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Article

Article

A Rapid UPLC Method for the Simultaneous Quantitation of Caffeic Acid Derivatives in Dried Extracts of *Echinacea Purpurea*

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Abstract

Echinacea purpurea is a traditional medicinal plant widely used as adjuvant for the treatment of respiratory and urinary infections. Caffeic acid derivatives are considered the main active markers, such as chicoric acid, caftaric acid and chlorogenic acid. An analytical method using ultra performance liquid chromatography (UPLC) and diode array detector was developed and validated, to quantify caffeic acid derivatives in commercial dried extracts of EP. UPLC method was developed using a C_{18} column (50 × 2.1 mm, 1.8 µm), at 30°C. Mobile phase was composed of acetonitrile and 0.05% (v/v) formic acid aqueous solution (10:90), flow rate 0.2 mL/min. Injection volume was 10 µL and detection was performed at 300 and 330 nm. The developed method complied with all required validation parameters, and showed to be linear, precise, accurate, selective and robust for all caffeic acid derivatives. Using the validated method, the levels of caftaric acid (0.110–0.507%w/w), chicoric acid (0.040–0.179%w/w) and chlorogenic acid (0.013–0.084%w/w) were determined in five commercial dried extracts of *E. purpurea*, with significant variation in the contents between different samples, indicating the need of standardization and control of individual caffeic acid derivatives in commercial extracts.

Introduction

Echinacea purpurea (L.) Moench (EP), commonly named purple coneflower, belongs to Asteraceae family and is widely used for medicinal purposes, especially in the treatment of some infections. The aerial parts and roots are used as adjuvant in antimicrobial treatments and to improve the cellular immune response (1, 2). The phytochemical constituents of EP include alcamides, caffeic acid derivatives, polysaccharides, glycoproteins, anthocyanins and flavonoids, whilst no echinacosides are present, unlike in other species of *Echinacea* genus (2, 3). Caftaric acid, chicoric acid and chlorogenic acid (Figure 1) are considered the main EP vegetal markers and are associated to the therapeutic properties of the plant (3). However, qualitative and quantitative composition of these markers can vary according to the part of EP employed, extraction methods, storage conditions and even adulteration of extracts (4, 5).

In addition, despite the well-established activities for EP, it is not clear if the commercial products based on this plant, such as dried extracts, have exactly the same properties, due to the extraction and storage conditions. The wide commercialization of herbal products based on EP evidences the need of an efficient quality control and evaluation of these products, aiming to assure adequate treatment efficacy and safety.

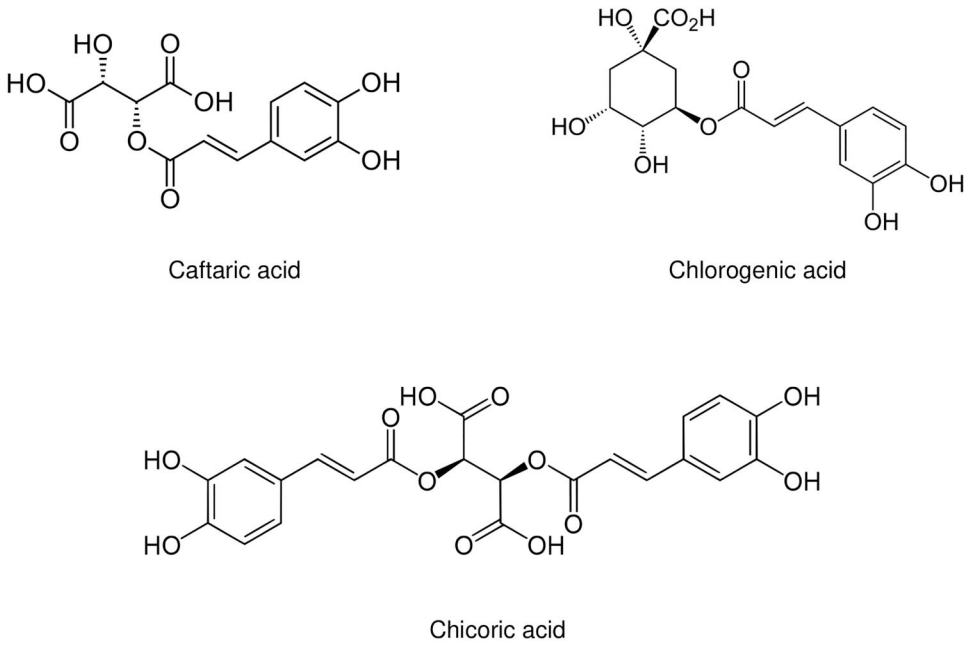

Figure 1. Chemical structures of caftaric, chlorogenic and chicoric acid, the main vegetal markers of *E. purpurea*.

Some studies have described the determination of caffeic acid derivatives in EP plant or products, usually by high performance liquid chromatography (HPLC) coupled to ultraviolet detection (6–8) and mass spectrometry (9–11), or using capillary electrophoreses (12). However, most methods are time-consuming and present long run times, gradient elution modes and complex sample preparation procedures, limiting the application for quality control routine analysis. Considering the advantages of ultra performance liquid chromatography (UPLC) such as improvement in efficiency, resolution, reduction of analysis time and solvent consumption (13, 14), the use of this technique has increased for the determination of vegetal markers in natural products. However, as far as we know, no UPLC analytical method have been described for the simultaneous quantitation of caffeic acid derivatives in herbal products based on EP.

Hence, the aim of this study was to develop and validate a novel UPLC method for simultaneous quantitation of caftaric acid, chicoric acid and chlorogenic acid in EP dried extracts. The validated method was employed for analysis of different commercial dried extracts.

Experimental

Chemicals and Reagents

Caftaric acid, chlorogenic acid and chicoric acid reference standards were purchased from Sigma-Aldrich®. Dried extracts samples of *E. purpurea*, usually employed as raw material for capsule production, were kindly donated by compounding pharmacies. Ultra-pure water was obtained from a Millipore® system. Acetonitrile, formic acid and methanol (HPLC grade) were obtained from Sigma-Aldrich®. All other reagents used in the study were of analytical grade.

Instrumental and analytical conditions

The UPLC analysis were carried out on Waters Acquity system, composed of binary pump, autosampler, diode array detector (DAD) and Empower® Pro 2 software (Milford, MA, USA). The column was Zorbax Eclipse Plus C_{18} (2.1 × 50 mm; 1.8 µm particle size) maintained at 30°C (Agilent, Santa Clara, CA, USA). For optimization of mobile phase composition, different proportions of organic solvents (methanol or acetonitrile) and acidic aqueous solvent (0.1% acetic acid, 0.05% formic acid and 0.05% trifluoroacetic acid) were tested, using isocratic and gradient elution modes. At each condition, chromatographic performance was evaluated, regarding retention time, resolution and peak symmetry. UV spectra from 200–400 nm were recorded for determination of maximum detection wavelength for each marker and for peak identification. The injection volume was 10 µL. Optimized mobile phase was composed of acetonitrile and 0.05% aqueous formic acid (10:90), at a flow rate of 0.2 mL/min and UV detection was performed at 300 and 330 nm.

Preparation of standard and sample solutions

For the preparation of standard stock solution, 3.0 mg of chlorogenic acid reference standard was weighed, transferred to a 25 mL volumetric flask and the volume was filled with water. An aliquot of 1 mL of this solution was transferred to a 10 mL volumetric flask, in which exactly weighed 1.6 mg of caftaric acid and 0.48 mg of chicoric acid reference standards were added. The volume was filled with water, so that final concentrations of 160 µg/mL caffaric acid, 48 µg/mL chicoric acid and 12 µg/mL chlorogenic acid were obtained.

Sample solutions were prepared by weighing 20 mg of EP dried extract sample and transferring to a 2 mL volumetric flask. The volume was filled with 0.05% (v/v) aqueous formic acid, resulting in a final concentration of 10 mg/mL.

All solutions were homogenized and filtered on a 0.22 µm PTFE membrane.

Validation of UPLC method

The developed method was fully validated according to the procedures described in ICH guideline Q2(R1) for the validation of analytical procedures (15).

Linearity

It was assessed by six or five-point calibration curves in triplicate. The curves were constructed by diluting the standard stock solutions and plotting the peak area versus the concentration of each marker. Evaluated concentration ranges were 8 to 68 µg/mL for caftaric acid, 2.4 to 17 µg/mL for chicoric acid and 1.5 to 5.1 µg/mL for chlorogenic acid. The obtained data were subjected to regression analysis using the ordinary least squares method and statistically analyzed to prove that they met the assumptions for a linear regression (16). Analysis of variance (ANOVA) was used to verify the significance of the regression and the deviation from linearity ($\alpha = 0.05$).

Precision

Intra-day precision was evaluated by means of six determinations of the same EP dried extract at 10 mg/mL in 0.05% (v/v) aqueous formic acid ($n = 6$). Similarly, the inter-day precision was evaluated in two consecutive days, with two different analysts ($n = 12$; 6 replicates x 2 days). The levels of each plant marker in sample solution were determined and the relative standard deviation (RSD) was calculated for the first day of analysis and for the consecutive days.

Accuracy

Recovery was investigated by means of a standard addition experiment. Different volumes of the standard stock solution were added to an EP dried extract sample solution, obtaining three levels for each marker: 20, 40 and 60 µg/mL for caftaric acid; 6, 12 and 17 µg/mL for chicoric acid and 1.5, 3.0 and 4.5 µg/mL for chlorogenic acid. At each level, samples were prepared in triplicate and the recovery percentage was determined.

Specificity

It was determined by the evaluation of the spectral purity of the chromatographic peaks of caftaric, chicoric and chlorogenic acids, obtained in chromatograms of standard and sample solutions, using UV spectra recorded by the DAD to evaluate possible interfering peaks.

Robustness

Standard solution and six independent sample solutions of EP dried extract were prepared at 10 mg/mL. These solutions were analyzed under the nominal conditions and the following analytical parameters were varied: proportion of acetonitrile and 0.05% formic acid (8:92 to 12:88), mobile phase flow rate (0.18 to 0.20 mL/min) and column temperature (27°C to 33°C). Concentrations of caftaric, chicoric and chlorogenic acids were determined for each condition and the obtained data were submitted to statistical analysis (ANOVA test). Statistical significance was set at $P < 0.05$.

Matrix effect

It was evaluated through the comparison of two calibration curves for chlorogenic acid, established in the same way as in linearity, with same concentration levels and number of replicates. The first curve was composed of standard solutions of chlorogenic acid, and the second was constructed by adding known amounts of chlorogenic acid to EP dried extract sample solution, to obtain the concentrations of linear range. After confirming the linearity of both curves, Student t test was employed to compare the slopes of the curves (statistical significance set at 5%). The parallelism of the curves indicates that the matrix effect was not significant.

Table I. Chromatographic parameters obtained for caffeic acid derivatives in EP sample solution using the developed UPLC method

Parameters	Results		
	Caftaric acid	Chlorogenic acid	Chicoric acid
Tailing factor	1.80	1.14	0.94
Resolution	3.65	2.47	2.30
Number of theoretical plates	6,261	5,776	4,363

Detection and quantitation limits

Limits of detection (LOD), and of quantitation (LOQ) were calculated based on the slope of the calibration curve, and on the standard deviation of the response, considering a relation of 3.3 for LOD and 10 for LOQ.

Quantification of caftaric, chicoric and chlorogenic acids in EP dried extracts

The levels of each plant marker in five samples of *E. purpurea* dried extract were determined using the validated method, in triplicate. Sample solutions were prepared at 10 mg/mL in 0.05% aqueous formic acid solution and filtered at 0.22 µm membranes. Concentrations of caftaric, chicoric and chlorogenic acids were determined in µg/mL and the content (w/w) was calculated in each EP dried extract.

Results and Discussion

Optimization of UPLC conditions

In spite of some methods previously described the separation of caffeic acid derivatives by HPLC, we developed a novel UPLC analytical method, aiming a better chromatographic efficiency, shorter analysis time and lower solvent consumption. Chromatographic parameters were initially evaluated for a standard solution at 40 µg/mL caftaric acid, 12 µg/mL chicoric acid and 3 µg/mL chlorogenic acid, as well as for a sample solution of EP dried extract 10 mg/mL. Acidification of aqueous phase showed to be important to improve peak symmetry. Acetic acid, formic acid and trifluoroacetic acid were tested in different concentrations. Comparing the obtained chromatograms, 0.05% aqueous formic acid provided better peak shape and resolution between caftaric acid, chicoric acid and chlorogenic acid. Acetonitrile instead of methanol, at 10% in isocratic mobile phase, showed to be a better choice to assure the adequate separation of peaks. Column temperature presented no impact in the chromatographic separation, so that 30°C was employed for all analysis. The chromatographic outcomes are presented in Table I for the three analytes.

To determine the optimal wavelength for analyte detection, UV spectra were obtained in the range of 200–400 nm, by means of DAD. Caftaric and chlorogenic acids presented adequate absorption at 300 nm, so that this wavelength was employed for their detection. However, chicoric acid peak presented higher intensity and symmetry at 330 nm, and it was detected at this wavelength. The use of DAD allowed the simultaneous quantitation of three analytes in two different wavelengths, with proper selectivity and sensitivity, essential for the quantitation of markers in herbal products. The chromatograms

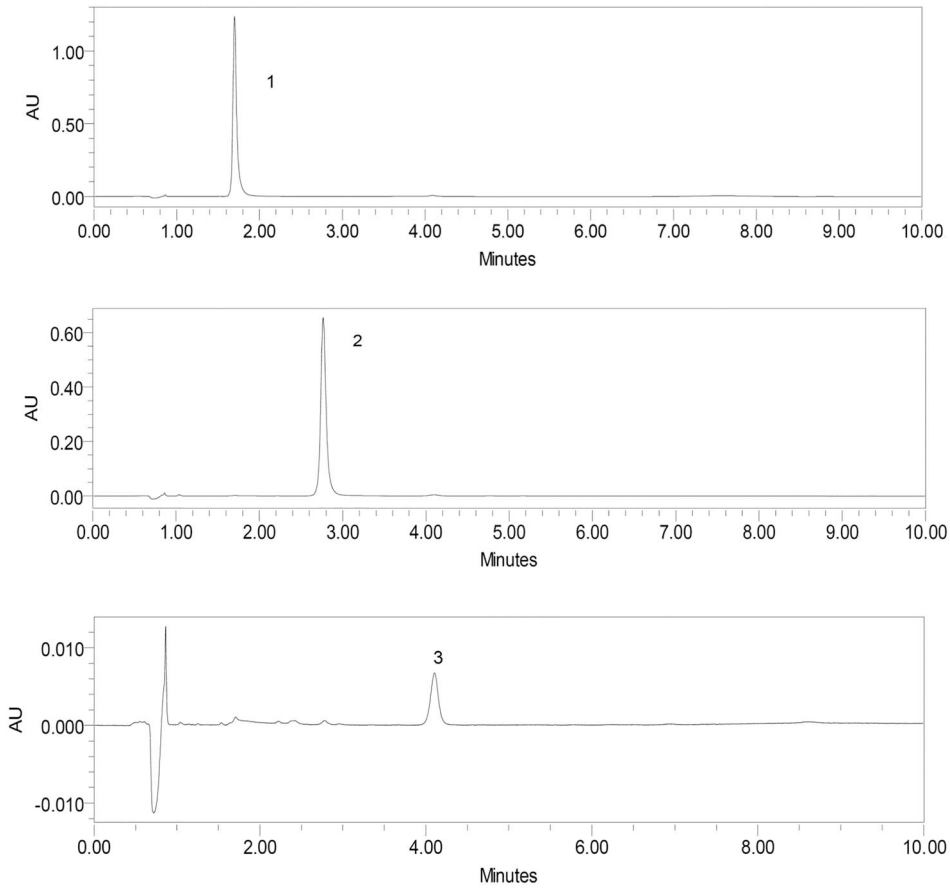

Figure 2. UPLC chromatogram obtained using the optimized conditions of the developed method for standards solutions of caftaric acid at 40 μg/mL (1), chlorogenic acid at 3 μg/mL (2) and chicoric acid at 12 μg/mL (3).

of standard solutions and sample solution are presented in Figures 2 and 3, respectively.

Compared with the previously described chromatographic methods for quantitation of caffeic acid derivatives in *E. purpurea* (6–11), the developed UPLC method presents a major advantage considering its short run time and consequent reduction in solvent consumption. The previous papers present total run time between 14 and 60 min, most of them employing gradient elution, while with the novel UPLC method, simultaneous analysis of caffeic acid derivatives was successfully performed in 6 min. These factors significantly reduce the time and cost of analysis. Also, the significant reduction in solvent use means that the method is a more environmentally friendly when compared to the previous reported HPLC methods.

Method validation

Linearity

A linear correlation was found between the peak areas and the concentrations of caftaric, chlorogenic and chicoric acids, in the assayed range. Outliers were excluded (Jackknife residual test), all residuals followed the normal distribution (Ryan-Joiner test), were homoscedastic (modified Levene's test) and independent (Durbin–Watson test) (16). Regression analysis data obtained for linearity evaluation are presented in Table II. The regression coefficient (r^2) values obtained were 0.9997, 0.9990 and 0.9964 for caftaric, chlorogenic and chicoric acid, respectively. No significant deviation of linearity was detected in the assayed range, confirmed by ANOVA ($\alpha = 0.05$), attesting the linearity of the method.

Precision and accuracy

RSD and recovery values obtained for caftaric, chlorogenic and chicoric acids by UPLC are showed in Table II. For intra-day and inter-day precision, RSD values ranged from 0.73 to 2.00% and from 1.61 to 3.70%, respectively. Recovery rates were from 100.65 to 104.74% for the three analytes. The results are in agreement with validation criteria established by Guidelines for Standard Method Performance Requirements from AOAC (17), proving the precision and accuracy of the developed method.

Specificity

Analysis of the spectral purity of the peaks allowed the evaluation of possible interfering peaks co-eluting with analytes. The obtained purity factors for the chromatographic peaks of each evaluated analyte were lower than the purity threshold limit, indicating adequate spectral purity of all caffeic derivative peaks. Therefore, other compounds were not co-eluted in the analyzed peaks for the chromatograms obtained with EP dried extracts. In addition, no carry-over effect was observed between injections.

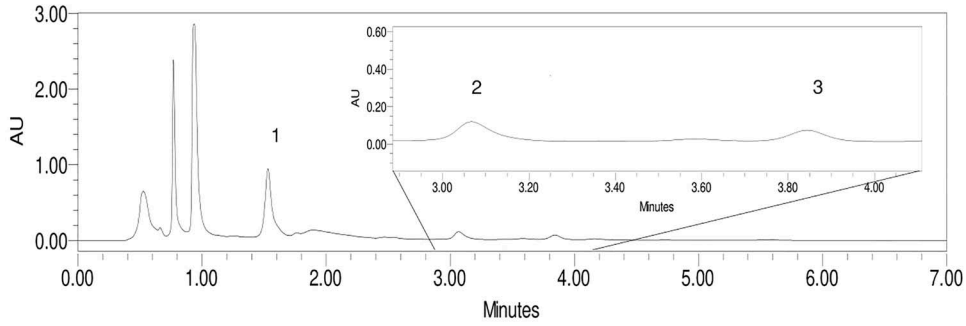

Figure 3. UPLC chromatogram obtained using the optimized conditions of the developed method for EP dried extract at 10 mg/mL. 1 = caftaric acid; 2 = chlorogenic acid; 3 = chicoric acid.

Table II. Validation data for caffeic acid derivatives obtained by UPLC method

Validation parameters	Caftaric acid	Chlorogenic acid	Chicoric acid
Linearity			
Concentration range (μg/mL)	8–68	1.5–5.1	2.4–17
Slope ± standard error	69589 ± 298	52587 ± 460	46812 ± 848
Intercept ± standard error	36293 ± 12866	−18902 ± 1628	16523 ± 9406
r^2	0.9997	0.9990	0.9964
Precision (R.S.D. %)			
Intra-day ($n = 6$)	0.73	2.00	1.31
Inter-day ($n = 12$)	1.61	3.70	2.42
Accuracy (recovery %) ($n = 9$)	101.83 ± 4.88	100.65 ± 8.12	104.74 ± 10.36
Limit of detection (μg/mL)	0.35	0.10	0.66
Limit of quantification (μg/mL)	1.06	0.31	2.01

Robustness

Statistical analysis showed no significant difference between results obtained employing the analytical conditions established for the method and those obtained in the experiments in which variations of some parameters were introduced (Table III). Thus, the method showed to be robust for changes in mobile phase flow rate from 0.18 to 0.20 mL/min, acetonitrile and 0.05% formic acid proportions from 8:92 to 12:88 and column temperature in the range of 27–33°C.

Matrix effect

Student *t* test indicated that the vegetal matrix did not affect the slope of the calibration curve of chlorogenic acid (t_{slope} 1.023 < $t_{critical}$ 2.160), demonstrating absence of matrix effect. Therefore, interfering matrix compounds had no significant influence on vegetal marker detection, assuring the reliability of the method throughout the concentration range.

Table III. Robustness results obtained for caffeic acid derivatives in EP sample solution using the developed UPLC method

Parameters	Obtained *F*-value			Critical *F*-value
	Caftaric acid	Chlorogenic acid	Chicoric acid	
Mobile phase flow rate	0.146	0.197	0.237	
Column temperature	0.346	0.113	0.546	3.467
Mobile phase composition	0.214	0.032	0.569	

Detection and quantitation limits

Limits of detection (LOD) and quantitation (LOQ) calculated based on the coefficients from the calibration curve are presented in Table II. Obtained LOQ were inferior to the lowest level of the calibration curve for all caffeic acid derivatives, demonstrating the adequate sensitivity of the developed method regarding the employed working range.

Quantification of caftaric, chicoric and chlorogenic acids in EP dried extracts

Samples of commercial EP dried extract, usually employed as pharmaceutical raw material, were analyzed by the validated method. All determined concentrations were within the validated linear ranges, for the three analytes. The marker contents in each sample are presented in Table IV. Caffeic acid derivative contents varied considerably between the different EP dried extracts, mainly in sample 3, which presented high contents of caftaric and chicoric acids. It is important to note that, according the quality reports of suppliers, sample 3 was the only obtained exclusively of *E. purpurea* roots. Other samples were obtained from aerial parts or aerial parts mixed with roots and stems of the plant. It seems that there is no consensus on which part of the plant should be used for extract preparation, so that European and United States Pharmacopeias presents monographs of both roots and aerial parts of *E. purpurea* (18, 19). The last also presents Powdered *E. purpurea* Extract monograph, which may be prepared from dried root, aerial parts or a mixture of them, by extraction with hydroalcoholic mixtures or other suitable solvents and should contain not <4.0% of total phenols. It is well established that contents of *E. purpurea* markers may significantly vary due to

Table IV. Content of caffeic acid derivatives in EP dried extracts by UPLC-DAD ($n = 3$)

Sample	Individual contents			Total content % (w/w)
	Caftaric Acid % (w/w)	Chlorogenic Acid % (w/w)	Chicoric Acid % (w/w)	
1	0.110	0.053	0.040	0.203
2	0.110	0.084	0.040	0.235
3	0.507	0.025	0.179	0.711
4	0.113	0.024	0.043	0.180
5	0.114	0.013	0.129	0.256

the influence of several factors, including part of the plant, extraction methods and solvents, as well as geographical location and storage conditions (3, 10).

In Brazil, National Health Surveillance Agency advocates that *E. purpurea* extracts obtained with aerial parts of the plant must provide a daily dose of 13 to 36 mg of the sum of caftaric and chicoric acids (20). Considering this specification and the results obtained with assayed EP dried extracts, patients should ingest 1.85 to 5.12 grams of sample 3, which is the most concentrated, to meet this recommended daily dose. This *E. purpurea* dried extract dose is considerably high, reflecting the low concentration of the markers verified in assayed commercial samples.

Since the biological activities are directly related to the qualitative and quantitative composition of the plant, this lack of standardization for commercial dried extracts may have an impact on the therapeutic efficacy expected for the *E. purpurea* based products.

Conclusion

In this work, we developed a novel UPLC method for simultaneous quantification of caffeic acid derivatives in *E. purpurea* extracts. The validated method proved to be fast and suitable for routine quantitative determinations, in quality control protocols. Variations in caftaric, chlorogenic and chicoric acid contents were verified between the assayed commercial samples, indicating the need of standardization and control of individual caffeic acid derivatives in *E purpurea* extracts used as pharmaceutical raw material.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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