Revista Brasileira de Farmacognosia 25 (2015) 513–521

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Response Surface Methodology IV-Optimal design applied to the performance improvement of an RP-HPLC-UV method for the quantification of phenolic acids in *Cecropia glaziovii* products

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ARTICLE INFO

Article history:
Received 5 May 2015
Accepted 26 May 2015
Available online 19 June 2015

Keywords:
RP-HPLC-UV method
Response Surface Methodology
Cecropia glaziovii
Vegetal extract
Chlorogenic acid
Caffeic acid

ABSTRACT

Chlorogenic and caffeic acids are bioactive phenolic compounds present in *Cecropia glaziovii* Snethl., Urticaceae, products that have been used as analytical markers. This paper reports a chemometric study aimed at improving chromatographic performance for quantification of these markers by RP-HPLC. The organic to aqueous content ratio, the acid content of the mobile phase, and the elution method were analyzed using a Response Surface Methodology IV-Optimal design. The resolution between peaks, retention time, tailing and retention factors, number of theoretical plates and peak widths were evaluated. The optimized conditions were mathematically determined as (A) trifluoroacetic acid 0.05% (v/v), (B) 12% (v/v) acetonitrile and (C) increasing gradient. The method was considered specific, fast, precise, reliable and linear in the ranges of 1.0–200.0 and 2.5–100.0 μg/ml for the chlorogenic and caffeic acids, respectively. The adequate conditions to separate and quantify both phenolic acids in *C. glaziovii* products were demonstrated. Satisfactory resolution was achieved when compared to a previously published chromatographic method which is unable to separate the chlorogenic acid and an interfering compound presented under certain extractive conditions, demonstrating the importance of systematic studies, specifically when analyzing complex plant matrices.

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Introduction

Cecropia glaziovii Snethl., Urticaceae, a plant species popularly known as "embaúba", is widely distributed in the Brazilian Atlantic Forest, and has been used in folk medicine as an antihyperlipidemic and hypotensive agent (Lorenzi and Matos, 2008; Silva et al., 2010). Over the past few years, it has been verified that *C. glaziovii* has activity as a hypotensive (Ninahuaman et al., 2007), antiasthmatic (Delarcina Jr. et al., 2007), anxiolytic-like (Rocha et al., 2002) and antidepressant-like (Rocha et al., 2007) agent. Due to these outstanding biological activities, researches have been carried out targeting at the development of products containing standardized extracts of *C. glaziovii* for pharmaceutical purposes (Arend et al., 2011; Beringhs et al., 2013; Santos, 2012). This growing interest is associated with an increasing need, in the academic and industrial areas, for analytical methods to quantify the active compounds present in pharmaceuticals derived from *C. glaziovii*.

Of the compounds which can be extracted from *C. glaziovii* leaves, phenolics constitute the major biologically active components. The chlorogenic (3-caffeoylquinic acid; CGA) and caffeic (3,4-dihydroxycinnamic acid; CFA) acids are present in significant quantities in *Cecropia* genus extracts (Arend et al., 2011), and are involved in several biological activities attributed to *C. glaziovii* (Bouayed et al., 2007; Cho et al., 2010; Ong et al., 2013; Takeda et al., 2002). For this reason, CGA and CFA have been widely used as chemical markers for this species. However, due to their chemical similarity, these phenolic compounds commonly elute in close proximity (Arend et al., 2011), which creates difficulties in developing chromatographic methods. In addition, several substances extracted concurrently may also interfere in the quantification of CGA and CFA. For this reason, special attention should be given to products of plant origin due to their chemical complexity. Arend et al. (2011) described the development of an RP-HPLC method for the quantification of these chemical markers. However, we recently noticed the presence of an interfering compound under new extractive conditions, and this method has proved to be unable to separate it from CGA. As result, it could lead to an overestimated quantification of CGA. For this reason, a multivariate approach to improving

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http://dx.doi.org/10.1016/j.bjp.2015.05.007
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the performance of chromatographic methods was adopted in this study.

The Response Surface Methodology (RSM) (Antony, 2014; Myers et al., 2009) experimental design using the IV-Optimal algorithm was applied as a chemometric tool to determine the influence of chromatographic conditions on various quality parameters. RSM is a multivariate technique that uses a set of mathematical and statistical tools to determine how different factors influence responses on a dataset. This technique allows the fitting of a polynomial equation to the experimental data (Myers et al., 2009). It also enables the extrapolation of a mathematical model to perform predictions and detect which factors, and their possible interactions, may influence the different experimental responses.

Materials and methods

Materials and chemical reagents

The materials were obtained from the following sources: chlorogenic (CGA; 3-caffeoylquinic acid) and caffeic acids (CFA; 3,4-dihydroxycinnamic acid) (Sigma–Aldrich, USA), HPLC-grade methanol and acetonitrile (J.T. Baker, USA), trifluoracetic acid (Vetec, Brazil), and ethanol (Labsynth, Brazil).

Raw plant material

Dried leaves of *Cecropia glaziovii* Snethl., Urticaceae, were purchased from the Pluridisciplinary Center of Chemical, Biological and Agronomic Studies (CPQBA) at the Universidade Estadual de Campinas, Brazil. The voucher specimen was deposited at the CPQBA herbarium, under number 78. Also, *C. glaziovii* leaves were randomly collected from six different geographic locations within South America, as follows: S1 (Florianópolis–Brazil; 27°42′33.7″ S 48°33′35.6″ W), S2 (Garopaba – Brazil; 28°01′18.3″ S 48°41′55.8″ W), S3 (Itanhaém – Brazil; 28°01′18.3″ S 48°41′55.8″ W), S4 (São Paulo – Brazil; 23°26′30.2″ S 46°39′15.1″ W), S5 (Rio de Janeiro – Brazil; 22°54′54.9″ S 43°20′10.6″ W) and S6 (Misiones – Argentina; 26°16′35.5″ S 53°43′00.5″ W). The plant material was ground in a knife mill (3.0 mm mash; Macmont) prior to use.

Chromatographic conditions

The chromatographic analysis was performed on a PerkinElmer high performance liquid chromatography system comprising the following modules: Series 200 Autosampler, binary pump and vacuum degasser and Series 600 LINK interface. The UV–Vis detector (Series 200) was used as the main detector due to its reduced cost when compared with the photodiode array detector (PDA) (Flexar). Unless otherwise specified, the detector wavelength was set at 330 nm (highest absorbance signal of CGA and CFA concomitantly). The stationary phase was a Luna C18(2) column (5 μm, 150.0 × 4.6 mm, 100 Å; Phenomenex, USA). The injection volume was 20.0 μl and the flow rate of the mobile phase was 1.0 ml/min. All analyses were performed in triplicate.

Design of experiments

A Response Surface Methodology (RSM) IV-Optimal design was set up to minimize the integrated prediction variance across the design space (Myers et al., 2009). A total of 22 experiments were carried out and the responses were collected based on the chromatograms obtained. The factors analyzed were the acid concentration in the aqueous portion of the mobile phase, the organic to aqueous solvent ratio in the mobile phase, and the elution methods. The binary mobile phases were comprised of different proportions of trifluoracetic acid (TFA) aqueous solution, an acidifier agent at several different concentrations, and acetonitrile (ACN). The elution method was evaluated as isocratic (ISO), increasing gradient (IG) or decreasing gradient (DG). The experimental domain is described in Table 1. The elution method was only employed during the first 12 min after sample injection. Next, a cleansing gradient was used to elute the remaining substances. The baseline program was an 11:89 ACN:TFA ratio for all experiments. Regression analysis of the data was carried out (Design-Expert®, Version 8.0.7.1, Stat Ease Inc., USA). Analysis of variance (ANOVA) was performed to identify the significance of single factors, binary interactions and quadratic terms in relation to their influence on the responses analyzed. Each factor was considered to be significant when the p value was <0.05.

Table 1
Experimental domain investigated (factors and respective levels).

Levels	Numeric continuous factors		Categoric nominal factor
	A – TFA concentration (%; v/v)	B – ACN: TFA	C – elution method
Low (−1)	0.01	12:88	Isocratic (ISO)
Center point (0)	0.05	14:86	Increasing gradient (IG)
High (+1)	0.10	16:84	Decreasing gradient (DG)

Chromatographic system suitability parameters

The responses analyzed in this study were retention time (RT), number of theoretical plates (NTP), tailing factor (TF), peak width (W) and retention factor (k') for both CGA and CFA, as well as the resolution between CGA and the interfering peak (Rs_{CGA-I}). All these suitability parameters were calculated and interpreted according to the US Food and Drug Administration (FDA) guidelines (FDA, 1994; Neue, 2005).

Chemometric optimization of the chromatographic method

The optimum conditions were determined by the desirability function (Myers et al., 2009), and experiments were performed to confirm the robustness of the predicted model under optimal conditions. The simultaneous objective function is a geometric mean of all transformed responses, and is calculated as follows (Antony, 2014):

$$D = (d_1 \times d_2 \times \ldots \times d_n)^{1/n} = \left(\prod_{i=1}^{n} d_i\right)^{1/n} \quad (1)$$

where D is the desirability function, d_i is the desirability ranges for each response, and n is the number of responses in the measurement.

Validation of the chromatographic method

The optimized chromatographic method was validated according to the International Conference on Harmonization (ICH) guidelines (ICH, 2005) and the US FDA guidelines (FDA, 1994). Calibration curves were prepared for both CGA and CFA. The slopes and intercepts were calculated by means of linear regression (ICH, 2005). The standard deviation of the y-intercepts of the regression lines and the slope of the calibration curves were used to calculate the detection limit (DL) and quantification limit (QL) of both CGA and CFA. The precision of the method was confirmed by means of repeatability and intermediate precision. The repeatability was calculated as the mean percentage and relative standard deviation (RSD) of detection of three distinct concentrations (2.5, 50 and 100 μg/ml) of each chemical marker injected three times. The intermediate precision was evaluated by injection in triplicate

Table 2
Mean responses obtained for chlorogenic and caffeic acids from the experimental design for retention time (RT), retention (k') and tailing factors (TF), peak width (W), number of theoretical plates (NTP), and resolution between CGA and interfering peak (Rs_{CGA-I}).

Run	Factors			Responses										
	A – TFA (%; v/v)	B – ACN (%; v/v)	C – elution method	Chlorogenic acid (CGA)						Caffeic acid (CFA)				
				RT (min)	k'	TF	W	NTP	Rs_{CGA-I}	RT (min)	k'	TF	W	NTP
#1	0.06	13.0	DG	7.488	2.200	1.051	0.700	1830	1.409	11.451	3.839	1.022	0.646	5027
#2	0.01	12.0	IG	7.363	1.821	0.995	0.677	1892	1.605	11.473	3.395	0.795	0.720	4063
#3	0.02	12.0	DG	7.559	1.852	0.935	1.070	798	1.434	11.459	3.324	0.970	0.683	4504
#4	0.01	14.0	ISO	6.205	1.332	0.831	0.590	1769	1.097	8.994	2.381	1.021	0.580	3847
#5	0.10	16.0	IG	8.603	2.162	1.194	0.867	1575	1.490	11.864	3.361	1.075	0.643	5447
#6	0.06	14.0	IG	8.254	2.161	1.085	0.864	1460	1.629	12.238	3.688	1.067	0.700	4890
#7	0.10	14.0	ISO	6.083	1.244	1.351	0.500	2368	1.045	8.665	2.197	1.037	0.699	2459
#8	0.06	12.0	ISO	8.002	2.031	1.285	0.769	1732	1.542	12.132	3.595	1.072	0.654	5506
#9	0.05	16.0	ISO	6.175	1.356	0.938	0.424	3393	0.946	8.740	2.335	1.128	0.601	3395
#10	0.01	16.0	IG	7.976	1.932	0.954	1.279	622	1.371	12.359	3.543	0.935	0.503	9659
#11	0.10	14.0	ISO	6.223	1.384	1.304	0.420	3512	0.946	8.802	2.372	1.059	0.678	2696
#12	0.06	15.0	DG	6.355	1.407	0.914	0.450	3190	1.276	9.358	2.544	1.098	0.415	8135
#13	0.06	14.0	IG	8.365	2.008	1.130	0.920	1322	1.449	12.435	3.473	1.100	0.726	4694
#14	0.10	12.0	DG	7.052	1.500	1.133	0.655	1854	1.364	10.631	2.769	0.904	0.508	7007
#15	0.01	14.0	ISO	6.223	1.357	0.821	0.580	1841	1.108	8.996	2.407	1.016	0.520	4789
#16	0.10	16.0	DG	5.664	1.420	0.794	0.305	5517	0.910	7.797	2.332	0.896	0.398	6141
#17	0.06	12.0	ISO	7.843	2.051	1.256	0.817	1474	1.440	12.082	3.701	1.021	0.678	5081
#18	0.06	14.0	IG	8.749	2.326	1.144	0.849	1699	1.680	12.670	3.817	1.096	0.673	5670
#19	0.06	12.0	IG	8.012	1.861	1.136	0.659	2365	1.956	12.012	3.290	0.959	0.744	4171
#20	0.01	16.0	ISO	5.299	1.061	0.741	0.497	1818	0.811	7.301	1.840	1.106	0.458	4066
#21	0.01	15.0	DG	6.029	1.318	0.854	0.485	2472	0.870	8.759	2.367	1.051	0.450	6058
#22	0.10	12.0	IG	6.749	1.884	1.239	0.451	3582	1.166	9.386	3.011	0.982	0.546	4728

of the same concentrations (2.5, 50 and 100 μg/ml) over 3 days, and the results were also expressed as percentages and RSD. The recovery and selectivity (FDA, 1994, 2003) were ascertained as indicated in the next subsection.

Preparation of C. glaziovii *dry extracts*

Quantification of the phenolics CGA and CFA was performed for different *C. glaziovii* dried products. Recovery was determined by adding measured amounts of CGA and CFA to the *C. glaziovii* extracts with known concentrations of each chemical marker and it was calculated as the percentage of the extra amount of CGA and CFA found in the samples, compared to the amount added.

The extractive solution #1 was prepared by a maceration method (Beringhs et al., 2013). The CPQBA plant material was macerated at room temperature for three days (18 g of plant material per 100 ml of 27 °GL ethanol). The suspension was filtered to obtain liquid extractive solution #1. The extractive solution #2 was prepared by the shear extraction method, using a high performance disperser device (Ultra-Turrax® T-25, IKA, USA) coupled to a dispersing tool (S20-25NK-19G, IKA, Germany) at 6500 rpm for 10 min (stator diameter = 19.0 mm; rotor diameter = 12.7 mm; gap between rotor and stator = 0.3 mm). In this case, CPQBA plant material (5.0 g) was submerged in 100 ml of a 80 °GL ethanol solution and submitted to the ultra-homogenization process, and the resulting suspension was filtered under reduced pressure to obtain liquid extractive solution #2.

Resulting extractive solutions (#1 and #2) were concentrated under vacuum (MA-120, Marconi, Brazil) and freeze-dried for 48 h (LD1500, Terroni, Brazil) to give the dried extracts DE#1 and DE#2, respectively. The preparation of the extract samples for HPLC analysis consisted of the dissolution of known amounts of each DE in a methanol solution in water (1:1 ratio; v/v). In order to demonstrate the ability of this method to separate the CGA and the interfering compound, when compared to another method, both extracts were also quantified employing the method published by Arend et al. (2011).

DE#3, 4, 5, 6, 7 and 8 were prepared using *C. glaziovii* plant samples from different geographic regions within South America (see *Raw plant material* section) in order to demonstrate the selectivity of the method. These extracts were prepared by shear extraction followed by freeze-drying, as described for DE#2.

Results

Chemometric analysis

The values for all the responses obtained for different chromatographic conditions are shown in Table 2. The results for the statistical analysis related to CGA and CFA are described in Tables 3 and 4, respectively. For the categorical nominal factor (elution method), two coefficients are provided due to the need to use multiple coefficients associated with multi-level factors. Adequate precisions were considered indicative of an adequate signal to noise ratio (Myers et al., 2009). The maximum absorption signal showed no significant change ($p > 0.05$) when analyzed under all the proportions of mobile phase and TFA concentrations studied.

Factors influencing the retention time and factor

The retention time (RT) of CGA (Fig. 1, first line) was affected by the proportion of ACN in the mobile phase (B) and the elution method (C) in combination (interaction BC, $p < 0.0001$; Table 3). During isocratic elution (ISO; Fig. 1a), the higher the ACN concentration, the shorter the CGA retention time due to the lower polarity of the mobile phase, leading to weaker interactions between the compound and the stationary phase. Similar behavior was observed for the decreasing gradient elution method (DG; Fig. 1c). On the other hand, the increasing gradient method (IG; Fig. 1b) caused an inverse behavior as the ACN concentration increased. It was noticed that intermediate concentrations of TFA (A) increased the CGA retention time in a non-linear manner (A^2, $p < 0.0001$; Table 3), as a result of the ionization suppression of the acidic solutes at low pH (Cai and Li, 1999). The behavior observed for CGA was also observed

Table 3
Analysis of variance (regression coefficients and significant *p* values) for the responses obtained for chlorogenic acid.

Polynomial term	Responses for chlorogenic acid											
	RT (min)[1]		k'[1]		TF[2]		W[1][a]		NTP[2][a]		Rs_{CGA-I}[1]	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Model	–	<0.0001	–	<0.0001	–	<0.0001	–	<0.0001	–	<0.0001	–	<0.0001
Intercept	7.51	–	1.87	–	1.04	–	−0.40	–	7.59	–	1.41	–
(A) TFA	−0.027	0.5639	0.017	0.7912	0.14	<0.0001	−0.17	<0.0001	0.34	<0.0001	−0.025	0.5756
(B) ACN	−0.38	<0.0001	−0.14	0.0325	−0.097	<0.0001	−0.14	0.0002	0.16	0.0227	−0.17	0.0021
(C) Elution method	−0.44/0.95	<0.0001	−0.09/0.31	0.0001	−0.11/0.06	<0.0001	−0.13/0.26	<0.0001	0.14/−0.25	0.0018	−0.07/0.23	0.0005
AB	0.22	0.0062	–	–	−0.031	0.0587	–	–	–	–	–	–
AC	–	–	–	–	−0.09/0.02	<0.0001	–	–	–	–	–	–
BC	−0.49/0.97	<0.0001	−0.08/0.28	0.0020	−0.02/0.07	0.0011	−0.30/0.45	<0.0001	0.47/−0.63	<0.0001	–	–
A^2	−0.75	<0.0001	−0.28	0.0024	–	–	−0.12	0.0022	–	–	−0.23	0.0056
B^2	–	–	–	–	–	–	–	–	–	–	–	–
R^2	0.9817		0.8687		0.9736		0.9756		0.9272		0.7969	
Adjusted R^2	0.9704		0.8030		0.9537		0.9634		0.9121		0.7334	
Predicted R^2	0.9469		0.6554		0.7955		0.9354		0.8554		0.5729	
Adeq. precision	28.671		11.534		23.279		36.589		26.691		11.242	

Process order = [1]Quadratic or [2]two factor interaction (2FI).
[a] Natural log transformation ($k = 0$).

Table 4
Analysis of variance (regression coefficients and significant *p* values) for the responses obtained for caffeic acid.

Polynomial term	Responses for caffeic acid									
	RT (min)[1]		k'[1]		TF[1]		W[1]		NTP[2][a]	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Model	–	<0.0001	–	<0.0001	–	<0.0001	–	<0.0001	–	0.0002
Intercept	11.14	–	3.25	–	1.09	–	0.63	–	8.50	–
(A) TFA	−0.31	0.0015	−0.080	0.2367	0.013	0.1159	0.008	0.2561	−0.089	0.0207
(B) ACN	−0.85	<0.0001	−0.32	0.0017	0.051	<0.0001	−0.062	0.0005	0.038	0.2094
(C) Elution method	−0.58/1.42	<0.0001	−0.12/0.46	0.0001	−0.018	0.0028	−0.08/0.05	0.0002	0.22/0.088	<0.0001
AB	0.26	0.0225	–	–	−0.027	0.0174	0.069	0.0010	−0.18	0.0061
AC	0.19/−0.34	0.0280	–	–	−0.064/0.069	<0.0001	−0.04/−0.02	0.0134	0.17/−0.016	0.0195
BC	−0.78/1.63	<0.0001	−0.14/0.50	0.0003	–	–	−0.07/0.02	0.0102	0.11/0.21	0.0004
A^2	−1.30	<0.0001	−0.49	0.0006	−0.090	<0.0001	−0.056	0.0136	–	–
B^2	–	–	–	–	−0.036	0.0179	–	–	–	–
R^2	0.9882		0.9011		0.9379		0.9242		0.8867	
Adjusted R^2	0.9776		0.8517		0.8913		0.8553		0.8017	
Predicted R^2	0.9188		0.7451		0.7245		0.5895		0.6404	
Adeq. precision	29.015		12.952		19.342		11.901		12.583	

Process order = [1]Quadratic or [2]two factor interaction (2FI).
[a] Natural log transformation ($k = 0$).

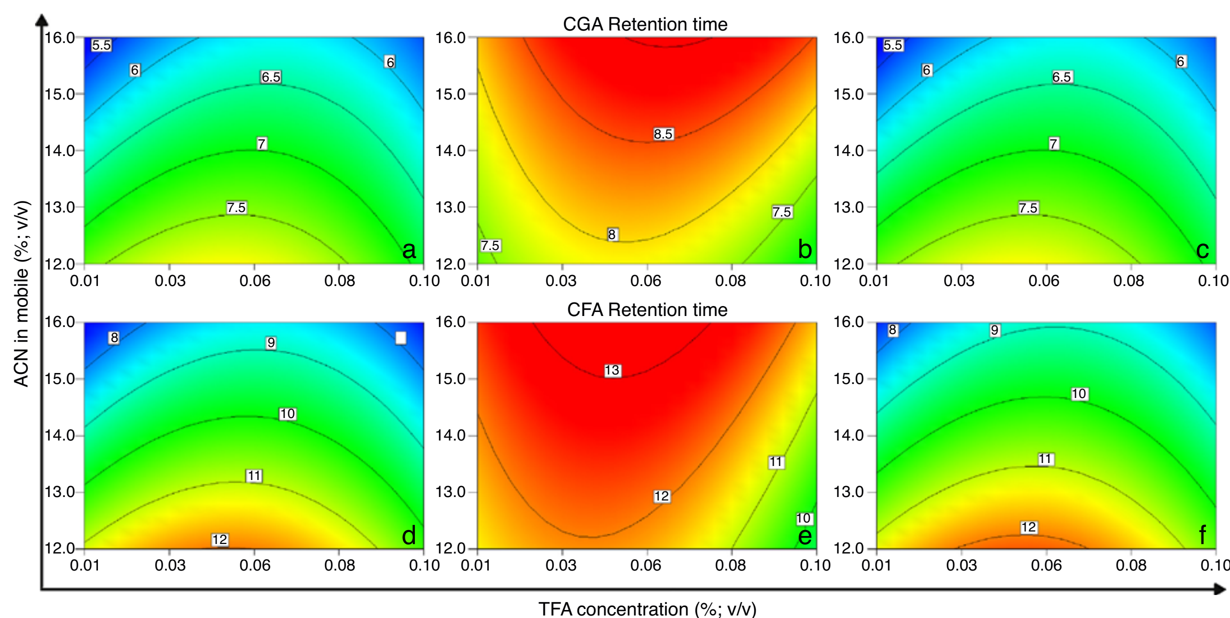

Fig. 1. Contour graphs of retention times for chlorogenic acid (CGA) and caffeic acid (CFA) with the (a/d) isocratic, (b/e) increasing gradient and (c/f) decreasing gradient elution methods, respectively.

for CFA (Table 4; Fig. 1, second line). The RSM results showed that the use of different gradient elution methods (DG or IG) led to different intensities in the responses. The retention factors (k') of both CGA and CFA showed the same behavior as the retention time (Tables 3 and 4), since this factor provides an estimate of the distance between the maximum signal of the peak and the void volume of the chromatographic run.

Factors influencing the tailing factor

The tailing factor (TF) for CGA (Fig. 2, first line) was clearly influenced by the TFA concentration (A) in the three elution methods. Peaks of symmetrical Gaussian shape were found in the range of 0.9–1.1 (ideal TF = 1.0). Regarding the CGA, an interaction between the TFA and elution method (interaction AC, $p < 0.0001$; Table 3) was observed. The ACN and the elution method in combination (interaction BC, $p = 0.0011$; Table 3) also had an important influence on the TF value. Under isocratic conditions (Fig. 2a), peak symmetry was found when TFA was used in the range of 0.01–0.08% (v/v), increasing proportionally to the increase in ACN. Under an increasing gradient (Fig. 2b), low concentrations of TFA (0.01–0.06%, v/v) provided good symmetry for all ACN proportions. The decreasing gradient (Fig. 2c) led to peak symmetry in the range of 0.03–0.08% TFA (v/v) and 12–14% ACN (v/v).

When analyzing the tailing factor for CFA, the quadratic factor was significant for TFA (A^2, $p < 0.0001$; Table 4), characterized by a parabolic response that achieving a plateau (Fig. 2, second line). However, the ideal TFA concentration is dependent on the other two factors (interactions AB, $p = 0.0174$ and AC, $p < 0.0001$; Table 4). Peak symmetry could be achieved with the three elution methods (Fig. 2d–f) by combining adequate concentrations of TFA with suitable ACN proportions.

The width (W) for both chemical markers showed similar results to that observed for the tailing factors (Tables 3 and 4).

Factors influencing the number of theoretical plates

The NTP for CGA was dependent on TFA concentration (A, $p < 0.0001$, Table 3) and the combination between ACN and elution method (BC, <0.0001, Table 3). The NTP increased as the proportion of ACN increased in both isocratic and decreasing gradient. Under increasing gradient, the response to the proportion of ACN was inverted (higher concentrations led to lower NTP) due to an increase in the gradient inclination. The TFA concentration (A) had a positive influence, promoting a slight increase in the NTP of CGA with all elution methods, due to the suppression of secondary interactions between the column and the analyte.

For CFA, low concentrations of TFA and ACN (interaction AB, $p = 0.0061$; Table 4) improved the NTP under isocratic elution. When increasing and decreasing gradients were used, high concentrations of ACN led to higher NTP (interaction BC, $p = 0.0004$; Table 4). With isocratic and increasing gradient methods, an increase in the TFA concentration (A) reduced the NTP. On the other hand, under decreasing gradient the NTP increased as the TFA concentration increased (interaction AC, $p = 0.0195$; Table 4).

Factors influencing the resolution between peaks

In the case of the separation of CGA and the interfering compound, the highest Rs value obtained was 1.956 (Table 3). It was noted that adequate separation for quantitative purposes was obtained when the Rs index was ≥1.3. The factors that influenced this response were the proportion of ACN (B, $p = 0.0021$; Table 3) and the elution method (C, $p = 0.0005$; Table 3). As shown in Fig. 3, the behavior of Rs was similar for all elution methods, but the intensity of the responses differed. Under increasing gradient (Fig. 3b), better Rs was obtained when compared to other elution methods. Isocratic elution (Fig. 3a) produced the worst Rs results, providing unsatisfactory separation of CGA from the interfering peak. In general, an increase in the ACN proportion led to a decrease in the Rs value. In this case, the CGA and the interfering compound elute in such close proximity so that the chromatography system integrator was unable to determine where one peak ended and the other started. Rs values under 1.0 were obtained when the highest proportion of ACN was used. The quadratic factor for TFA (A^2, $p = 0.0056$; Table 3) was also significant, indicating a non-linear concentration-response relationship, characterized by the parabolic shape of the contour graphs (Fig. 3). The influence of TFA

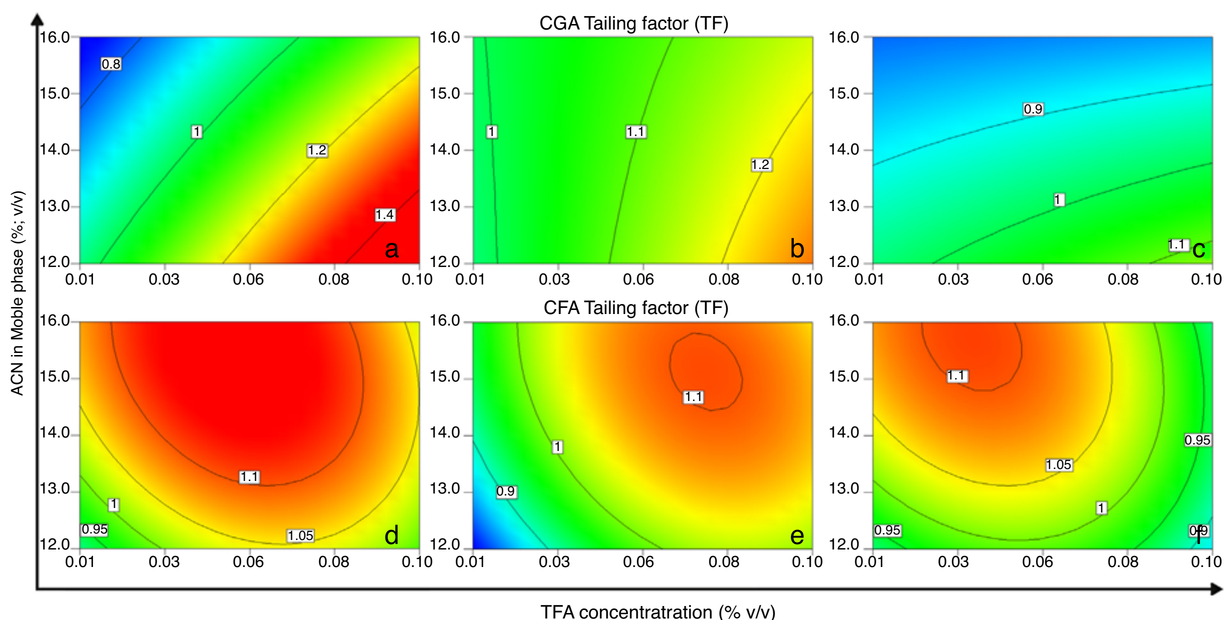

Fig. 2. Contour graphs of tailing factors of chlorogenic acid (CGA) and caffeic acid (CFA) with the (a/d) isocratic, (b/e) increasing gradient and (c/f) decreasing gradient elution methods.

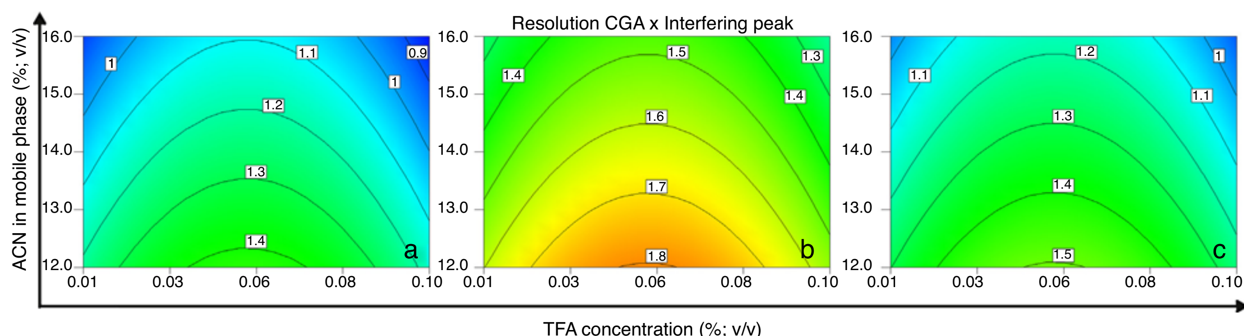

Fig. 3. Contour graphs of resolution between CGA and interfering peak (Rs_{CGA-I}) with the (a) isocratic, (b) increasing gradient and (c) decreasing gradient elution methods.

on this response is a reflection of its effect on peak width and retention time, with the resolution between peaks increasing when it was used in intermediate concentrations (0.04–0.07%; v/v).

Optimization of the chromatographic method

The optimization criteria were established as a targeted value of 1.0 for the tailing factors (importance = 4), an increase in the number of theoretical plates for both CGA and CFA (importance = 3) and the maximization of the resolution between the CGA and interfering peaks (importance = 5). The optimized conditions were mathematically determined as (A) TFA concentration 0.05% (v/v), (B) proportion of ACN in the mobile phase 12% (v/v), and (C) increasing gradient elution method. The responses predicted by the model were TF 1.11 and 0.99 and NTP 2204 and 4367 for CGA and CFA, respectively, and Rs_{CGA-I} 1.77. The combined desirability function was 0.598 (Fig. 4). The individual desirability functions for each response were 0.677 and 1.000 for TF and 0.323 and 0.265 for NTP, for CGA and CFA, respectively, and the Rs_{CGA-I} value was 0.843.

The conditions mathematically obtained were applied experimentally by preparing confirmation chromatographic runs ($n = 3$). The results obtained for the confirmation runs were within the 95% prediction and the 95% confidence intervals, indicating good predictability of the model for all the responses analyzed. The chromatogram obtained for DE#1 under the optimized condition is shown in Fig. 5a, and it clearly demonstrates an adequate resolution between the CGA, CFA and interfering peaks. The UV spectrum within the range of 200 to 400 nm was verified by means of a PDA detector, and the spectrum peaks were in agreement with those

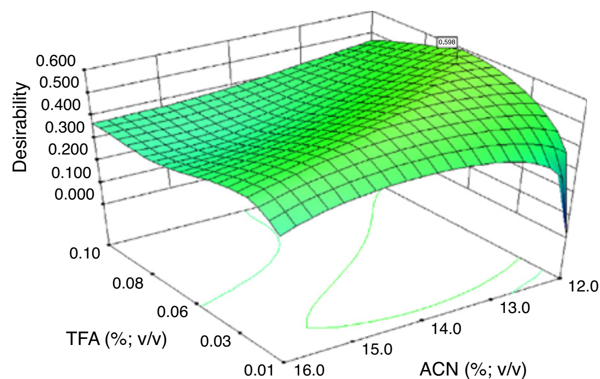

Fig. 4. Response surface graph of the optimized chromatographic conditions (prediction flag = 0.598).

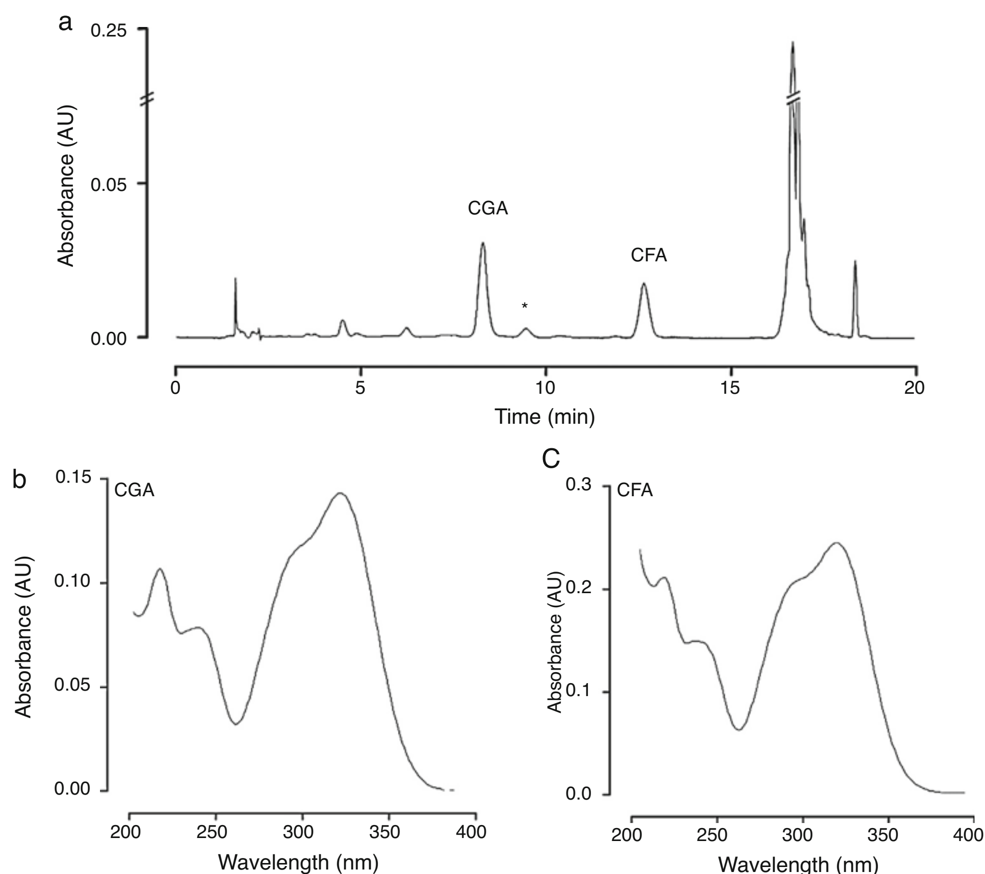

Fig. 5. (a) Typical chromatograms obtained for *Cecropia glaziovii* under the optimized chromatographic conditions. *Interfering peak; (b) and (c) PAD UV spectrum obtained for CGA and CFA, respectively.

obtained for the primary analytical standards (Fig. 5b and c), indicating good spectroscopic purity.

Validation of the chromatographic method

The results for the repeatability and the intermediate precision were considered satisfactory, since all RSD values were less than 0.8%, indicating that no significant variation in the quantification was detected. The analytical curves were plotted and a linear relationship ($r^2 > 0.999$ for both CGA and CFA) between the signal and concentration was found over the range of 1–200 μg/ml for CGA ($y = 53{,}422x - 85{,}614$) and 2.5–100 μg/ml for CFA ($y = 92{,}328x - 12{,}240$). The DL and QL values were determined as 0.006 and 0.020 μg/ml for CGA and 0.197 and 0.598 μg/ml for CFA, respectively.

Quantification of C. glaziovii *extracts*

A recovery assay was performed by adding known amounts of pure CGA and CFA standards to each sample in its solid state. The samples were spiked with 25, 50 and 75% of a known concentration of each chemical marker for each product (final concentrations of 125, 150 and 175%, respectively). The results obtained are shown in Table 5 and demonstrate excellent recovery of the chemical markers in each sample, indicating that the chromatographic method is able to quantify these compounds without any interference from the matrices. The method has proven to be selective when analyzing different plant samples (DE#3–8), indicating that no significant interferences were observed in products obtained from different geographic regions (FDA, 2003).

Also, the DE#1 and 2 were quantified employing a previously published RP-HPLC-UV method (Arend et al., 2011) and an increase on the CGA peak areas of 0.4 ± 0.2 and 3.4 ± 0.3%, respectively was noticed, when compared to that obtained employing the method developed in this work. Such increase can be due to the unsatisfactory resolution between CGA and the interfering compound under the method developed by Arend et al. (2011). The macerated extract (DE#1) presented no significant interference, but the high shear extract (DE#2) showed significant difference in peak area.

Table 5
Recovery data after contamination of each product with known amounts of CGA and CFA standards.

Product	Chemical marker	125%	150%	175%
		Mean recovery % (RSD)*		
DE#1	CGA	101.47 (0.74)	100.67 (0.77)	100.67 (0.73)
	CFA	100.61 (0.71)	100.45 (0.50)	100.38 (0.16)
DE#2	CGA	99.93 (1.25)	101.15 (0.35)	100.17 (0.06)
	CFA	100.50 (0.61)	100.92 (0.18)	100.05 (0.06)
DE#3	CGA	100.64 (0.57)	99.85 (0.70)	101.12 (1.13)
	CFA	99.77 (1.31)	100.68 (0.65)	100.46 (2.09)
DE#4	CGA	100.31 (0.11)	100.37 (0.15)	100.27 (0.09)
	CFA	99.91 (2.19)	99.82 (1.43)	99.85 (0.71)
DE#5	CGA	99.81 (0.67)	100.23 (0.06)	100.13 (1.10)
	CFA	100.17 (0.15)	100.60 (0.59)	101.15 (0.84)
DE#6	CGA	100.23 (1.09)	99.91 (1.28)	100.50 (0.43)
	CFA	101.39 (0.51)	100.86 (0.96)	100.69 (0.36)
DE#7	CGA	100.27 (0.06)	100.19 (0.98)	100.78 (0.55)
	CFA	101.08 (0.36)	99.83 (0.73)	100.10 (1.00)
DE#8	CGA	100.50 (0.28)	100.90 (0.64)	100.31 (0.11)
	CFA	100.54 (0.42)	101.12 (1.13)	99.98 (1.63)

* $n = 3$.

Discussion

C. glaziovii was chosen as the plant species for the development of the analytical method, due to its high content of phenolic compounds of biomedical interest. Among these compounds, the phenolic acids present an analytical challenge due to their relatively similar chemical structures, which leads to the elution of several compounds within the same chemical class in close proximity. In this case, parameters such as pH, polarity and ion pairing ability of the mobile phase have a direct influence on the retention, resolution and sharpness of the peaks, and thus strongly influence the chromatographic reliability. In the specific case of *C. glaziovii*, for example, unsatisfactory chromatographic conditions cause the elution of an interfering compound right after the CGA peak, diminishing its resolution and hindering the reliable quantification of this phenolic acid, since it is difficult for the chromatography system integrator to determine where the signal peak starts and ends. Depending on the chromatographic conditions, the two peaks may easily merge, creating a false impression of a pure signal, when in reality it is a spectroscopic impurity.

RP-HPLC is the method of choice for the analysis of phenolic compounds, due to the fast mass transfer, high mechanical strength and high separation capability, but some disadvantages should be taken into consideration. The C18(2) column used in this study is comprised of octadecyl silane ligands bound to silanol groups on the silica surface. These bonds are not complete, and there are residual free silanol groups that can generate a secondary interaction between the stationary phase and the analyte (Watson, 2005). In general, these interactions only occur to a small extent, due to a steric hindrance caused by the octadecyl silane, but the presence of these residual silanol groups may still affect the separation efficiency (Liu et al., 2008). These groups might interact with the analyte when both are ionized, and this interaction can result in undesirable broadening or tailing of the peaks (Liu et al., 2008). For this reason, the pH of the mobile phase may be acidified in order to prevent ionization of the silanol groups, and therefore suppress their interaction with the analyte. The addition of strong partially ionized acids, such as TFA, has two basic functions in the separation of acid compounds: to minimize the interaction between the silanol groups and the compound by protonating them, and suppress the ionization of acid solutes, altering the retention time of the molecules (Cai and Li, 1999), therefore justifying its use in these analyses.

In this study, the IV-Optimal Response Surface Methodology design has proved to be an excellent chemometric tool to evaluate the influence of different factors within the experimental domain. This experimental methodology, designed to minimize the integrated prediction variance using the IV-Optimal algorithm (Jones and Goos, 2012; Sambo et al., 2014), was efficient even when compared to other types of RSM design (Myers et al., 2009). The IV-Optimal design allowed the detection of slight changes in intensities of response that could not be observed through other experimental methodologies. This enabled the determination of adequate chromatographic conditions to satisfactorily analyze *C. glaziovii* products. The polynomial equations obtained through this algorithm are specially optimized, targeting the use of the model as a prediction tool, and delivering trustworthy results.

It was observed, through spectroscopic studies, that the maximum absorption of both CGA and CFA occurred concomitantly at 330 nm, and there was no significant change in the ultraviolet signal under all experimental conditions ($p < 0.05$). The UV–Vis detector was chosen to develop the chromatographic method because it is cheaper and more accessible when compared to other detectors such as the PDA detector, making this method useful for a higher number of laboratories. Evidently, the PDA detector could lead to higher sensitivity, since it would enable the analysis of several wavelengths, but it is also more expensive. Therefore, in cases where it is possible to obtain high absorption signals from all compounds under only one wavelength, the UV–Vis detector is a more interesting choice.

The optimization of the chromatographic method was performed using a numerical optimization method, which finds a point that allows maximization of the conditions related to the desirability function. The desirability function is a multiple response method where an objective function is calculated, ranging from zero (undesirable conditions) to one (target conditions). This tool allows the combination of a good set of conditions, obtaining the optimal experimental conditions that satisfy all the goals to the maximum degree possible. The desired characteristics may be modulated by altering the weight and importance of each response, allowing the mathematical model to give priority to the responses that are considered more important than others, but also taking them into consideration in the optimization process. As a result, a desirability index is calculated, indicating how well the optimization criteria were satisfied. Commonly, when there are a high number of responses involved in the optimization process, as in this study, the desirability index may drop. In this case, a low desirability index does not mean that the criteria were not satisfied. Rather it reflects the fact that when several responses are involved, it is difficult to satisfy all the optimization criteria with total efficiency. Therefore concessions must be made to allow the determination of an adequate optimized condition that fulfills as many of the criteria as possible (Myers et al., 2009). The optimized conditions were found under a desirability index of 0.598. This index is considered adequate when analyzing multiple responses using IV-Optimal RSM designs.

The high robustness of the model enabled successful determination of the ideal chromatographic conditions for the separation and quantification of both CGA and CFA in complex matrices derived from *C. glaziovii* in a relatively short period of elution time. The method was optimized based on a high amount of data, which enabled exact prediction of the behavior of the method when used to quantify *C. glaziovii* products. It was also proven to be a selective method when analyzing extracts prepared with plant samples from different geographic locations within the Atlantic Forest.

This chromatographic method, when compared to the one developed by Arend et al. (2011), has shown to be able to separate the interfering compound peak and the CGA peak, as shown in Fig. 5a. An increase in CGA's peak area was noticed when the other method was employed, which indicates that the previously published method is unable to satisfactorily separate these peaks and leads to a false impression of spectral purity. Even though the increase in the area of the peak is small (below 5%), this difference is highly relevant on the quantification of a chemical marker when the production of a pharmaceutical product is desired. It is also important to highlight that the differences were noticed only for the extract prepared by high shear extraction, and not for that obtained by maceration. This result may be due to the principle of each extraction method, once the shear extraction usually leads to highly concentrated extracts when compared to the maceration method.

The "one-variable-at-a-time" approach generally taken by researchers is not appropriate for the analysis of complex plant-based matrices, due to the high content of chemical substances with similar chemical characteristics that can easily promote inaccurate measurements. The information obtained in this study enhances our knowledge of how the development of analytical methods for plant-based products should be approached, since the results clearly show the importance of detailed improvements in the performance in order to guarantee the reproducibility and accuracy of the analysis. Therefore, the chemometric approach adopted here, using a RSM IV-Optimal design, proved to be much

more adequate when it comes to the development of analytical methods for complex matrices when compared to the typical "one-variable-at-a-time" experimental approach. Through this study, we demonstrated how to successfully apply the RSM IV-Optimal algorithm design to the optimization of chromatographic methods.

These findings can be useful for the reproduction of the optimized chromatographic conditions in industrial and academic laboratories for the quantification of chlorogenic and caffeic acids in *C. glaziovii* extracts and pharmaceutical products. The method developed has shown improved selectivity when compared to a method previously published. This study also provides preliminary information on the development of new analytical methods for the quantification of these compounds in other plant matrices, such as those based on other *Cecropia* species. Furthermore, the approach to improving the performance of chromatographic methods described herein may be adopted by researchers in the development of chromatographic methods for other plant species.

Authors' contributions

AOB (M.Sc. student) contributed in several steps of this work, including the chromatographic analysis, data analysis, experimental design and drafted the paper. MD contributed to the chromatographic analysis. TBCP collaborated with enriched discussions and correction of the paper draft. DS, coordinator of the research group, designed the study, supervised the laboratory work and analyzed all data. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

The authors express their appreciation to CAPES (Coordination for the Improvement of Higher Education Personnel) for their financial support (CAPES/DS fellowship).

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