Journal of Pharmaceutical and Biomedical Analysis 54 (2011) 653–659

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils

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ARTICLE INFO

Article history:
Received 14 August 2010
Received in revised form 6 October 2010
Accepted 7 October 2010
Available online 16 October 2010

Keywords:
Copaifera species
Oleoresins
Sesquiterpenes
Validated analytical method

ABSTRACT

Copaifera species (Leguminoseae) are popularly known as "copaiba" or "copaíva". The oleoresins obtained from the trunk of these species have been extensively used in folk medicine and are commercialized in Brazil as crude oil and in several pharmaceutical and cosmetic products. This work reports a complete validated method for the quantification of β-caryophyllene, α-copaene, and α-humulene in distinct copaiba oleoresins available commercially. Thus, essential oil samples (100 μL) were dissolved in 20 mL of hexanes containing internal standard (1,2,4,5-tetramethylbenzene, 3.0 mM) in a 25 mL glass flask. A 1 μL aliquot was injected into the GC-FID system. A fused-silica capillary column HP-5, coated with 5% phenylmethylsiloxane was used for this study. The developed method gave a good detection response with linearity in the range of 0.10–18.74 mM. Limits of detection and quantitation variety ranged between 0.003 and 0.091 mM. β-Caryophyllene, α-copaene, and α-humulene were recovered in a range from 74.71% to 88.31%, displaying RSD lower than 10% and relative errors between −11.69% and −25.30%. Therefore, this method could be considered as an analytical tool for the quality control of different *Copaifera* oil samples and its products in both cosmetic and pharmaceutical companies.

1. Introduction

The production of medicines requires the development of well-validated analytical methods to ensure its quality, as well as its safety and efficacy within different batches. For that, the analytical test method validation is completed to ensure that an analytical methodology is selective, accurate, reproducible, and robust over the specified range in which an analyte is analyzed [1]. For method validation, guidelines from the regulatory agencies provide a framework to perform such validations [2]. Essential criteria for the quality of natural compounds, pharmaceuticals, cosmetics, foods, and other products are ensured by method validation. Regarding phytotherapeutic agents development, a validated analytical method capable of analyzing natural complex matrices is required throughout all steps, which includes: the selection of a good plant cultivar and the determination of the best time of harvesting; the determination of extraction conditions and drying process; the development of an adequate formulation able to deliver the active compounds; the quantification of analytes during the production processes; the analyses of the final product and determination of its shelf life; and the quantification of the active compounds in biological matrices to follow up both pre-clinical and clinical assays, among others.

Copaifera species (Leguminoseae) are popularly known as "copaiba" or "copaíva", which grow mainly in the states of Amazonas, Pará and Ceará in Northern of Brazil. It can reach 5 to 40 meters in height and can live up to 400 years [3]. The oleoresins obtained from the trunk of *Copaifera* species have been extensively used in folk medicine as anti-inflammatory [4], anticancer [5], antioxidant [6], antihelmintic [7], repellent of insect [8], antimicrobial [9], antulcer [10], antitetanus [11], urinary antiseptic [11], as well as to treat gonorrhea, syphilis, bronchitis, skin diseases and wounds [11]. Veiga et al. [12] reported the chemical composition and anti-inflammatory activity of copaiba oils from three copaiba species. Copaiba oleoresins are largely used in alternative medicine, as a dietary supplement, in the production of flavoring agents, food additives, and it is extensively commercialized in Brazil as crude oil and in several pharmaceutical and cosmetic products, such as capsules, shampoos, soaps, capillary lotions and bathing foams [13].

Important studies on the chemical and biological characterization of *Copaifera* species have been reported [3–5,11,12]. *Copaifera* oleoresins are composed of a high amount of hydrocarbon

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doi:10.1016/j.jpba.2010.10.006

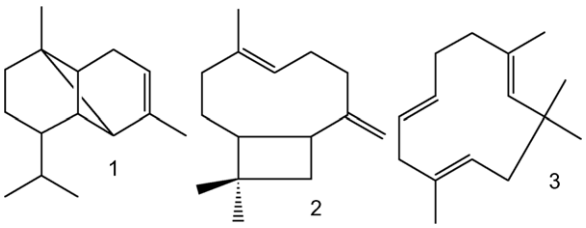

Fig. 1. Chemical structures of 1: α-copaene, 2: β-caryophyllene, and 3: α-humulene.

sesquiterpenes, contributing to almost 90% of the total oleoresins composition, followed by a small amount of diterpenes. The main constituent is β-caryophyllene, which is found as one of the most abundant sesquiterpene in copaiba oleoresins and appears to be ubiquitous in angiosperms [14]. β-Caryophyllene is a bi-cyclic hydrocarbon sesquiterpene [15] that occurs in essential oils of several plants, including *Piper nigrum* L. (Piperaceae) [16], *Baccharis* spp. (Asteraceae) [17] and *Copaifera* spp. (Leguminoseae) [12]. A variety of biological activities, including anti-carcinogenic [18], anti-inflammatory [19], and antioxidant [20] have been related to this hydrocarbon sesquiterpene. Moreover, due to its woody and spicy odor, β-caryophyllene is commonly used as a flavoring agent [21].

It is important to point out that β-caryophyllene, α-copaene, and α-humulene (Fig. 1) appear to be the chemical markers in *Copaifera* volatile oils [14], and over the last years, there has been a growing interest in these sesquiterpenes. Hence, a large diversity of studies involving β-caryophyllene, α-copaene, and α-humulene have been reported, demonstrating their high potential as anti-inflammatory [22], anti-allergic [23], antimicrobial [24], insecticidal [24], and anti-plasmodial [25].

Taking into account the importance of the copaiba oleoresins for the development of new natural products, and its large use in folk medicine and pharmaceutical and cosmetic industries, as well as the lack of validated analytical methods to accurately quantify these compounds in copaiba oleoresin raw material and its products, we report a complete validated method by gas chromatography for the quantitation of these three major sesquiterpenes: β-caryophyllene, α-copaene, and α-humulene, in distinct copaiba oleoresins commercially available.

2. Material and methods

2.1. Copaiba oils, reagents and standards

Authentic oleoresin from *Copaifera langdsdorffii* was provided by Apis-Flora Commercial and Industrial. This sample was collected in Ribeirão Preto, São Paulo, Brazil, following the recommendations described by Medeiros and Vieira, [26]. The sample was collected by perforating the trunk of the plant about 1 m above the soil level, by using a metal auger measuring 2.0 cm diameter and 1.5 m of length. After reaching the center of the trunk, the auger was removed, a 100 mL aliquot of the crude oleoresin was collected in a glass flask, and the hole was properly sealed. This sample was stored in a refrigerator (8 °C) and brought to room temperature before use. The plant material was identified by Professor Milton Groppo of the Biology Department of the Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo at Ribeirão Preto, São Paulo state, Brazil. A voucher specimen (SPFR 10120) is deposited at the Herbarium of the same Department. The copaiba oleoresins from Santos Flora Herbal Commercial – LTDA and Vid Amazon Commercial and Industrial (batches: n° 104-08 and 107-08) were bought in the local market of Ribeirão Preto-SP, Brazil.

Hexanes (chromatographic grade) supplied by Mallinckrodt Co. (Xalostoc, Mexico) were used in chromatography studies. Water was purified using Milli-Q-plus filter systems (Millipore, Bedford, MA, USA). The pure synthetic hydrocarbon sesquiterpenes β-caryophyllene, α-copaene, and α-humulene were purchased from Fluka Analytical Co. (St. Louis, MO, USA). The internal 1,2,4,5-tetramethylbenzene-TMB (IS) and secondary octadecane (SS) standards were acquired from Sigma–Aldrich INC. (St. Louis, MO, USA).

2.2. Oil distillation, sample preparation and chromatographic conditions

The copaiba oleoresins samples (2 mL) were poured into 250 mL of water in a 1 L round-bottom flask, and were submitted to hydrodistillation for 30 min using a Clevenger-type apparatus [27]. This procedure was undertaken in triplicate, and the mean yield of volatile oil was measured. The obtained volatile oil was conditioned in hermetically sealed glass containers with rubber lids, covered with aluminum foil to protect the contents from light, and kept under refrigeration at −20 °C until use.

Volatile oil samples (100 μL) were dissolved in 20 mL of hexane containing internal standard (3.0 mM) in a 25 mL glass flask, giving a final concentration of about 4.5 mg/mL of the oil. A 1 μL aliquot was injected into the GC system. GC analysis was carried out in Agilent Technologies GC equipment, model 6890N, equipped with split/splitless injector inlet and a flame ionization detector (FID). The output was recorded using the workstation. A fused-silica capillary column (HP-5, 30 m of length and 0.32 mm internal diameter) coated with 5% phenyl-methylsiloxane (0.25 μm film thickness) was used for this study. Hydrogen at a flow rate of 2.0 mL/min was employed as the carrier gas. The GC oven temperature was programmed from 100 to 140 °C at 10 °C/min, from 140 to 180 °C at 2.5 °C/min, and from 180 to 200 °C at 20 °C/min, finalizing the chromatographic run at 21 min. The temperatures of the injector and detector ports were kept at 240 and 280 °C, respectively. The injector was operated in a split mode of 80:1.

The chemical characterization of the oil samples was also carried out by gas chromatography mass spectrometer. For that, a Shimadzu GC/MS – QP2010 equipped with automatic sampler AOC – 20Si was used under similar conditions described above. All mass spectra were recorded in the scan mode at 70 eV (40–500m/z). β-Caryophyllene, α-copaene, and α-humulene were identified by comparing the retention index (RI relative to C_9–C_{22} *n*-alkanes) with those reported in the literature [28], as well as by comparison of the obtained mass spectra of the peaks with those either reported in the literature [28] or available in the Wiley 7.0 data system library.

2.3. Standard solution

A standard solution containing 2.84 mM of α-copaene, 2.75 mM of β-caryophyllene, 2.50 mM of α-humulene, 3.20 mM of 1,2,4,5-tetramethylbenzene (IS), and 7.00 mM of octadecane (SS) in hexanes was prepared in a 5 mL vial. It was stored in a freezer (−20 °C) and brought to room temperature before use.

2.4. Validation parameters

The selectivity was assessed by comparing the chromatographic profiles of authentic standards in relation to those obtained for the samples, besides the evaluation of the following responses: α separation factor, peak base width, and number of theoretical plate. Linearity was evaluated by calculation of a regression line using the least squares method. The analytical calibration curves were prepared in the concentration range expected for each sesquiterpene in the copaiba oil samples. These curves were obtained from eight different concentrations analyzed in triplicate.

Table 1
Factors and levels investigated in the robustness test.

Factors	Limits	Level (−1)	Level (+1)	Nominal
A Injector temperature (°C)	±5.0	235	245	240
B Detector temperature (°C)	±5.0	275	285	280
C Split	±2.0	78	82	80
D Flow rate (mL/min)	±0.2	1.8	2.2	2.0
E Oven initial temperature (°C)	±5.0	95	105	100
F Oven final temperature (°C)	±5.0	195	205	200
G Flow rate make-up N_2 (mL/min)	±3.0	42	48	45

The limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the response (σ) and on the slope of the calibration curve (S) using the following expressions: LOD = $3.3\sigma/S$ and LOQ = $10\sigma/S$. Precision was assessed by the evaluation of the repeatability and by intermediate precision. The repeatability (intra-day precision) was determined by a set of eight replicate analyses of a given sample in a day, which was carried out by the same technician. The intermediate precision (inter-day precision) was evaluated by a set of eight-fold preparation and analyses of the same sample in different days and by two technicians.

The accuracy and recovery of the method for the determination of the concentration of β-caryophyllene, α-copaene, α-humulene, and TMB (IS) were determined based on the results obtained for five replicates, which were analyzed three times each. Hence, three different concentrations consisting of low, medium and high content of β-caryophyllene, α-copaene, and α-humulene were dissolved in 20 mL of hexanes containing 3.0 mM of IS in all samples. Then, a 2 mL aliquot of each concentration level was submitted to hydrodistillation as described in Section 2.2. After this procedure, a 1 mL aliquot of each sample was transferred to small glass flasks and 500 μL of hexanes solution containing a known concentration (8.7 mM) of octadecane (SS) was added to all samples, just before the GC-FID analysis, allowing the quantification of β-caryophyllene, α-copaene, α-humulene, and TMB (IS).

The robustness of the chromatographic method was performed following the experimental design proposed by Plackett–Burman, which was described by Heyden et al. [29]. Briefly, it should be selected the operational factors that are related to the chromatographic method, such as: injector (A) and detector (B) temperatures, split (C), gas carrier flow rate (D), oven initial (E) and final (F) temperatures, and gas N_2 make up flow rate (G) (Table 1). Then, from nominal method described in Section 2.2, variation levels were determined to each one of these operational factors (Table 1). These levels were represented either as level (−1), when the factors were in the negative limit or (+1), when it were above the positive limit, and divided in 8 experiments, as shown in Plackett–Burman design [29] (Table 2). Each experiment was carried out for the pre-determined −1 and +1 variation limit of each factor. A response y was obtained at the end of each experiment (Table 2). The responses selected for this study were the peak area, retention time, peak height, and concentration of each component of interest detected in *Copaifera* essential oil sample. Upon the obtained eight responses, the estimated effect was calculated for each factor by taking into account the selected responses. To this end, the following equation was used:

$$E_x = \frac{\sum y(+)}{N/2} - \frac{\sum y(-)}{N/2}$$

where E is the estimated effect of the selected response x; x is the peak area, retention time, peak height, or component concentration; $\Sigma y(+)$ is the sum of responses at a positive level; $\Sigma y(-)$ is the sum of responses at a negative level; N is the number of experiments. To improve the interpretation of the results for the robustness study, the estimated effect E_x was converted using the equation RSD (%) = $(S/X)\cdot 100$ in which RSD % is the relative standard deviation, S is the value of E_x and X represents the mean of the responses of y, considering the different responses and factors.

Table 2
Plackett–Burman design for 7 factors and 8 experiments [29].

Factors	A	B	C	D	E	F	G	Response
Experiment N°								
1	+1	+1	+1	−1	+1	−1	−1	y_1
2	−1	+1	+1	+1	−1	+1	−1	y_2
3	−1	−1	+1	+1	+1	−1	+1	y_3
4	+1	−1	−1	+1	+1	+1	−1	y_4
5	−1	+1	−1	−1	+1	+1	+1	y_5
6	+1	−1	+1	−1	−1	+1	+1	y_6
7	+1	+1	−1	+1	−1	−1	+1	y_7
8	−1	−1	−1	−1	−1	−1	−1	y_8

3. Results and discussion

3.1. Chromatography and selectivity

GC-FID and GC/MS have been extensively used in the determination of essential oils, because of their advantages such as high efficiency and speed properties [30]. Recently, the validation of a GC-FID analytical method for the study of semiochemicals, E-β-farnesene and β-caryophyllene in slow release formulations was described [31]. *Copaifera langsdorffii* seeds and seed oil were characterized by GC-FID [32]. Veiga at al. [12] reported the same major compounds reported in this work in the copaiba oleoresins obtained from *Copaifera multijuga*, *Copaifera cearensis* and *Copaifera reticulate*, and they found that the major compound was β-caryophyllene, followed by α-humulene and α-copaene, with different amounts in each oleoresin.

Hydrodistillation using the Clevenger apparatus is the official AOAC method for the analysis of volatile oils from spices [33], and several researchers have used this technique to obtain volatile oils from different plant sources [27]. During the sample preparation studies, it was observed that the amount of volatile oil obtained from the copaiba oleoresin samples were quite significant, yielding between 30% and 40% of the total crude oil. Thus, the hydrodistillation extraction procedure for samples preparation was optimized using a representative aliquot of copaiba oleoresin (2 mL) in 250 mL of water, and distillation for 30 min. To get all the volatile fractions of the oleoresins and its replicates, a set of four Clevenger apparatus were set up for this purpose. The mean yields obtained for volatile fractions of the oleoresins from Apis Flora, Santos Flora Herbal Commercial and Vid Amazon Commercial and Industrial (batches: n° 104-08 and 107-08) were 0.8, 0.7, 0.7 and 0.6 mL, respectively. These versatile and rapid parameters, along with the low cost of the entire procedure are important features for the routinely analysis copaifera oleoresin and its products.

β-Caryophyllene, α-copaene, and α-humulene are non-polar compounds and, therefore, a non-polar solvent, hexanes, was selected as the diluting agent. 1,2,4,5-Tetramethylbenzene – TMB, octadecane, piperonal, veratraldehyde, trimethoxybenzene and benzophenone were also evaluated as possible internal and secondary standards. The chosen IS (TMB) and secondary standard (octadecane) met all the necessary requirements, considering its both retention times, which did not interfere with sample components, and detector response. The boiling point mean of the three hydrocarbon sesquiterpenes studied is 145 °C. Thus, the injector and detector temperatures, as well as the oven non-linear thermal program were properly developed with the purpose to achieve a baseline separation of the main compounds in a single run. Also, three non-polar capillary columns, HP – 1 coated with 100% of dimethylpolysiloxane, HP – 5 and DB – 5, both coated with

Table 3
Analytical parameters of selectivity, linearity, LOD and LOQ.

	TMB (IS)	α-Copaene	β-Caryophyllene	α-Humulene	Octadecane (SS)
Selectivity ($n = 5$)					
$\alpha \pm$ SD; RSD (%)	1.97 ± 0.004; 0.20	1.20 ± 0.001; 0.12	1.20 ± 0.001; 0.12	1.10 ± 0.001; 0.08	2.25 ± 0.013; 0.60
$W_b \pm$ SD; RSD (%)	0.02 ± 0.0005; 2.94	0.03 ± 0.0004; 1.38	0.03 ± 0.0005; 1.59	0.03 ± 0.0009; 2.70	0.06 ± 0.0021; 3.35
$N \pm$ SD; RSD (%)	443,005 ± 26,813; 6.05	656,321 ± 21,481; 3.27	652,877 ± 21,306; 3.26	675,429 ± 33,039; 4.90	1,012,812 ± 68,772; 6.80
Linearity ($n = 3$)					
LDR (mM)	0.10–9.80	0.15–7.66	0.78–18.74	0.13–6.63	0.10–10.53
LRE*	$y = 4{,}670{,}247x + 227{,}859$	$y = 6{,}790{,}941x + 25{,}593$	$y = 6{,}734{,}146x + 1{,}919{,}905$	$y = 7{,}269{,}075x + 189{,}331$	$y = 9{,}254{,}815x + 700{,}311$
r^2	0.9995	0.9993	0.9994	0.9994	0.9995
n	12	11	8	11	9
LOD, LOQ ($n = 3$)					
LDR (mM)	0.05–0.20	0.04–0.15	0.04–0.18	0.06–0.22	0.07–1.26
LRE*	$y = 4{,}789{,}101x + 5158$	$y = 6{,}692{,}892x + 21{,}309$	$y = 8{,}543{,}001x - 20{,}571$	$y = 8{,}433{,}519x - 65{,}679$	$y = 8{,}924{,}838x + 449{,}404$
r^2	0.9993	0.9995	0.9979	0.9992	0.9986
n	4	4	5	5	5
LOD (mM)	0.024	0.009	0.003	0.030	0.028
LOQ (mM)	0.074	0.028	0.008	0.091	0.085
RSD** (%)	8.82	6.30	5.75	5.33	7.40

α separation factor, N: number of theoretical plates, W_b: peak base width, IS: internal standard, SS: secondary standard, LDR: linear dynamic range; LRE: linear regression equation, *linear regression analysis with a regression equation of $y = ax + b$, in which x is the concentration in mM and y is the peak area, n: number of points (mM) of each calibration curve, LOD: limit of detection and LOQ: limit of quantitation. **Relative standard deviation considering LOQ values.

5% phenyl-methylsiloxane were assessed. The best resolution of peaks, which allowed the quantitation of the main volatile compounds of copaiba oleoresin was achieved according to Section 2.2, and within 21 min of run time. It is important to point out that the method was validated using an Agilent Technologies GC-FID equipment, and that the same chromatographic parameters were also applied for the Shimadzu GC/MS equipment, using a DB – 5 capillary column, furnishing good results for separation, detection and precision. In addition, considering the GC/MS data, the retention indexes calculated for β-caryophyllene, α-copaene, and α-humulene were correspondent to 1416, 1372 and 1450, respectively. These three sesquiterpenes accounted for 87% of the volatile constituents. β-Caryophyllene (60%) was the major compound, followed by α-copaene (17%) and α-humulene (10%). Therefore, the parameters validation described herein presented good reproduction response, since it could be applied to different GC equipments.

The selectivity of the method was determined by comparing the chromatographic profile of the standards with the one obtained for the samples. Taking into account the chromatographic profile of the standards, the relative standard deviations (RSD %) for α separation factor, peak base width and number of theoretical plates were calculated (Table 3). These results displayed great selectivity, since the maximum RSD was 6.80%. The chromatogram of the standards solution is shown in Fig. 2. The base line separation was obtained for all compounds, including both internal (TMB) and secondary (octadecane) standards. The chromatographic profiles of the volatile fractions of the copaiba oil samples are shown in Fig. 3A–D, in which a base line separation of the main sesquiter-

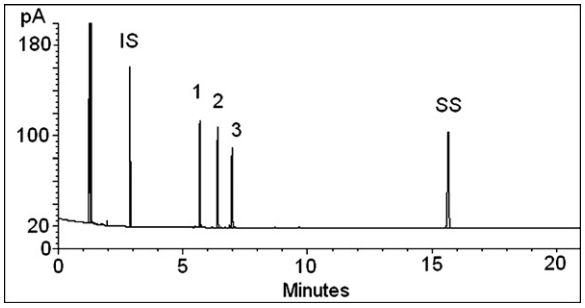

Fig. 2. Chromatographic profiles by GC-FID of the standard compounds 1,2,4,5-tetramethylbenzene: TMB (IS: internal standard), 1: α-copaene, 2: β-caryophyllene, 3: α-humulene, and octadecane (SS: secondary standard).

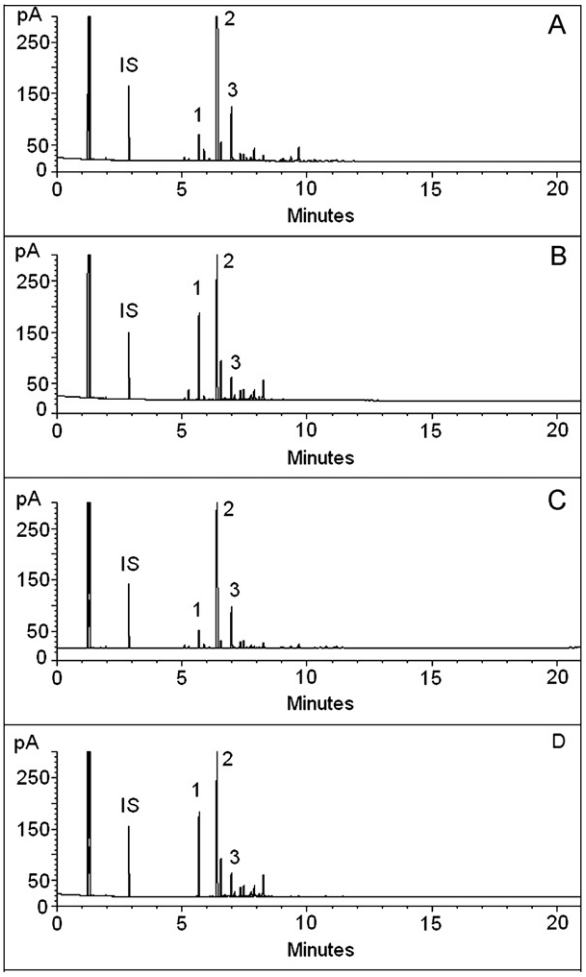

Fig. 3. Chromatographic profiles by GC-FID of three copaiba essential oil samples. (A) Authentic *Copaifera langsdorffii* oil from Apis Flora, (B) Vid Amazon Commercial and Industrial batch n° 104-08, (C) Santos Flora Herbal Commercial, and (D) Vid Amazon Commercial and Industrial batch n° 107-08. IS = 1,2,4,5-tetramethylbenzene: TMB, 1: α-copaene, 2: β-caryophyllene, and 3: α-humulene.

Table 4
Results for precision, accuracy and recovery.

Precision ($n = 8$)	RT	RSD (%)	Peak area	RSD (%)	Conc.	RSD (%)
Repeatability						
α-Copaene	5.7 ± 0.006	0.10	30,430,458 ± 1,617,189	5.29	4.25 ± 0.21	4.95
β-Caryophyllene	6.4 ± 0.006	0.09	74,339,513 ± 2,987,134	3.97	10.22 ± 0.50	4.85
α-Humulene	7.0 ± 0.004	0.06	9,798,720 ± 554,883	5.64	1.25 ± 0.07	5.79
Inter-day						
α-Copaene	5.7 ± 0.002	0.03	28,552,746 ± 2,140,886	7.47	4.16 ± 0.22	5.28
β-Caryophyllene	6.4 ± 0.002	0.04	71,437,916 ± 5,313,455	7.50	10.20 ± 0.44	4.37
α-Humulene	7.0 ± 0.003	0.04	9,448,821 ± 710,535	7.55	1.26 ± 0.06	4.56

Accuracy and recovery ($n = 5$)

	TC (mM)	RC (mM)	Rec. (%) ± SD	RSD (%)	Error (%)
α-Copaene					
High	2.20	1.82 ± 0.13	82.92 ± 5.96	7.19	−17.09
Medium	1.10	0.91 ± 0.03	82.86 ± 2.95	3.56	−17.14
Low	0.45	0.39 ± 0.02	86.43 ± 4.71	5.45	−13.57
β-Caryophyllene					
High	11.09	8.30 ± 0.58	74.71 ± 5.21	6.98	−25.30
Medium	4.30	3.40 ± 0.11	79.57 ± 2.47	3.11	−20.42
Low	2.10	1.67 ± 0.10	79.65 ± 4.52	5.68	−20.36
α-Humulene					
High	3.40	3.00 ± 0.28	88.31 ± 8.13	9.21	−11.69
Medium	1.70	1.46 ± 0.07	86.15 ± 3.88	4.51	−13.85
Low	0.70	0.59 ± 0.04	84.47 ± 5.58	6.60	−15.53
TMB (IS)	2.00	2.24 ± 0.40	112.40 ± 5.40	4.81	12.39

RT, retention time (min.); Conc., concentration (mM); TC, theoretical concentration; RC, real concentration; Rec, recovery.

penes β-caryophyllene, α-copaene, and α-humulene was also achieved. In addition, these chromatographic profiles displayed good resolution and reproducibility for the compounds of interest among more than 300 analyses undertook during the development of this method.

3.2. Linearity and limits of detection and quantitation

Table 3 shows that a good linearity response was obtained with the developed method for all compounds used as standards. The quantification of β-caryophyllene, α-copaene, and α-humulene was undertaken by using an IS to develop a simple, sensitive, and reproducible technique. It can be observed in Table 3 that the linear dynamic range was adequate for all the compounds, ranging from 0.10 to 18.74 mM and that the values of r^2 coefficients were higher than 0.999, giving a good linearity response for the developed method.

Limits of detection (LOD) and quantitation (LOQ) were determined considering the draw of the standard analytical curves from 0.04 to 1.26 mM furnishing correlation coefficients between 0.9979 and 0.9995. The results for the LOD and LOQ were between 0.003 and 0.091 mM, and the RSD value for the LOQ was less than 10.00% (Table 3). These values were considered low, which allowed us to assure that this method is capable of not only to quantify all the used standards, but also to detect trace amounts of these sesquiterpenes, either in copaiba oils or in its products.

3.3. Precision, accuracy, and recovery of the method

The precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same sample [31]. Normally, the precision is evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision), which are expressed by relative standard deviations. For repeatability variation, the RSD of the retention time was between 0.06% and 0.10%, the RSD of the peak area were less than 6.00%, and the RSD of the concentration varied between 4.85% and 5.79% (Table 4). For the inter-day variation, the RSD of the retention time, the peak area and concentration were respectively in the range of 0.03–0.04%, 7.47–7.55%, and 4.37–5.28% (Table 4). The results demonstrated that the developed method has a good precision.

With respect to accuracy and recovery studies, the results displayed in Table 4 allow to infer that the used hydrodistillation technique was able to recover β-caryophyllene, α-copaene, and α-humulene in a range from 74.71% to 88.31%, displaying RSD values lower than 10%, and relative errors between −11.69% and −25.30%. Therefore, accuracy and recovery were considered satisfactory. In addition, the use of the secondary standard octadecane allowed the determination of both recovery and accuracy of TMB (IS), which were of 112.40% with RSD of 4.81% and relative error of 12.39%. These results are adequate for the purposes of the developed method.

3.4. Robustness

The robustness test aims to examine sources potentially subject to variations through the evaluation of one or a set of responses inherent to the method. To examine these sources, a number of factors, which are inserted into the validation procedure, are selected and relatively mild variations are deliberated. In general, these variations mean to define the perspective of a given oscillation when the method is performed on instruments of other brands or transferred to another laboratory. Following the methodology described in the robustness parameter, the effects (E_x) were calculated and converted by the RSD % considering each response and all factors.

Assessing the RSD (%) for each response studied, regarding to β-caryophyllene, α-copaene, and α-humulene, the factor detector temperature (B) was sensible in relation to peak area, peak height and concentration responses with mean value of 17%. The split (C) variable presented mean value of 14% regarding the concentration response. Flow rate (D) and oven initial temperature (E) were susceptible as retention time response, displaying means of 12% and 14%, respectively. All other RSD values, evaluating factors and responses were lower than 10%. Taking into account the experimental design developed and the number of variables involved,

Table 5
Quantification of sesquiterpenes in distinct copaiba products ($n = 3$).

Samples	α-Humulene (mM)	RSD (%)	β-Caryophyllene (mM)	RSD (%)	α-Copaene (mM)	RSD (%)
1	1.12 ± 0.03	2.69	19.47 ± 0.53	2.73	2.56 ± 0.07	2.59
2a	4.24 ± 0.06	1.44	10.48 ± 0.17	1.66	1.33 ± 0.03	2.62
2b	4.40 ± 0.05	1.04	10.59 ± 0.15	1.46	1.30 ± 0.03	2.09
3	0.93 ± 0.01	1.40	18.47 ± 0.33	1.79	2.51 ± 0.05	2.03
4	0.82 ± 0.03	3.88	15.74 ± 0.70	4.43	2.05 ± 0.10	5.06
5	0.55 ± 0.01	1.46	6.37 ± 0.10	1.64	0.86 ± 0.02	1.86

1: volatile fraction of the oleoresin from *Copaifera langsdorffii* from Apis Flora; 2a, b: volatile fraction from the oleoresin commercialized by Vid Amazon Commercial and Industrial (batches: no 104-08[a] and 107-08[b]); 3: volatile fraction of the oleoresin commercialized by Santos Flora Herbal Commercial; 4: crude oleoresin from *Copaifera langsdorffii* from Apis Flora; 5: volatile fraction from *Copaifera langsdorffii* leaves.

these data may be acceptable within the limit range of 20% [34]. In the overall assessment of the results obtained during the GC-FID analysis, the developed method shall be considered suitable for use in different labs.

3.5. Analysis of β-caryophyllene, α-copaene, and α-humulene in commercial products

The Brazilian legislation on phytotherapic medicines requires that the products should be standardized chemically and evidence should be presented regarding its both efficacy and safety for human use [35]. The analytical method validation and chemical standardization of the oleoresins of *Copaifera* species is clearly essential if one is to relate chemical composition with biological activity.

In this work, the contents of β-caryophyllene, α-copaene, and α-humulene in the analyzed samples of copaiba oleoresins were determined by using a GC-FID method. The results are listed in Table 5, and the representative chromatograms of these samples are displayed in Fig. 3A–D. The contents of α-copaene, β-caryophyllene and α-humulene in the four commercially analyzed samples were between 0.93 and 4.40 mM, 10.48 and 19.47 mM, and 1.30 and 2.56 mM, respectively. In addition, the quantitation of these sesquiterpenes was also carried out for both the oleoresin obtained by Apis Flora and the volatile oil from *Copaifera langsdorffii* leaves (Table 5), being β-caryophyllene the major compound with contents of 15.74 and 6.37 mM, respectively. All the obtained results regarding to selectivity, linearity, limits of detection and quantitation, precision, accuracy, recovery, as well as robustness parameters, allowed a reliable quantitation of the major volatile compounds in distinct copaiba oil samples.

The development of well-validated method for the analyses of copaiba oil and its products is very important, because there is a lack of validated analytical methods reported in the literature. In this regard, Cascon and Gilbert [35] reported in 2000 the relative quantification of terpenoids in oleoresin of three *Copaifera* species by GC–MS. For that, the crude oleoresins were esterified with diazomethane in ether and were direct analyzed by GC/MS.

It should be also taken into consideration that the production of copaiba oleoresin was greater than 500 tons in 2008, considering only the production of three states of the Amazon region, being the Amazonas state responsible for more than 90% of the production [36]. There is an intense commercialization of it as crude oil, capsules, ointments, soaps, among others in Brazil, as well as these products have been exported to England, France, Germany and United States [3]. Therefore, we consider that the developed method will contribute for the quality assurance of not only the crude oleoresins, but also its products.

4. Conclusion

The developed GC-FID method is simple, reliable, and sensitive for the quantitation of β-caryophyllene, α-copaene and α-humulene in copaiba oleoresin. Acceptable values were obtained for the following validation parameters, such as: selectivity, linearity, LOD, LOQ, precision, accuracy, and recovery. The proposed robustness test allowed the simultaneous assessment of seven variables into the chromatographic method. Thus, this GC-FID method allows the unambiguously quantification of β-caryophyllene, α-copaene, and α-humulene. It should be considered suitable to be used in different labs for the routinely quality control of crude copaiba oleoresins and its products, in both cosmetic and pharmaceutical companies, taking into account not only the commercialization of more than 500 tons of this oleoresin annually, but also the potential for the development of new pharmaceuticals.

Acknowledgements

We thank Apis Flora Commercial and Industrial for kindly providing the oleoresin sample from *Copaifera langsdorffii*. We are thankful to CAPES and FAPESP for financial support (grant FAPESP no. 08/57775-6) and provided fellowships (FAPESP 04/13005-1; FAPESP 06/59893-0). We are also thankful to Professor Milton Groppo (FFCLRP - USP, Brazil) for his assistance in the identification of the plant.

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