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Original Article

An HPLC Method to Determine Phenolic Compounds of Plant Extracts: Application to *Byrsonima crassifolia* and *Senna alata* Leaves

Marília de A Cavalcante[1], Janyna dos S Oliveira[1], Mayra S da S Barreto[2], Lucas P Pinheiro[2], Patrick de C Cantuária[3], Wardsson L Borges[4,5], Gabriel A da Silva[2], Tiago Marcolino de Souza[2]

ABSTRACT

Background: The Amazonian Region has a variety of medicinal plants with bioactive compounds, whose characterization could present the potential for sustainable development. **Objectives:** A method for separating, identifying, and quantifying a mixture of nine phenolic compounds (gallic acid, 3-hydroxybenzoic acid, p-coumaric acid, catechin, myricetin, rutin, quercetin, kaempferol, and cyanidin) was developed, validated, and applied to analyze aqueous and hydroethanolic extracts from *Byrsonima crassifolia* (L.) Kunth and *Senna alata* (L.) leaves. **Materials and Methods:** The separation was carried out by HPLC, using a Shim-pack VP-ODS C_{18} column (5 µm, 150 x 4.6 mm) at 40°C. Detection was performed at 254 nm and separation occurred in 35 min. **Results:** The optimized method was validated for each of the nine phenolic compounds. The calibration curve for the phenolic compound standards showed suitable linear fitting and exhibited correlation coefficients greater than 0.990. The LOD and LOQ varied between 6.2807 - 14.8851 µg mL^{-1} and 6.8002 - 16.0071 µg mL^{-1}, respectively. The method was found to be robust for changes of ±2 ml in mobile phase composition. *Byrsonima crassifolia* aqueous extracts indicated contents of gallic acid, catechin, rutin, and cyanidin whereas hydroethanolic one did not show the first substance. *Senna alata* aqueous extract presented only 3-hydroxybenzoic acid and rutin whereas myricetin, cyanidin, quercetin, and kaempferol were also identified in the hydroethanolic one. **Conclusion:** The HPLC method is efficient, precise, accurate, and sensitive to determining phenolic compounds in plant extracts and it is recommended for efficient assays in routine work.

Keywords: Bioactive compounds, Chromatographic method, Plant extracts.

Marília de A Cavalcante[1], Janyna dos S Oliveira[1], Mayra S da S Barreto[2], Lucas P Pinheiro[2], Patrick de C Cantuária[3], Wardsson L Borges[4,5], Gabriel A da Silva[2], Tiago Marcolino de Souza[2]

[1]Federal Institute of Amapá, Macapá, BRAZIL.
[2]State University of Amapá, Macapá, BRAZIL.
[3]Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá, Macapá, BRAZIL.
[4]Embrapa Amapá, Macapá, BRAZIL.
[5]Embrapa Agroindústria Tropical, Fortaleza, BRAZIL.

Correspondence

Ms. Marília de Almeida Cavalcante
Federal Institute of Amapá, Rodovia Br-210, km 03, s/n, Brasil Novo, 68909-398, Macapá, AP, BRAZIL.
Email id: marilia.cavalcante@yahoo.com.br
ORCID iD: 0000-0002-1447-5723

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INTRODUCTION

Traditional communities in the Amazon region use a significant number of plant species for food and medical purposes. Nonetheless, many of these plants' chemical compositions, bioactive contents, biological properties, and medicinal efficacy are unknown.[1] In this context, Paula Filho (2018)[2] realized a phytochemical characterization of medicinal plants used by the population living on Cajari riverbanks in Amapá state. Among the studied plant species, the author reported the use of *Acmella oleracea* L., *Byrsonima crassifolia* (L.) Kunth, *Cuminum cyminum* L., *Eryngium foetidum* L., *Inga edulis* Mart., *Pterocarpus rohrii* Vahl, *Senna alata* (L.) Roxb and *Talinum paniculatum* (Jacq.), leaves.

Previous studies have investigated extraction methods, identification, isolation, toxicity, bioactive efficacy, and applications of Amazonian plant species.[3-5] Silva *et al.* (2007)[6] evaluated the phenolic compounds content in extracts obtained from 15 plant species from the Brazilian Amazon. These authors prepared extracts rich in phenolic compounds, highlighting the plants' potential as sources of bioactive agents.

Bysornima crassifolia (L.) Kunth is a plant from Malpighiaceae family commonly known as muruci. The leaves, bark, and fruits of murici are reported to present diuretic and anti-inflammatory properties, according to traditional use in folk medicine in the Amazonian region.[6] The fruits are consumed in natura or used to produce sweets, ice cream, and juice.[2] Some studies indicated the content of phenolic compounds and the antioxidant and antimicrobial activities of extracts obtained from this species.[6,7]

Senna alata is a plant from the Fabaceae family used in traditional herbal medicine for different skin problems. This plant species, found in Ghana and Brazil, is reported to present antimicrobial, antifungal, and antioxidant activities, besides nutritional and other values.[8,9] The medical effect of *Senna alata* is attributed to the presence of active

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components, such as anthraquinone (aloe emodin) and flavonoids (kaempferol 3-gentiobioside, and kaempferol).[10]

Quantification of phenolic compounds is commonly performed by spectrophotometric and chromatographic methods. Spectrophotometry is used to estimate the total phenolic content, whereas the individual classes of these substances can be identified by high-performance liquid chromatography (HPLC).[11] Folin-Ciocalteu is an example spectrophotometric method widely used for phenolic compound quantification. It consists of reduction reactions using tungsten and molybdenum-containing reagents followed by absorbance evaluation.[12-14] In contrast, chromatography consists of techniques for separating and quantifying the analytes present in plant matrices. The retention time allows the separation of different constituents, analytes, and interferents. In this case, the quantification is based on the analyte adsorption by the stationary phase.[15-17]

Some factors, such as the type of column, detectors, mobile phase, and properties of the tested substances, influence the analysis of phenolic compounds by HPLC.[11] For example, reverse-phase liquid chromatography coupled with an ultraviolet detector or a diode array is widely used for routine quantitation analyzes of phenolic compounds.[18,19] Thus, several studies are commonly carried out to develop chromatographic methods that enable the separation and identification of phenolic compounds in plant species, considering the type and number of phenolic compounds found in various matrices.

This study developed and validated a simple, sensitive, and selective method for separating and identifying phenolic compounds using HPLC. This method was also applied to analyze aqueous and hydroethanolic extracts obtained from the leaves of the following Amazonian plants: *Byrsonima crassifolia* (L.) Kunth and *Senna alata* (L.) Roxb.

MATERIALS AND METHODS

Chemicals and reagents

The standards of gallic acid (97%), ellagic acid (95%), 3-hydroxybenzoic acid (99%), p-coumaric acid (98%), catechin (99%), myricetin (98%), rutin (95%), quercetin (95%), kaempferol (97%), and cyanidin (95%) were purchased from Sigma-Aldrich (Sao Paulo, SP, Brazil). Methanol and acetonitrile were HPLC gradient grade (Exodo Científica, Brazil), whereas ethanol was analytical grade (REATEC, Brazil). Ultrapure water was obtained from a water purification system.

Plant material

Byrsonima crassifolia (L.) Kunth and *Senna alata* (L.) Roxb usage was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge, No. AE536D5. The Fresh leaves were obtained from the Brazilian Agricultural Research Corporation (EMBRAPA, Amapá, Brazil). The vegetal species' excicatas were identified by a taxonomist and deposited in the Amapaense Herbarium (HAMAB) under the codes 019453-HAMAB and 019458-HAMAB, respectively. The geographic coordinates of collection sites were the following: *Byrsonima crassifolia* (N 0°23'16.73732"; W 0.387983), and *Senna alata* (N 0°0'57.77517'; W 0.0160049"). Fresh leaves were selected, washed, and dried in an air circulation oven at 60°C for 4 hr. The dried samples were ground to obtain a particle size of approximately 5 mm and then were stored at 4°C until analysis.

Preparation of the extract

Extracts of the plant species were prepared by suspension of 1 g of the dry plant powder in 6 mL of extraction solvent, water or ethanol:water mixture (1:1 v:v), at 30°C and 200 rpm for 1 hr, followed by filtration using Whatman paper No. 4. The residue was then extracted by adding 6 mL of the extraction solvent under the same condition. After this process, extracts were rotary evaporated under reduced pressure (LUCA-EV 01, Lucadema). The liquid extracts obtained at the end of evaporation were frozen and subsequently lyophilized (freeze dryer, Terroni, Enterprise I-D) to attain dried crude samples (Figure 1).[15,20-22]

Preparation of sample solutions

For the *Senna alata* aqueous and hydroethanolic extracts, approximately 0.01 g of the dried sample was resuspended in 10 mL of water and ethanol:water (1:1 v:v) solution, respectively. For *Byrsonima crassifolia* aqueous and hydroethanolic extracts, 0.05 g of the dried sample was resuspended in 10 mL of water and ethanol:water (1:1 v:v) solution, respectively. The samples were stored at 4°C until analysis.

Preparation of standard solutions

The standard stock solution (1 g L^{-1}) of ellagic acid, 3-hydroxybenzoic acid, p-coumaric acid, myricetin, quercetin, and kaempferol were prepared by dissolution in methanol. Rutin, catechin, and cyanidin standard stock solutions (1 g L^{-1}) were produced by dissolution in ethanol whereas gallic acid was dissolved in acidified water (1% formic acid in ultrapure water). All solutions were stored at 4°C until analysis. Working solutions of the standards were prepared by dilution of stock solutions according to desired concentrations.

Ranges of the calibration curve

Aliquots of the standard stock solution were transferred to volumetric flasks and diluted to get concentration in the linear range described in Table 1.

HPLC instrumentation and chromatographic conditions

A reversed-phase C_{18} HPLC column was used to separate phenolic compounds chosen among phenolic classes to draw a composition profile of the studied matrices. HPLC analysis was performed using a liquid chromatographer (Shimadzu, Prominence LC2030C) equipped with a binary pump, an online vacuum degasser, a diode array detector (DAD), an autosampler, a thermostatted column compartment, which accommodates 216 samples at a time and features a direct access

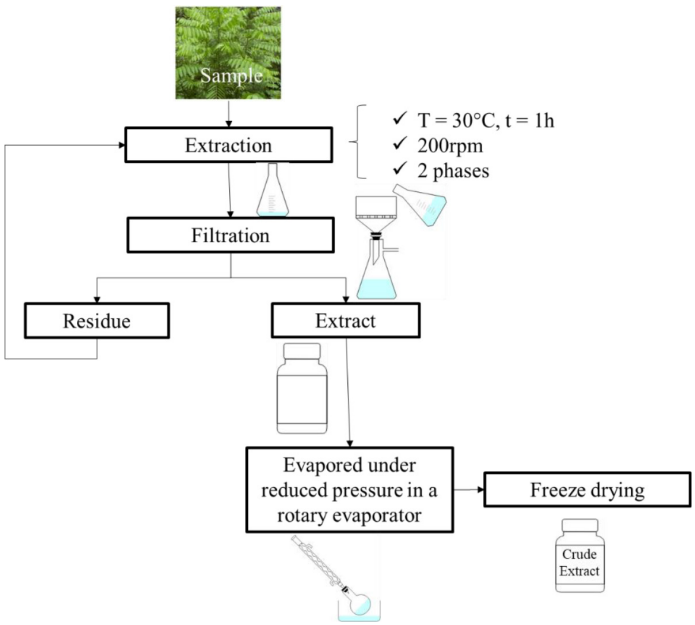

Figure 1: Extraction process employed for the studied plant species.

Table 1: Linearity range values of the external standards.

Compound	Linear range (μg mL^{-1})
Gallic Acid	5 – 50
Catechin	5 – 50
3-Hydroxybenzoic acid	10 – 50
p-Coumaric acid	5 – 50
Rutin	10 – 50
Myricetin	10 – 50
Cyanidin	5 – 50
Quercetin	5 – 50
Kaempferol	5 – 50

rack system. System control and data analyses were performed using Labsolution software (Shimadzu). Before choosing the chromatographic conditions, several trials were carried out using different types of solvents and mobile phase concentrations to check the retention time (RT), peak shape, and tailing factor (peak symmetry) of the analyte. Separation was carried out in a Shim-pack VP-ODS C_{18} column (5 μm, 150 x 4.6 mm). The mobile phase consists of a gradient elution using the proportions of solvent A (1% formic acid in water) to solvent B (acetonitrile) as follows: initial 5% B; 0 - 7 min, 5% B; 7 - 18 min, 5 – 30% B; 18 - 35 min, 30 - 60% B; 35 - 40 min, 60 – 95% B with a flow-rate of 1 mL min^{-1} and the injection volume of 0.02 mL of samples and standards. The total running and post-running times were 40 and 5 min, respectively. The column temperature was maintained at 40°C throughout the analysis and the spectra were acquired in the 210 - 800 nm range. Chromatograms were plotted at 254 and 277 nm. Using these chromatographic conditions, it was possible to confirm the retention time of phenolic compounds by injection of the corresponding standard separately.

Identification of phenolic compounds in the extracts was based on the comparison of spectra and retention times of the samples against the external standards. The quantification was made by external standardization using analytical curves and limits of detection (LOD) and quantification (LOQ).

Validation of the Method

The chromatographic method was validated considering the guidelines recommended by the International Conference on Harmonisation (ICH).[23] Parameters such as specificity, linearity, repeatability, precision, LOD, LOQ, and robustness were evaluated to determine the method's performance. Since the test of extraction-method efficiency was out of the purpose of this work, the recovery was not calculated.

Specificity

The method specificity is the ability to distinguish between investigated analytes and other components, such as impurities, degradants, or excipients. The specificity was demonstrated by running a blank procedure, standard and sample. The chromatographic parameters such as column efficiency and peak symmetry were determined for the standards, according to the ICH guidelines

Linearity

The curve's linearity was determined by different known concentrations (Table 1) of the mix standards solution. For the linearity study, the standard solutions were injected five times (n = 5), and the peak area was measured. The calibration curve for each phenolic compound was constructed by plotting peak areas against concentration. Linear regression equations and correlation coefficient were determined.

Limit of detection and limit of quantification

The LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions whereas the LOQ is the lowest amount of analyte in a sample, which can be quantitatively determined with accuracy.[24] The LOD and LOQ were determined from a signal-to-noise ratio of 3 and 10, respectively, for the injection of standard solutions at low concentrations. In addition, the regions before and after chromatographic peaks were considered for calculating background noise.

Precision

Method precision was determined by repeatability, inter and intra-day precision. The inter and intra-day precision were determined using a minimum of three and six replicates for three different concentration levels, respectively: 10, 30, and 45 μg mL^{-1} for gallic acid, catechin, p-coumaric acid, cyanidin, and kaempferol, and 15, 30, 45 μg mL^{-1} for 3-hydroxybenzoic acid, rutin, myricetin, and quercetin. The inter-day precision variations were studied by different analysts. Peak area was measured and the results for precision were expressed as the percentage relative standard deviation (%RSD).

Robustness

The robustness of the HPLC method was evaluated considering the optimized parameters and slight and viable variations. The evaluated parameters were mobile phase composition (8 mL, 10 mL, and 12 mL of formic acid) and wavelength (250 nm, 254 nm, and 258 nm, except for catechin). Retention times and peaks area were checked for ruggedness by injecting the mixture of standards at 30 μg mL^{-1}.

Statistical Analysis

All the measurements were made in triplicates, and the results were expressed as mean values and standard deviation. Linear adjustment and determination of coefficients were realized in Excell software. Experimental data were subjected to analysis of variance (ANOVA) and Tukey's test at 5% significance using the Minitab® 19 software.

RESULTS

Chromatographic Method

For the method development and validation, a mixture of 10 phenolic compounds standards (4 phenolic acids, and 6 flavonoids) was injected into the HPLC system. For the mobile phase selection, different acetonitrile initial concentrations (ranging from 10% to 5%) were evaluated with a 50 min total running time. After optimizing the initial concentration (5%, v/v), the gradient time was decreased to obtain the best separation condition. It comprised a 40-min separation using gradient elution with linear steps, where the acetonitrile concentration varied from 5% to 95% (see details in the methodology section). Under these conditions occurred complete co-elution between ellagic acid and other compounds. To solve this problem, ellagic acid was eluted separately in the same method. Even though, this standard was not separated due to its solubilization in the mobile phase and interaction with the column (Figure 2). Therefore, it was not possible to use the ellagic acid standard in this method.

Chromatograms of the nine external standards mixture (gallic acid, catechin, 3-hydroxybenzoic acid, p-coumaric acid, rutin, myricetin, cyanidin, quercetin, and kaempferol), were recorded at 254 nm and 277 nm, are presented in Figure 3. The investigated compounds showed suitable responses and were successfully separated at 254 nm, except for the catechin which presented a better definition at 277 nm. As the article's objective was to develop an optimized chromatographic method,

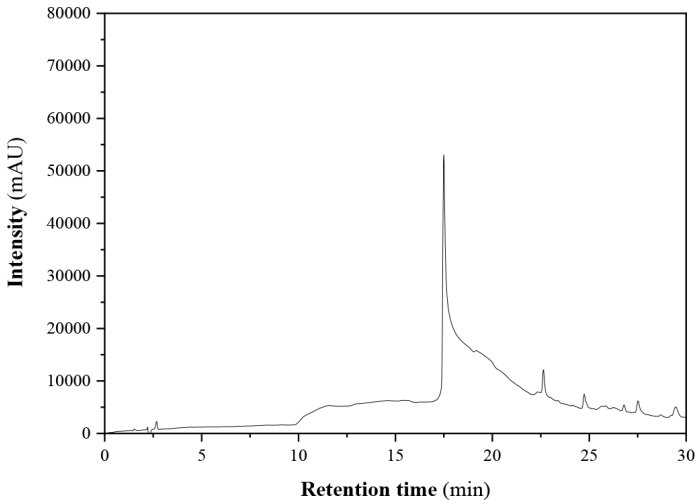

Figure 2: Ellagic acid chromatogram obtained by HPLC.

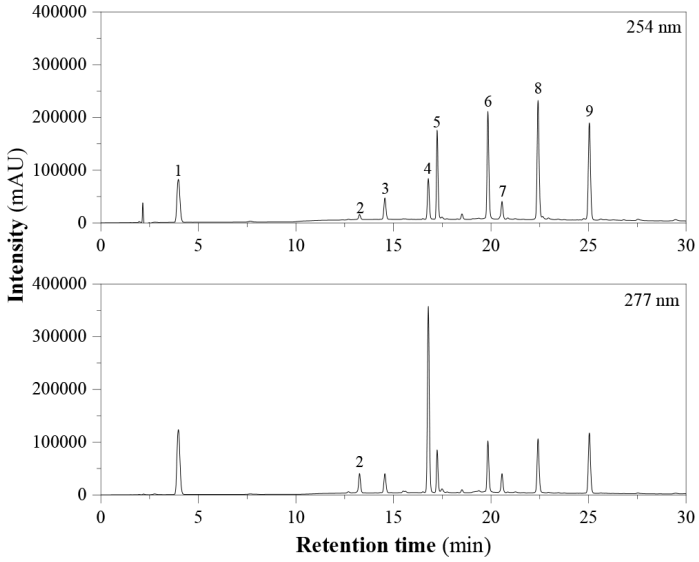

Figure 3: Chromatogram of the nine external standards mixture at 254 nm and 277nm.
Standards: 1. Gallic acid, 2. Catechin, 3. 3-Hydroxybenzoic acid, 4. p-Coumaric acid, 5. Rutin, 6. Myricetin, 7. Cyanidin, 8. Quercetin, 9. Kaempferol.

catechin was evaluated at 277 nm, whereas the other compounds were analyzed at 254 nm.

The retention times of the gallic acid, catechin, 3-hydroxybenzoic acid, p-coumaric acid, rutin, myricetin, cyanidin, quercetin, and kaempferol were 3.927, 13.071, 14.340, 16.600, 17.170, 19.787, 20.426, 22.338, and 24.943 min, respectively.

Method Validation

For each of the nine phenolic compounds, calibration curves based on the area under chromatographic peaks were plotted at five different concentrations to check the method's linearity (Figure 4). The calibration plots for the nine phenolic compound standards showed suitable linear fitting, as indicated by regression parameters in Table 2. The curves for all the investigated compounds exhibited correlation coefficients greater than 0.990.

The peak area was checked for repeatability and reproducibility by injecting the mixture of standards at concentrations of 10, 30, and 45 µg mL^{-1} for gallic acid, catechin, p-coumaric acid, cyanidin, and kaempferol and 15, 30, and 45 µg mL^{-1} for 3-hydroxybenzoic, myricetin, rutin, and quercetin using six replicates on consecutive days. Table 3

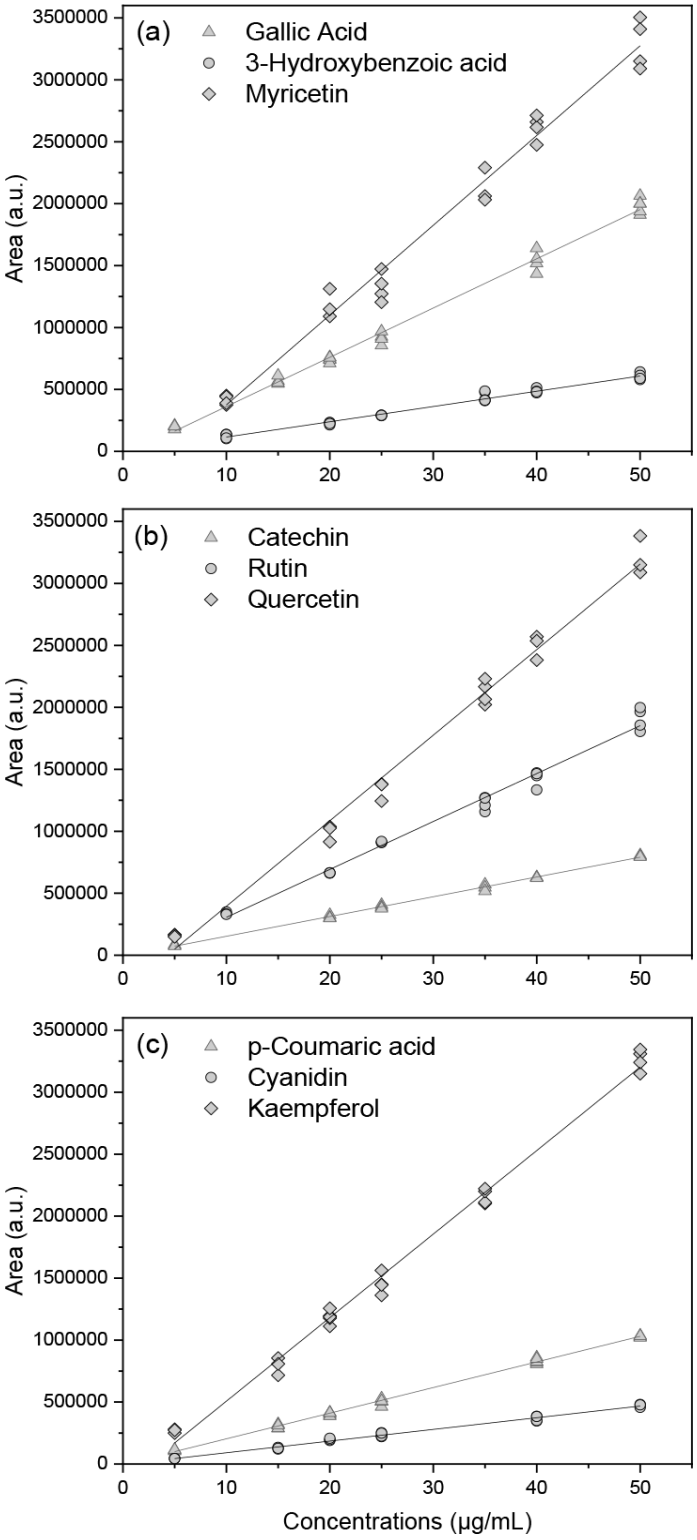

Figure 4: Calibration curve for (a) Gallic acid, 3-Hydroxybenzoic acid, and Myricetin; (b) Catechin, Rutin, and Quercetin; and (c) p-Coumaric acid, Cyanidin and Kaempferol.

shows that in repeatability and reproducibility at three different concentration levels of phenolic compounds standard solution. Precision was evaluated based on the %RSD value.

The method was evaluated for changes of ±2 ml and ±4 nm in mobile phase composition and wavelength, respectively. The analytes' retention times were significantly affected by variations in mobile phase composition, as indicated by the one-way analysis of variance at a 5%

Table 2: Calibration curves and linearity test result (r) of the external standards (*n* = 5).

Compound	λ (nm)	Calibration curve	r	LOD* (μg mL^{-1})	LOQ* (μg mL^{-1})
Gallic Acid	254	y = 39774.1934x – 36110.4394	0.9965	10.0033	10.9063
Catechin	277	y = 15947.6575x – 6564.7322	0.9984	7.1625	7.9378
3-Hydroxybenzoic acid	254	y = 12322.5575x – 7649.1186	0.9912	14.2393	15.5105
p-Coumaric acid	254	y = 20674.8543x – 3274.6140	0.9985	6.2807	6.8002
Rutin	254	y = 38668.0611x – 81051.4188	0.9935	12.31385	13.6395
Myricetin	254	y = 72486.6381x – 349583.1972	0.9910	14.8851	16.0071
Cyanidin	254	y = 9416.4116x – 3203.7950	0.9953	8.6337	10.7002
Quercetin	254	y = 69042.8615x – 295727.6557	0.9941	12.2495	13.4257
Kaempferol	254	y = 67407.1510x – 166941.1141	0.9965	9.23880	10.0514

*99.9% probability level, except for cyanidin (99.0%).

Table 3: Repeatability and reproducibility of external standards.

Compound	Concentration (μg mL^{-1})	Repeatability (n=3)		Reproducibility (n=6)	
		Mean (μg mL^{-1})	RSD (%)	Mean (μg mL^{-1})	RSD (%)
Gallic acid	10	10.6006	1.6237	10.4195	3.1600
	30	29.0511	0.8663	29.1162	1.3927
	45	44.6870	2.7492	45.2773	2.2247
Catechin	10	10.7968	1.8675	7.8130	5.9272
	30	29.8366	3.2913	30.4362	4.7397
	45	45.9066	0.7939	45.8401	1.9318
3-Hydroxybenzoic acid	15	15.1438	2.1084	14.9122	1.9541
	30	29.1937	3.6589	28.3192	2.9255
	45	41.8027	0.9001	45.7216	5.4903
p-Coumaric acid	10	10.1758	2.2121	10.2683	1.8735
	30	29.6214	1.9695	30.0508	2.5442
	45	44.7343	0.8578	44.1608	2.8898
Rutin	15	14.1691	0.6866	14.3566	2.1982
	30	25.9303	1.3810	30.8347	2.0285
	45	43.9643	1.1832	42.2231	3.0224
Myricetin	15	15.4711	2.4199	15.3799	2.1346
	30	28.8304	3.8610	29.4715	2.6016
	45	42.3508	0.7397	43.8421	4.0754
Cyanidin	10	9.7734	3.5025	9.7875	3.0326
	30	31.7841	3.4029	30.2532	3.2717
	45	47.0165	0.4236	45.6995	5.7703
Quercetin	15	15.2272	2.6712	14.9713	1.5140
	30	31.7987	4.7377	28.1478	4.6594
	45	41.5736	1.7037	42.6002	5.7360
Kaempferol	10	10.4489	4.1253	10.4562	2.6107
	30	29.3732	1.6488	29.9574	2.5487
	45	45.4916	1.3398	45.2431	2.3741

*RSD: relative standard deviation.

level (Table 4). A slight reduction in retention times was detected for all compounds increasing the formic acid content (Table 5). In addition, Tukey tests showed a significant difference among means retention time at different mobile phase compositions for each external standard. ANOVA results also showed that retention times were not affected by the wavelength increase (Table 4).

For most of the external standards, the peaks' area changed significantly with mobile phase composition and wavelength (Table 6). However, the increase in formic acid content did not considerably affect cyanidin's peak area at a 5% level (p= 0.099). For quercetin (p= 0.338) and kaempferol (p= 0.518), no significant change in the peaks' area was also verified with the wavelength variation. As indicated by the mean values (Table 7), Tukey tests did not show any pattern of increase or decrease of peaks' area with the changes in mobile phase composition and wavelength.

The developed and validated method allowed the quantification of the nine distinct phenolic compounds in extracts of plant species. The mobile phase composition, column temperature, and wavelength parameters selected for the method were 1% formic acid aqueous solution, 40°C, and 254 and 277 nm, respectively.

Analysis of amazonian medicinal plants extracts

Identification of phenolic compounds was performed by comparing the extracts' retention times and spectra with those of the corresponding external standards. Quantification was based on external standards calibration. The chromatograms for the aqueous and hydroethanolic extracts of *Senna alata* and *Byrsonima crassifolia* are presented in Figure 5. The number and sort of phenolic compounds identified in the samples varied among the studied extracts. None of the extracts presented the peak associated with p-coumaric acid, but other peaks associated with unknown compounds were also detected.

The mean phenolic compounds contents in aqueous and hydroethanolic extracts of *Byrsonima crassifolia*, and *Senna alata*, expressed as mg g^{-1} of extract ± standard deviation, are presented in Table 8. *Byrsonima crassifolia* aqueous extract presented four phenolic compounds, whereas *Senna alata* hydroethanolic one presented six. Rutin was detected in the four evaluated extracts and its contents were significantly lower for *Byrsonima crassifolia* (2.51 and 5.97 mg g^{-1}) when compared to *Senna alata* (12.17 and 11.34 mg g^{-1}). Hydroethanolic extract of *Senna alata*

Table 4: ANOVA results for the effect of mobile phase composition and wavelength of retention times of standards compounds.

Compound	Source	DF	Retention times							
			Mobile phase composition*				Wavelength*			
			Sum of Squares	Mean Square	*F* value	*p*-value	Sum of Squares	Mean Square	*F* value	*p*-value
Gallic acid	Par*	2	0.031940	0.015970	29.24	0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.003277	0.000546			0.008811	0.001469		
	Total	8	0.035217				0.008812			
Catechin	Par*	2	0.089548	0.044774	35.77	<0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.007511	0.001252			0.022118	0.003686		
	Total	8	0.097060				0.022118			
3-Hydroxybenzoic acid	Par*	2	0.074662	0.037331	162.23	<0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.001381	0.000230			0.002888	0.000481		
	Total	8	0.076042				0.002888			
p-Coumaric acid	Par*	2	0.036817	0.018408	373.98	<0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.000295	0.000049			0.000416	0.000069		
	Total	8	0.037112				0.000416			
Rutin	Par*	2	0.011348	0.005674	282.14	<0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.000121	0.000020			0.000078	0.000013		
	Total	8	0.011469				0.000078			
Myricetin	Par*	2	0.017798	0.008899	355.95	<0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.000150	0.000025			0.000056	0.000009		
	Total	8	0.017948				0.000056			
Cyanidin	Par*	2	0.017560	0.008780	303.93	<0.001	0.000001	0.000000	0.02	0.985
	Error	6	0.000173	0.000029			0.000131	0.000022		
	Total	8	0.017734				0.000132			
Quercetin	Par*	2	0.015162	0.007581	274.01	<0.001	0.000000	0.000000	0.01	0.994
	Error	6	0.000166	0.000028			0.000106	0.000018		
	Total	8	0.015328				0.000106			
Kaempferol	Par*	2	0.012896	0.006448	214.93	<0.001	0.000000	0.000000	0.00	1.000
	Error	6	0.000180	0.000030			0.000104	0.000017		
	Total	8	0.013076				0.000104			

DF: Degree of freedom; *Par: parameter affecting retention times (mobile phase composition, column temperature, or wavelength).

Table 5: Retention times of external standards ± standard deviation.

Compound	Retention times (min)					
	8 mL	10 mL	12 mL	250 nm	254 nm	258 nm
Gallic acid	4.09^{a} ± 0.01	4.03^{b} ± 0.04	3.95^{c} ±0.01	4.03^{a} ± 0.04	4.03^{a} ± 0.04	4.03^{a} ± 0.04
Catechin	13.39^{a} ± 0.01	13.27^{b} ± 0.06	13.15^{c} ± 0.00	13.27^{a} ±0.06	13.27^{a} ± 0.06	13.27^{a} ± 0.06
3-Hydroxybenzoic acid	14.62^{a} ± 0.01	14.51^{b} ±0.02	14.40^{c} ± 0.01	14.51^{a} ± 0.02	14.51^{a} ± 0.02	14.51^{a} ± 0.02
p-Coumaric acid	16.84^{a} ± 0.01	16.76^{b} ±0.01	16.68^{c} ± 0.01	16.76^{a} ± 0.01	16.76^{a} ± 0.01	16.76^{a} ± 0.01
Rutin	17.32^{a} ± 0.00	17.27^{b} ± 0.00	17.23^{c} ± 0.01	17.27^{a} ± 0.00	17.27^{a} ± 0.00	17.27^{a} ± 0.00
Myricetin	19.09^{a} ± 0.00	19.84^{b} ± 0.00	19.80^{c} ± 0.01	19.84^{a} ± 0.00	19.84^{a} ± 0.00	19.84^{a} ± 0.00
Cyanidin	20.59^{a} ±0.00	20.53^{b} ± 0.01	20.48^{c} ± 0.01	20.53^{a} ± 0.00	20.53^{a} ± 0.01	20.53^{a} ± 0.00
Quercetin	22.46^{a} ± 0.00	22.40^{b} ± 0.00	22.36^{c} ± 0.01	22.40^{a} ± 0.00	22.40^{a} ± 0.00	22.40^{a} ± 0.00
Kaempferol	25.08^{a} ± 0.00	25.02^{b} ± 0.00	24.99^{c} ± 0.01	25.02^{a} ± 0.00	25.02^{a} ± 0.00	25.02^{a} ± 0.00

*Except for catechin: 273nm, 277nm e 281nm; Means classified by the same letter for the same variable (mobile phase composition, column temperature or wavelength) and external standard did not differ according to the Tukey test at 5% significance.

Table 6: ANOVA results for the effect of mobile phase composition and wavelength of peaks' area of standards compounds.

Compound	Source	DF	Peak areas							
			Mobile phase composition*				Wavelength*			
			Sum of Squares	Mean Square	*F* value	*p*-value	Sum of Squares	Mean Square	*F* value	*p*-value
Gallic acid	Par*	2	1189066341	594533170	20.04	0.002	3.83529x10^{11}	1.91765x10^{11}	200780.52	<0.001
	Error	6	177967667	29661278	-	-	5730575	955096		
	Total	8	1367034008	-	-	-	3.83535x10^{11}			
Catechin	Par*	2	9823561974	4911780987	58.45	<0.001	3198238638	1599119319	5.74	0.040
	Error	6	504235806	84039301			1670274334	278379056		
	Total	8	10327797780				4868512972			
3-Hydroxy-benzoic acid	Par*	2	960110950	480055475	7.32	0.025	4.22412x10^{11}	2.11206x10^{11}	145057.09	<0.001
	Error	6	393294170	65549028			8736115	1456019		
	Total	8	1353405120				4.22421x10^{11}			
p-Coumaric acid	Par*	2	1311061551	655530775	5.88	0.039	2.65419x10^{11}	1.32710x10^{11}	4494.66	<0.001
	Error	6	668795785	111465964			177156514	29526086		
	Total	8	1979857336				2.65596x10^{11}			
Rutin	Par*	2	9018782296	4509391148	5.14	0.050	319488642	159744321	14.14	0.005
	Error	6	5267861321	877976887			67793830	11298972		
	Total	8	14286643618				387282472			
Myricetin	Par*	2	32159751413	16079875706	119.50	<0.001	6792609447	3396304723	15.26	0.004
	Error	6	807388609	134564768			1335375093	222562515		
	Total	8	32967140022				8127984540			
Cyanidin	Par*	2	4241730210	2120865105	3.48	0.099	22170159939	11085079969	318.81	<0.001
	Error	6	3657875548	609645925			208622480	34770413		
	Total	8	7899605758				22378782419			
Quercetin	Par*	2	5.71789x10^{11}	2.85895x10^{11}	138.95	<0.001	10933482422	5466741211	1.31	0.338
	Error	6	12345139330	2057523222			25065375166	4177562528		
	Total	8	5.84134x10^{11}				35998857588			
Kaempferol	Par*	2	2.39882x10^{12}	1.19941x10^{12}	141.25	<0.001	36513438233	18256719116	0.73	0.518
	Error	6	50949901776	8491650296			1.49162x10^{11}	24860292095		
	Total	8	2.44977x10^{12}				1.85675x10^{11}			

DF: Degree of freedom; *Par: parameter affecting peaks' area (mobile phase composition, column temperature, or wavelength).

Table 7: Peaks area of external standards ± standard deviation.

Compound	Peaks area					
	8 mL	10 mL	12 mL	250 nm	254 nm	258 nm
Gallic acid	1134683^{b} ± 9338	1160078^{a} ±1188	1136853^{b} ± 611	908897^{a} ± 874	1160078^{b} ± 1188	1414548^{c} ± 831
Catechin	422010^{b} ±2553	469259^{a} ± 15661	388736^{c} ± 577	423636^{b} ± 16226	469259^{a} ± 15661	452614^{ab} ± 18072
3-Hydroxybenzoic acid	360080^{b} ±13173	383948^{a} ± 1100	364750^{ab} ± 4679	726031^{a} ± 1349	383948^{b} ±1100	203649^{c} ± 1157
p-Coumaric acid	708294^{b} ±11370	736736^{a} ± 5979^{ab}	715527^{ab} ± 13014	579387^{c} ± 7098	736736^{b} ±5979	995909^{a} ± 1564
Rutin	1157229^{b} ±50115	1232433^{a} ±2470	1211190^{ab} ± 10787	1143309^{b} ± 8009	1232433^{b} ± 2470	1245072^{a} ± 4658
Myricetin	1894562^{b} ± 4844	1994518^{a} ± 16867	1851877^{c} ± 9784	1955123^{b} ± 17919	1994518^{a} ± 16867	1927573^{b} ± 7878
Cyanidin	255945^{a} ± 41160	303152^{a} ± 7844	300749^{a} ± 8558	244913^{c} ± 5919	303152^{b} ± 7844	366452^{a} ± 2783
Quercetin	1667146^{b} ± 27570	2142479^{a} ±66663	1563582^{b} ± 31120	2058175^{a} ± 67228	2142479^{a} ± 66663	2088650^{a} ± 59742
Kaempferol	739892^{b} ± 13518	1779103^{a} ±158249	635446^{b} ± 15794	1714533^{a} ± 152157	1779103^{a} ± 158249	1869821^{a} ± 162439

*Except for catechin: 273nm, 277nm e 281nm; Means classified by the same letter for the same variable (mobile phase composition, column temperature, or wavelength) and external standard did not differ according to the Tukey test at 5% significance.

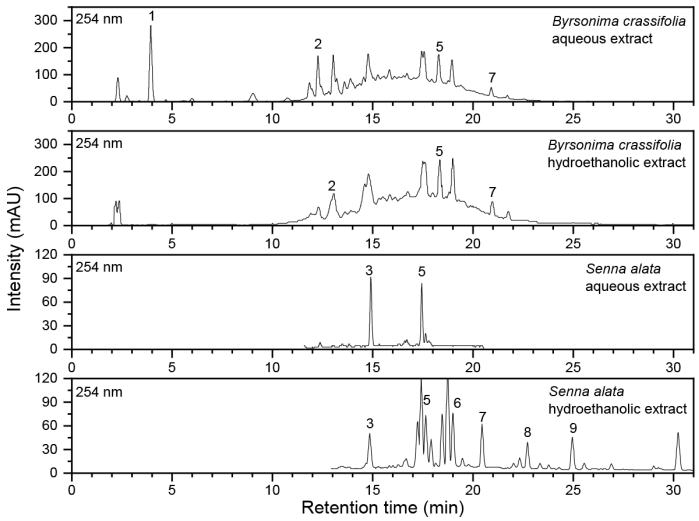

Figure 5: Chromatogram of the aqueous and hydroethanolic extracts of *Senna alata* and *Byrsonima crassifolia* at 254 nm.
Identified compounds: 1. Gallic acid, 2. Catechin, 3. 3-Hydroxybenzoic acid, 5. Rutin, 6. Myricetin, 7. Cyanidin, 8. Quercetin, 9. Kaempferol.

Table 8: Content of plant phenolics in aqueous and hydroethanolic extracts of *Byrsonima crassifolia* and *Senna alata* species was expressed as mg g-1 of extract ± standard deviation.

Compounds	*Byrsonima crassifolia*		*Senna alata*	
	Aqueous extracts	Hydro-ethanolic extracts	Aqueous extracts	Hydro-ethanolic extracts
Gallic Acid	11.21 ± 0.48	-	-	-
Catechin	49.02 ± 0.86	65.35 ± 0.62	-	-
3-Hydroxybenzoic acid	-	-	41.51 ± 1.09	27.54 ± 0.65
p-Coumaric acid	-	-	-	-
Rutin	2.51 ± 0.04	5.97 ± 0.22	12.17 ± 0.44	11.34 ± 0.19
Myricetin	-	-	-	11.75 ± 0.13
Cyanidin	4.79 ± 0.11	9.21 ± 0.28	-	45.02 ± 0.68
Quercetin	-	-	-	7.67 ± 0.16
Kaempferol	-	-	-	7.68 ±0.12

also presented kaempferol, quercetin, myricetin, 3-hydroxibenzoic acid, and cyanidin, but the two last substances were most abundant. Catechin (49.02 mg g^{-1}) was the most abundant phenolic content in both *Byrsonima crassifolia* extracts (Table 3).

DISCUSSION

Validation of an analytical method consists of an evaluation of the procedure's suitability for the intended purpose. Data from method validation are required to ensure the quality, reliability, and consistency of analytical results.[25] A reversed-phase HPLC method was developed and validated to determine different phenolic compounds in aqueous and hydroethanolic extracts of *Byrsonima crassifolia* and *Senna alata* leaves. We used a mobile phase composed of acetonitrile gradients and 0.1% formic acid in water (v/v) which is typical in separating complex samples.[26,27] The developed method can be considered rapid as it allowed the separation of compounds at 254 nm in only 35 min.

The optimized method was validated based on ICH guidelines.[23] According to ICH, linearity is associated with the ability to obtain test results that are proportional to the concentration of analyte in the sample within a specified range. The LOD and LOQ varied between 6.2807 - 14.8851 µg mL^{-1} and 6.8002 - 16.0071 µg mL^{-1}, respectively (Table 2). Considering the used equipment and column, attained LOD and LOQ indicated that this method enabled an adequate detection and quantification of the phenolic compounds in the specified concentrations.

The precision of the analytical method demonstrates agreement between a series of measurements obtained by multiple sampling of the same homogeneous sample under the prescribed conditions.[23] Repeatability tests determined the precision of the experiments through solutions prepared and measured by the same analyst. Measurements made by distinct analysts, considering systematic errors, were also conducted to calculate the reproducibility (inter-day precision). Analyzing the results of the repeatability and reproducibility revealed that the relative standard deviation (RSD) of the peak area was less than 5% and 6%, respectively. The highest values of reproducibility were recorded for cyanidin and quercetin solution with a concentration of 45 µg mL^{-1}. %RSD values below 10% are acceptable by the AOAC manual for the Peer-Verified Methods Program,[28] so the method was found to be precise and reproducible.

The phase reverse HPLC method was succsesfully applied for analysis of the phenolic compounds in plant extracts, enabling the quantification of eight phenolic compounds present in the aqueous and hydroethanolic extracts of *Byrsonima crassifolia* and *Senna alata* matrix samples.

Rutin, quercetin 3-O-xyloside, hesperidin, and quercetin contents of 4.4 mg kg^{-1}, 12 mg kg^{-1}, 0.7 mg kg^{-1}, 1.4 mg kg^{-1}, respectively, were detected in methanolic extracts of *Byrsonima crassifolia* aerial parts by HPLC.[29] Sobrinho *et al*. (2020)[30] reported the presence of catechin (1.00 ± 0.40 mg of quercetin equivalents g^{-1}) and epicatechin (1.00 ± 0.35 mg of quercetin equivalents g^{-1}), and non-quantified amounts of quercetin and ferulic acid in hydromethanolic extracts of *Byrsonima crassifolia* leaves. The extracts evaluated in our work also indicated the presence of gallic acid and Cyanidin.

Oladeji *et al*. (2016)[8] detected cinnamic acid in the ethanolic and methanolic extracts of *Senna alata* leaves whereas Okapuzo *et al*. (2009)[31] identified naringin and apigenin as the major phenolic components in ethyl acetate fraction. Our results indicated that *Senna alata* leaves can also contain 3-Hydroxybenzoic acid, rutin, myricetin, cyanidin, quercetin, and kaempferol.

The total phenolic content and extract composition depend on the extraction method and solvent's type and polarity.[32] Such aspects might explain the differences between obtained results and literature data. Furthermore, growing conditions and differences in plant genotypes also affect plant phenolic content and composition.[33]

CONCLUSION

A simple and precise HPLC method, using the usual 5 µm columns, was developed and validated to determine nine phenolic compounds found in plant extracts (gallic acid, catechin, 3-hydroxybenzoic acid, p-coumaric acid, rutin, myricetin, cyanidin, quercetin, kaempferol). It showed efficiency, precision, accuracy, and sensitivity, allowing time-saving during analysis. Therefore, this method is recommended for efficient assays in routine work. The developed method was successfully applied to analyze aqueous and hydroethanolic extracts of *Byrsonima crassifolia* and *Senna alata* leaves, respectively. Catechin was the main phenolic compound identified for the *Byrsonima crassifolia* extracts whereas 3-hydroxibenzoic acid and cyanidin were the most abundant substances in aqueous and hydroethanolic extracts of *Senna alata*, respectively. Further HPLC analyzes of extracts from these plants using other solvents will still be evaluated to investigate other phenolic compounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPLC: High performance liquid chromatography; **LOD:** Limits of detection; **LOQ:** Limits of quantification; **RT:** Retention time; **ICH:** International Conference on Harmonisation; **RSD:** Relative standard deviation; **ANOVA:** Analysis of variance; **AOAC:** Association of Official Agricultural Chemists.

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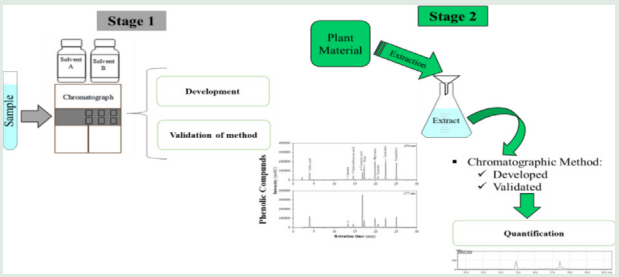
GRAPHICAL ABSTRACT



SUMMARY

The development and validation of a high-performance liquid chromatography (HPLC) method is the first step to identifying and quantifying phenolic compounds in plant extracts. The developed method was validated for linearity, specificity, precision, limits of detection, and limits of quantification, according to ICH guidelines. Attained results for aqueous and hydroethanolic extracts of *Byrsonima crassifolia* and *Senna alata* revealed that the proposed method allowed the quantification of eight phenolic compounds in these plant extracts. This method could be recommended for routine quality work analysis.

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