previous publications, such as the afore mentioned, reported the CAR content by GC analysis, this present work has shown a CAR amount percentage in accordance with the related about *Copaifera reticulata* Ducke. Indicating that the method developed by HPLC in this study could quantify one the most found volatile compound present in the CO samples, the CAR, in addition the method showed robustness about the oven temperature (20 – 24 °C).

Further, analyzing the CAR content and CAR trapping efficiency (TE) of formulations containing CO (EM and SEDDS), it was possible to observe that the emulsion systems showed good TE results, indicating their capacity to trapping the CAR in the systems. The EM displayed 3.99 ± 0.18 % (w.w-1) of CAR content, with a recovery of 94.04 ± 4.18 %, and the SEDDS displayed 3.76 ± 0.11 (w.w-1) of CAR amount, and a recovery of 88.62 ± 2.70 %.

In addition, compared to another HPLC method for CAR analysis in CO samples (Borges et al., 2013), the method developed in this work has an additional benefit that of not using phosphate buffer as part of mobile phase. The use of buffer in the liquid chromatographic methods can cause precipitation in the column, reducing the column stability (Moldoveanu & David, 2017). Also, the method developed in this study has shown a lower CAR retention time compared to the optimized HPLC methods of other studies (Borges et al., 2013; Shah et al., 2020), causing a smaller analysis time and use of low quantity of mobile phase by time.

4. Conclusion

The proposed HPLC method utilized as marker one of most found CO compounds, the sesquiterpene CAR, and it has been the majority analyte (around 42%) reported in the CO (*Copaifera reticulata* Ducke) samples used in this study. This CAR amount found was in accordance with others studies performed using GC methods for CAR analysis in CO samples, demonstrating that the developed HPLC method can determine the CAR efficiently. In addition, this method could also be utilized for the analysis of CAR in emulsion systems (an emulsion and a self-emulsifying drug delivery system) containing CO, which showed a good trapping efficiency. Furthermore, the developed method showed suitable values of the system suitability parameters (capacity factor, repeatability in relation of retention time, relative retention, resolution, tailing factor, theoretical plate number and height of the theoretical plate), indicating a good separation of the components in the CO samples and in complex formulations (emulsion systems) containing CO. The method demonstrated good CAR selectivity, linearity, accuracy, precision and robustness in relation to temperature of analysis. It alo showed advantages in relation to other HPLC methods for CAR analysis, such as the absence of buffer in the mobile phase and the lower CAR retention time. Emphasizing that HPLC method developed showed robustness in relation to oven temperature, being an important parameter to volatile compounds as CO samples. Therefore, this reversed-phase liquid chromatographic method showed to be valid, versatile and applicable for CAR analysis in CO samples and in samples with different and complex matrices, such as emulsion systems.

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