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Determination by GC–MS-SIM of furanoditerpenes in *Pterodon pubescens* Benth.: Development and validation

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ABSTRACT

The crude hydroalcoholic extract from fruits of *P. pubescens* is widely used because of its anti-rheumatic, antinociceptive and anti-inflammatory activities. Furanoditerpenes have a vouacapan skeleton and are involved with the pharmacological activity of the oil extracted from *P. pubescens* fruits. Furanoditerpenes methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate and methyl 6α-hydroxy-7β-acetoxyvouacapan-17β-oate from *P. pubescens* were isolated and identified. The present study developed and validated a GC–MS-SIM method for the separation and quantification of vouacapan constituents in a semipurified extract from *P. pubescens* fruits. The GC–MS analyses were carried out using a system equipped with a HP-5 capillary column (30 m × 0.25 mm). Temperature program: 100 °C (4 °C min^{-1})–270 °C (5 min), injector 260 °C, detector 270 °C. Helium was used as the carrier gas (0.7 bar, 1 mL min^{-1}). The MS was taken at 70 eV. Scanning speed was 0.5 scans s^{-1}, from 50 to 650. Sample volume was 1 μL. Split 1:20. Analyses for validation of methodology were conducted by GC–MS-SIM (Single Ion Monitoring), where the ions monitored were 131, 145 and 146 (between 43 and 44.5 min), 105, 145 and 197 (from 44.5 to 45.3 min) and 131, 178 and 312 (from 45.3 to 48.5 min).Validation of the analytical method was based on the following parameters: linearity, robustness, limits of detection and quantification, precision (within-day and between-day variabilities), recovery and stability. The method was linear over a range of 12.81–2.56 $\mu\gamma\mu\Lambda^{-1}$ of vouacapan 1, 112.78–22.56 μg mL^{-1} of vouacapan 2, and 333.34–66.67 μg mL^{-1} of vouacapans 3 and 4, with detection limits of 0.39, 3.45 and 9,44 μg mL^{-1} and quantification limits of 1.19, 10.47 and 28.62 μg mL^{-1}, respectively. Recovery values were 100.69%, 97.48% and 96.98% for vouacapans 1, 2 and 3–4, respectively. Thus, the method was efficient to separate and quantify furanoditerpenes in the extract or fraction.

1. Introduction

Trees of the genus *Pterodon*, commonly known as "sucupira branca" or "faveiro," are native to Brazil [1–3]. The crude hydroalcoholic extract from fruits of *P. pubescens* is widely used in folk medicine because of its anti-rheumatic [4,5], antinociceptive [6–8] and anti-inflammatory activities [9,10].

Furanoditerpenes have a vouacapan skeleton and are involved with the pharmacological activity of the oil extracted from *P. pubescens* fruits [7,9,11–13]. Transformation of plant raw materials into a drug should aim to preserve the pharmacological and chemical integrity of the plant, ensuring the constancy and security of its biological effect and use, as well as its potential therapeutic value [14]. Chemical integrity can be guaranteed by the standardization of analytical techniques, which in turn must be evaluated and validated to meet the requirements of analytical applications for experimental studies, ensuring the reliability of results [15].

Although the biological effects of oil from *P. pubescens* fruits are scientifically proven, analytical methodologies have not yet been developed to quantify furanoditerpenes in extracts from plants of this genus. The present study developed and validated a GC–MS-SIM method for the separation and quantification of vouacapan constituents in a semipurified extract from *P. pubescens* fruits. The main validation parameters of the methods were also determined.

2. Experimental

2.1. Chemicals and reagents

Chromatography Column: Silica gel 60 (Merck, 70–230 mesh) and Sephadex® LH-20 (Pharmacia); Silica gel plates F254 (Merck, 0.25 mm thick). The crystals isolated from subfractions A (**1**) and B (**2**) of the hexane fraction, with a purity of 97.3% and

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identified as furanoditerpenes, and the crystals isolated and identified from subfraction C as methyl 6α-acetoxy-7β-hydroxy-vouacapan-17β-oate (**3**) and methyl 6α-hydroxy-7β-acetoxy-vouacapan-17β-oate (**4**), with a purity of 98%, were used as standards for validation of the analytical methodology.

2.2. Apparatus

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz): Varian Gemini 300 (7.05T) spectrometers in $CDCl_3$, internal standard TMS (^{1}H NMR) and solvent signal (^{13}C NMR); GC–MS-Thermo Electron Corporation DSQ II; TLC.

2.3. Plant material

Pterodon pubescens Benth. fruits from Nossa Senhora do Livramento, Mato Grosso, Brazil (15°89′ S; 56°41′ W) were collected in May 2010. The specimen was identified by Dr. Germano Guarim Neto from the Herbarium of the Federal University of Mato Grosso, and the voucher specimen was deposited in the Herbarium of Maringá State University, under no. 20502.

2.4. Extraction and isolation of the constituents

The dried fruits (30 g) were extracted with 600 mL of ethanol by turbo extraction (Ultra-Turrax UTC115KT, IKA Works, Wilmington, NC, USA) and were filtered. The filtrate was added to 700 mL of water and partitioned with 700 mL of hexane, dichloromethane and ethyl acetate. The organic solvent was evaporated in a vacuum evaporator and the aqueous fraction was lyophilized to yield the hexane fraction (FH) (5.70 g), dichloromethane fraction (FD) (0.76 g), ethyl acetate fraction (FEAc) (0.44 g) and aqueous fraction (FA) (1.07 g).

The FH (5 g) was chromatographed in a column with Silica Gel 60 (70–230 mesh ASTM) eluted with 150 mL of hexane, hexane:ethyl acetate (9:1; 8:2; 7:3; 6:4; 1:1 and 2:8), ethyl acetate and methanol, affording 114 fractions. The fractions were analyzed by TLC, affording 24 subfractions. Fraction 20 (0.36 g) was purified on a Sephadex LH-20 gel eluted with methanol:chloroform (1:1) to yield subfractions A (2.1 mg), B (7.4 mg) and C (20.7 mg). The compounds present in subfractions A and B were not identified. However, the majority compound from the FH fractions was identified as 14,15-epoxygeranylgeraniol.

Methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate (**3**) white solid;. EI-MS, *m/z* (rel. int.): 404 $[M]^+$. (2), 344(30), 313(28), 312(96), 269(27), 183(42), 178(70), 171(32), 163(29), 159(25), 157(25), 153(26), 152(25), 147(26), 146(26), 145(54), 137(33), 133(30), 131(100), 123(60), 121(44), 119(61), 117(26), 115(26), 109(54), 107(30), 105(34), 95(70), 93(30), 91(69), 85(28), 81(58), 79(26), 71(35), 69(71), 57(31), 55(54); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.24 (s; H-16), 6.17 (d, J=1.8 Hz; H-15), 5.23 (dd, J=11.4 and 9.0 Hz; H-6), 3.73 (s; OCH_3), 3.43–3.40 (m; H-14), 3.34 (dd, J=19.8 and 9.9 Hz; H-7), 2.66 (m; H-11β), 2.48 (m; H-11α), 2.39 (m; H-8), 2.03 (s; $OCOCH_3$), 1.70 (m; H-1β), 1.50 (m; H-2), 1.43 (m; H-9), 1.43 (m; H-3β), 1.32 (d, J=11.4 Hz; H-5), 1.22 (m; H-3α), 1.09 (s; H-20), 1.03 (s; H-18), 0.99 (m; H-1α) and 0.95 (s; H-19). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 175.67 (C-17), 172.20 ($OCOCH_3$), 150.68 (C-12), 141.66 (C-16), 113.56 (C-13), 108.67 (C-15), 81.53 (C-7), 75.71 (C-6), 55.12 (C-5), 52.50 (OCH_3), 48.09 (C-9), 46.50 (C-14), 43.56 (C-3), 42.72 (C-8), 39.44 (C-1), 38.91 (C-10), 36.42 (C-18), 33.33 (C-4), 22.69 (C-19), 22.16 (C-11), 22.03 ($OCOCH_3$), 18.48 (C-2) and 15.67 (C-20).

Methyl 6α-hydroxy-7β-acetoxyvouacapan-17β-oate (**4**) white solid;. EI-MS, *m/z* (rel. int.): 404 $[M]^+$. (2), 313(26), 312(100), 269(28), 267(27), 183(42), 178(52), 171(33), 147(31), 146(25), 145(57), 137(31), 133(31), 131(94), 123(56), 121(46), 119(58), 118(32), 109(56), 107(34), 105(36), 95(60), 93(29), 91(78), 83(25), 81(61), 79(26), 77(28), 69(75), 67(28), 55(50); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 7.24 (s; H-16), 6.37 (d, J=1.8 Hz; H-15), 5.42 (dd, J=11.7 and 8.7 Hz; H-6), 4.12 (dd, J=14.4 and 7.2 Hz; H-7), 3.73 (s; OCH_3), 3.43-3.40 (m; H-14), 2.66 (m; H-11β), 2.48 (m; H-11α), 2.39 (m; H-8), 2.04 (s; $OCOCH_3$), 1.70 (m; H-1β), 1.50 (m; H-2), 1.43 (m; H-9), 1.43 (m; H-3β), 1.36 (m; H-5), 1.22 (m; H-3α), 1.09 (s; H-20), 1.03 (s; H-18), 0.99 (m; H-1α), and 0.95 (s; H-19). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 175.67 (C-17), 172.20 ($OCOCH_3$), 150.68 (C-12), 141.66 (C-16), 113.56 (C-13), 107.44 (C-15), 81.53 (C-7), 74.28 (C-6), 54.22 (C-5), 52.50 (OCH_3), 48.09 (C-9), 46.50 (C-14), 43.56 (C-3), 42.72 (C-8), 39.44 (C-1), 38.91 (C-10), 36.42 (C-18), 33.33 (C-4), 22.69 (C-19), 22.16 (C-11), 22.03 ($OCOCH_3$), 18.48 (C-2) and 15.67 (C-20).

14,15-epoxygeranylgeraniol yellow oil; EI-MS, *m/z* (rel. int.): 288 $[M^-H_2O]$ (2), 119(27), 107(35), 25(26), 93(64), 81(100), 79(38), 71(63), 70(47), 68(30), 55(33); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 5.41 (td, J=6.9 and 1.2 Hz; H-2), 5.13 (2 H; m; H-6, H-10), 4.15 (d, J=6.9 Hz; H-1), 2.71 (t, J=6.3 Hz; H-14), 2.05 (3 H; m; H-4, H-8, H-12), 1.64 (3 H; m; H-5, H-9, H-13), 1.60 (s; H-8′), 1.30 (2 H; s; H-16, H-16′) and 1.26 (2 H; s; H-4′, H-12′). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C 139.91 (C-3), 135.42 (C-7), 134.25 (C-11), 125.02 (C-10), 124.09 (C-6), 123.56 (C-2), 64.45 (C-14), 59.60 (C-1), 57.49 (C-15), 39.80 (C-4), 39.73 (C-8), 36.50 (C-12), 27.65 (C-13), 26.75 (C-9), 26.45 (C-5), 25.11 (C-16), 25.11 (C-16′), 18.95 (C-4′), 18.55 (C-12′) and 16.22 (C-8′).

2.5. Analysis by GC–MS

The GC–MS analyses were carried out using a system equipped with a HP-5 capillary column (30 m × 0.25 mm). Temperature program: 100 °C (4 °C min^{-1})–270 °C (5 min), injector 260 °C, detector 270 °C. Helium was used as the carrier gas (0.7 bar, 1 mL min^{-1}). The MS was taken at 70 eV. Scanning speed was 0.5 scans s^{-1}, from 50 to 650. Sample volume was 1 μL. Split 1:20. The samples were diluted in chloroform.

Analyses for validation of methodology were conducted by GC–MS-SIM (Single Ion Monitoring), where the ions monitored were 131, 145 and 146 (between 43 and 44.5 min), 105, 145 and 197 (from 44.5 to 45.3 min) and 131, 178 and 312 (from 45.3 to 48.5 min).

2.6. Method validation

The method was validated according to the guidelines established by the ICH (International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) [15]. All results were expressed as percentages. For the statistical analysis, the program Statistica® was used. A 5% level of significance was selected.

The crystals isolated from subfractions A, B and C of FH, showing a purity of 97.3% and 98% and identified as furanoditerpenes, were used as standards for validation of the analytical methodology. A mixture of subfractions A (2.79%), B (24.57%) and C (72.64%) was used as the analytical standard.

2.6.1. Linearity

The calibration curve was constructed in triplicate, using seven levels of concentration between 12.81 and 2.56 $\mu\gamma\mu\Lambda^{-1}$ of vouacapan 1, 112.78 and 22.56 μg mL^{-1} of vouacapan 2, and 333.34 and 66.67 μg mL^{-1} of vouacapans 3 and 4. The slope and other statistics of the calibration curves were calculated by linear regression.

2.6.2. Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) and the slope (S) of the calibration curves, using Eqs. (1) and (2).

$$LOD = 3.3(SD/S) \quad (1)$$

$$LOQ = 10(SD/S) \quad (2)$$

2.6.3. Precision

A stock solution of FH was prepared and diluted in three concentrations (0.4 mg mL^{-1}, 0.2 mg mL^{-1} and 0.1 mg mL^{-1}). Each concentration was prepared independently in triplicate. Repeatability was assessed by the amount of furanoditerpenes obtained at each concentration. After 12 days, the procedure was repeated for evaluation of the intermediate precision. The relative standard deviation (RSD) was calculated for repeatability and intermediate precision.

2.6.4. Accuracy

Recovery was evaluated by the standard addition method, adding the mixture of vouacapans isolated in three different known concentrations to the extractive solution of FH. All concentrations were prepared independently in three replicates. The recovery data were determined by dividing the value obtained for the sample prepared with the added standards, by the amount added, and then multiplying by 100% [15].

2.6.5. Robustness

Robustness was assessed by changing the initial temperature of the oven from 60 °C min^{-1} to 270 °C (5 min), with an increase of 4 °C. The temperatures of the injector and detector were kept at 260 °C and 270 °C, respectively.

2.6.6. Stability

A standard solution of vouacapans from *P. pubescens*, with known concentration, was stored at −20 °C for 12 days and analyzed by GC–MS. To be considered stable, the analytes should remain chemically unchanged during the period.

3. Results and discussion

After preparation of the ethanolic extract and its partition, the subsequent experiments used only the hexane fraction (FH), since it is beyond the high yield shown, and its low polarity, which facilitates the extraction of nonpolar compounds such as diterpenes. GC–MS analysis of FH (Fig. 1) showed the presence of a predominant compound with retention time of 34.41 min, which was identified using NMR as 14,15-epoxygeranylgeraniol. This diterpenoid was previously isolated and identified by Mors et al. [18] and was effective as a chemoprophylactic against schistosomiasis.

Because of a lack of commercially available reference standards, several chromatographic techniques were employed to isolate furanic diterpenes present in the FH of *P. pubescens* fruits. Subfractions A, B and C of FH were used as analytical standards to validate the analytical method. The retention time of vouacapan **1** (isolated from subfraction A), **2** (from subfraction B) and **3–4** (from subfraction C), as well as the mass spectrum, was used to identify the peak in the *P. pubescens* extractive solutions (Fig. 2).

Compound **4** was previously identified by Fascio et al. [17] in *P. pubescens*. However, compound **3** has so far been identified only in the species *P. apparicioi* and *P. polygalaeflorus* [16,17]. This is the first identification of methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate (**3**) in *P. pubescens*.

Inspection of the fragmentation data revealed that the fragments generated by compounds **3** and **4** were identical, varying only in intensity, which is a strong indication of isomerism. These data were confirmed from the NMR spectra, where the compounds from subfraction C were identified as methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate (**3**) and methyl 6α-hydroxy-7β-acetoxyvouacapan-17β-oate (**4**) by comparison of experimental ^{1}H and ^{13}C-NMR spectroscopy, g HSQC and g HMBC spectral analysis, and comparison of literature data [16,17]. Compound (**4**) was characterized by the presence of an acetoxyl group at C-7 and the hydroxyl function at C-6. Acetylation at position 6 in compound (**3**) resulted in modest downfield shifts in the range of 0.9 ppm–1.43 ppm compared with compound (**4**) C-5 [$\Delta\delta_c$=55.12 (**3**)–54.22 (**4**)=0.9 ppm], C-6 [$\Delta\delta_c$=75.71 (**3**)–74.28(**4**)=1.43 ppm].

Since they are isomers of position, the GC–MS analysis did not show good resolution in the separation of compounds **3** and **4**, which had overlapping peaks. Therefore these were considered as

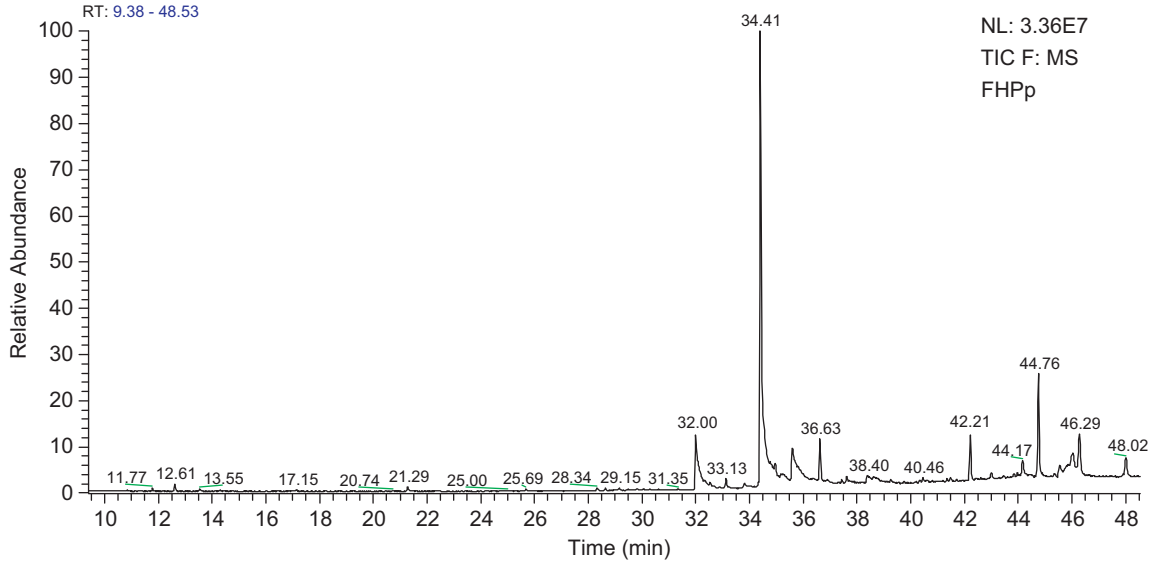

Fig. 1. Chromatographical profile of FH of *Pterodon pubescens* obtained by GC–MS.

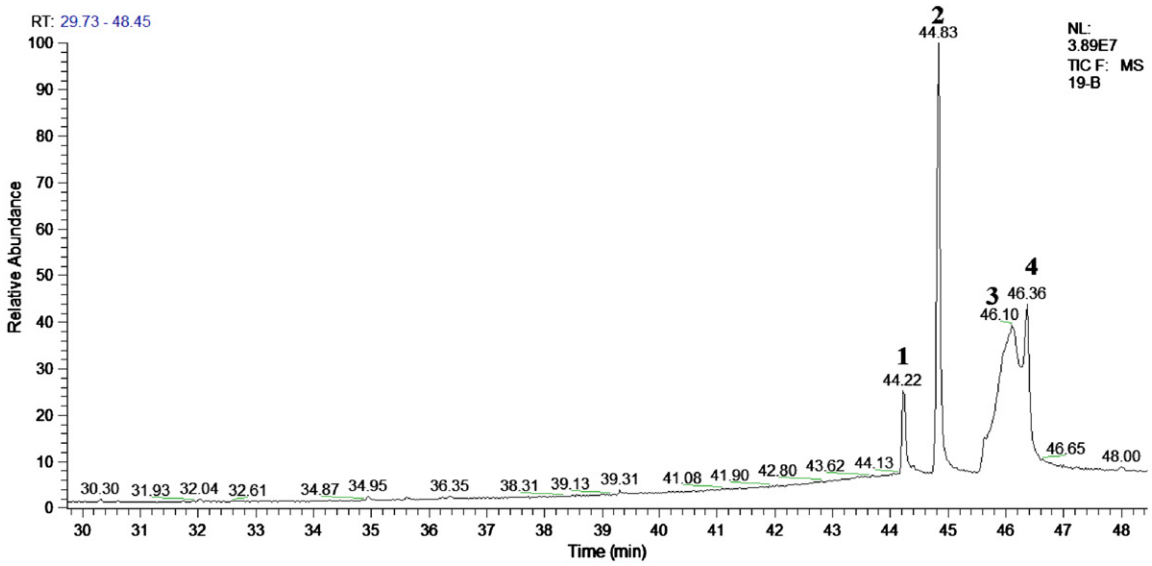

Fig. 2. Chromatogram of furanic diterpenes isolated from FH. Vouacapans 1 (retention time: 44.22), 2 (retention time: 44.83) and 3–4 (retention time: 46.10 and 46.36).

Table 1
Overview of the results about the calibration model.

	Vouacapan 1	Vouacapan 2	Vouacapan 3–4
Range (μg/mL)	12.81–2.56	112.78–22.56	333.34–66.67
Number of standards	7	7	7
Correlation coefficient	0.9924	0.9924	0.9935
Intercept ± standard error	48472.32 ± 10923.42	−749194.83 ± 107334.2	−1610689.55 ± 288015.7
Slope ± standard error	91752.18 ± 1839.32	102540.6 ± 2052.8	100632.42 ± 1863.7
F cal. regres. (F crit.=4.38)	2488	2495	2915
F cal. residue (F crit.=2.96)	0.79	0.57	0.51
D.L. (μg/mL)	0.39	3.45	9.44
Q.L. (μg/mL)	1.19	10.47	28.62

a single peak in the analytical validation, comprising 72.64% of the total area of the furanoditerpenes present in the standard.

Compounds **1** and **2** were not identified because they were present in small quantities in the sample (2.79% and 24.57%, respectively) and were not detected by NMR. However, fragmentation is not a randomized process. It can be predicted and is related to the charge location, the cleavage mechanisms, and the stability of the compound, allowing interpretation of the spectrum [19]. The mass spectra of peaks **1** and **2** indicated that these compounds belong to the class of furanoditerpenes. The accurate identification of these compounds is being investigated.

Knowledge of the amount of the compound of interest in the sample, the variability of this content due to climate and season of collection, and the lack of commercially available reference compounds are the main challenges for the development of new quantitative methods for determination of compounds present in medicinal plants. The creation of a new method involves both development and validation of the proposed methodology [20].

For qualitative and quantitative analysis of FHPp, an analytical method for determination of furanic diterpenes was developed. GC–MS-SIM analysis was proposed, because this method is commonly used to detect furanoditerpenes. Gas chromatography allows a prior separation of the constituents of the sample, which provides some specificity in the assay of the constituents of extracts and/or herbal drugs, as these are not analyzed as a mixture, but rather singly or in less-complex mixtures.

After this separation, each constituent is analyzed individually by mass spectrometry. The selected ion monitoring allows a more sensitive and accurate assay, which ensures more accurate and reliable qualitative and quantitative analytical results [21].

The linearity was examined by the analysis of vouacapans **1**, **2** and **3–4** at seven concentration levels (12.81–2.56 $\mu g mL^{-1}$ of vouacapan **1**, 112.78–22.56 $\mu g\ mL^{-1}$ of vouacapan **2** and 333.34–66.67 $\mu g\ mL^{-1}$ of vouacapans **3–4**). The curves showed an excellent correlation coefficient (r). The regression model was significant for the three analytical curves. The lack of fit of the linear model was also analyzed, demonstrating that the model is adjusted. The LOD, the lowest absolute concentration of the analyte in a sample that can be detected, but not necessarily quantified in the experimental condition, and the LOQ, the lowest concentration of analyte in a sample that can be accurately determined and with acceptable quantification, were calculated (Table 1).

Precision was investigated at two levels: Repeatability (under the same conditions over a short period of time) and intermediate precision (the effect of carrying out analyses on different days). The values obtained are summarized in Table 2.

According to the AOAC [22], values of the relative standard deviation (RSD) up to 3.7%, 5.3% and 7.3% are accepted for testing the precision of analyte concentrations of 0.1%, 100 ppm and 10 ppm, respectively. For tests of repeatability and intermediate precision, the concentrations 400 ppm (0.4 $mg\ mL^{-1}$), 200 ppm (0.2 $mg\ mL^{-1}$) and 100 ppm (0.1 $mg\ mL^{-1}$) had RSD values less than 5.22%, within the maximum allowed (5.3%) for solutions at a concentration of 100 ppm.

These results demonstrate that the method has acceptable repeatability and intermediate precision, regardless of the level

Table 2
Data on the precision of the method.

Concentration (mg/mL)	*Repeatability*		*Intermediate precision*	
	Mean ± (μg/μL) S.D.	R.S.D. (%)	Mean (μg/μL) ± S.D.	R.S.D. (%)
Vouacapan 1 (n=3)				
0.4	6.07 ± 0.00	2.20	5.93 ± 0.00	7.77
0.2	3.50 ± 0.00	4.28	3.50 ± 0.00	5.72
0.1	2.63 ± 0.00	4.65	2.68 ± 0.00	4.76
Vouacapan 2 (n=3)				
0.4	36.07 ± 0.00	2.13	36.88 ± 0.00	4.71
0.2	26.75 ± 0.00	2.22	27.95 ± 0.00	5.14
0.1	23.56 ± 0.00	1.64	23.41 ± 0.00	1.72
Vouacapan 3–4 (n=3)				
0.4	73.13 ± 0.00	1.67	69.36 ± 0.00	4.46
0.2	69.75 ± 0.00	2.72	66.37 ± 0.00	0.86
0.1	67.47 ± 0.00	0.39	64.66 ± 0.00	3.46

Table 3
Results of the recovery experiment.

Solution (n=3)	Mean of determined amount (μg/mL)	Theoretical amount (μg/mL)	Recovery ± R.S.D. (%)
Vouacapan 1[a]	2.21	2.17	101.67 ± 2.78
Vouacapan 1[b]	1.99	1.82	108.81 ± 1.86
Vouacapan 1[c]	1.43	1.56	91.58 ± 4.04
Vouacapan 2[a]	13.30	13.82	96.21 ± 0.74
Vouacapan 2[b]	13.48	12.74	105.80 ± 2.74
Vouacapan 2[c]	10.58	11.70	90.44 ± 2.84
Vouacapan 3–4[a]	35.68	39.02	91.44 ± 0.62
Vouacapan 3–4[b]	35.06	34.20	102.51 ± 0.74
Vouacapan 3–4[c]	29.63	32.53	91.08 ± 0.37

[a] Large amount of standard addition.
[b] Addition of intermediate quantity of standard.
[c] Addition of small amount of the FHPp standard solution.

tested, for vouacapans **2** and **3–4**, with a relative standard deviation less than 5%, as established by ICH [15]. Vouacapan **1** showed a wider variation, which can be explained by the low concentration (2.79%) of this compound; however, the RSD of vouacapan **1** did not exceed the limits set by the AOAC [22]. These results can be considered satisfactory, since most herbal drugs show coefficients of variation of 3–6% [23]. Therefore, the method showed accuracy for the FHPp derived vouacapan analysis.

Recovery was also evaluated at three levels, and mean recoveries were calculated (Table 3). Recovery was between 90% and 107%, indicating the accuracy of the analytical method for the concentration of 100 ppm (0.1 mg mL^{-1}). These findings indicate that this method is accurate for vouacapans **2–4**, which showed recoveries between 90.4% and 105.8%, within the range set by the AOAC [between 91.5% and 108.81%, again within the AOAC limit [22] of 80–110% for 22].

Similarly, vouacapan **1** showed recoveries solutions with a concentration of 10 ppm (0.01 mg mL^{-1}).

The robustness of the method was tested by changing the temperature of the device. The retention time of peaks **1, 2, 3** and **4** increased to 50.97, 51.85, 53.49 and 53.82 min, respectively.

With increasing temperature, generally the solubility of the sample in the liquid phase is altered, decreasing the residence time of the analyte in the liquid phase, which leads to a decrease in residence time in the gas chromatograph [24]. The reverse occurs when the temperature is decreased, explaining the longer residence time of the compound in the liquid phase and the consequent increase in retention time of the compounds. However, despite the increased retention time, the chromatographic profile did not change appreciably, indicating that the method is robust and stable under small variations of the analytical parameters. To evaluate the stability, a sample was evaluated after 12 days, and the vouacapan-derived compounds remained chemically unchanged, indicating their stability.

4. Conclusion

The diterpenes 14,15-epoxygeranylgeraniol; methyl 6α-acetoxy-7β-hydroxyvouacapan-17β-oate and methyl 6α-hydroxy-7β-acetoxyvouacapan-17β-oate of *Pterodon pubescens* were isolated and identified. The analytical method was validated according to the ICH guidelines. In this study, the GC–MS-SIM method proved to be sensitive, accurate, linear, precise, reproducible, repeatable, and with robust stability. These results indicate that this method is suitable for the determination of vouacapans in *P. pubescens* extracts and oil fraction.

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References

[1] R.C. Dutra, R. Trevizani, F. Pittella, N.R. Barbosa, Lat. Am. J. Pharm. 27 (2008) 865–870.
[2] D. Hansen, M. Haraguchi, A. Alonso, Braz. J. Pharm. Sci. 46 (2010) 605–616.
[3] D.M.T. Oliveira, E.A.S. Paiva, Braz. J. Biol. 65 (2005) 483–494.
[4] M.G. Coelho, K.C. Sabino, S.R. Dalmau, Clin. Exp. Rheumatol. 22 (2004) 213–218.
[5] K.C. Sabino, F.A. Castro, J.C. Oliveira, S.R. Dalmau, M.G. Coelho, Phytother. Res. 13 (1999) 613–615.
[6] L.P. Coelho, P.A. Reis, F.L. Castro, C.R.M. Gayer, C.S. Lopes, M.C.C. Silva, K.C.C. Sabino, A.R. Todeschini, M.G.P. Coelho, J. Ethnopharmacol. 98 (2005) 109–116.
[7] H.M. Spindola, L. Servat, C. Denny, R.A.F. Rodrigues, M.N. Eberlin, E. Cabral, I.M.O. Sousa, J.Y. Tamashiro, J.E. Carvalho, M.A. Foglio, BMC Pharmacol. 10 (2010).
[8] H.M. Spindola, L. Servat, R.A.F. Rodrigues, I.M.O. Sousa, J.E. Carvalho, M.A. Foglio, Eur. J. Pharmacol. 656 (2011) 45–51.
[9] J.C.T. Carvalho, J.A.A. Sertié, M.V.J. Barbosa, K.C.M. Patrício, L.R.G. Caputo, S.J. Sarti, L.P. Ferreira, J.K. Bastos, J. Ethnopharmacol. 64 (1999) 127–133.
[10] R.C. Dutra, M.B. Fava, C.C. Alves, A.P. Ferreira, N.R. Barbosa, J. Pharm. Pharmacol. 61 (2009) 243–250.
[11] E.A. Nunan, M.G. Carvalho, D. Piloveloso, Braz. J. Med. Biol. Res. 15 (1982) 450–451.
[12] M.C.C. Silva, C.R.M. Gayer, C.S. Lopes, N.O. Calixto, P.A. Reis, C.P.B. Passeas, M.C. Paes, S.R. Dalmau, K.C.C. Sabino, A.R. Todeschini, M.G.P. Coelho, J. Pharm. Pharmacol. 55 (2004) 135–141.
[13] H.M. Spindola, J.E. Carvalho, A.L.T.G. Ruiz, R.A.F. Rodrigues, C. Denny, I.M.O. Sousa, J.Y. Tamashiro, M.A. Foglio, J. Braz. Chem. Soc. 20 (2009) 569–575.
[14] M.D. Miguel, O.G. Miguel, Desenvolvimento de fitoterápicos, Robe, São Paulo, 1999.
[15] International Conference on Harmonization, ICH Topic Q2 (R1) Validation of analytical procedures: text and methodology. Available from: 〈http://www.ich.org〉, 2005.
[16] A.M.C. Arriaga, M.A.B. Castro, E.R. Silveira, R. Braz-Filho., J. Braz. Chem. Soc. 11 (2000) 187–190.
[17] M. Fascio, W.B. Mors, B. Gilbert, J.R. Mahajan, M.B. Monteiro, D. Dos Santos Filho, W. Vichnewski, Phytochemisty 15 (1976) 201–203.
[18] W.B. Mors, M. Fascio, H.J. Monteiro, B. Gilbert, J. Pellegrino, Science 157 (1967) 950–951.
[19] Agilent, Interpretation of Mass Spectra: Course Number H4063A, 2000, 253.
[20] Mart H.B.L. Theunis, K. Foubert, J. Pollier, M. Gonzales-Guzman, A. Goossens, A.R. Vlietinck, L.A.C. Pieters, S. Apers, Phytochemistry 68 (2007) 2825–2830.
[21] C.F. Peng, H. Kaung, X.Q. Li, C.L. Xu, Chem. Pap. 61 (2007) 1–5.
[22] AOAC Peer Verified Methods Program , Manual on Policies and Procedures, Arlington, VA, 1993.
[23] W. Andlauer, M.J. Martena, P. Furst, J. Chromatogr. A 849 (1999) 341–348.
[24] G.P. Sabin, 2007. Desenvolvimento e validação de método utilizando SPE e CG-MS para a determinação multirresíduo de pesticidas em água potável, Dissertação, 2007.