Journal of Chromatography A, 1219 (2012) 147–153

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Assay of caffeoylquinic acids in *Baccharis trimera* by reversed-phase liquid chromatography

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ARTICLE INFO

*Article history:*
Received 15 July 2011
Received in revised form 4 November 2011
Accepted 13 November 2011
Available online 28 November 2011

*Keywords:*
*Baccharis trimera*
Caffeoylquinic acids
Dicaffeoylquinic acids
Tricaffeoylquinic acids
RP-LC
Photostability

ABSTRACT

*Baccharis trimera* commonly named 'carqueja', is wide-spread in South America and are used as raw material for herbal medicines. A reversed-phase liquid chromatography (RP-LC) method coupled to diode array detector was developed for the analysis of caffeoylquinic acids (CQAs), the main compounds responsible for its digestive activity. The identity of the quinic acids was established by mass spectrometry and were them: 5-*O*-[*E*]-caffeoylquinic acid, 3,4-*O*-[*E*]-dicaffeoylquinic acid, 3,5-*O*-[*E*]-dicaffeoylquinic acid, 4,5-*O*-[*E*]-dicaffeoylquinic acid and a tricaffeoylquinic acid. The RP-LC method for the quantitation of the caffeoylquinic acids was validated according to ICH guidelines, based on the following parameters: linearity, selectivity, robustness, limits of detection and quantification, precision and recovery. Hydroalcoholic extracts were prepared by the maceration of the plant material with ethanol:water 1:1 (v/v) in a 0.1:25 g $mL^{-1}$ plant:solvent ratio in a water bath at 40 °C. Validation data indicated that the HPLC method proposed is suitable for the analysis of caffeoylquinic acids in *B. trimera* raw material. The results of the LOD and LOQ analyses for the 5-CQA were 4.1 μg $mL^{-1}$ and 12.5 μg $mL^{-1}$, respectively, 1.3 μg $mL^{-1}$, 3.9 μg $mL^{-1}$ for 4,5-diCQA and 1.7 μg $mL^{-1}$, 5.1 μg $mL^{-1}$ for triCQA. The levels of total CQAs ranged from 2.1 to 4.0 g% (w/w). The influence of season harvest and site collection was also evaluated and variations were observed in the results and can be related to phonologic phase, different locations, seasons and soil. Long term and photostability of plant material were carried out and was observed a stable behavior during the time of the experiments.



## 1. Introduction

In recent years despite the growth in global trade of herbal products, these are often unable to enter in international markets, due to inconsistency in quality and effectiveness. It is therefore essential the development of analytical methods that enable the standardization of extracts, as well as the stability study of herbal medicines [1]. In this context and considering the great biodiversity in South America, the countries from this region are important centers for marketing of medicinal plants, such as *Baccharis trimera*.

*B. trimera* (Less.) DC. (Asteraceae) is a shrub widespread in South American countries (Brazil, Argentina, Uruguay and Paraguay) that belongs to the section Caulopterae DC. [2]. This complex genus is characterized by the presence of longitudinal wings. The plant is used in folk medicine for multiple indications, mainly digestive disorders and as a diuretic [3,4]. The drug is widely used as a raw material for herbal medicines and as a food supplement. The main reported constituents for this species are flavonoids, caffeoylquinic acids and terpenoids [5–9]. Caffeoylquinic acids are considered to be the main compounds responsible for the digestive and hepatoprotective activities in some medicinal plants [10,11].

Many factors contribute with the quality of the herbal drugs, such as seasonal variation, collection period, site of collection, post-harvesting processing, procedures of extraction and storage location. Also, herbal pharmaceutical preparations are frequently dispensed as strikethrough raw material and taking in account the lability of the constituents, it is of great concern to monitor the behavior of the chemical constituents against thermal and photostability as well as the phytochemical variation related with the site of collection and seasonal variation.

Considering the wide use of *B. trimera*, appropriate quality control methods need to be developed in order to comply with regulatory requirements. Keeping these points in view, the aim of the current study was the development of a simple, effective and reliable liquid chromatography (LC) method for the quantification

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doi:10.1016/j.chroma.2011.11.042

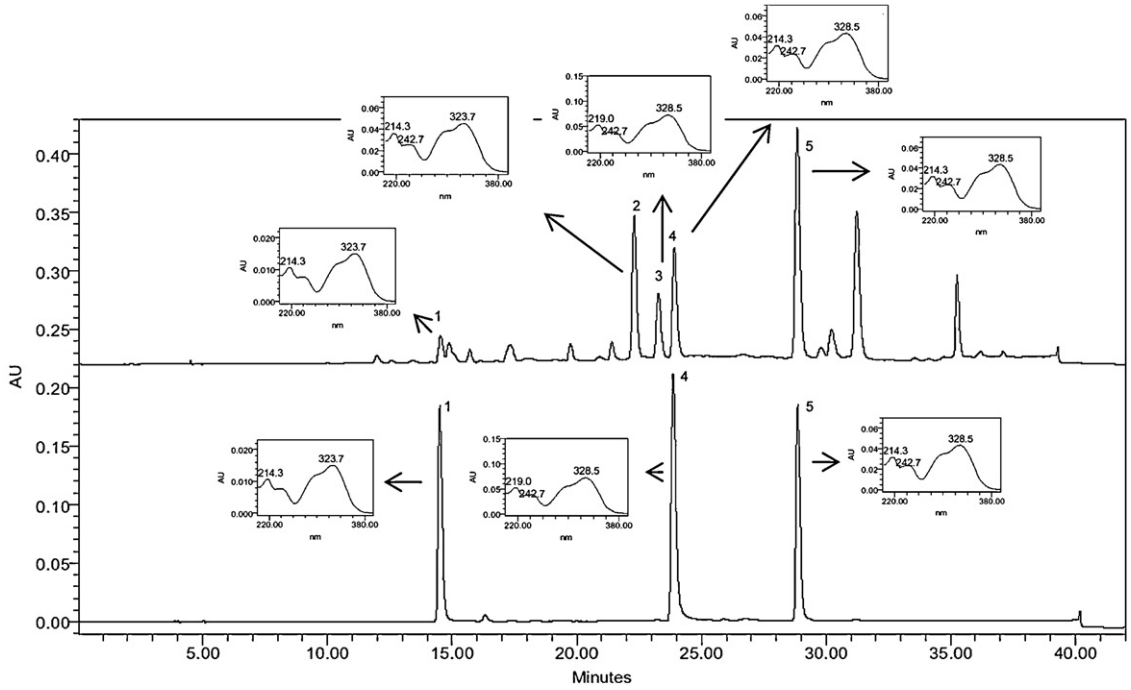


**Fig. 1.** Typical HPLC chromatogram for *B. trimera* ethanol extract (325 nm). The UV spectra corresponding to the main CQAs are shown above the chromatogram. Peaks: (1) 5-*O*-[*E*]-caffeoylquinic acid; (2) 3,4-dicaffeoylquinic acid; (3) 3,5-dicaffeoylquinic acid; (4) 4,5-dicaffeoylquinic acid; (5) triCQA.

of the CQAs in extracts of *B. trimera*. Optimization of the extraction conditions required to obtain the highest yield of bioactive compounds in the extracts was performed. In addition, the photostability and the influence of the phenological phase of the plant were also evaluated.

## 2. Experimental

### 2.1. Plant material

To evaluate seasonal variation, samples of the aerial parts of *B. trimera* were collected in São Jerônimo (RS-Brazil) in different phenological phases, covering the blooming and vegetative phases. A voucher specimen (ICN 128440) was deposited in the Herbarium of the UFRGS. In addition, samples of different origins were analyzed to compare their CQA contents. Samples were collected in Argentina were identified by Dr. Etile Spegazzini.

The plant material was dried at room temperature for 10 days and reduced to a fine powder (0.250 mm), using a knife mill (model A11 Basic S10, IKA® WERKE), immediately before the preparation of the extracts.

### 2.2. LC equipment and conditions

LC analysis were performed on a Waters Alliance 2695 chromatograph using a UV detector (UV/VIS Waters 2487) and a C18 reversed-phase column (Luna® Phenomenex; 5.0 μm, 250 mm × 4.6 mm) with a security guard column packed with Lichrosorb $RP_{18}$ (10 × 4 mm; Merck), operated at 25 ± 2 °C. A photodiode array detector (DAD; UV/UV Waters 996) was used to check the purity and verify the specificity of the evaluated compounds. UV spectra were recorded in a range 210–400 nm. Equipment control, data acquisition and integration were performed with Waters Empower software.

The mobile phase consisted of a gradient elution of acetonitrile and water acidified with trifluoracetic acid (5:95:0.08, v/v/v) (solvent A, pH 2.1) and acetonitrile (solvent B). The gradient profile was: 0–30 min from 0 to 57% of B, 30–35 min 57–100% of B, 35–38 min 100% of B, 38–42 min from 100% to 0% of B at 0.8 mL $min^{-1}$. The injected volume was 10 μL and detection wavelength was 325 nm. All solutions were filtered through a 0.45 μm membrane (Millipore) prior to use.

For long term storage and photostability assays, LC analysis were performed in the same Waters HPLC apparatus, using a C18 reversed-phase column (Luna® Phenomenex; 3.5 μm, 75 mm × 4.6 mm) with the same gradient profile, but with a flow of 0.6 mL $min^{-1}$.

*LC–MS analysis*. Analyses were carried out using a Agilent (Karlsruhe, Germany) 1200 series HPLC equipped with a G1312B SL binary pump, a G1367D high-performance autosampler (HiP ALS SL+), a G1316B SL thermostated column compartment, and a Phenomenex (Torrance, CA, USA) Luna C18(2) column (250 × 4.6 mm i.d., 5 μm particle). The mobile phase consisted of 0.3% (v/v) formic acid in water (eluent A) and acetonitrile (eluent B). The gradient profile was: 5–45% B (0–30 min), 45–90% B (30–35 min), and 90% B (35–40 min). Flow rate of 0.8 mL $min^{-1}$, injection volume of 10 μL, and oven at 30 °C.

The mass detector was an Agilent model G6460A Triple Quadrupole fitted with an ESI source. Equipment control, data acquisition and processing were performed using MassHunter Workstation Software. Negative ion mass spectra of the column eluate were recorded in the range *m*/*z* 50–1000. The instrument was operated with the capillary voltage at 3500 V, and charging voltage at 500 V. Nitrogen was used as nebulizer gas of 45 psi, a carrier gas of 6 L $min^{-1}$ at 350 °C, and a sheath gas of 11 L $min^{-1}$ at 350 °C.

*Semi-preparative HPLC conditions for triCQA isolation*: For the semi-preparative HPLC, it was used a Waters Alliance 2695system equipped with a PrepPak 25 × 100 mm C18 6 μm column and radial compression at 700–800 lb $in^{-2}$, eluted at 5 ml $min^{-1}$ from 0 to 40 min with a linear gradient solvent system from 40:60:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid to 80:20:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid (system A), or from 0 to 40 min with a linear gradient solvent system

**Table 1**
UV spectra and negative ion $MS^2$ fragmentation data of caffeoylquinic acids in *Baccharis trimera*.

| Peak[a] | $t_R$ (min)[a] | UV spectra ($\lambda_{máx}$)[a] | Negative ion mode ($m/z$)[b] | | Compound |
|---|---|---|---|---|---|
| | | | MS $[M-H]^-$ | MS–MS (RA%) | |
| 1 | 14.976 | 214, 243, 323 | 353.0 | 191 (100), 179 (2.8), 85.1 (1.2), 84.7 (1.25) | 5-*O*-[*E*]-caffeoylquinic acid (5-CQA) |
| 2 | 22.242 | 214, 243, 323 | 515.0 | 515.1 (1), 352.8 (15.4), 334.8 (6.5), 191 (46.9), 179 (88.5), 173 (100), 161 (6) | 3,4-*O*-[*E*]-dicaffeoylquinic acid (3,4-diCQA) |
| 3 | 23.554 | 219, 243, 328 | 515.0 | 352.8 (12.7), 191 (100), 178.9 (48.9), 172.9 (4.7) | 3,5-*O*-[*E*]-dicaffeoylquinic acid (3,5-diCQA) |
| 4 | 24.260 | 216, 243, 326 | 515.0 | 352.9 (26.9), 191 (25.1), 179 (85), 173 (100) | 4,5-*O*-[*E*]-dicaffeoylquinic acid (4,5-diCQA) |
| 5 | 29.290 | 214, 243, 328 | 677.0 | 677 (3.3), 515 (100), 353 (57), 334.7 (2.9), 179 (7.44), 173.2 (5) | Tricaffeoylquinic acid (triCQA) |

Compounds conclusively identified by comparison with authentic standard. Compounds tentatively identified by UV and mass spectral data.
[a] Samples analyzed by HPLC-DAD.
[b] Samples analyzed by HPLC–ESI-MS in negative ion mode, at collision energy of 25 V.

from 45:55:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid to 50:50:0.1 (vol/vol/vol) methanol:water:trifluoroacetic acid (system B). Eluting compounds were monitored with a Waters system controller and UV detector (UV/VIS Waters 2487), and every peak was separated.

### 2.3. Chemicals and reagents

Acetonitrile (HPLC grade; Merck), trifluoracetic acid (analytical grade; Nuclear), and ultra pure water from a Milli-Q system (Millipore) were used for the mobile phase preparation. 5-*O*-[*E*]-caffeoylquinic acid (5-CQA), purchased from Sigma–Aldrich (≥95% purity; St Louis, MO, USA) and 4,5-*O*-[*E*]-dicaffeoylquinic acid (4,5-diCQA, ≥90%) previously isolated and unambiguously identified by HNMR-500 MHz [9,12,13], were used as the authentic chemical references.

*4,5-O-[E]-dicaffeoylquinic acid*. 1H NMR (CD3OD, 500 MHz) d 2.04–2.28 (2H, m, H-2), 5.52 (1H, m, H-3), 3.90 (1H, m, H-4), 5.37 (1H, m, H-5), 2.07–2.25 (1H, m, H-6), 7.04 (1H, broad s, H-2′), 6.76 (1H, d, $J$ = 8.3 Hz, H-5′), 6.95 (1H, d, $J$ = 8.3 Hz, H-6′), 7.56 (1H, d, $J$ = 15 Hz, H-7′), 6.25 (1H, d, $J$ = 15 Hz, H-8′), 7.04 (1H, broad s, H-2″), 6.76 (1H, d, $J$ = 8.3 Hz, H-5″), 6.96 (1H, d, $J$ = 8.3 Hz, H-6″), 7.61 (1H, d, $J$ = 15 Hz, H-7″), 6.32 (1H, d, $J$ = 15 Hz, H-8″).

### 2.4. Standard solution preparation

A standard solution was prepared at a concentration of 1.0 mg mL$^{-1}$ in methanol and five dilutions in methanol were made (17–280 μg mL$^{-1}$ of 5-CQA, 15–250 μg mL$^{-1}$ of 4,5-diCQA and 15–250 μg mL$^{-1}$ triCQA). The solutions were stored at 4 °C in a flask protected from the light. All solutions were filtered through a 0.45 μm filters (PVDF; Millipore).

### 2.5. Sample preparation

Powdered dried aerial parts (0.1 g) were extracted with a hydroalcoholic solvent (BtE) (2 × 10 mL, ethanol:water, 1:1, v/v), in a water bath at 40 °C for 10 min, stirring every 3 min. The extracts were vacuum-filtered and transferred quantitatively to a 25 mL volumetric flask, which was then taken to volume with the extraction solvent. Before injection the extracts were filtered with a membrane filter (0.45 μm; PVDF; Millipore). Each sample was prepared and analyzed in triplicate.

*Loss on drying*: water content was performed with a Bell Mark infrared balance.

### 2.6. Validation of the analytical method

The HPLC method was validated for the quantitative analysis of 5-CQA, diCQA and triCQA in agreement with International Conference on Harmonization guidelines [16], using the following analytical parameters: specificity, linearity, precision (repeatability), limit of detection (LOD) and limit of quantitation (LOQ). Linearity was evaluated by the calculation of a regression line using the least squares method. Precision was assessed by analyzing the full range of the extractive solution six times in the same day (intra-day precision, repeatability) and by analyzing the same extract three times in three different days (inter-day or intermediate precision). Accuracy was tested by determining the recovery of 5-CQA, diCQA and triCQA at three different concentrations and by the calculation of the relative standard deviation (% R.S.D.) of the recovery. The selectivity was determined by checking peak purity of all the peaks, using a PDA detector. Quantification limits were determined based on the standard deviation of the response compared to the allowed (≤5%) inter-assay precision at the specified 100% concentration. Robustness was evaluated by applying the method on *B. trimera* plant material while introducing variations deliberately flow rate of the mobile phase, pH and column.

## 3. Results and discussion

A RP-LC method for the quantitation of caffeoylquinic acids in plant material has been developed, providing a simple

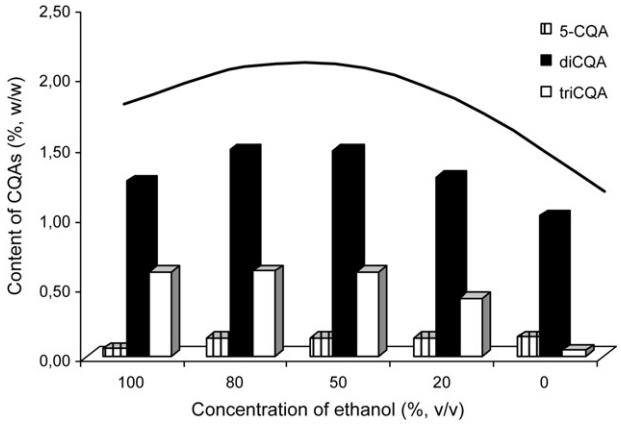


**Fig. 2.** Evaluation of total CQA content on extracts of *B. trimera* by HPLC. The highest levels of caffeoylquinic derivatives (%) in the extractive solutions were obtained from ethanol 50% and 80% at 40 °C. The line above the columns represents the variation of the total content of CQAs.

**Table 2**
Experimental values obtained for intra-day repeatability ($n = 9$) and inter-day precision ($n = 3$) in HPLC analysis for the five CQAs in the extractive solution of *B. trimera* (BtE).

| | Repeatability | | | Day 1 | | | Day 2 | | | Day 3 | | | Mean (inter-day) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Calculated concentration (mean ± S.D.) | R.S.D. (%) | Retention time | R.S.D. (%) | Calculated concentration (mean ± S.D.) | R.S.D. (%) | Retention time | R.S.D. (%) | Calculated concentration (mean ± S.D.) | R.S.D. (%) | Retention time | R.S.D. (%) | Calculated concentration (mean ± S.D.) | R.S.D. (%) | Retention time | R.S.D. (%) |
| 5-CQA | 0.050 ± 0.003 | 5.8 | 14.86 ± 0.168 | 1.13 | 0.051 ± 0.002 | 3.9 | 14.64 ± 0.141 | 0.97 | 0.051 ± 0.006 | 11.7 | 14.70 ± 0.053 | 0.36 | 0.049 ± 0.000 | 0.2 | 14.46 ± 0.009 | 0.06 | 0.050 ± 0.002 | 4.0 | 14.60 ± 0.121 | 0.83 |
| 3,4-diCQA | 0.484 ± 0.007 | 1.4 | 22.34 ± 0.137 | 0.61 | 0.500 ± 0.040 | 8.0 | 22.37 ± 0.093 | 0.42 | 0.449 ± 0.009 | 2.0 | 22.44 ± 0.00 | 0.00 | 0.416 ± 0.001 | 0.2 | 21.48 ± 0,016 | 0.08 | 0.455 ± 0.004 | 0.8 | 22.09 ± 0.537 | 2.43 |
| 3,5-diCQA | 0.401 ± 0.004 | 0.9 | 23.52 ± 0.044 | 0.19 | 0.411 ± 0.035 | 8.0 | 23.39 ± 0.148 | 0.63 | 0.372 ± 0.008 | 2.1 | 23.38 ± 0.153 | 0.65 | 0.360 ± 0.004 | 1.1 | 22.50 ± 0.013 | 0.04 | 0.381 ± 0.027 | 7.0 | 23.09 ± 0.513 | 2.22 |
| 4,5-diCQA | 0.445 ± 0.006 | 1.3 | 24.22 ± 0.053 | 0.22 | 0.446 ± 0.036 | 8.0 | 24.01 ± 0.124 | 0.52 | 0.424 ± 0.008 | 1.8 | 24.18 ± 0.117 | 0.48 | 0.394 ± 0.006 | 1.5 | 23.14 ± 0.009 | 0.04 | 0.435 ± 0.026 | 5.0 | 23.78 ± 0.555 | 2.33 |
| triCQA | 0.746 ± 0.026 | 3.4 | 29.16 ± 0.178 | 0.61 | 0.766 ± 0.011 | 1.4 | 28.94 ± 0.154 | 0.46 | 0.787 ± 0.005 | 0.6 | 29.16 ± 0.178 | 0.61 | 0.762 ± 0.017 | 2.2 | 29.16 ± 0.178 | 0.61 | 0.772 ± 0.013 | 1.7 | 19.09 ± 0.127 | 0.44 |
| Total CQAs | 2.12 ± 0.033 | 1.38 | | | 2.202 ± 0.011 | 0.50 | | | 2.083 ± 0.005 | 0.23 | | | 1.982 ± 0.044 | 2.2 | | | 2.089 ± 0.111 | 5.3 | | |

procedure, without significant sample preparation. Application of the proposed gradient elution profile results in the separation of analytes with good resolution, in a runtime of 42 min (including re-equilibration). A gradient system was chosen because of the similarity of the compounds analyzed. The choice of 325 nm as detection wavelength enabled a high sensibility for all CQAs. Fig. 1 shows a typical chromatogram of the hydroalcoholic extract obtained, as well as the corresponding on line UV spectra. Peak assignment was confirmed by their mass spectrometric behavior (Table 1). Peaks **1–5** showed characteristic UV absorptions of caffeoyl moiety and could be assigned as follows: Peak **1** was identified as 5-*O*-[*E*]-caffeoylquinic acid (5-CQA) and peak **4** as 4,5-*O*-[*E*]-dicaffeoylquinic acid (4,5-diCQA) by comparison with reference standards. Peaks **2** and **3** were assigned by RP-LC-DAD as the other two isomers of isochlorogenic acid 3,4-*O*-[*E*]-dicaffeoylquinic acid (3,4-diCQA) and 3,5-*O*-[*E*]-dicaffeoylquinic acid (3,5-diCQA), respectively. Peak **5** was identified as a tricaffeoylquinic acid. The elution sequence is in accordance with previous literature [13–15].

Quantification of CQA, diCQAs and triCQA in the analyzed samples were based on analytical curves of authentic standards of the five dilutions of 5-CQA, 4,5-diCQA and triCQA. The highest levels of caffeoylquinic derivatives (%) in the extractive solutions were obtained from ethanol 50% and 80% at 40 °C (Fig. 2). No significant differences were observed among the higher levels, so it was chosen to work with the concentration of 50%.

The levels of 5-CQA, diCQA and triCQA in the extracts obtained at extraction temperatures ranging from 40 to 100 °C was evaluated and the highest content (2.23%) was obtained at 40 °C. For higher temperatures, a decrease in the content was observed (1.20% at 100 °C).

In order to establish the best drug/solvent (ethanol 50%) ratio, six different proportions were tested. The increase of drug:solvent ratio from 0.1:25 to 1.0:25 (w/v) did not present differences in the content of CQAs. For the 1.5:25 (w/v) rate a slight decrease in the level was observed.

To ensure that the proposed analytical method is suitable for the determination of the caffeoylquinic acid content in *B. trimera* and is capable of providing useful and valid analytical data, the specificity linearity, precision, accuracy, recovery, limit of detection (LOD) and limit of quantitation (LOQ) and robustness were evaluated. The LC method was validated for the quantitative analysis of caffeoylquinic acids and is in agreement with International Conference on Harmonization guidelines [16].

The specificity of the developed LC method for CQAs was carried out in the presence of other compounds in the extract. Peak purity test was determined for the five CQAs present in the extract using the spectral analysis provided by the DAD detector. No interfering peaks were observed in the peaks of interest in the chromatogram of the crude extract of *B. trimera*. Peak purity was confirmed by comparing the spectra on the upslope and downslope of caffeoylquinic derivatives peaks in the sample. The chromatogram of the extractive solution presented high resolution of all peaks, confirmed by LC–MS spectra, indicating that the proposed method could be applied for the determination of caffeoylquinic derivatives in *B. trimera* preparations (Fig. 1).

The linearity between peak area and concentration was evaluated by the calculation of a regression line using the least squares method. Analytical curves were obtained by triplicate on three different days with five concentrations ranging from 17 to 280 $\mu g\,mL^{-1}$ of 5-CQA, 15 to 250 $\mu g\,mL^{-1}$ of 4,5-diCQA and 15 to 250 $\mu g\,mL^{-1}$ triCQA for the evaluation of both standards in the BtE. Standard deviation and ANOVA calculations were performed using Microsoft Office Excel 2003. Linearity was evaluated from these data by plotting the peak area versus concentrations of compounds. Regression lines were calculated using the least-squares regression equation, and linearity was expressed by the $r^2$-value.

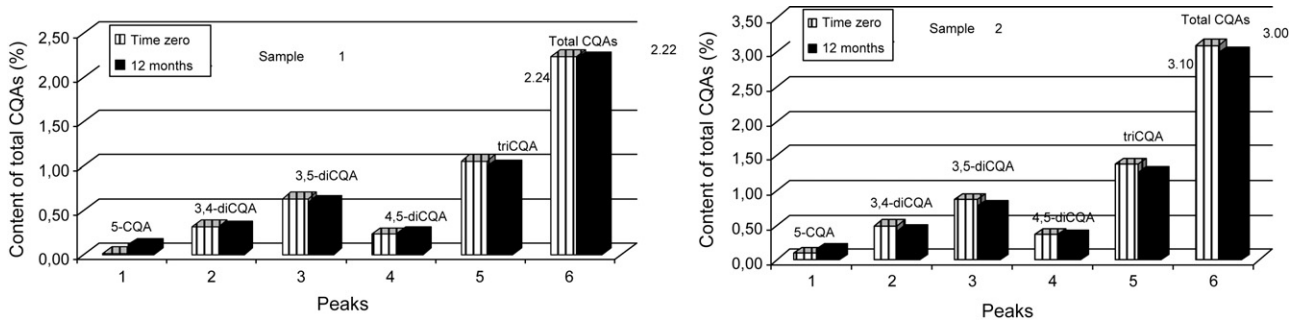


**Fig. 3.** Stability of two powdered *B. trimera* samples evaluated for long term storage. The samples remained stable for a year under these storage conditions (30 ± 3 °C).

The % relative standard deviation (R.S.D.) value for slope and *y*-intercept of the analytical curve was calculated and the minimally acceptable correlation coefficient ($r^2$) is 0.99 or greater [17,18]. A mean analytical curve was obtained and used to determine analyte concentrations in all further experiments. The correlation coefficients ($r$) of 5-CQA, 4,5-diCQA and triCQA were not less than 0.9998 ($Y = 59771x + 78333$), 0.9999 ($Y = 67246x + 71695$) and 1 ($Y = 67035x + 109114$), respectively.

The repeatability was determined by nine consecutive injections of the same sample solution at 100% test concentration. Intra- and interday variation and standard deviation of all results over three days are summarized in Table 2. Precision was assessed by analyzing the full range of the standard solutions 3 times in the same day (intra-day precision, repeatability) and by analyzing the same standard on 3 different days (inter-day or intermediate precision). The precision of the assay was determined by calculating the % R.S.D. of the results of six independent assays of the sample solution. Concentration of the total caffeoylquinic derivatives peaks from the experiments were individually calculated using the linear regression equation of the standard curve for chlorogenic acid. The corresponding results indicated that the analytical method for quantitation of the CQAs from crude extracts revealed adequate precision of the analytical method within the tested range.

Quantification limits were determined based on the standard deviation of the response compared to the allowed (≤5%) inter-assay precision at the specified 100% concentration. The limits of detection (LOD) and quantification (LOQ) were calculated applying equations [16] by triplicate for all tested samples. The results of the LOD and LOQ analyses for the 5-CQA were 4.1 μg mL$^{-1}$ and 12.5 μg mL$^{-1}$, respectively, 1.3 μg mL$^{-1}$, and 3.9 μg mL$^{-1}$ for 4,5-diCQA and 1.7 μg mL$^{-1}$, and 5.1 μg mL$^{-1}$ for triCQA, respectively, indicating that the analytical method for the quantification of these compounds in the hydroethanolic extracts exhibited good sensitivity.

The accuracy was assessed by determining the recovery of known amounts of 5-CQA, 4,5-diCQA and triCQA standards added to samples of BtE and the calculation of the R.S.D. of the recovery. Each recovery experiment was performed by triplicate at three different concentrations points (100%, 150%, and 200% of specified concentrations), so that the mean recovery and R.S.D. were calculated from 9 determinations (Table 3). A mean recovery ranging from 99.41% to 101.42% was found for 5-CQA, 97.87% to 105.38% for 4,5-diCQA and 100.54% to 102.30% for triCQA, fully complying with the accuracy requirement which is >95%.

Robustness was evaluated by applying the method to *B. trimera* plant material while introducing variations in sample amount. Three solutions of hydroalcoholic extracts were prepared under the experimental conditions, and variations were introduced in the following analytical parameters: chromatographic column (batch, porosity and length), flow, pH of the mobile phase and different brands of the mobile phase. The robustness of the method was evaluated considering the peaks resolution and the level of total CQAs in the extractive solution. Some of the intentional alterations in the method changed the retention times, without affecting the chromatographic resolution. The different conditions did not significantly affect the results, so the method was considerate to be robust.

### 3.1. Seasonal and regional variation of caffeoylquinic acid content

After validation of the LC method, the seasonal variation in caffeoylquinic acid content in six samples of aerial parts of *B. trimera* was evaluated using HPLC-DAD. Each sample was evaluated by triplicate and the level of total caffeoylquinic acid content was found to range between 2.19% and 4.03% (Table 4), showing significant differences in the content of caffeoylquinic derivatives. The highest amounts were detected during the vegetative period. This suggests that *B. trimera* should be collected in the same phenological phase to avoid variations in the caffeoylquinic acid content. Samples were also collected in different sites in South Brazil (Table 5), all of them in the sterile phase that correspond to the best period of caffeoylquinic acid content. The levels of these compounds ranged from 1.28 to 4.09%. The variation, besides the phonologic phase, can be related to the soil and whether conditions. Additionally, two commercial samples analyzed and presented similar results between them (1.42 and 1.55%) and not so different from to some others samples, demonstrating a homogeneous distribution of this group of compounds in *B. trimera*.

In order to evaluate the possible variation of caffeoylquinic acids according to its origin, samples collected in Argentina were also analyzed, and different levels of these compounds were found (0.40–2.06%). The variations observed in the results can be related to the phonologic phase and site of collection (Table 5).

### 3.2. Long term storage and photostability

The stability of the powdered *B. trimera* was evaluated for long term storage and photostability. Two samples of powdered stems (0.5 g) collected in March and October in South Brazil were stored in ambar bottle tightly closed, protected from the light and moisture, at room temperature (30 ± 3 °C, relative humidity of 52%, annual media in the storage room), simulating storage conditions. The samples for analysis were prepared according to the method previously outlined. The initial concentration ($T_0$) of total CQAs (%) for both samples was 2.24 ± 0.010% and 3.10 ± 0.04%, respectively, and 2.22 ± 0.065% and 3.00 ± 0.009%, respectively, after 12 months of storage (Fig. 3), indicating that the samples remained stable for a year under these storage conditions, considering the caffeoylquinic acid content. Changes in sensorial properties of the dried powder were observed during the test, when the samples change the color from green to brownish green. Additionally, the assay was conducted using paper bags, simulating commercial conditions. After

**Table 3**
Recovery values for caffeoylquinic acids added to extracts of *Baccharis trimera*.

| Substance | Added concentration (μg mL$^{-1}$) | Measured concentration (μg mL$^{-1}$) | % recovery | Mean % recovery | R.S.D. (%) |
|---|---|---|---|---|---|
| 5-CQA | 2.28 | 2.30 | 101.29 | | |
| | 3.00 | 2.98 | 99.41 | 100.70 ± 1.116 | 1.10 |
| | 4.00 | 4.05 | 101.42 | | |
| 4,5-diCQA | 2.17 | 2.29 | 105.58 | | |
| | 3.09 | 3.02 | 97.87 | 100.51 ± 3.450 | 3.40 |
| | 4.12 | 4.05 | 98.28 | | |
| triCQA | 2.20 | 2.21 | 100.57 | | |
| | 2.80 | 2.78 | 100.54 | 101.13 ± 1.00 | 0.99 |
| | 4.50 | 4.56 | 102.30 | | |

**Table 4**
Variation in caffeoylquinic derivatives content in *Baccharis trimera* (g%, w/w), collected in different months.

| Month collection | Phenologic phase | 5-CQA | 3,4-diCQA | 3,5-diCQA | 4,5-diCQA | triCQA | Total |
|---|---|---|---|---|---|---|---|
| October | Sterile | 0.379 ± 0.015 | 1.252 ± 0.063 | 1.263 ± 0.049 | 0.554 ± 0.028 | 0.586 ± 0.015 | 4.034 |
| November/2006 | Flower | 0.264 ± 0.013 | 0.773 ± 0.067 | 0.693 ± 0.058 | 0.370 ± 0.054 | 0.581 ± 0.089 | 2.681 |
| December/2006 | Flower | 0.088 ± 0.004 | 0.639 ± 0.012 | 0.494 ± 0.030 | 0.378 ± 0.003 | 1.249 ± 0.033 | 2.848 |
| March/2007 | Flower | 0.008 ± 0.004 | 0.314 ± 0.001 | 0.634 ± 0.009 | 0.229 ± 0.001 | 1.010 ± 0.002 | 2.195 |
| April/2007 | Sterile | 0.095 ± 0.006 | 0.516 ± 0.001 | 0.915 ± 0.009 | 0.382 ± 0.001 | 0.421 ± 0.002 | 2.331 |
| July/2007 | Sterile | 0.592 ± 0.015 | 1.018 ± 0.015 | 1.376 ± 0.162 | 0.424 ± 0.088 | 0.483 ± 0.011 | 3.896 |

Values expressed in g% (w/w). Data are the mean of three determinations ± S.D. 5-CQA: chlorogenic acid; 3,4-diCQA: 3,4-dicaffeoylquinic acid; 3,5-diCQA: 3,5-dicaffeoylquinic acid; 4,5-diCQA: 4,5-dicaffeoylquinic acid; triCQA: tricaffeoylquinic acid.

**Table 5**
Contents of caffeoylquinic acids in samples of *B. trimera* (g%, w/w) collected in Argentina and Brazil.

| | 5-CQA | 3,4-diCQA | 3,5-diCQA | 4,5-diCQA | triCQA | Total |
|---|---|---|---|---|---|---|
| Brazil | | | | | | |
| Sapiranga | 0.582 ± 0.007 | 0.274 ± 0.005 | 1.296 ± 0.002 | 1.051 ± 0.005 | 0.893 ± 0.002 | 4.096 |
| Santo Augusto | 0.235 ± 0.004 | 0.692 ± 0.001 | 0.522 ± 0.002 | 0.593 ± 0.008 | 0.792 ± 0.003 | 2.834 |
| Caxias do Sul | 0.234 ± 0.002 | 0.316 ± 0.002 | 0.708 ± 0.002 | 0.310 ± 0.006 | 0.236 ± 0.001 | 1.804 |
| Tramandaí | 0.139 ± 0.001 | 0.433 ± 0.003 | 0.457 ± 0.006 | 0.365 ± 0.001 | 0.138 ± 0.003 | 1.532 |
| Dom Pedro de Alcantara | 0.217 ± 0.004 | 0.628 ± 0.001 | 0.575 ± 0.007 | 0.453 ± 0.003 | 0.569 ± 0.008 | 2.442 |
| Caxias do Sul | 0.154 ± 0.001 | 0.207 ± 0.001 | 0.466 ± 0.001 | 0.306 ± 0.001 | 0.155 ± 0.003 | 1.288 |
| São Jerônimo | 0.260 ± 0.001 | 0.584 ± 0.001 | 1.446 ± 0.003 | 0.504 ± 0.001 | 0.442 ± 0.002 | 3.236 |
| Comercial 1 | 0.282 ± 0.001 | 0.257 ± 0.001 | 0.638 ± 0.004 | 0.229 ± 0.003 | 0.153 ± 0.001 | 1.559 |
| Comercial 2 | 0.512 ± 0.004 | 0.210 ± 0.006 | 0.383 ± 0.007 | 0.249 ± 0.001 | 0.072 ± 0.003 | 1.426 |
| Argentina | | | | | | |
| Cordoba | 0.022 ± 0.002 | 0.137 ± 0.003 | 0.199 ± 0.005 | 0.116 ± 0.001 | 0.186 ± 0.009 | 0.660 |
| Buenos Aires | 0.040 ± 0.003 | 0.097 ± 0.003 | 0.201 ± 0.004 | 0.070 ± 0.004 | 0.076 ± 0.0018 | 0.484 |
| Buenos Aires | 0.015 ± 0.001 | 0.270 ± 0.002 | 0.183 ± 0.002 | 0.139 ± 0.002 | 0.180 ± 0.001 | 0.787 |
| Buenos Aires | 0.129 ± 0.002 | 0.244 ± 0.002 | 0.397 ± 0.001 | 0.236 ± 0.002 | 0.400 ± 0.001 | 1.406 |
| La Plata | 0.364 ± 0.003 | 0.027 ± 0.003 | 0.373 ± 0.002 | 0.056 ± 0.002 | 0.012 ± 0.009 | 0.832 |
| La Plata | 0.011 ± 0.003 | 0.097 ± 0.002 | 0.079 ± 0.002 | 0.084 ± 0.002 | 0.129 ± 0.002 | 0.400 |
| La Plata | 0.180 ± 0.002 | 0.409 ± 0.003 | 0.945 ± 0.001 | 0.404 ± 0.001 | 0.130 ± 0.001 | 2.068 |
| La Plata | 0.162 ± 0.002 | 0.328 ± 0.002 | 0.755 ± 0.001 | 0.242 ± 0.001 | 0.447 ± 0.001 | 1.934 |

six months, CQA content decreased about 40%, indicating that this storage condition for sale is not appropriate.

The photostability of samples (1 g of the sample collected in December) of dry and ground stems was performed by triplicate in three different storage conditions: amber flask, transparent flasks or open-dishes. The samples were submitted to UV-C radiation (light express LE UV, 254 nm, 30 W) in light chamber, with an internal cover mirror, in order to detect the stability of each caffeoylquinic acid (Fig. 4). The loss on drying of the samples was measured before and after the assay in order to determine variation in water content according to the storage conditions. The results obtained were 15.70% to 13.40% in amber flasks; 15.70% to 13.90% in transparent flasks and 15.70% to 11.70% in open-dishes. For the three chromatograms it was observed a stable behavior during the time of the experiment. The LC analyses were carried out at 0, 12, 24 and 48 h. Fig. 5 shows the photostability of each CQA reflected in the area of its corresponding peak in the chromatogram, 48 h after UV-C irradiation. It was observed that the content remained within an acceptable range for 48 h (±10%) and only for peak 3, when stored in open-dishes, was a slight decrease in the value observed.

Although the trans–cis isomerization is to be expected when CQAs are in aqueous or aqueous alcoholic solution, it was not observed in the *Baccharis* extracts as described in the literature [19]. It is also described [20,21] that chlorogenic acids present in plant

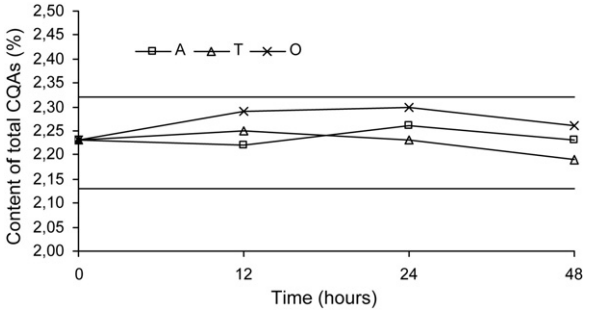


**Fig. 4.** Total caffeoylquinic acid content in powdered *B. trimera* samples evaluated for photostability. A: amber flasks; T: transparent flasks; O: open-dishes. Lines represent 10% of the onset value.

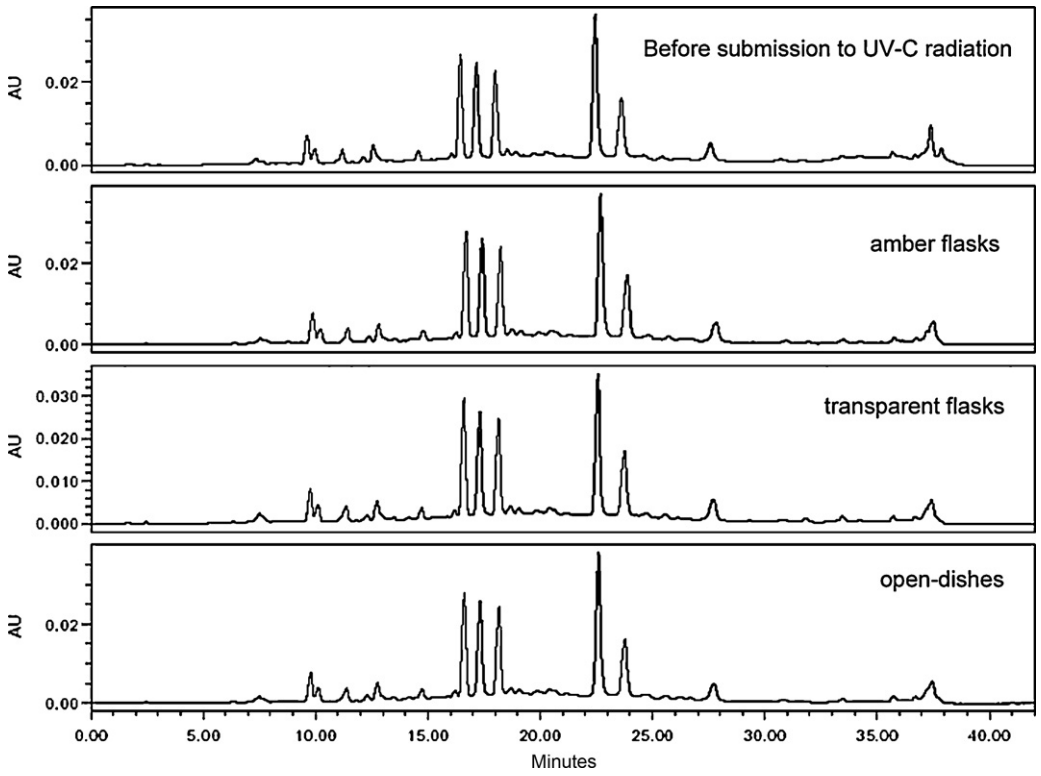


**Fig. 5.** Representative HPLC profiles of irradiation of the extract in ethanol 50%: (a) before submission to UV-C radiation and after 48 h of submission to UV-C (254 nm) in (b) amber flask, (c) transparent flask and (d) open dish.

tissue exposed to natural UV light undergo trans–cis isomerization. In our case, the plant material was exposed during its growth to fairly intense UV light. After drying, it was expected the trans–cis isomerization, but it was also not observed. It is subject of interesting discussion if the trans–cis isomerization has any physiological importance such as protection from UV light, and it must also be considered the presence of structures in the *B. trimera* that could avoid this kind of isomerization, such as lignans and waxes. These considerations remain subject to speculation and require further detailed investigations.

## 4. Conclusion

The results of this study show that the proposed method is appropriate for the analysis of total caffeoylquinic derivative in *B. trimera* samples. The validation study undertaken indicated that the method is reproducible, specific, linear and accurate and can be used for standardizing extracts, herbal products of *B. trimera* and other species of the same section Caulopterae.

## Acknowledgment

This work was supported by CNPq.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.11.042.

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