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Article

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Development of a Gas Chromatography Method for the Analysis of Copaiba Oil

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Abstract

A rapid, simple, precise and economic method for the quantification of main compounds of copaiba resin and essential oils (*Copaifera langsdorffii* Desf.) by gas chromatography (GC) has been developed and validated. Copaiba essential oil was extracted by hydrodistillation from the copaiba resin. Resin derivatization allowed the identification of diterpenes compounds. A gas chromatography–mass spectroscopy (GC/MS) method was developed to identify compounds composing the copaiba resin and essential oil. Then the GC/MS method was transposed to be used with a flame ionization detector (FID) and validated as a quantitative method. A good correlation between GC/MS and GC/FID was obtained favoring method transposition. The method showed satisfactory sensitivity, specificity, linearity, precision, accuracy, limit of detection and limit of quantitation for β-caryophyllene, α-humulene and caryophyllene oxide analyses in copaiba resin and essential oils. The main compounds identified in copaiba essential oil were β-bisabolene (23.6%), β-caryophyllene (21.7%) and α-bergamotene (20.5%). Copalic acid methyl ester (15.6%), β-bisabolene (12.3%), β-caryophyllene (7.9%), α-bergamotene (7.1%) and labd-8(20)-ene-15,18-dioic acid methyl ester (6.7%) were diterpenes identified from the derivatized copaiba resin. The proposed method is suitable for a reliable separation, identification and quantification of compounds present in copaiba resin and essential oil. It could be proposed as an analytical method for the analysis of copaiba oil fraction in raw and essential oil parent extracts and after they have been incorporate in pharmaceutical formulations.

Introduction

Compounds obtained from vegetable sources are usually complex mixtures of plant's secondary metabolites bearing protective activity against microorganisms and animal predators. They were found to have pharmacological activities motivating their used in folk medicine since ancient times (1, 2). Among natural compounds of interest, different extracts of copaiba oil show biological activities that would be worth to be used in medicine. In folk medicine, the

resin and the essential oil extracts are used for their antibacterial, antifungal, anti-inflammatory, anti-leishmania and anti-cancer activities (3–7). These extracts consist in complex mixtures composed of a majority of diterpenes and sesquiterpenes that gives the wide panel of biological activities (8–11).

The use and development of pharmaceuticals originating from natural sources require analytical methods able to identify and quantify those products in the raw materials and final formulations (13). Several techniques are available to analyze drugs, impurities, intermediates, degradation products, mixtures of compounds, phytoextracts, etc. (14). Among these techniques, chromatography stands out due to its performance for separation, identification and quantification when applied to the analysis of complex products (15–17).

In this context, validated analytical method are needed to accurately quantify the various compounds find in copaiba oil extracts if one want to develop pharmaceutical formulations from these natural oils being able to verify that the composition of the oil extract was not modified by the formulation. Sousa *et al.* have suggested a gas chromatographic (GC) method that they have validated for the quantification of three sesquiterpenes including β-caryophyllene, α-copaene and α-humulene by gas chromatography–flame ionization detection (GC/FID) system (8). The method was suggested to perform quality controlled analysis in distinct commercially available copaiba oleoresins. However, copaiba oil includes many other compounds and fractions which can be identified by gas chromatography–mass spectroscopy (GC/MS) especially if one considers the resin extract.

The aim of the present work was to validate a GC method achieving the separation, the identification and the quantification of components composing both copaiba resin and essential oils. Separation and identification of the main eluted components were achieved by GC/MS. Although GC/MS is the most powerfull method to carry on both qualitative and quantitative analysis of natural compounds of complex composition, the quantitative method was developed using GC/FID that is more generally accessible in laboratories. Thus, one part of the work was aimed to establish a correlation between analyses performed using an apparatus equipped with a MS detector and an apparatus equipped with a FID. In a second part of the work, the validation of the quantitative performance of the method was achieved using standards of β-caryophyllene, α-humulene and caryophyllene oxide with GC/FID according ICH and FDA guidelines. The method was then applied on the absolute quantification of these three components and a semi-quantitative approach based on the official guidelines for the quantitative GC of volatile flavoring substances that have been provided by the Working Group on Methods of Analysis of the International Organization of the Flavor Industry (IOFI) was applied to determine the composition of the other identified components that composed the oil extracts.

Materials and Methods

Materials

Copaiba resin (*Copaifera langsdorffii* Desf.) was obtained from Flores & Ervas (Piracicaba, SP, Brazil). (Trimethylsilyl) diazomethane solution (2.0 M in diethyl ether), β-caryophyllene (purity = 92.4%), α-humulene (purity ≥ 99%), caryophyllene oxide (purity ≥ 99%) and *n*-alkane standard solution (C_8–C_{20}) were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). *n*-Hexane and ethyl acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was obtained from a Millipore purification system (Milli-Q® plus, Millipore, St Quentin en Yvelines, France). All chemicals were of reagent grade and used as received.

Copaiba essential oil extraction

Copaiba essential oil was produced by the hydrodistillation method. About 400 mL of copaiba resin with four times the volume of ultrapure water were placed in a Clevenger-type apparatus for 3 h to extract essential oil. The obtained essential oil extracted was dried with sodium sulfate, filtered through 0.22 µm cellulose membrane (Merck Millipore, Billerica, MA, USA) and stored in borosilicate glass vial at −20°C until further use.

Copaiba resin derivatization

Copaiba resin was submitted to methylation derivatization before GC analysis. These procedures were used to prepare detectable low vapor pressure compounds and thermally stable derivatives (18). Methylation reaction was achieved by diluting 20–30 mg of copaiba resin with 2 mL of ethyl acetate. This mixture was placed in an ice bath and 2 mL of (Trimethylsilyl)diazomethane solution (0.4 M in ethyl acetate) were slowly added. The reaction was allowed to continue for 30 min at a low temperature to prevent evaporation of the diazomethane reagent. After reaction, the solvent was completely evaporated under nitrogen flow. A rather viscous blank residue was obtained. The volume was adjusted to 1.5 mL with ethyl acetate prior analysis by GC. Completeness of copaiba oil derivatization reaction was confirmed by thin layer chromatography (TLC) (see Supporting information S1).

Copaiba oil analysis

Gas chromatography–mass spectrometry

Identification of copaiba resin and essential oil constituents was performed by GC/MS using Hewlett-Packard 6890 gas chromatograph with split/splitless injection port and HP-5975 mass selective detector. The column used was a HP-5MS cross-linked fused silica capillary column (30 m × 0.25 mm × 0.25 µm). (Agilent J&W, Santa Clara, CA, USA). Chromatographic parameters for copaiba resin and essential oil analysis are described in Table I. The injected volume for all samples was 1 µL. The split ratio was 1:25 and the electron ionization system was set at 70 eV. Helium was the carrier gas. Data acquisition and integration were carried out using the MSD ChemStation software. The retention indices from essential oil were determined by co-injection of *n*-alkane standard solutions (C_8–C_{20}), in the same chromatographic conditions. Copaiba resin and essential oil components were identified by comparing their fragmentation pattern with both the National Institute of Standards and Technology (NIST-05) mass spectral library data, retention index from co-injection of *n*-alkane patterns, and analysis of data described in the literature (19). β-caryophyllene, α-humulene and caryophyllene oxide were also used to further confirm and quantify these compounds in the samples.

GC/FID detector

Quantification of volatile constituents were performed using a PR2100 GC/FID instrument with split/splitless injection port (Alpha MOS, Toulouse, France). A fused silica capillary column (25 m × 0.32 mm i.d., 0.5 µm) coated with cross-linked 5% phenyl polysilphenylene-siloxane (SGE Analytical Science Pty Ltd, Victoria, Australia) was used. Chromatographic parameters are described in Table I. The volume

Table I Chromatographic Parameters for Copaiba Resin, Methylated and Essential Oil Analysis by GC

Parameters	GC/MS		GC/FID
	Copaiba resin and methylated oils	Copaiba essential oil	Copaiba resin, methylated and essential oil
Oven initial temperature (°C)	110	60	90
Ramp rate 1 (°C min^{-1})	5	3	2
Oven final temperature 1 (°C)	280	240	150
Ramp rate 2 (°C min^{-1})	5	5	20
Oven final temperature 2 (°C)	300	250	300
Final hold	300°C for 20 min	250°C for 5 min	300°C for 20 min
Injector (°C)	250	220	250
Detector (°C)	300	250	300

injected for all samples was 2.5 µL. The split ratio was 1:80. Nitrogen was the carrier gas at a pressure of 166 kPa. Data acquisition and integration were carried out using Winilab three software. β-Caryophyllene, α-humulene and caryophyllene oxide were selected as the standard for the quantification of the main components presented in the copaiba resin and essential oil.

Validation of the method for quantitative analysis

Validation of the quantification method was achieved using standards of β-caryophyllene, α-humulene and caryophyllene oxide. The ICH (20) and FDA (21) guidelines were followed. All equipments and volumetric glassware were evaluated and calibrated before analysis. The balance (Sartorius MSA-224S-000-DU Cubis Analytical Balance, Elk Grove, USA) was calibrated to minimal measures of 0.1 mg. Specificity, selectivity, linearity range, accuracy, precision, detection and quantification limits were evaluated using the GC/FID method.

Preparation of stock solutions

Three individual stock solutions of β-caryophyllene, α-humulene and caryophyllene oxide were prepared in ethyl acetate at 1 mg mL^{-1}, placed in an amber vial hermetically sealed and kept at −20°C until use. These stock solutions were diluted to obtain the concentrations required for preparation of standard working solutions ranging from 40 to 160 µg mL^{-1} (40, 70, 100, 130 and 160 µg mL^{-1}) and prepared in 1 mL of ethyl acetate.

Specificity and selectivity

The specificity and selectivity of the analytical method were confirmed by injecting solutions containing 100% of the normal working concentration of β-caryophyllene, α-humulene and caryophyllene oxide. The ability to separate all the compounds (related substances, degradation products and excipients) from standard samples was confirmed.

Linearity

Calibration curves for β-caryophyllene, α-humulene and caryophyllene oxide were prepared by injecting standard solutions ranging from 40 to 160 µg mL^{-1} (40, 70, 100, 130 and 160 µg mL^{-1}). Peak area of the standards were individually plotted against the analyte concentrations. Standard calibration curves of the compounds were developed by calculation of the regression line using the least squares method. Linearity curves were performed on three different days.

Determination of the limit of detection and quantification

Limit of detection (LOD) was determined based on the ratio between the standard deviation of the response and the slope estimated from the calibration curve of the standards multiplied by 3.3. The limit of quantitation (LOQ) was determined as the lowest amount of analyte that was reproducibly quantified. This parameter was calculated by the ratio of the standard deviation of the response and the slope of the calibration curve of the standards multiplied by 10.

Accuracy

To determine the accuracy of the method, recovery studies were carried out by adding different amounts (80%, 100% and 120%) of bulk samples of β-caryophyllene, α-humulene and caryophyllene oxide along with the linearity range taken in triplicate. Then percentages of recovery values were determined by the absolute percentage of deviation at each concentration of the standard solutions.

Precision

Precision was estimated by intra-day (repeatability) and inter-day precision. Intra-day precision was investigated by injecting triplicate samples of β-caryophyllene, α-humulene and caryophyllene oxide solutions of three different concentrations (40, 100 and 160 µg mL^{-1}). Inter-day precision was assessed by injecting the same three samples over three consecutive days. Inter- and intra-day precisions were expressed as the relative standard deviation (RSD).

Determination of the β-caryophyllene, α-humulene and caryophyllene oxide in copaiba resin and essential oil by GC/FID

Stock solutions (10 mg mL^{-1}) of copaiba resin and essential oil were prepared in triplicate with ethyl acetate. These solutions were diluted if necessary to obtain solutions with concentrations that fall within the calibration range. Samples were injected in GC/FID in the same conditions as the validation studies. Quantitation of β-caryophyllene, α-humulene and caryophyllene oxide was performed based on the standard calibration curves of individual compounds.

Statistical analyses

All experiments were conducted in triplicates. All values were expressed as their mean and standard deviation. Means of two groups were compared using non-paired Student's *t*-tests. When comparing multiple groups, one-way analysis of variance was applied with the Tukey multiple comparison procedure. The statistical data were considered significant at $P < 0.05$

Results

Extraction of the essential oil

Copaiba essential oil was obtained by hydrodistillation from the copaiba resin using Clevenger apparatus to separate the colorless volatile fraction of the viscous residue. The yield of this extraction was calculated as the ratio (w/w) between the volatile oil obtained and the resin material used initially for extraction. Thus, the copaiba essential oil yield was of 11.0 ± 0.8%.

Development of GC methods for copaiba oil analysis

The characterization of copaiba oil was performed based in the flowchart presented in Figure 1. The identification of compounds composing the resin and essential oils was achieved by GC/MS. Identification of acid compounds in the copaiba resin was achieved after a derivatization (the success of the reaction was suggested by TLC due to the modification of the TLC profile, see Supplementary material Figure S1). Finally, the method was applied using an FID detector to achieve the transposition for a quantitative method. The chemical composition of the columns used in the two modalities of GC method was identical although column length, diameter and film thickness characteristics were different. No difference in the peak area of the eluted compounds was observed comparing analysis performed on the two columns. Differences occurred on the retention times only but not on the order of elution of the compounds. The quantitative GC/FID method was validated using the β-caryophyllene, α-humulene and caryophyllene oxide.

Development of the GC method and identification of components composing copaiba oil extracts

In order to identify compounds that are present in the copaiba resin and essential oil, a GC/MS method was developed. Chromatograms highlighted a series of peaks indicating a good separation of the compounds (see Supporting information S2). In addition, compounds in trace amounts can be also observed. To achieve a good separation between compounds, chromatographic conditions were slightly different for copaiba essential and copaiba resin oils. Essential oil analysis was performed at a lower initial temperature (60°C instead of 110°C for the resin) and heating rate (3°C min^{-1} instead of 5°C min^{-1} for the resin) in order to better separate the different compounds and to prevent possible degradation of unstable molecules. Therefore, the slight changes introduced in the method promoted a different retention time of the sesquiterpenes but the order of elution of the different sesquiterpenes was kept identical. Chemical composition of copaiba essential and resins were obtained by GC/MS considering components with a relative peak area greater than 0.1%. The method could detect 62 individual compounds. Only those represented at a percentage above 0.1% of the composition were further considered including a list of 38 compounds in the copaiba resin that represented 98.1% of all volatile compounds present in the resin (Table II). Most of the identified compounds found in the copaiba resin were sesquiterpenes representing 95.9% of the composition of the identified compounds. In the copaiba essential oil, only sesquiterpenes were detected, corresponding to 97.5% of all peak areas on the chromatogram. The retention indexes calculated from the sesquiterpenes in the copaiba essential oil were consistent with the data described in the literature (19). The major compounds identified in the copaiba essential oil were β-bisabolene (23.6%), β-caryophyllene (21.7%) and α-bergamotene (20.5%). The major compounds in the copaiba resin were β-bisabolene (25.2%), β-caryophyllene (18.7%) and α-bergamotene (16.0%).

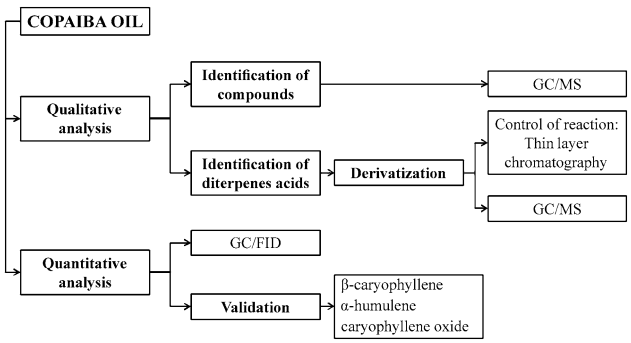

Figure 1. Flowchart of the quantitative and qualitative aspects for the copaiba oil characterization.

When analyzed by GC/MS, the derivatization reaction confirms the preservation of the same sesquiterpene compound profile but resulted in a large amount of compounds eluted in a region typical of diterpenes (retention time: from 18.58 to 31.06 min) (see Supporting information S2) (11). Same chromatographic profiles were also obtained for the essential oil after methylation reaction as expected (data not shown). By the methylation reaction, it was attempted to detected 54 compounds in the copaiba resin oil, in which 44.8% of the relative peak area corresponded to sesquiterpenes and 48.2% to diterpenes, totaling 93% of detected compounds (Table II). In the methylated copaiba resin oil, copalic acid methyl ester (15.6%), β-bisabolene (12.3%), β-caryophyllene (7.9%), α-bergamotene (7.1%) and labd-8(20)-ene-15,18-dioic acid methyl ester (6.7%) were the main compounds that could be identified. Small area variations among each compound in the resin, essential and methylated oils were expected on GC/MS qualitative analysis.

The residue obtained after extraction of the essential oil possessed a lower amount of sesquiterpenes (less than about 4.0 ± 0.3% for each individual compound), indicating that most of the sesquiterpenes compounds were extracted by hydrodistillation and concentrated in the essential oil (Figure 2). Consistently, an increase in the concentration of diterpene compounds in the methylated residue fraction was observed (Figure 2B).

Development and validation of the quantitative analytical method

To validate the method, the same analytical profile of the copaiba essential oil and the copaiba resin was developed using GC/FID. The goal was to find the correlation between the two methods to allow the dosing of copaiba resin and essential oil components with a simple and readily available instrument. Figure 3 shows the difference between all percentage peak areas detected on chromatograms analyzed by GC/MS and GC/FID of copaiba resin (Figure 3A) and copaiba essential (Figure 3B) oil, respectively. There were no major changes in the percentage areas of the compounds analyzed by both methods (maximum variation of 2.5% for each individual compound). The differences between the two methods were not statistically significant ($P > 0.05$). In addition, the main compounds detected by GC/MS were also identified by the GC/FID at the same retention time, indicating correspondence between both methods.

GC/FID method for copaiba resin and essential oils were transposed and optimized to increase resolution and to reduce analysis time. This adjustment caused only a slight change in the retention times but did neither alter peak elution order nor the relative peak area of the compounds. Therefore, in order to quantify

Table II GC/MS Analysis of *C. langsdorffii*

Compounds*	MW	RT	RO area (%)	mRO area (%)	RT	EO area (%)	RI	RI_{lit}
δ-Elemene	204	6.90	0.4	0.2	21.17	0.6	1337	1337
α-Cubebene	204	7.13	0.1	0.1	21.67	0.2	1349	1351
Cyclosativene	204	7.53	0.5	0.2	22.31	0.7	1365	1368
α-Copaene	204	7.66	1.0	0.4	22.77	1.4	1375	1376
ni	204	7.81	0.3	0.1	23.39	0.3	1390	–
β-Elemene	204	7.92	1.5	0.6	23.46	2.0	1392	1390
Cyperene	204	8.19	0.4	0.1	23.74	0.5	1399	1398
(Z,β)-Farnesene	204	8.33	0.2	–	–	–	–	–
β-Caryophyllene	204	8.57	18.7	7.9	24.63	21.7	1421	1419
α-Bergamotene	204	8.75	16.0	7.1	25.29	20.5	1437	1435
α-Guaiene	204	8.84	1.2	0.5	25.38	0.9	1439	1439
Aromadendrene	204	8.95	0.3	0.1	25.54	0.4	1442	1440
α-Humulene	204	9.04	2.9	1.3	25.96	2.9	1453	1454
(E,β)-Farnesene	204	9.23	1.6	0.8	26.11	1.7	1457	1456
ni	204	–	–	–	26.81	0.3	1474	–
τ-Muurolene	204	9.60	0.8	0.4	26.89	0.5	1476	1476
Germacrene D	204	9.75	4.8	2.2	27.06	1.7	1481	1481
β-Selinene	204	9.88	4.4	2.0	27.27	6.1	1486	1486
α-Selinene	204	–	–	–	27.62	2.3	1494	1494
β-Guaiene, trans-	204	–	–	–	27.79	0.5	1499	1499
α-Bisabolene, cis-	204	10.04	5.7	2.7	27.95	0.9	1503	1503
β-Bisabolene	204	10.21	25.2	12.3	28.24	23.6	1510	1509
γ-Cadinene	204	10.40	0.3	0.1	–	–	–	–
δ-Cadinene	204	10.51	2.4	1.2	28.76	1.4	1524	1523
ni	204	10.86	2.7	1.4	–	–	–	–
ni	204	11.34	0.3	0.2	29.50	1.2	1543	–
Caryophyllene oxide	220	11.89	0.4	0.2	31.04	4.1	1583	1581
ni	220	12.30	0.3	0.2	–	–	–	–
ni	220	12.72	0.2	0.1	–	–	–	–
ni	220	12.86	0.1	–	–	–	–	–
ni	204	13.03	1.8	1.4	33.40	0.4	1646	–
Aromadendrane <dehydro>	206	13.21	0.7	0.4	–	–	–	–
ni	204	–	–	–	33.72	0.5	1655	–
α-Cadinol	222	13.31	0.2	0.2	–	–	1651	1652
α-Bisabolol, epi	222	13.84	0.5	0.4	35.11	0.2	1693	1685
Hexadecanoic methyl ester	270	18.58	–	0.2	–	–	–	–
Kaur-16-ene	272	20.99	0.4	0.3	–	–	–	–
ni	272	21.45	0.3	–	–	–	–	–
ni	286	21.66	0.3	0.2	–	–	–	–
Linoleic acid methyl ester	294	21.75	–	0.5	–	–	–	–
ni	296	21.82	0.2	–	–	–	–	–
ni	286	22.45	0.3	0.2	–	–	–	–
ni	286	24.22	–	0.5	–	–	–	–
ni	320	24.28	–	1.9	–	–	–	–
ni	320	24.44	–	0.8	–	–	–	–
ni	320	24.77	–	3.3	–	–	–	–
ni	286	24.95	0.5	0.3	–	–	–	–
Kaur-16-en-18-oic acid methyl ester	318	25.38	–	2.4	–	–	–	–
Methyl copalate	318	25.59	–	3.5	–	–	–	–
Kauran-19-oic acid methyl ester	318	25.67	–	1.9	–	–	–	–
ni	318	26.38	–	3.8	–	–	–	–
Copalic acid methyl ester	330	26.51	–	15.6	–	–	–	–
ni	332	26.79	–	0.4	–	–	–	–
ni	318	26.94	–	0.2	–	–	–	–
ni	332	27.26	–	0.5	–	–	–	–
ni	330	27.66	–	2.3	–	–	–	–
ni	336	28.11	–	0.4	–	–	–	–
Labd-8(20)-ene-15,18-dioic acid methyl ester	364	28.58	–	6.7	–	–	–	–
ni	364	28.76	–	0.2	–	–	–	–
ni	362	29.40	–	0.6	–	–	–	–
ni	376	30.55	0.2	0.1	–	–	–	–

(*Continued*)

Table II Continued

Compounds*	MW	RT	RO area (%)	mRO area (%)	RT	EO area (%)	RI	RI_{lit}
ni	376	31.06	–	1.4	–	–	–	–
Total of sesquiterpenes			95.9	44.8		97.5		
Total of diterpenes			2.2	48.2		0.0		
Total detected			98.1	93.0		97.5		

Peak identification, retention time (RT, min) and relative area percentage of RO: copaiba resin, mRO: methylated copaiba resin and EO: copaiba essential oil. MW, molecular weight; RI, retention index calculated; RI_{lit}, retention index obtained with literature data; ni, not identified; –, absent.

*Names of compounds were provided according the NIST mass spectral library. The isomer was specified when possible.

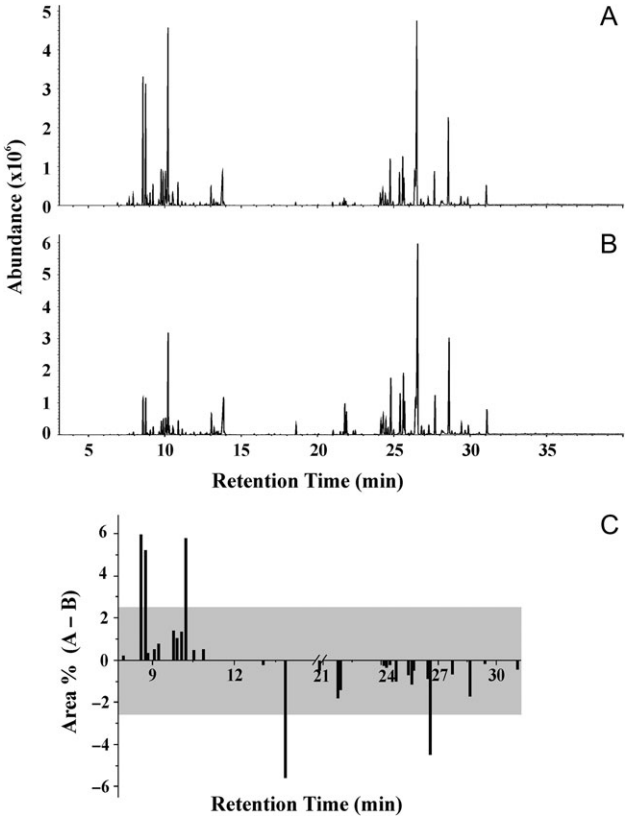

Figure 2. Difference between (A) the methylated copaiba resin before hydrodistillation, (B) residue obtained after hydrodistillation and (C) the copaiba essential oil. The gray area represents the precision of the method.

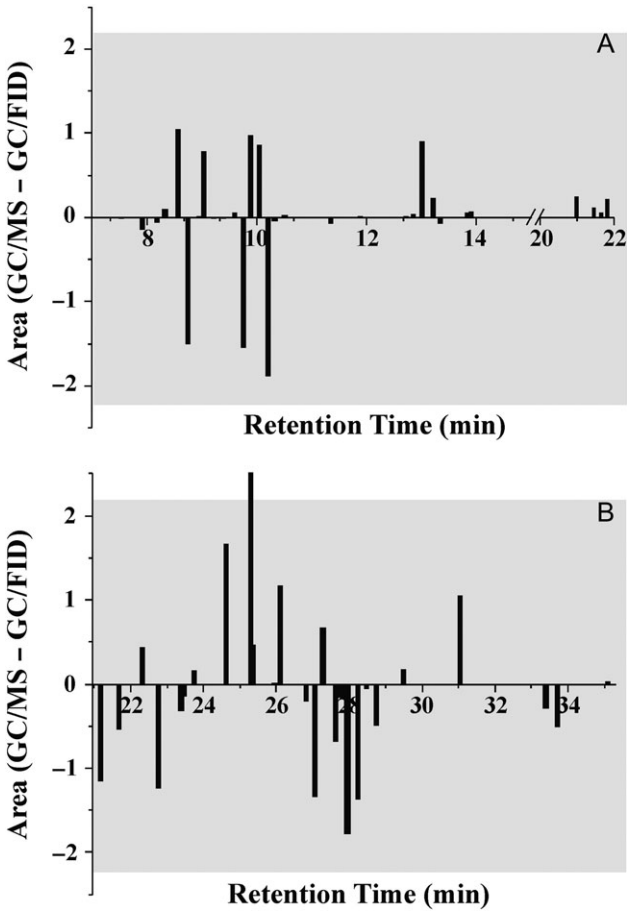

Figure 3. Main differences between peak areas detected on the chromatograms analyzed by GC/MS and GC/FID of (A) copaiba resin and (B) copaiba essential oil. Results were calculated based on the percentage area (%) difference between the compounds. The gray color represents the precision of the method.

the compounds, a validation procedure was performed using β-caryophyllene, α-humulene and caryophyllene oxide as reference substances. These sesquiterpenes were selected due to their presence in every previously analyzed copaiba oil samples. Although being all sesquiterpene, they showed different characteristics having very different retention time and Kovats retention index. The method showed good resolution for these compounds, indicating high specificity and selectivity (Figure 4). This method was specific for the standards with no interference with the peaks at the retention time. Purity of the peaks was confirmed by mass fragmentation in GC/MS. The retention times measured by GC/FID analyses for β-caryophyllene, α-humulene and caryophyllene oxide were 13.15, 14.87 and 21.52 min, respectively. Chromatogram of β-caryophyllene (peak at 14.9 min) revealed the presence of an impurity eluted after β-caryophyllene (Figure 4A). This small peak corresponds at α-humulene according the mass fragmentation profile detected in GC/MS. Therefore, the proposed method was considered adequate for β-caryophyllene, α-humulene and caryophyllene oxide quantification because peaks of standards were well separated from each other compounds and no peaks interfered with the observed analyte peaks.

A linear regression curve was established to characterize the concentration/response relationship. Linearity of the analytical procedure was evaluated by plotting detector response (peak area) against analyzed concentration. Calibration plots were constructed after analysis of β-caryophyllene, α-humulene and caryophyllene oxide solutions at concentrations of 40, 70, 100, 130 and 160 μg mL^{-1}.

Regression equation parameters are presented in Table III. The regression coefficients for the β-caryophyllene, α-humulene and caryophyllene oxide were 0.993, 0.997 and 0.998, respectively. These regression coefficients were not statistically different from 1. Linearity of the methods should be demonstrated by the slope of the linear calibration curves were statistically different from 0. The intercept was very small and they were not statistically different from 0 indicating that the calibration curves passed through the origin. In addition, the residues were homoscedastic indicating a higher normal distribution of values found around each regression line of the model. All these results indicated that the methods to all standards were linear over the range of 40–160 μg mL^{-1}.

LODs for β-caryophyllene, α-humulene and caryophyllene oxide were 11.95, 10.51 and 8.22 μg mL^{-1}, respectively (Table III). LOQs for β-caryophyllene, α-humulene and caryophyllene oxide were 39.85, 35.02 and 27.40 μg mL^{-1}, respectively (Table III). Standards accuracy was determined in the range of 80–120% by calculating recovery. The accuracy for β-caryophyllene, α-humulene and caryophyllene oxide ranged from 98.8 to 102.5%, 99.9 to 103.7%, and 98.6 to 101.1%, respectively, with an RSD value of 3.21, 3.46 and 2.24%, respectively (Table III).

Precision was estimated by the intra-day (repeatability) and the inter-day precision. Intra-day precision was investigated by injecting triplicate samples of β-caryophyllene, α-humulene and caryophyllene oxide solutions at three different concentrations (40, 100 and 160 μg mL^{-1}). Inter-day precision was determined by evaluating the repeatability of the analytical method, if reproduced in the same laboratory, but on another day. The results obtained for the inter- and intra-day precision studies are presented in Table IV. Based on these results, the method was considered satisfactory, presenting low random errors ($P < 0.05$).

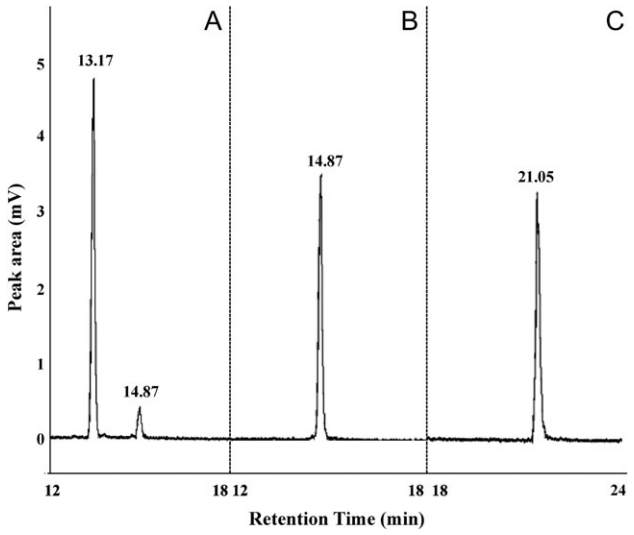

Figure 4. Representative GC/FID chromatograms of the β-caryophyllene (A), α-humulene (B) and caryophyllene oxide (C) standards at 160, 130 and 130 μg mL^{-1}.

Copaiba oil characterization

In this study, the validated analytical method was used as quality control in order to quantify the amount of the three compounds in the raw materials of copaiba resin and copaiba essential oil by GC/FID (Figure 5). The stock solution at 10 mg mL^{-1} of copaiba essential oil contained 1982 ± 13, 279 ± 25 and 24.2 ± 0.9 μg mL^{-1} of β-caryophyllene, α-humulene and caryophyllene oxide, respectively. In contrast, copaiba resin showed values of 808 ± 25, 97 ± 6 and 16.0 ± 0.6 μg mL^{-1} for β-caryophyllene, α-humulene and caryophyllene oxide, respectively.

Discussion

Copaiba oil is an important source to medicine due its complex mixture of diterpenes and sesquiterpenes compounds with numerous pharmacologic activities (2, 3, 5, 7, 11, 12). In this work, a new analytical method was proposed for a reliable separation, identification and quantification of main compounds present in copaiba resin and essential oils (*C. langsdorffii*). Copaiba essential oil was extracted by hydrodistillation from the copaiba resin with a satisfactory yield. Compared with previous work, the yield of recovery was low in the present work. Gelmini *et al.* obtained a yield of 22.5% for *C. langsdorffii* using steam distillation as the extraction method (10). However, the hydrodistillation method used in this work was considered as relevant since it generally allows a minimal loss of volatile substances due to the operation conditions applied in a closed circuit. In addition, it was applied directly on the raw extract of oil. This in contrast with other methods that required additional extraction steps that are time-consuming (up to 3 h), labor-intensive methods and that need relatively large volumes of organic solvents (22).

The GC/MS method was developed to identify compounds composing the copaiba resin and essential oil. It is noteworthy that in natural resin there are low vapor pressure compounds that are part of the complex composition. To make possible their detection by GC, the method generally consists to modify the analyte through a chemical reaction performing a derivatization to enable chromatographic separations. This approach is generally helpful in order to identify low vapor pressure compounds such as diterpenoic acids and their derivatives. A derivatization reaction was then applied to the copaiba oil extracts. The reaction of (trimethylsilyl) diazomethane

Table III Validation Parameters of β-Caryophyllene, α-Humulene and Caryophyllene Oxide by GC/FID

Parameters	β-Caryophyllene	α-Humulene	Caryophyllene oxide
Retention time (min)	13.15 ± 0.02	14.87 ± 0.02	21.52 ± 0.04
Linearity			
a (slope)	0.204 ± 0.010	0.201 ± 0.006	0.227 ± 0.006
b (intercept)	−0.903 ± 0.813	−0.501 ± 0.704	−2.347 ± 0.622
R^2	0.993	0.997	0.998
LOD (μg mL^{-1})	11.95	10.51	8.22
LOQ (μg mL^{-1})	39.85	35.02	27.40
Accuracy (%RSD)	3.21	3.46	2.24

Table IV Intra and Inter-day Variations of β-Caryophyllene, α-Humulene and Caryophyllene Oxide by GC/FID

Spiked concentration (μg mL^{-1})	Measured concentration								
	β-Caryophyllene			α-Humulene			Caryophyllene oxide		
	Mean (μg mL^{-1})	SD	RSD (%)	Mean (μg mL^{-1})	SD	RSD (%)	Mean (μg mL^{-1})	SD	RSD (%)
Intra-day variation									
40	41.4	1.0	2.3	40.3	1.2	3.0	41.1	1.7	4.2
100	99.4	2.4	2.4	103.5	2.0	1.9	100.5	3.9	3.9
160	162.3	3.2	2.0	161.3	1.9	1.2	161.1	2.5	1.5
Inter-day variation									
40	40.8	1.2	2.9	41.5	1.8	4.3	41.5	1.8	4.3
100	98.8	4.3	4.4	105.5	2.5	2.4	105.5	2.0	1.9
160	159.1	4.9	3.1	162.1	2.1	1.3	162.1	1.9	1.2

Values are for $n = 3$ observations.

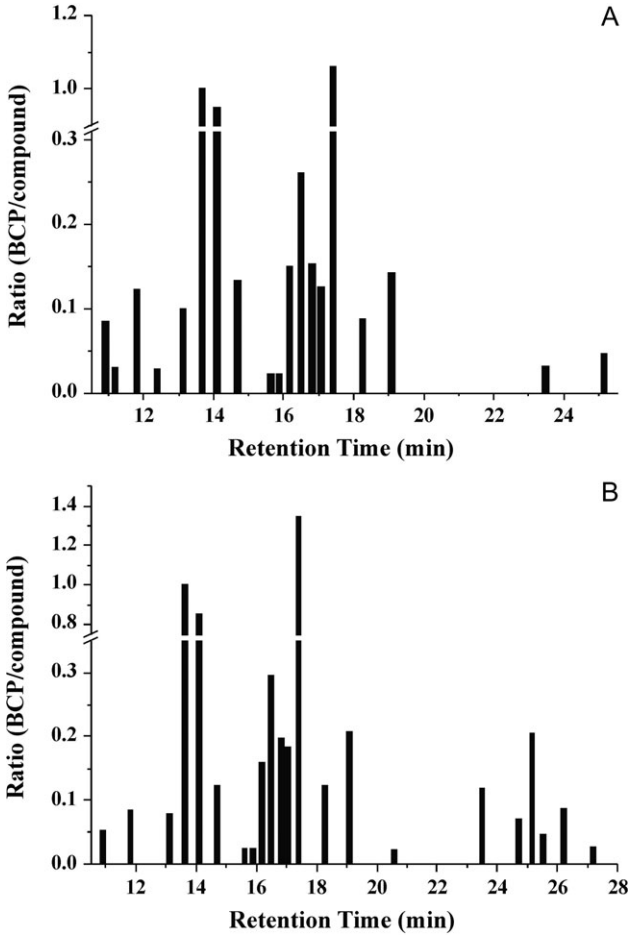

Figure 5. Ratio between peaks areas of β-caryophyllene (BCP) and other compounds for copaiba essential oil (A) and copaiba resin (B).

with a carboxylic acid instantly forms methyl esters compounds. Derivatives show lower polarity than their parent substances due to the replacement of the hydrogen by a methyl group (18).

The GC method developed here was capable to separate and identify the main copaiba oil compounds. However, the data obtained in this work were different from those previously reported by different authors. Gramosa *et al.* reported that the major component in the copaiba essential oil was β-caryophyllene (53%) (9). Soares *et al.* also detected a high level of β-caryophyllene (42.3%) in the essential oil and a high amount of copalic acid (49.9%) in the non-volatile fraction (23). Another study revealed the presence of large amounts of α-bergamotene (48%) in the essential oil and copalic acid (22%) in the resin (10). Variations of composition of copaiba oil found in the different studies may be attributed to several factors including the influence of the geographical origin of the plants, environmental factors such as the amount of light received by the tree, the temperature, the soil composition and the season, the period and harvest time, as well as the plant organ, age and stage in the vegetative cycle (24). Besides these factors, it is not known yet whether copaiba trees might be sub-classified into several chemotypes that may also be a possible source of heterogeneity in the oil composition. Further work is required to clarify the possible origin of variability of the copaiba oil composition.

Although mass spectrometer is considered as the most powerful detector for chromatographic methods, flame ionization detector is still widely used due to its technical performances including adequate sensitivity, large linear response range and high signal to noise ratio for most needed analysis and because the equipment is more accessible to all analytical laboratories based on economic considerations (15, 17, 25, 26). Applying the method developed by GC/MS with FID as the detection method, a good correlation was established on the order of elution of the different compounds detected by GC/MS and the GC/FID. Variations of areas of the peaks of the different components also showed a good correlation between the two methods. This allowed transposition of the method developed on GC/MS into a validated quantitative method applying GC/FID as a relevant detector that is simpler and based on easily available instruments proposed by official guidelines for the quantitative GC of volatile flavoring substances provided by the Working Group on Methods of Analysis of the International Organization of the Flavor Industry (IOFI) (26).

Validation of the GC/FID method was performed to quantify β-caryophyllene, α-humulene and caryophyllene oxide in copaiba essential and resin oils following a protocol described in the ICH recommendations. The method showed good resolution for β-caryophyllene, α-humulene and caryophyllene oxide compounds, indicating high specificity and selectivity with low random errors ($P < 0.05$). In addition, it can be inferred that the method demonstrated a good correlation between the theoretical and the experimental values, satisfying drug level requirements. The sensitivity of the method

was higher than that of previously described method (8) and the accuracy and precision were within acceptable ranges given in the ICH.

The validated analytical method was applied to quality control of copaiba resin and essential oils. Furthermore, as chromatograms always show reproducible ratios between peak of individual compounds and the peak given by a well define compound for which the concentration can be precisely determined with the validated method, these ratios can be used to extrapolate the relative concentration of all the individual components found in the analyzed copaiba oil extract samples. This methodology is a recognized method that can be applied to determine the composition of complex natural extracts that is described in the official guidelines for the quantitative GC of volatile flavoring substances provided by the Working Group on Methods of Analysis of the IOFI (26). The semi-quantitative analysis explained above was applied to determine the composition in all identified compounds found in the copaiba oil extracts that were analyzed in the present work. Peak area ratios of individual compounds were calculated using the peak area of β-caryophyllene as the concentration of this component in the extract could be absolutely determined with the validated method. The different ratios are given in Figure 5. The composition of the extracts of copaiba oil analyzed in the present work was found quite different from those described in the literature (9, 10). Analyzing extracts from natural resources lead to a certain variability due to the nature of the sample. Additionally, the difference found on the essential oil extract can be also explained by the difference in the methods of extraction that were used in the different work and that can also influence the composition of the final extract.

Conclusion

Compounds present in the copaiba resin and essential oil were identified by GC/MS analysis. A derivatization was performed to allow tentative identification of diterpenes compounds from copaiba resin. The elution profile of compounds of copaiba resin and essential oils were consistent between the GC/MS and GC/FID methods allowing a transposition of the method from GC/MS to GC/FID. The GC/FID method that was further developed and validated following ICH and FDA guidelines is rapid, simple, accurate, and precise. It was found suitable to access absolute quantitation of β-caryophyllene, α-humulene and caryophyllene oxide in copaiba oil samples and its sensitivity was found superior to that of previously proposed methods. As shown in this work, the method can be applied to quantify β-caryophyllene, α-humulene and caryophyllene oxide in copaiba resin and essential oil samples. Due to reproducible ratios between the peak given by β-caryophyllene and peaks given by other compounds present at a percentage above 0.1%, the method can be applied to determine the composition of the oil extracts in these components according to the official guidelines for the quantitative GC of volatile flavoring substances that have been provided by the Working Group on Methods of Analysis of the IOFI. The methods developed in the present work can be proposed to achieve quality control analysis of different products from copaiba oil extracts on both qualitative and quantitative analytical basis. They should be suitable to control that compositions of copaiba oil extracts are not modified while incorporated in pharmaceutical formulations for instance.

Highlights

- New gas chromatographic methods for copaiba oil analysis.
- A GC/MS method resolving the complex composition analysis of copaiba oil.
- Transposing a GC/MS analytical method of copaiba oil to CG/FID.
- Validated GC/FID to quantify main compounds of copaiba oil extracts.
- Simple, rapid, sensitive GC/FID method for quantitative analysis of copaiba oil.

Supplementary Data

Supplementary data are available at *Journal of Chromatographic Science* online.

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