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Experimental design as a tool to evaluate chlorogenic and caffeic acids extracted from *Cecropia glaziovii* Sneth

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ABSTRACT

The effects of different parameters, including ethanol concentration, time of drug:solvent contact, temperature and the presence of a preservative, on chlorogenic acid (CGA) and caffeic acid (CFA) yields in *Cecropia glaziovii* Sneth extracts were investigated using an experimental design. In order to quantify the phenolic acids in these extracts a high-performance liquid chromatography–diode array detection (HPLC–DAD) method was developed and validated. Extracts with 80% ethanol presented a higher CGA content, but low amounts of CFA. Extracts with 20% ethanol showed a higher CFA concentration, but a sharp reduction in CGA extraction yield. The presence of a preservative, under the same extraction conditions, resulted in a slight difference or no difference in the CGA and CFA extraction yields. When the temperature was controlled at refrigerator or room temperature, a slight alteration in the concentrations of the phenolics studied was observed. The present approach can be applied in order to determine the optimum conditions for the preparation of *C. glaziovii* Sneth extracts based on CGA or CFA extraction yield as a chemical marker.

1. Introduction

The analysis of plants and herbal formulations presents several problems arising from their complex nature and the inherent variability of their constituents. Plants are complex mixtures of a variety chemicals, which poses a problem in terms of standardization and quality control. However, this very fact is responsible for their feature of being therapeutically effective. Consequently, the herbal drug preparation itself, as a whole, is regarded as the active substance. Hence, the extraction procedure and the stability of the extract are important since they determine the quality and the yield of the individual constituents. Also, the economical feasibility of an industrial process requires that it works in such a way that high efficiency values are attained. Some factors can contribute to achieving this aim: (1) correctly choosing the raw materials to extract; (2) subjecting these materials to appropriate pretreatment; and (3) optimizing the values of the variables which have a direct influence on the process [1]. *Cecropia* species are extensively used in traditional medicine in Latin America as cardiotonic, diuretic, hypotensive, and anti-inflammatory agents. Moreover chlorogenic acid (CGA), also found as a major compound in other plants, has been related to the pharmacological properties of *Cecropia* sp. [2–11]. Although *Cecropia glaziovii* is widely used in Brazilian folk medicine, there are no reports concerning the influence of its extraction conditions on the phenolic acids content. In addition, little information is available on the quality evaluation and standardization methods for both qualitative and quantitative determination of this component [12–14]. In addition, studies related to the intermediate pharmaceutical products have been reported [15–17], and the results are not associated with the stability during the extraction process. Extraction efficiency is commonly a function of the process conditions. Temperature, time of contact, and plant:solvent ratio are some of the most important factors that influence the extraction efficiency in terms of quality and yield [1,17–20]. On the other hand, the role of each factor in the mass transfer of the process is not predictable since the chemical characteristics of the solvent and the diverse structure and composition of the natural products lead to each material–solvent system showing different behavior [1].

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In the present study, a specific extraction condition allowed us to obtain caffeic acid (CFA), a substance not yet reported for this species. Thus, we evaluated the extraction and stability of CGA and CFA during the extraction process using a Response Surface Methodology (RSM). Ethanolic solvent concentration, extraction time and the use of temperature or a preservative were chosen as variables. All data were obtained using a validated high-performance liquid chromatography/diode array detection (HPLC/DAD) method for analysis of the crude extract of *C. glaziovii* Sneth leaves.

2. Experimental

2.1. Materials

2.1.1. Chemical reagents

Chemicals and reagents were obtained from the following commercial sources: chlorogenic acid and caffeic acid (Sigma–Aldrich, St. Louis, MO, USA), methanol and acetonitrile (HPLC grade) (J.T. Baker, Phillipsburg, NJ, USA), acetic acid (Qhemis, São Paulo, Brazil), LC-grade water obtained with a Milli-Q system (Millipore, Bedford, MA, USA), and methylparaben (DEG, São Paulo, Brazil). All samples and solutions were prepared from purified water. All other reagents and solvents were analytical grade.

2.1.2. Raw material characterization: plant material

Dried leaves of *C. glaziovii* were obtained from the Pluridisciplinary Center of Chemical, Biological and Agronomic Studies of the University of Campinas, SP, Brazil. The dry leaves were ground in a knife mill (Macmont) with a mesh of 3 mm. The ground material was submitted to a particle size distribution test, as described below.

2.2. Methods

2.2.1. Particle size distribution test

Particle size distribution was evaluated by a standard sieving method, for a period of 15 min (Sieve shaker Bertel 1400), with 30 g of the dried milled plant material, using a series of sieves with screen sizes corresponding to 180, 355, 500, 710, 1000 and 1700 μm. The average particle size was calculated by means of Probito's evaluation [21,22].

2.2.2. Preparation of extracts

The extraction solutions (ES) were prepared by maceration at 5.0% (plant:solvent ratio; w/v) of crushed leaves (average diameter 780 ± 410 μm). Three ethanol concentrations (20, 50, and 80%; v/v) and three extraction times (4, 6, and 8 days) were evaluated. The extraction process was carried out at two different temperatures (refrigerator: $4\,^{\circ}C \pm 0.5$ and room temperature: $25\,^{\circ}C \pm 1.0$) and in the presence or absence of the preservative (methylparaben, MP, 1.0 mg/mL). The experiment that evaluated the effect of the presence of the preservative was carried out at room temperature. All extractions were prepared in duplicate in the dark.

2.2.3. Analytical procedures: sample preparation

In order to evaluate the linearity of phenolic acids concentration in relation to the extract dilution, extraction solution curves [8d20, 6d50 and 8d80] were obtained. Three milliliters of the ES were diluted to 10 mL with methanol:water solution (50:50; v/v). The samples were filtered through a 0.45 μm HVLP membrane (Millipore).

2.3. Chromatography conditions (HPLC)

The chromatographic analysis was performed on a PerkinElmer chromatograph equipped with a Series 200 auto sampler, Series 200 binary pump, Series 200 UV-Vis detector or Series 200 EP Diode Array Detector and Series 200 vacuum degasser. A Zorbax C HP C18 column (5 μm, 150 mm × 4.6 mm, Agilent Technologies) was used. The gradient elution consisted of acetonitrile (A)–1.0% acetic acid (B) with a flow rate of 1 mL/min and was programmed as follows: 0–15 min, 87% B; 15–25 min, 87–60% B; 25–34 min, 60% B. Detection was at 330 nm. The mobile phase was prepared daily and degassed by sonication before use. The injection volume was 20 μL. The data were gathered using TotalChrom® Workstation software. All chromatographic analyses were performed in triplicate.

2.4. Quantitative analysis

The quantification of chemical markers, CGA and CFA, was carried out by comparison of their retention times and by co-injection of standard solutions. Standard curves were plotted for chlorogenic (2.5–200 μg/mL) and caffeic (2.5–100 μg/mL) acids. The quantification of the individual compounds was performed using a validated regression curve ($r^2 > 0.9999$). All standard solutions and the ES were analyzed in triplicate. The average areas of the peaks were calculated.

2.5. Validation of analytical procedures

The parameters were validated according to International Conference on Harmonization (ICH) guidelines [23]. The linearity was determined for the calibration curves obtained by HPLC analysis of CGA and CFA. The slope and the other statistics of the calibration curves were calculated by linear regression. The detection limit (DL) and quantification limit (QL) were based on the standard deviation (SD) and the slope (*S*) of the calibration curves [23]. The precision of the method was determined as repeatability and intermediate precision. To evaluate the repeatability, the percent relative standard deviation (%RSD) of three distinct concentrations of the curve (2.5, 50 and 200 μg/mL for CGA; 2.5, 25 and 100 μg/mL for CFA), with each injection carried out three times, was considered. The intermediate precision was evaluated in triplicate over 3 days. Trueness was determined by recovery, adding measured amounts of CGA and CFA to the extraction solutions. The recovery experiment was performed for one concentration of each phenolic acid level (25.0 μg/mL) in three different extraction solutions (8d20, 6d50 and 8d80) injected in triplicate. The recovery was determined by subtracting the values obtained for the control matrix preparation from those obtained for the samples that were prepared with the standards added, divided by the amount added and then multiplied by 100 [23].

2.6. Experimental design

Two Response Surface Methodology (RSM) designs were used to evaluate 2 numerical variables at 2 levels and a central point (levels −1, 0 and +1): ethanol concentration (A) (20, 50 and 80%) and extraction time (B) (4, 6 and 8 days). A preliminary study was carried out in order to verify the lower limit of both the ethanol concentration and extraction time variables for detection of the polyphenolic compounds. For each RSM design a categorical variable (*C*) related to the stability was introduced. One design evaluated the effect of temperature (refrigerator: $4 \pm 0.5\,^{\circ}C$ and room temperature: $25 \pm 1\,^{\circ}C$) and the other of preservative (absence and presence), using −1 as a coded value for refrigerator or absence of preservative and +1 for room temperature or presence of preservative. Two replicates of all experiments and four replicates of

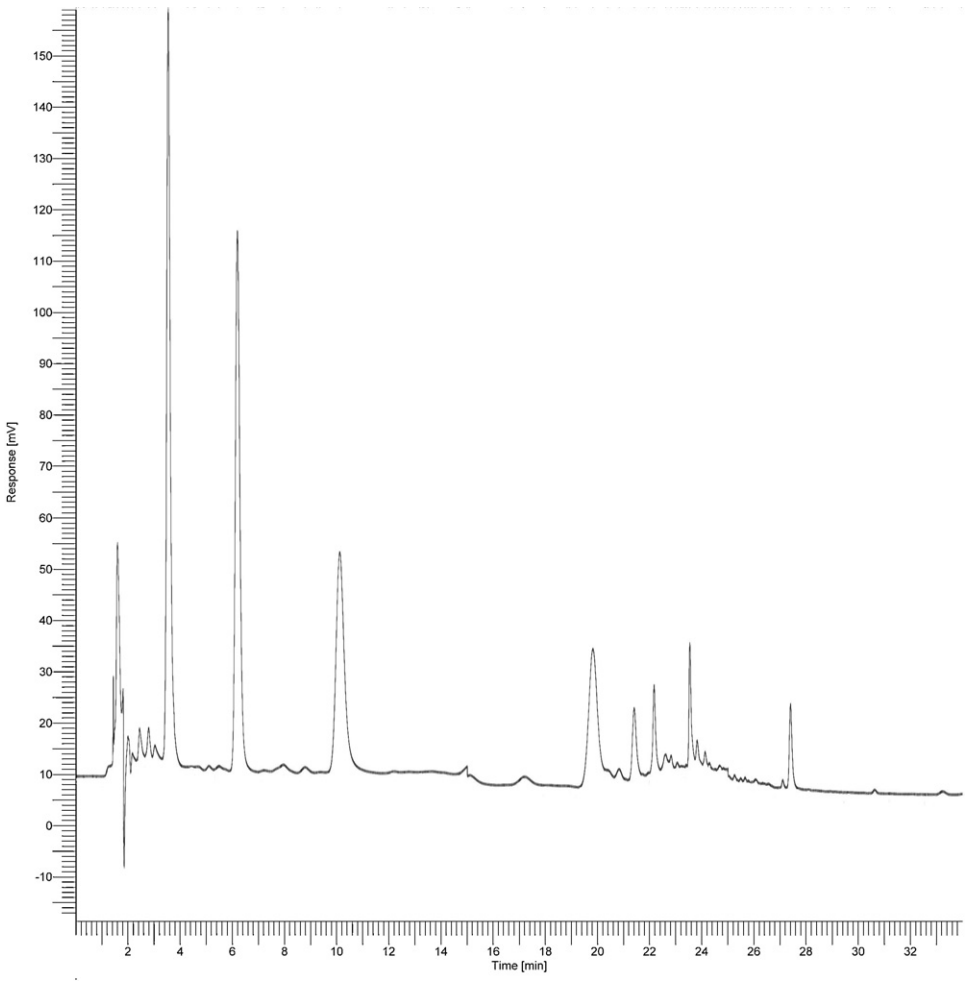

Fig. 1. Typical UV chromatogram of *Cecropia glaziovii* Sneth extraction solution. 20 μL was injected in an HPLC system with Zorbax C HP C18 column (5 μm, 150 mm × 4.6 mm). The gradient elution consisted of acetonitrile (A)–1.0% acetic acid (B) with a flow rate of 1 mL/min and was programmed as follows: 0–15 min, 87% B; 15–25 min, 87–60% B; 25–34 min, 60% B. Detection was at 330 nm.

the center point (level 0, i.e. 50% and 6 days) were run for both stability conditions. All factor level combinations were applied in a randomized order. The results were evaluated with the program Design-Expert®, Version 8.0.1 (StatEase Inc., Minneapolis, MN, USA).

The effects were calculated presuming a quadratic model with interaction among the factors. The equation model was defined as:

$$Y = M + aA + bB + cC + abAB + acAC + bcBC + aaA^2 + bbB^2 \quad (1)$$

where Y is the measured response associated with each variable level combination, M is the mean value, A, B, C are the main factors (A = ethanol concentration; B = extraction time; C = temperature or presence of a preservative), AB, AC, BC, are the binary interactions between the factors, and A^2 and B^2 are the quadratic numerical factors, and a, b, c or ab, ac, bc, or aa and bb are the coefficients of the main factors, interaction factors, and quadratic numerical factors, respectively, employing a probability error of $p < 0.05$. Analysis of variance (ANOVA) was performed to support the polynomial equations and to identify the significance of single factors, their binary interactions, and quadratic numerical factors.

3. Results and discussion

3.1. Analytical method validation

Reversed-phase high-performance liquid chromatographic (HPLC) was proposed as a suitable method for the simultaneous determination of CGA and CFA in ES. The method developed was then validated in terms of trueness, precision, linearity, quantification limit and detection limit, in accordance with the ICH guidelines [23]. The nature of the sample determines which parameters should be evaluated, especially when the samples are complex biologic matrices, as in the case of plant extraction solutions [24]. Fig. 1 shows a typical chromatogram for the ES. The retention times investigated (3.5 min for CGA and 6.0 min for CFA) showed a sharp and symmetrical peak, with good baseline resolution and minimal tailing, thus facilitating the accurate measurement of peak area ratios.

3.1.1. Precision and trueness

The precision and trueness were determined by spiking the sample with a known quantity of the standard. The mean recovery was calculated for one assay for each standard. Good trueness was observed with satisfactory recovery in the range of 98.00–102.30% (Table 1). Measurement of intra- and inter-day variability was used to determine the precision of the newly developed method. The intra-day variation (repeatability) was determined by analyzing in triplicate the mixed standard solution three times within 1 day. For the inter-day variability test (intermediate precision), the solution was examined in triplicate on 3 separate days. The percentage relative standard deviation (%RSD) was taken as a measure of precision. The results for the precision showed low values (less than 5.0%) for intra and inter-day %RSD as shown in Table 2.

Table 1
Trueness data for phenolic compounds.

Extracts[a]	Compound	Extract concentration (μg/mL)	Recovery[b]	
			Mean (%)	RSD (%)
8d20	Chlorogenic acid (25 μg/mL)	65.9	100.6	0.4
	Caffeic acid (25 μg/mL)	41.7	100.8	0.5
6d50	Chlorogenic acid (25 μg/mL)	155.9	98.0	0.2
	Caffeic acid (25 μg/mL)	9.4	101.9	0.5
8d80	Chlorogenic acid (25 μg/mL)	138.7	100.1	0.5
	Caffeic acid (25 μg/mL)	5.3	102.3	0.1

[a] 8d20: extraction for 8 days with 20% ethanol; 6d50: 6 days with 50% ethanol; 8d80: 8 days with 80% ethanol.
[b] Recovery was determined by injection of spiked samples, in triplicate, with standard solutions.

Table 2
Repeatability and intermediate precision data for phenolic standards.

Compound	Repeatability[a]		Intermediate precision[a]	
	Concentration (μg/mL)	RSD (%)	Concentration (μg/mL)	RSD (%)
Chlorogenic acid	2.5	0.2	50.0	0.6
	50	0.4		
	200	0.2		
Caffeic acid	2.5	0.4	25.0	0.7
	25	0.3		
	100	0.2		

[a] Limits: RSD < 5%.

3.1.2. Calibration curves, linearity and detection and quantification limits

The calibration curves were found to be linear over the range of 2.5–200 μg/mL for CGA and 2.5–100 μg/mL for CFA. The ranges and correlation coefficients are given in Table 3. All calibration curves showed good linear regression in the range of r^2 = 0.999974–0.999990. The DL and QL values for CGA were found to be 0.3460 and 1.05 μg/mL, and for CFA were 0.0732 and 0.22 μg/mL, respectively (Table 3).

3.2. Statistical analysis for stability study

Initially, CGA was chosen as the chemical marker because phenolic acid has been reported as the major compound in aqueous and/or butanolic extracts of *Cecropia* species [6,25–29]. However, in this study, mixtures of ethanol and water were chosen as nontoxic and environmentally friendly solvents, which have been shown to be effective in the extraction of polyphenolic compounds. Since the type and magnitude of the extraction variables can affect the analyte recovery, a suitable design (response surface) was used. The experimental parameters analyzed initially were ethanol concentration and time. Surprisingly, in the 20% ethanolic extracts we observed a considerable amount of some other compound that was not detected in the extracts with higher ethanolic concentrations. Later, we identified it from the DAD spectrum as being caffeic acid (Fig. 2), and confirmed this finding through it having the same retention time as the CFA standard solution. In addition, when comparing CGA concentrations at 4d20 and 8d20 ES, a significant reduction in the yield from 4 to 8 days was observed. This effect might be related to a chemical and/or enzymatic and microbiological degradation, considering that chlorogenic acid is an ester of caffeic acid and quinic acid [30]. However, there are no reports of the degradation of CGA during the extraction process. In order to investigate this possible degradation, the influence of temperature and the presence of a preservative on CGA content in the extraction solutions were evaluated. For this, these two variables, each with two levels, were introduced into the experimental design.

The experimental design allows the maximum amount of information to be obtained from the data collected in the smallest number of experimental runs. The basic idea is to change all relevant factors simultaneously over a set of planned experiments and then connect and interpret the results using mathematical models [31]. To provide a statistical verification of the response curvatures, four center points were added to each categorical variable. Also, each experiment was repeated twice. All 24 values are the average of three determinations. The CGA and CFA content of the 24 runs of each experimental design are presented in Table 4. The experiments resulted in concentrations of CGA ranging from 62.76 to 173.59 μg/g and of CFA from 4.90 to 46.21 μg/g.

3.2.1. Influence of extraction temperature

Statistical analysis of the experimental data was used to establish the best-fit models for the independent variables. The adequacy of the model was verified by an *F*-test and the determination coefficient R^2. The quadratic model was established for CGA content (R^2 = 0.9582) and CFA content (R^2 = 0.9954). In the case of CFA, due to the considerable variation between the contents of the ES, a log transformation was required. The analysis of variance for the extraction temperature showed that this regression model was highly significant ($p < 0.0001$) for CGA and CFA responses (Table 5),

Table 3
Calibration data of phenolic standards.

Compound	Linearity range (μg/mL)	Calibration equation[a]	Correlation coefficient (r^2)	QL[b] (μg/mL)	DL[b] (μg/mL)
Chlorogenic acid	2.5–200	$y = 54{,}291x - 16{,}595$	0.999990	1.05	0.3460
Caffeic acid	2.5–100	$y = 91{,}599x - 6746.9$	0.999974	0.22	0.0732

[a] Chlorogenic acid and caffeic acid (n = 3).
[b] QL: quantification limit; DL: detection limit.

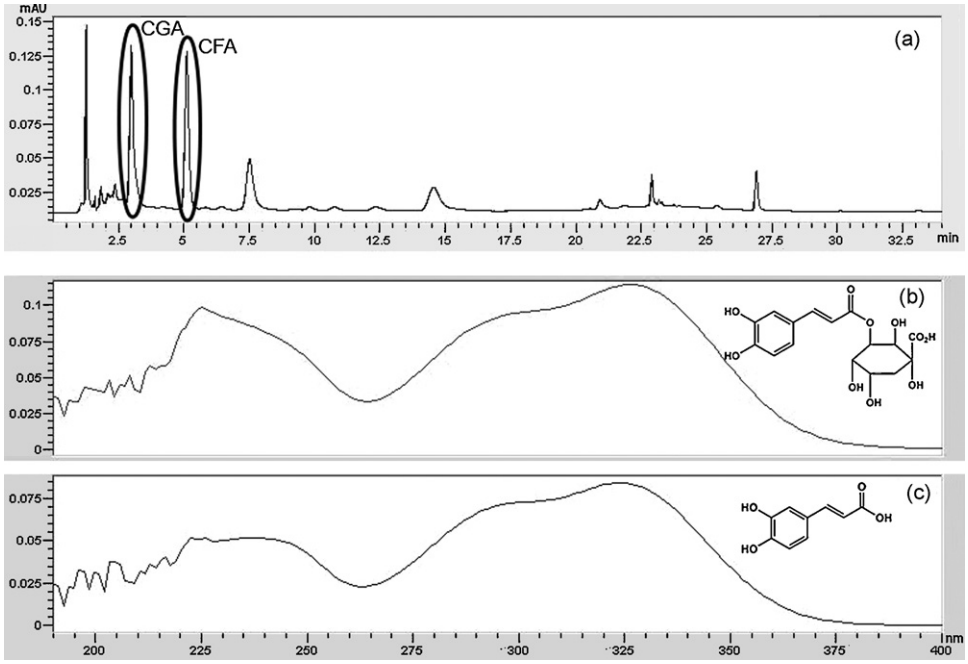

Fig. 2. (a) DAD chromatogram of *Cecropia glaziovii* Sneth extraction solution. (b) DAD spectrum and chemical structure of chlorogenic acid (CGA). (c) DAD spectrum and chemical structure of caffeic acid (CFA).

with values of 52.37 and 499.67, respectively. The predicted values Pred R^2 of 0.8815 (CGA) and 0.9871 (CFA) are in reasonable agreement with the adjusted values Adj $R^2 > 0.9399$ (CGA) and Adj $R^2 > 0.9935$ (CFA).

The lack-of-fit for two responses was not significant ($p > 0.05$) (Table 5), which implies adequacy of the model.Adequate precisions of 19.826 (CGA) and 61.011 (CFA) indicate an adequate signal. This is the signal-to-noise ratio. It compares the range of the predicted values at the design points to the average prediction error. Ratios greater than 4 indicate adequate model discrimination. Thus, these models can be used to navigate the design space.

Our results provide strong evidence of interaction between factors AB and AC for both CGA and CFA extraction yields (Table 5). In the case of CGA this implies that at room temperature and with short extraction time (4 days) an increase in the percentage of EtOH improves the extraction yield (Fig. 3a and b). On the other hand for a longer extraction time (8 days) and lower percentage of EtOH the CGA extraction yield drops. This behavior was very similar in the case of refrigerator conditions but the yield was slightly lower.

For the CFA, a lower percentage of EtOH and longer extraction time (Fig. 3c and d) improves the extraction yield. Under these conditions, a higher CFA extraction is obtained at room temperature (Fig. 3d).

3.2.2. Influence of extraction preservative

In the same way as for the temperature design, the model established to analyze the response surface design was a quadratic model for CGA content ($R^2 = 0.9710$), and CFA content ($R^2 = 0.9914$). For CFA, also with a considerable variation in content, a transformation (inverse square) was required.

The ANOVA for the extraction preservative design indicates that of the models for the CGA and CFA responses are significant ($p < 0.0001$) (Table 5), with Model *F*-values of 76.41 and 262.81, respectively. The values of Pred R^2 of 0.9165 (CGA) and 0.9771 (CFA) were in reasonable agreement with the Adj R^2 values of 0.9582 (CGA) and 0.9876 (CFA). The lack-of-fit for two responses was not statistically significant ($p > 0.05$), as observed in Table 5. The interaction between the factors A (EtOH%) and B (extraction time) are significant in terms of the CGA yield. In this case we observed the same behavior as that in the previous temperature design. With an increase in the EtOH% there was an increase in the CGA concentration (Fig. 4a). On the other hand with 20% ethanol after 8 days we observed a decrease in the CGA content, as previously discussed. The C factor is not significant (Table 5), indicating that the presence of a preservative does not modify the CGA concentration when compared to the ES without preservative. This suggests that the decrease in CGA content in 8d20 when compared to 4d20 is not due to microbiological degradation during the extraction period. However, chemical instability, not identified in our analytical system, might be present. Concerning this result more studies are in progress in our group in order to understand this effect. While for CFA content, the influence of A and B was observed, without any interaction, the CFA content was affected on changing the levels of the factors, as shown in Fig. 4b. A lower EtOH% and higher extraction time raises the CFA concentration. However, there is no difference in the extraction yield when we compare the presence and absence of preservative.

3.3. Optimization of extraction conditions

The aim of optimization is to find a good set of conditions that will meet all the goals.

The final equations in terms of coded factors for CGA and CFA contents in the two experimental designs are:

$$\text{Temperature CGA} = +162.92 + 8.93A - 8.69B + 0.23C + 8.69AB + 6.37AC - 1.89BC - 40.23A^2 - 40.23B^2 \quad (2)$$

$$\text{Temperature ln(CFA)} = +2.18 - 0.86A + 0.11B + 0.13C - 0.072AB - 0.093AC - 0.015BC + 0.39A^2 + 0.39B^2 \quad (3)$$

Table 4
CGA and CFA content in *Cecropia glaziovii* extraction solutions using two response surface designs.

STD	EtOH (%)	*t* (days)	Stability conditions[a]	CGA (μg/g) ± SD		CFA (μg/g) ± SD	
T1	20	4	4	112.24	1.22	21.38	0.06
T2	20	4	4	116.12	0.03	18.95	0.20
T3	80	4	4	152.89	0.82	5.60	0.39
T4	80	4	4	139.65	0.40	4.94	0.07
T5	20	8	4	84.81	0.87	31.32	0.04
T6	20	8	4	90.32	0.02	30.95	0.17
T7	80	8	4	145.86	0.20	5.45	0.12
T8	80	8	4	145.56	0.18	5.59	0.43
T9	20	4	25	97.01	0.96	32.65	0.62
T10	20	4	25	119.15	0.89	34.64	1.34
T11	80	4	25	173.59	1.12	6.17	0.12
T12	80	4	25	140.35	0.69	4.90	0.07
T13	20	8	25	62.76	0.03	46.21	0.47
T14	20	8	25	67.62	0.52	42.79	0.18
T15	80	8	25	155.19	0.48	5.90	0.20
T16	80	8	25	159.86	0.36	6.12	0.02
T17	50	6	25	166.13	7.08	9.98	1.70
T18	50	6	4	156.12	7.08	7.58	1.70
T19	50	6	4	163.39	1.11	8.12	1.32
T20	50	6	25	166.51	2.76	10.03	1.42
T21	50	6	25	161.83	1.11	9.99	1.32
T22	50	6	4	162.61	2.76	8.03	1.42
T23	50	6	25	165.87	3.53	10.31	1.88
T24	50	6	4	160.88	3.53	7.64	1.88
MP1	20	4	Absence	119.15	0.89	34.64	0.25
MP2	20	4	Absence	97.01	0.96	32.65	0.11
MP3	80	4	Absence	173.59	1.12	6.17	0.13
MP4	80	4	Absence	139.86	0.69	4.9	0.07
MP5	20	8	Absence	62.76	0.03	46.21	0.47
MP6	20	8	Absence	67.62	0.52	42.79	0.18
MP7	80	8	Absence	155.19	0.50	5.9	0.20
MP8	80	8	Absence	159.86	0.36	6.12	0.02
MP9	20	4	Presence	100.18	1.70	25.82	0.10
MP10	20	4	Presence	101.45	0.07	24.81	0.09
MP11	80	4	Presence	140.15	0.03	4.92	0.04
MP12	80	4	Presence	148.64	0.31	5.53	0.03
MP13	20	8	Presence	72.04	0.15	34.53	0.03
MP14	20	8	Presence	74.45	0.21	34.49	0.23
MP15	80	8	Presence	155.14	0.24	6.02	0.09
MP16	80	8	Presence	152.43	0.29	5.93	0.03
MP17	50	6	Absence	166.13	0.19	9.98	0.02
MP18	50	6	Absence	166.51	3.31	10.03	0.03
MP19	50	6	Presence	166.45	0.58	10.01	0.02
MP20	50	6	Presence	165.63	0.58	10.05	0.18
MP21	50	6	Presence	167.03	0.08	10.67	0.03
MP22	50	6	Absence	161.83	3.31	9.99	0.03
MP23	50	6	Presence	166.92	0.08	10.63	0.03
MP24	50	6	Absence	165.87	0.19	10.31	0.18

[a] T1–T24: temperature (°C); MP1–MP24: preservative.

Table 5
Analysis of variance: temperature and preservative.

Source	*p*-Value			
	Temperature (experiments T1–T24)		Preservative (experiments MP1–MP24)	
	CGA	CFA	CGA	CFA
Model	<0.0001	<0.0001	<0.0001	<0.0001
A (EtOH)	<0.0001	<0.0001	<0.0001	<0.0001
B (Time)	0.0007	<0.0001	0.0012	0.0006
C (temperature or preservative)	0.8954	<0.0001	0.5180	0.0949
AB	0.0007	0.0003	<0.0001	0.9031
AC	0.0072	<0.0001	0.2886	0.1580
BC	0.3737	0.3623	0.1391	0.5076
Lack-of-fit	0.4420	0.2991	0.6941	0.1866
R^2	0.9582	0.9954	0.9710	0.9914
Adj R^2	0.9399	0.9935	0.9582	0.9876
Pred R^2	0.8815	0.9871	0.9165	0.9771
Adeq precision	19.826	61.011	22.932	41.734

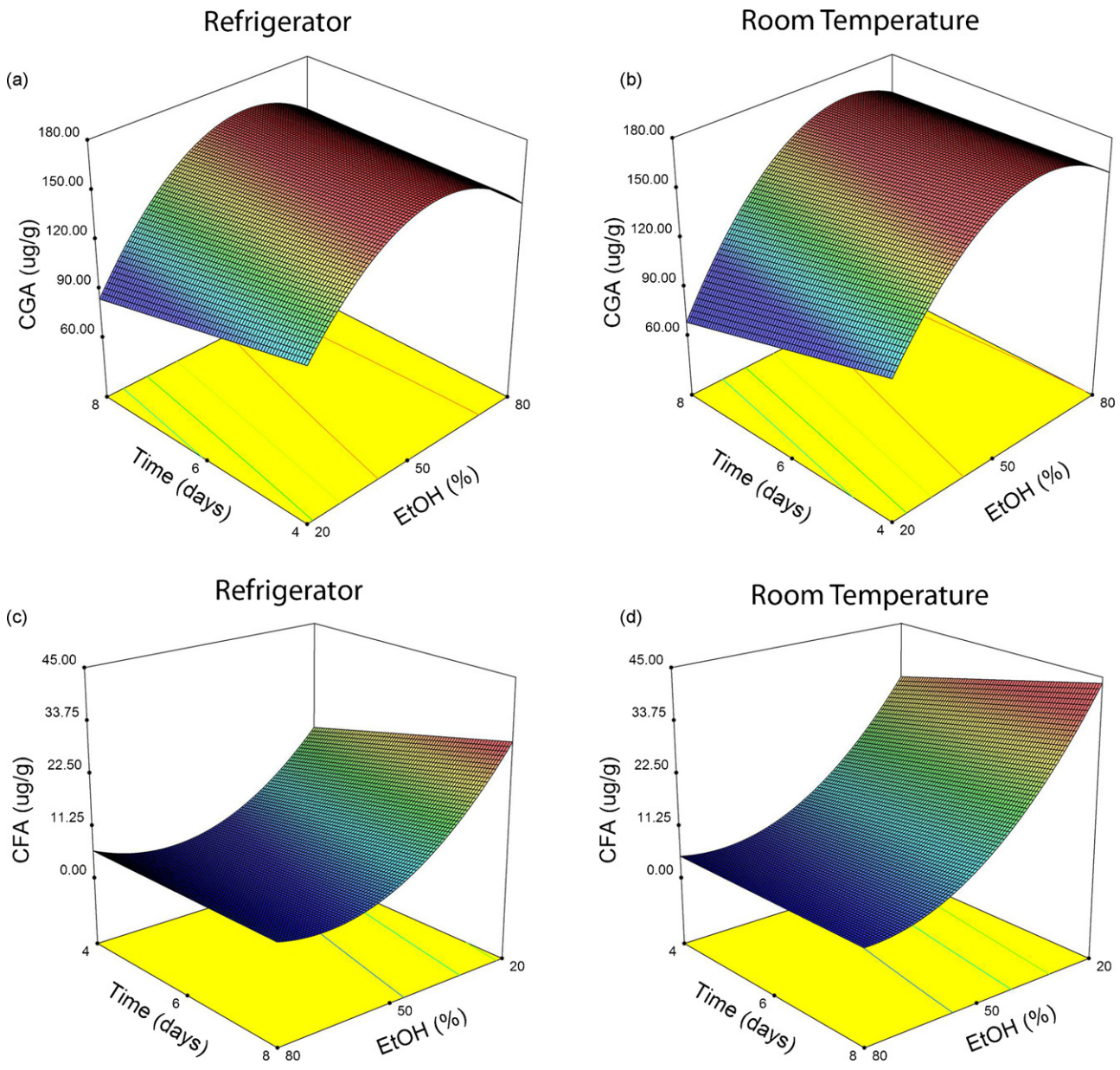

Fig. 3. Influence of extraction temperature (experiments T1–T24). (a) Refrigerator influence on CGA extraction. (b) Room temperature influence on CGA extraction. (c) Refrigerator influence on CFA extraction. (d) Room temperature influence on CFA extraction.

$$\text{Preservative CGA} = +165.80 + 33.14A - 7.53B - 1.04C + 10.08AB - 2.11AC + 2.99BC - 45.83A^2 - 45.83B^2 \quad (4)$$

$$\text{Preservative } 1/\text{Sqrt(CFA)} = +0.31 + 0.12A - 0.012B + 0.0042C + 0.0004AB - 0.0043AC - 0.0020BC - 0.016A^2 - 0.016B^2 \quad (5)$$

These equations allow us to establish excellent conditions of extraction for each one of the acids, or both. The mathematically obtained conditions of extraction were applied and showed agreement with the theoretical data.

The optimum conditions of extraction in terms of obtaining an ES of *C. glaziovii* with a higher concentration of CGA or of CFA were selected based on the constraints of the extraction parameters established to perform the response surface design. The optimization was established from the models related to the temperature (experiments T1–T24). The numerical optimization finds a point that maximizes the desirability function. The characteristics of a goal can be altered by adjusting the weight and importance. For several responses and factors all goals are combined into one desirability function. Desirability is an objective function that ranges from zero outside of the limits to one at the goal. The value is completely dependent on how close the set lower and upper limits are in relation to the actual optimum (Fig. 5).

At this point the desired result must be clearly defined. Are we interested in obtaining an ES with a higher concentration of CGA or of CFA? Alternatively, should both be maximized?

The optimization of the extraction conditions for the contents of CGA and CFA were determined considering the yield of both phenolic compounds. However, it is important to highlight that it would be impossible to obtain a maximum concentration of both pheno-

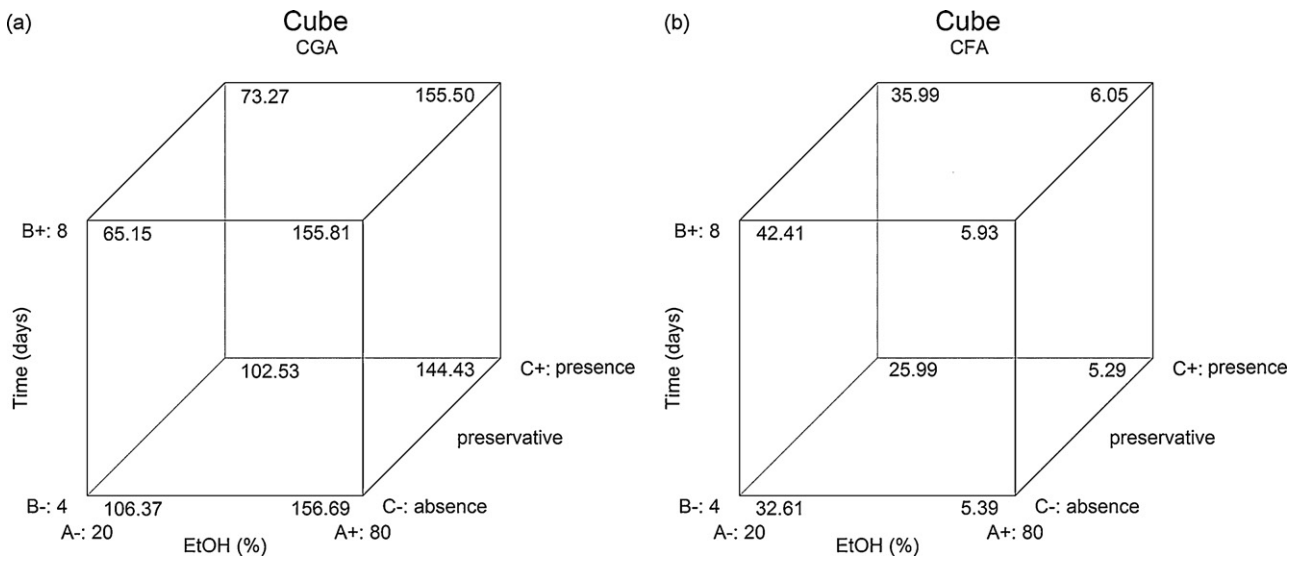

Fig. 4. Influence of presence of preservative (experiments MP1–MP24). (a) CGA extraction. (b) CFA extraction.

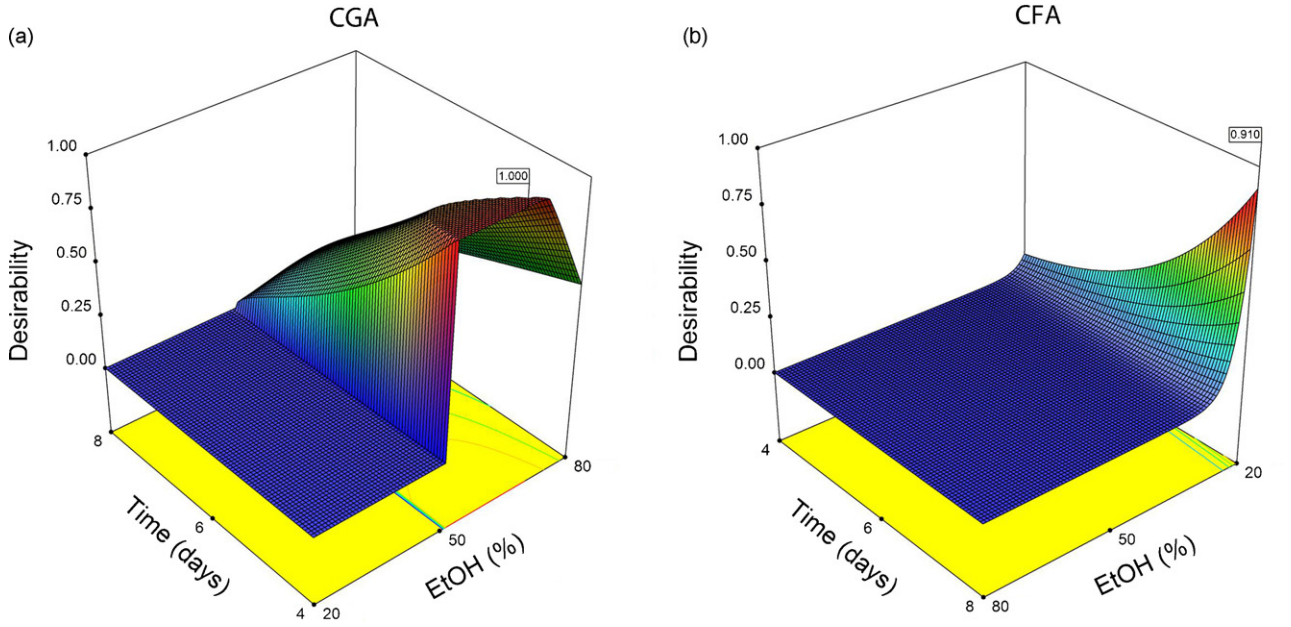

Fig. 5. Optimization of extraction conditions from temperature design (experiments T1–T24). (a) CGA maximum. (b) CFA maximum.

lics in the same extract, even if we give more importance to one than the other. If both are maximized with the same importance, the desirability is 0.523, but if CFA becomes the more important compound, even if both are maximized, we have an improvement in the desirability of 0.661.

In the design only space limits were used. In this way the extract that permits maximization of only one phenolic acid has greater desirability. To obtain the maximum for CGA, the conditions selected by the design space were 63% ethanol for 4 days, at room temperature, with a desirability of 1.00. In contrast, to obtain a maximum concentration of CFA, the conditions of 20% ethanol (or less) for 8 days, at room temperature are selected, with a desirability of 0.910 (Table 6). However, extrapolating outside of the design space is not recommended due to the increased prediction error. The presence of a preservative does not modify the CGA or CGA concentration when compared to the optimized ES without preservative. Table 6 shows predicted and experimental results for CGA and CFA for both sets of conditions described above. We observed an excellent reproducibility of the results ranging between 1.00 and 2.53%, suggesting good predictability of the established model.

Table 6
Extraction process optimization and statistical model validation.

Phenolic acids (μg/mL)	Predicted properties	Experimental properties	Deviation (%)
CGA (μg/g) maximum	177.87	173.37	−2.53
CFA (μg/g) maximum	45.59	45.64	+1.0

4. Conclusions

The extraction and HPLC–DAD methods described herein were applied to the determination of two phenolic acids extracted from *C. glaziovii*. An important result was the detection of CFA, considering that this compound has not yet been reported for this species. The validated method is simple, selective, accurate, precise, specific and has the ability to separate and quantify CGA and CFA from *C. glaziovii* hydroethanolic extraction solutions. These results are of great interest since these phenolic acids show a broad spectrum of pharmacological activities and are widely distributed in nature. Therefore, the developed method can give rational support to evaluating the extraction procedures of this and other medicinal plants, and could also be used as a marker in studies for establishing dose–response relationships.

A statistical regression model allowed the determination of an optimized set of extraction conditions, in the design space studied, in order to obtain a high quantity of CGA or CFA. The statistical model was experimentally validated, with the variation observed between predicted and measured properties being below 3%.

In conclusion, it was verified that it is possible to obtain ES with high concentrations of CGA or CFA using specific extraction conditions. Thus, it is necessary to clarify the intended use of the extracts obtained and the desired activity. In this regard, other simultaneous biological and analytical assays are necessary in order to obtain better results. Improved knowledge of these aspects would increase the possibility for the industrial use of *C. glaziovii*, potentially enhancing the overall profitability of its medicinal use.

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