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Original Article

ANALYTICAL METHOD BY HPLC-DAD ALLOWS QUANTIFICATION OF QUERCETIN MARKER IN STANDARDIZED EXTRACT OF *ANADENANTHERA COLUBRINA* VAR. CEBIL

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ABSTRACT

Objective: The *Anadenanthera colubrina* (Vell.) Brennan var. cebil is a medicinal plant that has been used for the treatment of many diseases in the northeastern region of Brazil. This plant contains secondary metabolites such as quercetin, a flavonoid that is known by its antioxidant and anti-inflammatory effects. The aim of this work is to propose the validation of an analytical method using high-performance liquid chromatography with diode array detector (HPLC-DAD) for the quantification of quercetin and standardization of the hydroalcoholic extract (HAE) of *A. colubrina.*

Methods: The *A. colubrina* extracts were prepared by the maceration process with powdered leaves at 20% weight: volume (w/v) and a hydroalcoholic solution at 50% volume: volume (v/v) for 120 h at room temperature. After pretreatment of the hydroalcoholic extract, the quercetin marker was used for quantification and proceeded to the evaluation of validation parameters for the method using HPLC-DAD.

Results: The analytical method proved to be specific. Linear over the range 1.4–26.6 µg/ml, regression analysis showed a good correlation coefficient (R2= 0.999); the limit of detection (LOD) and the limit of quantification (LOQ) were 0.27 and 0.81 µg/ml respectively. The relative standard deviation (RSD) did not exceed 2.5% for precision. The proposed method was validated with an average recovery of 92.5–97.5%.

Conclusion: The method was validated using HPLC-DAD, allowing the quantification of quercetin in the standardisation process of extracts and quality control of the herbal drug containing *A. colubrina* Phyto complex.

Keywords: Validation, Analytical, Method, Quercetin, Flavonoid, *Anadenanthera colubrina*

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INTRODUCTION

Anadenanthera colubrina (Vell.) Brenan var. cebil (Griseb) Altschul (*Fabaceae*/*leguminosae* is a native plant detected in the South American continent and distributed throughout Paraguay, Argentina, Bolivia, and Brazil [1-2]. Popularly known by the names yopo, cohoba, vilca, and angico, this plant has economic, cultural, and medicinal importance [3-4]. Hydroalcoholic extracts and decoctions of the leaves and bark of *A. colubrina* are used in the treatment of gastric and respiratory tract infections and others inflammatory diseases. It is classified among one of ten medicinal plants most frequently used by the communities living in the bioma named Caatinga located in northeastern Brazil [5-6].

Preclinical studies of the healing and antimicrobial activities of *A. colubrina* extracts endorse their popular usage [7-9]. These assays also demonstrated anti-inflammatory mechanisms, such as the decrease of the production of pro-inflammatory cytokines, inflammatory mediators, increased tyrosine kinase production, and healing action by reducing the synthesis of polymeric collagen and acidic glycoproteins that make healing difficult, and thus contributing to the development of a phytotherapeutic medicinal products with safety and therapeutic efficacy [10-13]. Research on the quality of a herbal drug is not based solely on clinical effects but also on the standardization of plant extracts through quantitative analyses using active ingredients and secondary metabolites called biomarkers [14-16].

The development of analytical methods must be based on fundamental conditions such as sampling, pretreatment sampling, and the evaluation of validation parameters in order to guarantee the quality, consistency, and reliability of the analytical results [17-18]. The pretreatment of samples is a decisive step in the development of the analytical methods of constituents in biological matrices and must guarantee the release of the analytes of the complex matrix, to be monitored by analytical signals with appropriate instrumentation [19-21]. Some matrices of plant´s components may behave as interferers, which impair the identification and quantification of biomarkers, in addition, these interferers may compromise the performance of chromatographic systems [18, 21-23].

A sample clean-up procedure improves analytical sensitivity, enables more robust reproducible results, and preserves the life of chromatographic columns [24-26]. Chromatographic techniques such as HPLC (High-Performance Liquid Chromatography) present advantages for efficiency separation and the identification of compounds when associated with detection systems appropriate to the chemical nature of the analytes, providing qualitative and quantitative information about Phyto-complex components [27-28]. *A. colubrina* has a large amount and class of secondary metabolites: tannins; alkaloids; flavonoids such as quercitrine; isoquercetrine; apigenin; quercetin; and other phenolic compounds [29-33].

Several analytical methods have been reported for the analysis of flavonoids in the extracts of medicinal plants [34-35]; however, they cannot be used as general methods for analysis due to the complexity of each vegetable plant species and the variability of factors that could influence their chemical composition [36-37]. Until now, no method validated by HPLC-DAD for quercetin quantification of HAE of *A. colubrina* has been reported in the literature. Properly validated analytical methods allow reproducible qualitative and quantitative analyses and ensure consistency of the analytical quality of a medicinal product [29, 38]. In this research, a methodology will be developed for extracting the biomarker quercetin in the phytocomplex of *A. colubrina* and an analytical method for the quantification of quercetin in that plant's extract will be validated, according to the ICH (International Conference on Harmonisation) Q2 (R1) guidelines for HPLC-DAD [39-40].

MATERIALS AND METHODS

Reagents and materials

The quercetin standard (Cas 117-39-5) was purchased from Sigma Alldrich®, Brazil, methanol HPLC grade was purchased from Tedia®, USA, orthophosphoric acid was purchased from Merck®, Germany, ethanol 96% was purchased from Toscano®, Brazil, dichloromethane hplc grade and hexane were purchased from Vetec®, Brazil.

Drug identification plant

The *Anadenanthera colubrina* var. cebil leaves were collected from cacimbas farm, Caraúbas county located in the micro region of cariri in the state of Paraiba (Lat. 7 °30´48.1´´S; Long. 36 °41´81.0´´O) in May 2015. The research project with *A. colubrina* received authorization from the Ministry of Environment of Brazil (ICMBio\SISBio\MMA-Brazil) for activities with scientific purpose n ° 41277-2. The exsiccatae of the plant species was deposited in the herbarium Lauro Pires Xavier, UFPB Joao Pessoa, Brazil, under the number NC262.

Plant drug drying

The *Anadenanthera colubrina* var. cebil leaves were dried in a greenhouse with circulating air (Tecnal®, model TE 394-4), under a temperature of 40±2 degrees *celsius* (°C) by 3 d.

Obtaining the sprayed plant drug

The dry plant drug was submitted to a vertical rotor mechanical mill (Tecnal® Modelo TE–631–3). The powdered vegetable drug was packed in a hermetically sealed plastic bag protected from light and moisture.

Obtaining hydroalcoholic extract (HAE)

The extraction was established from the ratio of 20% weight: volume (w/v) from the mass of the sprayed vegetable drug and the extractive solvent system. The extraction system was defined using a proportion of hydroalcoholic solution at 50:50 volume: volume (v/v), and the extraction method utilised was maceration.

Chromatographic conditions

We used HPLC-DAD system prominence series by SHIMADZU®, Japan, control system was performed by software LC Solutions®; octodecilsilano C-18 stationary phase Gemini nx 5 micrometers (µm) 150 x 4.6 millimeters x 0.5 µm; pre-column gemini C-18 4 x 3.0 mm; membrane-filtered mobile phase PTFE 0.45 µm and degassed: methanol: phosphoric acid 1% (47: 53%). mobile phase flow: 1.2 ml/min; Oven temperature at 40 °C; monitored wave number at 370 nm; injection volume 20 microliters (µL); chromatographic run time 30 min.

Development of the analytical method

An analytical method was validated by HPLC-DAD for the quantification of quercetin marker in extract samples of *A. colubrina*. The ICH Q2-R1 guidelines [34] were observed in the evaluation and validation parameters for analytical procedures: specificity, selectivity, linearity, limit of quantification and detection, precision, accuracy, robustness, and stability. Deviations of up to 5% were considered in the statistical evaluation of the data.

The choice of quercetin marker was based on bibliographical researches of phytochemical compositions [31-32] and preliminary tests of characterization of the variant *A. colubrina* extract, which indicated the same as the majority. An analytical extractive procedure was developed to guarantee clean-up, pre-concentration, exhaustive extraction of the marker, and elimination of possible interferences of the fraction to be injected into the chromatographic system.

In the development of the marker recovery method, continuous multiple extraction was employed and liquid-liquid extraction (ELL) was used by the agitation system (Vortex model AP-56 Phoenix®) of threated tubes with caps of 10 milliliter (ml) n ° 9825 (Pirex®, Mexico) and the use of centrifugation (Centrifuge 80-2B, Macro®) with rotation of 4000 revolutions per minute (RPM) during 10 min to separate the phases.

Determination of the analysis factor

The analysis factor was determined due to successive dilutions made in multiple extraction continued and allowed to quantify the content of the chemical marker present in the extract in micrograms per milliliter (µg/ml).

The Equation 1 below shows how to determine the concentration of the marker in the HAE.

$$Ca = Cp \times FA \times Aa \div Ap \quad (1)$$

Hence:

Ca is the concentration of the quercetin biomarker in the HAE sample, expressed in micrograms per milliliters (µg/ml),

Aa is the area of the chromatographic peak marker on HAE,

Ap is the quercetin standard chromatographic peak,

Cp is the concentration of the chemical reference substance quercetin,

FA is the analysis factor and is calculated by equation 2:

$$FA = Ta \div \{[(Ta \div Fec) \times Fevap] \div Vrec\} \quad (2)$$

Hence:

Ta = HAE Sample Taking

Fevap = Organic Fraction Aliquot

Fec = Fraction Continuous extraction in 3 batches

Vrec = Volume of Reconstitution in the mobile phase.

Specific assessment

In order to demonstrate specificity and selectivity of the method, it was performed in triplicate runs of *A. colubrina* extract samples subjected to analytical screening, standard marker solution, and mobile phase.

Determination of linearity

A quercetin stock solution of 200 µg/ml prepared with methanol 70% was obtained and diluted solutions with concentrations corresponding to 1.4 µg/ml, 2.1 µg/ml, 5.6 µg/ml, 9.8 µg/ml, 14µg/ml, 18.2 µg/ml, 22.4 µg/ml, 26.6 µg/ml to aim linearity. This procedure was performed in triplicate for each concentration level.

Quantification and detection limits

The lowest amount of the analyte detected in a sample that but not necessarily measured as an exact value is known as the limit of detection (LOD). The lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy is the limit of quantification (LOQ).

The determination of the limits of quantification and detection was based on the treatment of linearity data by statistical methods. The limit of quantification calculation was obtained from the ratio of the deviation of the linear regression by the inclination of the line, multiplied by 10. The limit of detection calculation was based on the ratio of the deviation of the linear regression by the slope of line, multiplied by 3.3.

Precision assessment

Repeatability

Repeatability was demonstrated by the injection of 06 quercetin-content samples close to the mean value (100%) of the standard linearity curve. The acceptance criterion for this parameter was a relative standard deviation (RSD) with a maximum of 5%.

Intermediate precision

Intermediate precision was determined from the analysis of 6 samples of the extract, from different analysts and days, with quercetin content close to the mean value (100%) of the standard linearity curve. A total of 18 chromatographic runs were performed,

and the acceptance criterion was at the maximum of 5% and evaluated the RSD.

Accuracy assessment

The accuracy of the analytical method was assessed by the post-addition recovery of the quercetin marker standard in extract samples. Three concentration levels (low, medium, and high) were monitored with values corresponding to 20%, 100%, and 180% opposite the accurate concentration obtained. Samples were prepared in triplicate, and injections were performed in duplicate for each concentration level; relative standard deviations (RSD) and recoverability were assessed.

The recovery capacity was calculated from the average concentration of the marker on the standardized extract. Table 1 shows how to calculate standard addition values and percentages of value recovery of the biomarker for all levels.

Table 1: Process of evaluating accuracy of the method

Recovery of standard added				
Level	**A (µg/ml)**	**B (µg/ml)**	**C (µg/ml)**	**% recovery**
20%	[W_1]	[0.2 W_1]	[W_2]	Rec 20% = $\frac{[C]}{[A+B]} x\ 100$
100%	[W_1]	[W_1]		Rec 100% = $\frac{[C]}{[A+B]} x\ 100$
180%	[W_1]	[1.8 W_1]		Rec 180% = $\frac{[C]}{[A+B]} x\ 100$

Note: A (initial concentration of quercetin in HAE); B (concentration of standard substance chemistry added); C (Recovered concentration of quercetin in HAE); [W_1] equivalent to the average of the intermediate precision; [W_2] equivalent to the average of the experimental recovery.

Robustness assessment

In the robustness assessment, variations of oven temperatures in the range ±2 °C were evaluated, as well as variations in the pH of the mobile phase varying ±0.1 and changes in the mobile phase flow±0.1. Furthermore, variations in the chromatographic profiles such as retention time, peak area of chromatograms, and ultraviolet spectrum profile of the monitored samples were evaluated. The quantification of the marker in the extract of *A. colubrina* samples was determined with a standard quercetin substance in the same analysis condition of parameter robustness. The RSD was calculated and evaluated.

Stability assessment

To evaluate the chemical storage stability of solutions of the marker and the extract samples, these were analyzed at zero to 24 h at room temperature. The chromatographic profile, the analysis of the areas of the chromatographic peaks, the retention time, and the spectral profile of the marker were verified to demonstrate stability.

Statistical analysis

The statistical analysis of the data was determined through analysis of variance (ANOVA) in software Prism® 6.01, considering a level of significance α = 0.05.

RESULTS AND DISCUSSION

Development of the analytical method

Evaluation of analyte separation parameters

The main pharmacopoeias contains few monographs related to medicinal plants obtained in tropical regions. The standard methods in pharmacopeias are the most indicated for the evaluation quality control of herbal drugs but not exists any reference related to *Anadenanthera colubrina* [41-42]. Although there are several analytical methods for the analysis of quercetin in extracts, these methodologies cannot be directly applied for any medicinal plant due to the complexity effect of the plant matrix [43-45]. Moreover, not exists any validated method reported to quantify quercetin in *A. colubrina* by HPLC.

Chabariberi and cols. when validated a spectrophotometric methodology for flavonoid analysis in extracts of *Maytenus (Celastraceae)* and *Passiflora (Passifloraceae)* have found differences between the absorbances maximum levels with those recorded in the official pharmacopoeias method which quantified total flavonoids expressed in rutin. This variation can be attributed to modifications in the sample pretreatment, as well as to the complexity of the chemical matrix of the analyzed fraction [46].

The evaluation of the validation parameters in this work was preceded by the developing of samples preparation method and extraction of quercetin marker, then the separation conditions by HPLC of the extract fraction of *A. colubrina* was obtained. The chromatographic analysis of the marker was performed initially with a mobile phase system composed by methanol and phosphoric acid, with pH 2.5 varying the ratio of the organic component. Three proportions methanol with 0.01% phosphoric acid were tested in the order of 30:70, 50:50, and 70:30.

In the analytic method development, the HAE sample and the quercetin standard was evaluated under the same separation conditions. Under the conditions of 30:70 and 70:30 were generated overlapping peaks, already under the 50:50 condition it was showed co-elution with the marker peak when compared to the quercetin standard peak, this interference in the co-elution can be characterized by the absence spectral similarity of the main peak under the evaluated conditions. The fig. 1 shows the chromate-graphic profiles of each investigated condition.

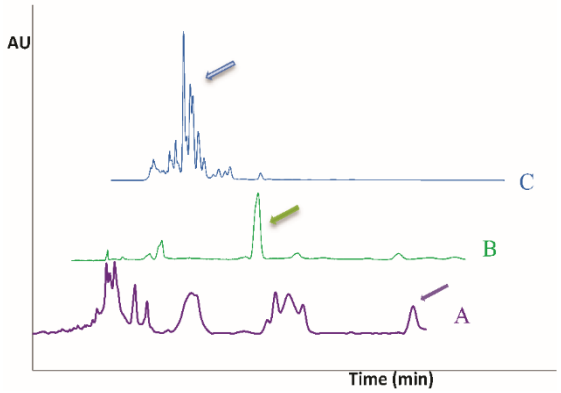

Fig. 1: The chromatographic profile obtained from mobile phase system conditions in the development of the method

According to a review about recent advances in analytical technology applied to quality control of medicinal plants, the complex matrix, the compounds interferences and co-elutions in the chromatographic separations do not allow a reproducible characterization of the extracts [47-48]. In another work with polyphenols, evaluating the chromatographic profiles of natural product, it was demonstrated that there was a great variability in the

content of the secondary metabolites in real matrices of these products [49].

According to Paiva and cols., in a study evaluating the chromatographic profile of two species of the *Plumbaginaceae* taxonomic family, the data showed that these species presented common chemical markers, however the chromatographic profiles were different among of the analyzed species, the data, also demonstrated which there are chemical variability on the constituents of the extracts which can differentiate so close species [50].

Xie and cols. validated a method for the simultaneous quantification of three flavonoids and evaluated the chromatographic fingerprints of *Flos sophorae immaturus* which were collected from different areas from China. The data showed that although the samples shared similar chromatographic patterns, the similarity analysis demonstrated that difference in the constituent's ratios and integrated areas resulted in low similarity values, even if all samples show similar chromatographic fingerprint profiles [51].

The matrix effect is not usually evaluated in validation guidelines, and has been omitted in most of the methods here consider. However, this parameter is a very important parameter to obtain a properly validated and accurate analytical method [52].

The chromatographic analysis of the HAE sample presented interferents that precludes a direct analysis, thus, it was necessary to perform a series of pretreatments in order to remove the interferents such as, fats and pigments which are very common in complex matrices of plants [53-55]. These interfering substances may influence the parameters of selectivity and specificity of analytical validation and can be confirmed when it is seen no clean chromatograms by co-elution of other substances with the main peak and when apolar compounds are retained in the column and chromatographic runtimes are extensive [56-58].

The selection of the best separation condition was based on peak resolution parameters (Rs), retention index (k'), tailing factor (T), separation efficiency (α), and number of theoretical plates (N) in relation to the main peak monitored, showing the need to optimize the elution condition 50:50 to at 47%:53% methanol: phosphoric acid 0.01%, pH 2.5. The pH value is justified and consistent with the literature through the employs low pH and suppression of ionization, assuring complete elution of analyte [59-61].

All evaluated parameters shown in table 2 demonstrate the efficiency of the separation. The peak of the quercetin chemical marker with retention time of 10.14 min was considered as the main peak to calculate the values obtained.

Table 2: Parameters of chromatographic separation efficiency

Standard peak (RT = 10.1 min)	Mobile phase 47:53	Recommendation
Resolution (Rs)	2.07	Rs>1.5
Retention factor (K')	3.76	$1 \leq K' \leq 10$
Tailing factor (T)	1.020	$0.5 \leq T \leq 2$
Separation efficiency (α)	1.23	α>1
Theoretical plates (N)	3878	N>2000

Note: Mobile phase system consisting of methanol 47% and 53% Phosphoric acid 0.01%. RT (real time).

As shown in table 2, the optimized conditions generate chromatograms with an ideal range for retention factors (1.0<k<10), separation efficiency values greater than 1.0, and resolution above 2.0, confirming the quality of separation. The efficiency of the column was optimum, and the peaks of the rejection factor between 0.5 and 2.0 demonstrate the run. Analytical methods confirm good results in the efficiency parameters, as obtained in the value of 3878 theoretical plates higher than 2000.

Timóteo and cols. In validating ~~a~~ method for analysis of herbal teas found that a single chromatographic peak corresponded to three constituents. The accurate separation of the constituents was only possible after varying the separation conditions using stationary phase with different characteristics and dimensions and monitoring of the spectral purity, as realized in the present [62].

Evaluation of analyte recovery

The recovery of the marker investigated the extractive potential of solvents with different polarities: dichloromethane, ethyl acetate, and chloroform on the fluid extract matrix. Extraction with ethyl acetate showed a single-phase system with difficult separation, extraction not being possible. In contrast, the chloroform solvent was able to extract with limitation and variation in the recovery and reproducibility of the analyte, even with the use of continuous multiple extractions. Otherwise, dichloromethane was suitable for extracting the analyte efficiently after three extractions using a continuous multiple-extraction system.

The recovery of the method was also evaluated through different aqueous fractions of 0.5 to 2 ml of HAE, in which the recovery and the selectivity of the marker in the extraction were determined. The best recovery was obtained in 0.5 ml fraction with three consecutive extraction steps, obtaining a separation of the marker with selectivity and adequate recovery. The final step of the sample preparation was the evaporation at 50 °C of 4.0 ml dichloromethane fraction followed by reconstitution of the same in the mobile phase. The reconstituted solution was filtered and transferred to vials to be injected into the chromatographic system.

A study that evaluated extraction of quercetin and kaempferol in plant matrix concluded that ethyl acetate solvent was insufficient to extract the flavonoids by liquid-liquid extraction, which is consistent with the data obtained in this work [63]. Several papers present the methanol solvent as optimum in the extraction of flavonoids [51, 62], but due to the aqueous nature of the extract preference was given to a solvent that produced a heterogeneous system with the fluid extract. In this work, the choice by chloroform and dichloromethane was based on the principle of miscibility between the fluid extract and these solvents and still on the chemical affinity these solvents have for flavonoid compounds [64-65].

Evaluation of the method validation parameters

Assessment of specificity

The specificity of the method was based on comparing its retention times and UV-spectra in the 200–400 ηm range with the quercetin standard. Also compared were the spectral and chromatographic profiles of blank from the mobile phase, quercetin standard, and sample extract.

The choice of an appropriate detection wavelength was of great importance to ensure accurate detection of the quercetin and to achieve specificity. The UV spectrum of the compound was detected at 370 ηm by diode array detector under the chromatographic conditions as described in methodology. The specificity analysis was demonstrated as shown in fig. 2 and the results are consistent with those reported in the literature [52].

The analysis of chromatographic profiles of the standard and sample showed that the method was specific in separation and identification marker quercetin by overlapping chromatographic profiles. The spectral analysis of the sample and standard quercetin, shown in fig. 2 and 3 demonstrates the specificity by the similarity of the spectral

profile and identification of the wave number with maximum absorbance at 370 ηm.

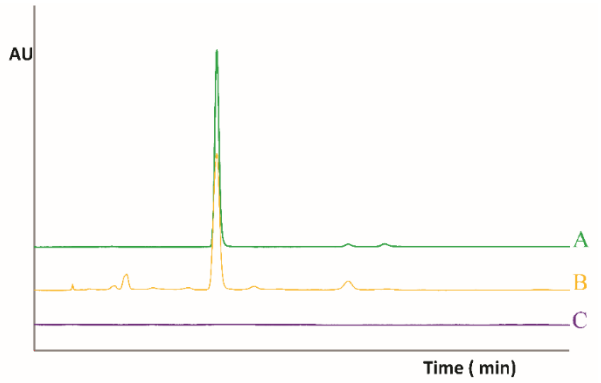

Fig. 2: Chromatographic profiles of the quercetin solution standard (A), solution sample (B) and blank (C)

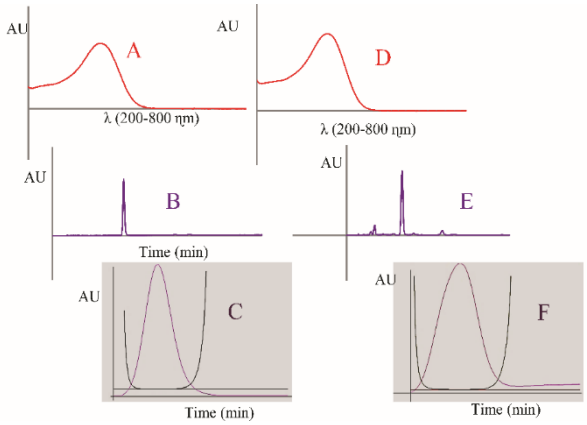

Fig. 3: Spectral profile of the quercetin standard (A, B, C) and the marker monitored in the extract of the leaves of *A. colubrina* (D, E,F)

The ultraviolet spectrum indicated on graphics A and D were evidence of similar spectral profiles with a maximum absorbance of 370 ηm. The graphics C and F demonstrate spectral purity for both chromatographic peaks, shown in profiles B and E, which represent the chromatographic run of the sample and standard. In addition, specificity was confirmed by detector photodiode arrangement, which demonstrated that quercetin peaks have no co-elution of any additional peak, with values of peak purity greater than 0.999. The confirmation of the identification of the monitored peak in the chromatographic profiles is visualized in fig 3.

Based on the chromatographic and spectral profiles observing peak retention time, spectral profiles similarity, and spectral purity, it was possible to demonstrate the specificity of the same proposed method.

Determination of sensitivity

Both the limit of detection (LOD) and the limit of quantitation (LOQ) were also determined with linearity and statistical tool applications; the limits of quantification and detection were respectively calculated to obtain values of 0.81 µg/ml and 0.27 µg/ml. similar results were reported in the development of the analytical method for quercetin determination in the *Platycladus orientalis* (L.). At cited work obtaining LOD values of 0.005 µg/ml and LOQ values equivalent to 0.01 µg/ml [66]. The values difference in relation to the proposed method can be associated with several factors such as extractive matrix complexity, the method of determination of LOD parameters and LOQ up to detector sensitivity. Mattonai and cols. validated a method by HPLC-DAD and HPLC-MS for determination of polyphenols in monofloral honey, obtaining LOD and LOQ for quercetin equivalent to 0.2 and 0.7 ug/ml respectively. These results of cited article are in agreement with data of the analytical method validation proposed in this work [67].

Determination of linearity

Table 3 shows the plotted reference values of linearity with average areas obtained for each concentration level. The linearity of the method was demonstrated by evaluating the linear correlation R^2 coefficient obtained by linear regression and analysis of single factor variance, where it was shown that the F Tabulated (Critical) is greater than the calculated F value, for a level of significance of α>0.05. Based on ANOVA, the variations between values obtained from the three curves were not significant.

Table 3: The Mean linearity obtained from triplicate injections of solutions containing quercetin as reference standard

Level (%)	Concentrations (µg/ml)	Mean área[a]±RSD
10	1.4	75218±4.4
15	2.1	116212±3.1
40	5.6	308288±3.1
70	9.8	552019±1.3
100	14	796704±1.7
130	18.2	1037895±0.1
160	22.4	1268748±0.2
190	26.6	1504493±1.2

Note: [a] n = 3 injections, RSD (Relative standard deviation).

The mean linearity curve and linear regression equation are shown in fig. 4. The linearity met the requirement of R^2 greater than 0.99 as recommended in the validation guides of analytical methods. The data that generated average linearity are available in the complementary information. The analysis of variance as a single factor showed a value of F (critical) that was higher than the value of F calculated, indicating that the variations among the data for the three curves were not significant. The statistical data of linearity were in agreement with ICH guidelines for analytical methods. Similar results were reported by Blainski and cols. when validating a method by HPLC-DAD for gallocatechin and epigallocatechin quantification in rhizomes from *Limonium brasiliense* obtained linearity results similar to this work. The regression coefficient (R2) for two markers were above 0.99, calculated F-value is lower than critical F-value, the analysis of variance showed p-value below 0.001[68].

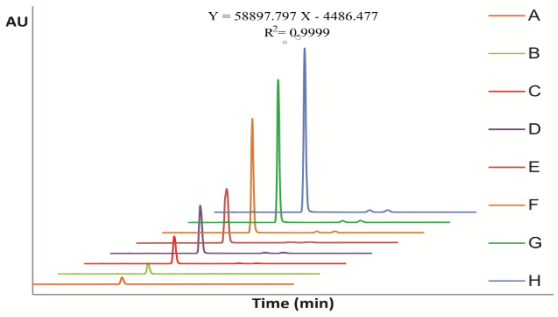

Fig. 4: Chromatographic profiles of linearity obtained with 8 points at increasing concentrations and linear regression equation with R^2 value

Precision assessment

Repeatability and intermediate precision

Repeatability was evaluated with the same analyst, day, and chromatograph with six runs from the extract sample, allowing the proposed method to exhibit adequate precision for quercetin, with the percentage of RSD overall better that 5%.

The intermediate precision was evaluated in three days with a total of 18 runs of the extract obtained from the extract sample. The results obtained from the precision are plotted in table 4. These data demonstrated intermediate precision and met the requirements for the analytical method validation guidelines. According to the statistical analysis, there were no significant variations among the data, where *p* value between rows and columns is above 0.05, and critical F values are smaller than the values of the tabled F, proving that the method developed is accurate under the established conditions. The overall composition of the extract was the same in all the samples analyzed, assure reproducibility and highlighted the repeatability and intermediate precision as an important parameter for quantitative analysis of *A. Colubrina.* These results are according to the official literature for methods of validating [69] and demonstrate that the developed method can be satisfactorily used for determination of quercetin in the *A. colubrina* samples extracts.

Table 4: Precision assessment

Repeatability		**Concentration of quercetin (µg/ml)**	**RSD (%)**
	Mean[a]±SD	114.96±2.23	1.9
Day	**Intermediate precision (intra day)**		
1	Mean[b]±SD	127.01±4.06	3.2
2	Mean[b]±SD	125.05±1.10	0.88
3	Mean[b]±SD	125.67±2.92	2.32
Intermediate precision (inter day)			
	Average[c]±SD	125.92±2.90	2.30

Note: [a,b]n = 6; [c]n =18, SD (Standard deviation) and RSD (Relative standard deviation).

Accuracy assessment

The accuracy of the analytical method was evaluated by adding known amounts of the marker quercetin in three concentrations: low, medium, and high. The standard addition had values of 20%, 100%, and 180% compared with mean values of the concentration obtained in the matrix. In table 5, the data are plotted for the accuracy assessment of the proposed method. It contains the values of average areas (duplicate) obtained for each level of accuracy with their respective values, information about the true value concentration of samples with standard addition, and experimental values obtained after recovery of the marker expressed in percentages. Statistical analysis confirmed that the data were within the acceptance criteria.

Table 5: Evaluate of recovery and accuracy

Level of addition (%)	**A (µg/ml) ±SD**	**B (µg/ml) ±SD**	**C (µg/ml) ±SD**	**Recovery (%) ±SD**
20 %[a]	125.9±2.90	25.2	147.3±4.3	97.5±2.7
RSD (%)	2.3		2.9	2.7
100 %[b]		125.9	238.15±4.3	93.8±4.1
RSD (%)			1.8	4.4
180 %[c]		226.6	327.5±11.5	92.5±3.4
RSD (%)			3.4	3.7

Note: A (concentration of quercetin in matrix of the extract); B (concentration of standard quercetin added); C (amount recovered). [a,b,c] n = 18, SD (Standard deviation) and RSD (Relative standard deviation).

The quercetin marker was recovered by partitioning fluid extract and dichloromethane solvent using liquid liquid extraction. The pretreatment of the sample was based on the principle of chemical similarity, where the solvent removed interfering compounds and recovered the quercetin from the phytocomplex of *A. colubrina* [50].

The assessment of recovery is a determining parameter in the safety of analytical measures and critical step in the validation [39]. Mattila and colleagues determined the amount of quercetin in apple, red wine and green tea obtaining recovery of 79%, 104% and 82% respectively, compared to 93% reference sample, which demonstrates the variability of extraction in different matrices and the importance of establishing strategies for the removal of interferences and total extraction of the analytes of interest [70]. The recovery of analytes in complex matrices by methodology similar to the one performed in the present research presented recovery range between 92 and 94% with RSD<5% [71]. Tang and cols. evaluating the recovery of the quercetin marker in *Ginkgo biloba* extract samples, obtained recovery values between 97.2 and 101.4% with RSD = 2% [72]. Another study, evaluating different techniques of extracting the flavonoids quercetin and kaempferol in plant matrices, presented values of recovery of these analytes after standard addition between 96.8 and 98.2% [63]. These researches confirm the recovery values of quercetin obtained for samples of *A. colubrina* extract.

Robustness assessment

The robustness was determined using the overall mean, standard deviations, relative standard deviations (RSD %) for each parameter. Samples were prepared in triplicate and the analyses were performed in duplicate for each condition evaluated.

The most typical variations on robustness considered in analytical methods validation are the column oven temperature, the composition, the pH and the flow of the mobile phase [52].

In the evaluation of the influence of variations in the column oven temperature, on the areas of the peaks, it was observed that this parameter in the evaluated conditions±2 °C did not influence the results and therefore, of marker content.

In the influence of variations in the flow of the mobile phase on the peak areas, it was observed that the parameter evaluated under the conditions of 0.1 ml into more or less did not influence the results area of the analyte.

In the evaluation of the influence of variations in the pH of the mobile phase on areas of the peaks, it was observed that this parameter in the evaluated conditions±0.1 influenced the results of area of the peak analyte. This variation can be attributed to

ionization problems of the marker molecule, and therefore pH is a parameter to be strictly monitored for the proposed method.

In the statistical analysis, ANOVA was chosen with a 95% of confidence interval due to the biological matrix studied being complex. Therefore, at a level α of 0.05, there is a significant difference in the robustness dataset, considering that F value is calculated higher than F (Critical) should be necessary evaluated possible variations in the robustness parameter. The results of the robustness evaluation are presented in table 6.

Table 6: Evaluation of conditions of column oven temperature, flow of the mobile phase and pH in robustness of the method

Normal Condition	**Temperature (°C)**		**Flow (ml/min)**		**pH**	
	40		**1.2**		**1.6**	
Average concentration[a] (µg/ml)±SD;	125.1±1.4;					
RSD (%)	1.2					
Tested condition	38	42	1.1	1.3	1.5	1.7
Average concentration[a] (µg/ml) ±SD	124.2 ±1.8	125.6±1.3	124.3±4.4	126.1±5.0	114.8±2.3	115.9±0.6
RSD (%)	1.4	1.0	3.5	4.0	2.0	0.5

Note: [a]n = 6. SD (Standard deviation) and RSD (Relative standard deviation).

Table 7: Stability assessment of sample extracts solutions

	Concentration of quercetin (µg/ml)	
	T_0	T_{24}
Average concentration [a]	115.0±2.23;	114.0±1.92;
(µg/ml)±SD; RSD (%)	1.9	1.7

Note: [a]n = 6, SD (Standard deviation) and RSD (Relative standard deviation). T_0 (Sample solution in time zero), T_{24} (Sample solution after 24 h the time zero).

Evaluation stability

The stability of the sample extract was analyzed at 0 and 24 h after sample preparation. The data showed that the samples in these conditions were stable for at least 24 h. The stability data are plotted in table 7. The sample solution analyzed exhibited values of concentrations of quercetin at 115.0 and 114.0 µg/ml, respectively for the test time. The coefficient of variation of the stability data of the analyte did not exceed 2.0%.

These results are according to the minimal official guides requirements for methods of validating [39] and demonstrate that the developed method can be suitably used for analysis of quercetin in the *A. colubrina* extract samples.

CONCLUSION

In this paper, the analytical method of HPLC-DAD was validated for quantitative determination of quercetin on an extract of *A. colubrina* allowing selective analysis free of interferents. The analytical method proved to be specific, selective, linear, precise, accurate, robust, fast, and reliable. The data obtained suggest that the method can be applied as an appropriate analytical tool in the quality control of herbal medicines obtained from extracts of *A. colubrina*.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

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