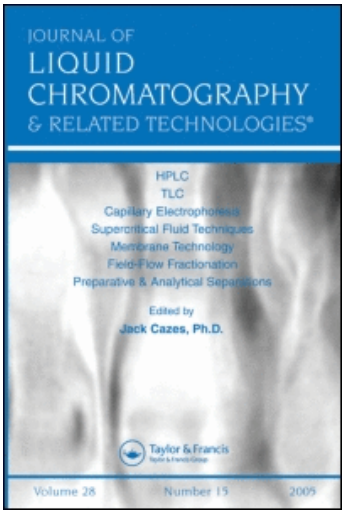

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Development and Validation of an HPLC Method for Analysis of *Picrasma crenata*

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Abstract: *Picrasma crenata* is a Brazilian tree, which is utilized to treat *Diabetes mellitus*, gastric perturbation, and hypertension. Quassinoids are the main constituents obtained from the wood of this plant. The relationship between the quassinoids and the biological effects reveal the interest of quantifying these constituents in *P. crenata* wood, as well as of the validation of the analytical methodologies. This work was designed, therefore, to develop an HPLC system to separate quassin and quantify it in extractive solution from *P. crenata*. The main validation parameters of the method are also determined. The method showed linearity for quassin in the range 13.13–100 μg/mL. The aqueous extractive solution showed linear response in the range 0.978–6.520 mg/mL. Precision and accuracy were determined for the extractive solution in the concentration of 6.52 mg/mL. The HPLC method showed an excellent performance in separating the quassinoids in *P. crenata* extracts, since the presence of interference has been previously evaluated.

Keywords: High performance liquid chromatography, *Picrasma crenata*, Quassin, Quassinoids, Validation

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INTRODUCTION

The vegetable specie *Picrasma crenata* (Vell.) Engl. (Simaroubaceae), popularly named lieutenant stick, fox herb, or bitter taste stick, is a Brazilian medium size tree, which possesses yellow and very bitter tasting wood.[1] It is used in traditional medicine to treat *Diabetes mellitus*, gastric perturbation, and hypertension.[1,2]

Plants of the Simaroubaceae family are well known to contain alkaloids and quassinoids.[3] Some quassinoids received renewed attention because of their biological activity as potential antitumor as well as antiulcer agents.[4,5]

The investigations on the wood of *P. crenata* have demonstrated that the quassinoids are the main constituents. The quassinoids quassin, α-neoquassin, β-neoquassin, α-dihydro-norneoquassin, β-dihydro-norneoquassin, 11-dihydro-norneoquassin, 16-β-*O*-methyl-neoquassin, 16-β-*O*-ethyl-neoquassin, parain, isoparain, and 12-norquassin have been isolated from the wood of *P. crenata*.[3,6,7]

The relationship between the quassinoids and the biological effects reveal the interest of quantifying these constituents in *P. crenata* wood, as well as of the validation of the analytical methodologies. Thus, the aim of this work was to develop and validate a high performance liquid chromatography (HPLC) method in order to analyse the quassinoids from *P. crenata* wood.

EXPERIMENTAL

Chemicals and Reagents

Dried and grounded wood of *P. crenata* (Vell.) Engl. (Simaroubaceae) was obtained from Copo Medicinal (Carangola,, Brazil). Methanol (HPLC grade, Merck, Darmstadt, Germany), and HPLC grade water (Milli-Q system, Millipore, Bedford, MA) were used for the mobile phase preparation. Quassin, α-neoquassin, β-neoquassin, parain, and α-dihydro-norneoquassin were isolated from *P. crenata* (Vell.) Engl. (Simaroubaceae), identified by different methods including UV, IR, and NMR as previously reported,[3] and used as external standards (purity higher than 99%).

Instrumentation and Chromatographic Conditions

High performance liquid chromatography analyses were performed using a Gilson HPLC system (Middleton, WI, USA) equipped with two pumps

(Gilson 321), an automatic flow controller, an Gilson Ultraviolet–Visible 156 variable wavelength detector (set at 254 nm), Rheodyne injection valve with a 20 μL loop, a Gilson column oven, a Gilson solvent degasser system 184, and an UNIPOINT LCNT controller and integrator system.

Chromatographic separation was accomplished using a LiChrospher 100 column RP C18 (Merck, 250 mm × 4 mm i.d., 5 μm), connected with a precolumn LiChrospher RP C18 (Merck, 30 mm × 4 mm i.d., 5 μm), with mobile phase consisting of methanol (pump A) and water (pump B), previously filtered through a 0.45 μm filter (PALL-Gelman). Gradient separation (Table 1) was performed at flow rate of 1.0 mL/min, with the temperature column set at 30 ± 01°C. The sensitivity was 0.05 AUFS and the chart speed was 10 mm/min. All calculations concerning the quantitative analysis were performed with external standardization by measurement of peak areas.

Quassinoid Calibration Curves

Quassin standards were dissolved in methanol-water (50:50, v/v) yielding concentrations of 7.5; 13.13; 17.14; 24; 40; 60; 80; 100, and 120 μg/mL. The solutions were filtered through a 0.50 μm membrane filter (Millipore-FHLP01300). Evaluation of each point was repeated four times and each calibration curve was fitted by linear regression.

Preparation of Extractive Solution (ES)

The aqueous extractive solution was prepared by turbo extraction. The plant-water ratio of 5:95 was employed. The ES was filtered through filter paper (grade 1: 11 μm, Whatman, UK), the volume was made up to 100 mL with water and freeze dried.

Table 1. Gradient elution system used in analysis of quassinoids

Time (min)	Methanol (%)	Water (%)
0	10	90
2	10	90
10	40	60
20	50	50
25	50	50
30	10	90

Preparation of ES Curve

Exactly 1.63 g of dried ES was diluted in methanol–water (50:50, v/v) to 200 mL (Principal Solution – PS). Samples of 3, 4, 5, 7, 10, and 20 mL of the PS were diluted in methanol–water (50:50, v/v) to 25 mL, yielding concentrations of 0.978, 1.304, 1.630, 2.282, 3.260, and 6.520 mg/mL. The solutions were filtered through a 0.50 μm membrane filter (Millipore-FHLP01300). A 20 μL aliquot of each solution was analysed by HPLC. Evaluation of each point was repeated three times.

Method Validation

The specificity was evaluated by analysis of the ES chromatogram compared with the chromatogram of quassinoid external standards.

The linearity was determined for the calibration curves obtained by HPLC analysis of quassin standard and for the ES curve. The range of the appropriate amount of samples was then determined. The slope and the other statistics of the calibration curves were calculated by linear regression using the software STATISTICA® (StatSoft, OK, USA).

The detection limit (DL) and quantification limit (QL) were calculated based on the S.D. and the slope (S) of the calibration curves.[8]

Precision of the method was determined following International Conference of Harmonization (ICH) guidelines.[8] For evaluation of the repeatability, solutions of PS at the concentrations of 2.5, 5, and 10%, prepared according to the above section were analyzed. The peak areas of quassin were determined and the S.D. and R.S.D. of five injections were considered.[9] The intermediate precision was evaluated in triplicate for 3 days.

Accuracy was determined by recovery, adding measured amounts of quassin to ES.[8,9] The recovery experiment was performed at three concentration levels (120, 600, and 960 μg) of quassin standard added in the ES. The recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that were prepared with the added standards, divided by the amount added, and then multiplied by 100%.[8,9]

RESULTS AND DISCUSSION

In this study, a method based on reversed phase HPLC separation combined with UV spectrometric detection was developed to analyse and quantify quassinoids in extracts of *P. crenata* wood. A gradient system with methanol and water, as well as the other chromatographic

conditions, showed high performance in the separation of the quassinoids quassin, α-neoquassin, β-neoquassin, parain, and α-dihydro-norneoquassin in vegetal species analyzed.

The specificity of the method was evaluated by analysis of ES chromatograms compared with the chromatograms of quassinoid external standards (Figure 1). The chromatograms showed high definition of peaks, indicating the method may be utilized for selective determination of these quassinoids in *P. crenata*.

For validation of the analytical methods, the guidelines of ICH[8] and USP 24[9] recommend the accomplishment of tests for accuracy, precision, specificity, linearity, work strip, and robustness of the method.

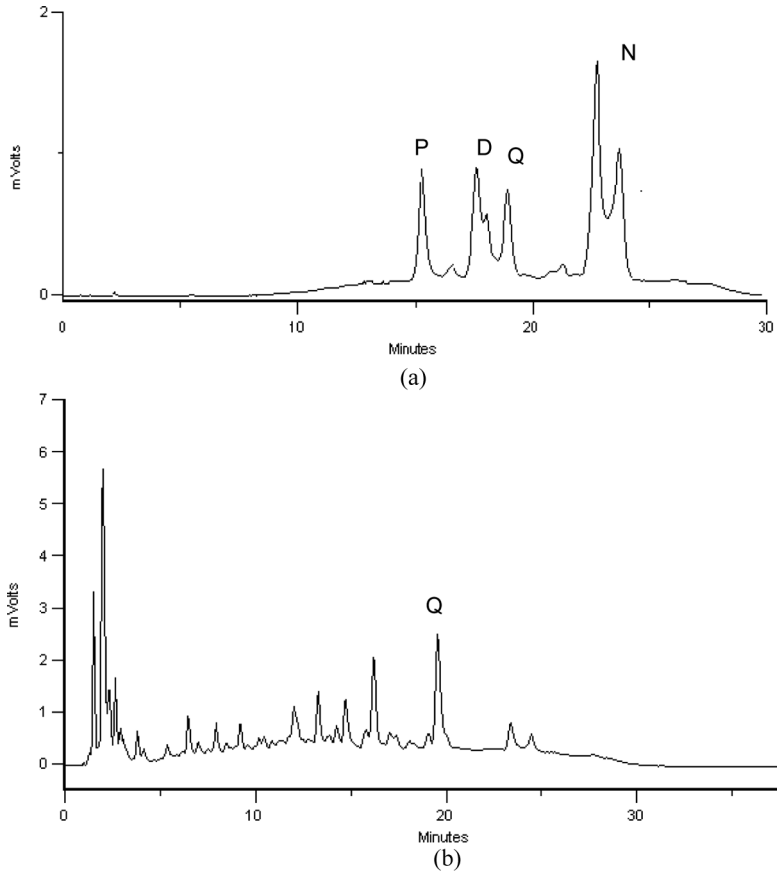

Figure 1. High performance liquid chromatography chromatograms: (a) of quassinoids parain (P), α-dihydro-norneoquassin (D), quassin (Q), and mixture of α-neoquassin and β-neoquassin (N); (b) of the *P. crenata* wood extractive solution.

The type of method and its respective use determines what parameters should be evaluated, especially when the samples are complex biologic matrices, as in the case of extractive solutions from plants.[10,11] Thus, the ES curves were utilized to determine the amount of quassin in the sample, between the linearity range. Linearity of the HPLC method was investigated for quassin in the range 7.5–120 μg/mL at nine concentration levels. The linearity of the method was also investigated employing different amounts of ES, obtaining the calibration curve in the range 0.978–6.520 mg/mL.

Quassin presented the retention time of 19.35 min and the calibration curve was linear in the range 13.13–100 μg/mL, with excellent correlation coefficients (r). The representative linear equation for quassin was $y = 1112.71\ x – 5129.12$ ($n = 6$; $r = 0.9987$) and the R.S.D. of the slope was 10.94. The retention time of quassin (authentic sample) was used to identify the corresponding peak in the *P. crenata* extractive solution.

The DL, taken as the lowest absolute concentration of analyte in a sample, which can be detected but not necessarily quantified under the stated experimental condition, was 1.68 μg/mL for quassin. The QL, taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, was 5.59 μg/mL for quassin.

The selectivity of the proposed method was evaluated by the analysis of the ES chromatogram. The chromatogram presented high resolution of the peak of quassin, indicating that the proposed method could be applied for the selective determination of this quassinoid in the *P. crenata* liquid preparations (Figure 1).

Preparations of *P. crenata* are biological samples and, therefore, complex matrices; in these cases the presence of interference is possible. Thus, the extractive solution curve was used to determine the sample amount of ES, where linearity is observed. The calibration curve of the quassin in this extractive solution was $y = 35526\ x – 10708.30$ ($n = 6$; $r = 0.9967$) and the R.S.D. of the slope was 788.70.

Table 2 shows the concentration of the quassin in six levels of *P. crenata* extract.

Table 2 also shows that ES presents high quassin concentration, which is why the precision (repeatability and intermediary precision) and accuracy (recovery) were determined for this solution. The repeatability of the HPLC analysis of ES was demonstrated with R.S.D. from 0.90 to 6.0% and medium R.S.D. of 3.65%. This result could be considered satisfactory since the majority of phytochemicals present a range of 3–6%.[12] The R.S.D. of the areas obtained by HPLC was 2.33% for the analyst I and 3.65% for the analyst II, showing high reproducibility between areas for the quassin.

The accuracy of the HPLC method for the assay analysis of recovery was determined by preparing samples adding 120, 600, and 960 μg of

Table 2. Calibration curve of the extractive solution ES by HPLC with the corresponding concentrations (μg/mL) of quassin

ES (mg/mL)	Quassin (μg/mL)	R.S.D. (%)
0.978	29.18	3.99
1.303	37.41	4.04
1.630	48.74	4.03
2.282	62.79	0.90
3.260	98.46	2.89
6.520	204.48	6.00

quassin in the ES. The recoveries of added quassin standard were, respectively, 108.6%, 102.9%, and 108%. These results referred to the average of three assays and they are in good agreement with the results (80–120%) required.[8]

CONLCUSIONS

In this work, an HPLC method was developed allowing the separation of quassin present in *P. crenata*. The extractive solution curve showed linear response in the range 0.978–6.520 mg/mL. Precision and accuracy were demonstrated for the high concentration of quassin in the extractive solution.

In conclusion, the proposed HPLC method shows an excellent performance to separate and quantify the quassinoids in *P. crenata* preparations.

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