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Article

Evaluation of the Analytical Conditions for the Determination of Chlorogenic Acid in Coffee Silverskin

Elisa A. Beltran-Medina [1], Guadalupe M. Guatemala-Morales [1,*], Rosa I. Corona-González [2], Eduardo Padilla-Camberos [1], Pedro M. Mondragón-Cortéz [1], Priscilla Ruiz-Palomino [1] and Enrique Arriola-Guevara [2,*]

[1] Tecnología Alimentaria, Biotecnología Médica y Farmacéutica, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C., Normalistas 800, C.P. 44270, Guadalajara, Jalisco, Mexico; es_ebeltran@ciatej.mx (E.A.B.-M.); epadilla@ciatej.mx (E.P.-C.); pmondragon@ciatej.mx (P.M.M.-C.); prruiz_al@ciatej.edu.mx (P.R.-P.)

[2] Departamento de Ingeniería Química, Centro Universitario de Ciencias Exactas e Ingenierías, Universidad de Guadalajara. Blvd. Marcelino García Barragán #1421, esq. Calzada Olímpica. C.P. 44430, Guadalajara, Jalisco, Mexico; rosa.corona@academicos.udg.mx (R.I.C.-G.)

* Correspondence: enrique.arriola@academicos.udg.mx (E.A.-G.); gguatemala@ciatej.mx (G.M.G.-M.); Tel.: +33-33455200 (ext. 1501) (G.M.G.-M.)

Abstract: Chlorogenic acid or 5-Caffeoylquinic acid is a polyphenolic component present in coffee and its by-products. Chlorogenic acid has been shown to exert biological properties, particularly in relation to glucose and lipid metabolism, including antibacterial, antioxidant, anti-inflammatory activities, among others. Due to its importance, it is necessary to evaluate the reliability of the analytical method for its determination in complex matrices such as food. In this work, different methods of chlorogenic acid extraction in coffee Silverskin were studied, as well as its quantification by HPLC. The results showed that the method of extraction with a mixture of methanol:water (3:1 v/v) in an ultrasonic bath, favored the recovery of chlorogenic acid with a recovery of 77.44%. The instrument detection limit for chlorogenic acid was 3.311 µg/mL and the quantification limit was 11.037 µg/mL. For coffee Silverskin, the concentration obtained of chlorogenic acid by the three extraction methods evaluated was in the range of 57 to 224 mg/kg of coffee silverskin (dry basis).

Keywords: chlorogenic acid; extraction; coffee Silverskin; analytical method

1. Introduction

Coffee is one of the most consumed beverages in the world and is the second largest traded commodity after petroleum [1]. The coffee production chain begins with the harvest of the ripe coffee berries that will be treated in order to separate the pulp, from the coffee bean, by one of the following two processes: (a) wet process, or (b) dry process, where the green coffee bean is obtained. Finally, the bean is heat treated by a process called roasting, thus obtaining the coffee that will be used for the preparation of the drink [2]. Since coffee is a very popular and appreciated beverage around the world, the coffee industry is responsible for generating large amounts of by-products, which include the coffee Silverskin (CS), that represents 4.2% (w/w) of the coffee bean [3]. CS is a yellowish transparent endosperm that covers coffee beans [2] and is currently used as fuel and fertilizer [4]. However, coffee by-products have been reported to possess bioactive compounds, mainly secondary metabolites such as phenolic acids, for example, hydroxycinnamic acids and flavonoids, desired for their beneficial antioxidant properties [5]. Chlorogenic acid or 5-Caffeoylquinic acid (5CQA) belongs to the family of hydroxycinnamic acids and is one of the most

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abundant polyphenolic compounds in the human diet, is part of the group of secondary phenolic metabolites produced by certain plant species and it is an important component in coffee, and in the CS [6,7]. 5CQA is of special interest due to the wide spectrum of potentially beneficial effects on health, including antidiabetic, anti-obesity, antioxidant, anti-hypertension, anti-inflammatory and antibacterial effects. Its study could provide an approach to the treatment or prevention of some chronic diseases [8,9].

CS has been reported as a source of chlorogenic acids, however, until now, there are few reports concerning the content of 5CQA in CS, and those that exist show controversial results, since the reported concentration are in the range of 1,000 to 11,678 mg of 5CQA/kg of CS [4,10-12]. This could be due to the extraction methods used, since different procedures have been used to obtain the 5CQA, such as aqueous, alcoholic and acidified-water extracts, applying temperatures between 25 to 100°C by conventional extraction [3,10-13]. In addition, techniques such as ultrasonic bath extraction and subcritical water extraction have been employed [4,10,13]. 5CQA quantification has been carried out using techniques such as UPLC-MS [11], UHPLC-LITMS [4], HPLC [10,12], nevertheless, there is no evidence of the evaluation of the analytical method, through parameters as recovery, linearity, precision, and in some cases, of the limits of quantification and detection. These are important indicators, considering that there are few studies where the content of 5CQA is determined in this matrix (CS), which would ensure the reliability of the determinations.

The aim of this work was to analyze different extraction procedures and evaluate the method for the quantification of 5CQA in CS by HPLC.

2. Materials and Methods

2.1. Materials

CS produced by roasting coffee beans (*C. Arabica* 100%) was obtained from Don Balbino Company (Talpa de Allende, Jalisco, Mexico). CS was milled prior to its extraction (Average Particle Size = 0.28 ± 0.004 mm).

5-Caffeoylquinic acid powder reference standard (USP 12601) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) was HPLC grade (Sigma-Aldrich, St. Louis, MO, USA). Phosphoric acid was reagent grade (Karal, Leon, Guanajuato, Mexico).

The standard of 5CQA was diluted in MeOH to obtain a stock solution at 1,000 μg/mL, from where the calibration curve was prepared. All solutions remained refrigerated at 4°C in amber vials.

2.2. HPLC analysis

Sample analysis was performed on liquid chromatograph Alliance 2695, equipped with 2998 Diode Array Detector (Waters, Milford, MA, USA), Software Empower 3. The separation was carried out on a 5 micron (100 Å, 250 x 4.6 mm) C-18 reverse phase Kromasil column (Bohus, Sweden) at room temperature. The mobile phase was phosphoric acid 5 mM (solvent A) and Methanol (solvent B), at a flow rate of 1 mL/min. The elution gradient was carried out as follows, a linear gradient of 85-80% solvent B (0-5 min), 60% B (6-10 min), 70% B (11-20 min), 80% B (21-25 min) and finally 85% B (26-30 min). The injection volume was 20 μL and the 5CQA was detected at a wavelength of 325 nm. This method was adapted from Fujioka and Shibamoto (2008) [14]. Sample chromatograms were compared with those of the 5CQA standard for identification. The measurements were carried out in triplicate.

2.3. Instrumental calibration

Eight different levels of concentration were employed for 5CQA: 10, 40, 80, 100, 200, 300, 400 and 500 μg/mL. Each point of the calibration curve was injected in triplicate and prepared by diluting the solution of 1,000 μg/mL. Initially, the *Pearson* correlation coefficient (r) was calculated to estimate the type of adjustment of the experimental points in the calibration curve and subsequently a statistical contrast of student's t-test [15] and variance analysis were performed, to verify its significance.

2.4. Extractrion process

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The ground coffee Silverskin was treated to extract the 5CQA; three procedures were evaluated. Blanks of the three extraction processes were prepared by applying the same treatment but, instead of adding CS, a 200 ppm 5CQA standard concentration was used. **Method 1 (M1)** was taken from Ballesteros et al. (2014) [3]. Briefly, 1 g of CS was mixed with 40 mL of MeOH at 60% (v/v). The mixture was heated during 90 min in a water bath at 60-65°C under magnetic stirring. After this time, the extracts were separated by centrifugation (Luzeren TDL-40B, Mexico) at 3800 rpm for 20 min. The extract was reduced and filtered (0.45 μm). **Method 2 (M2)** was followed from Del Castillo et al. (2013) [13] with some modifications, briefly; 2 g of CS were added to 10 mL of demineralized water. It was heated for 10 min in a boiling water bath. The mix was centrifugated at 2900 rpm for 10 min. The extract was reduced and filtered (0.45 μm). **Method 3 (M3)** was adapted from Del Río et al. (2014) [16]; 0.5 g of CS were weighed and 5 mL of MeOH:water 3:1 (v/v) were added. The mixture was sonicated (Branson 5800, USA) for 15 min, removed and stirred in a Vortex-Genie (Scientific Industries, Bohemia, NY, USA) for another 15 min. Afterwards; it was centrifuged at 3400 rpm for 10 min. The supernatant was transferred to another container and the residue was re-extracted. The second extract was added to the first. It was filtered (0.45 μm) and the extract was reduced. All extracts were reserved in amber vials, under refrigeration, until analysis.

2.5. Evaluation of analytical conditions

The method was evaluated by calculating the extraction recoveries, the accuracy and the determination of the detection limit and quantification limit [15]. These results are used to verify the performance of the analytical conditions.

2.6. Statistical Analysis

The STATGRAPHICS Centurion XV package (Statpoint Technologies; Warrengton, VA, USA) was used for the analysis of variance of the recovery in the evaluation of the analytical method.

3. Results and Discussion

3.1. Extraction

According to Regulation (EC) No. 333/2007 [17], if an analytical method includes an extraction step, the result of the analysis must be corrected based on the recovery, so the level of recovery or the analyte recovery must be recorded. The recovery percentage (%R) was calculated according to Equation (1):

$$\%R = (CF / CA) * 100 \quad (1)$$

Where CF is the concentration of the analyte measured in the blank and CA is the concentration of the analyte added (measured value, not determined by the method). The results suggest that the efficiency of extraction of 5CQA was favored with M1 extraction process, where the recovery was 84.63%, followed by M3 procedure with 77.44% and finally, by M2 procedure, with 63.26%.

3.2. Method performance

3.2.1. Linearity evaluation

The linearity of the calibration curve for the 5CQA at eight concentration levels in the range of 10-500 μg/mL was evaluated. The peak area ratios for each solution were measured against their corresponding concentration and the calibration curve was obtained. The determination coefficient (r^2) was 0.9996, in the concentration range studied.

3.2.2. Accuracy

Accuracy was assessed through the repeatability and reproducibility of the HPLC equipment. The repeatability was determined by calculating the percentage of the coefficient of variation (%CV)

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of the relative areas in the triplicate analysis of the same concentration level, thus repeatability was calculated according to Equation (2):

$$\% \text{ Repeatability} = 100 - \%CV \quad (2)$$

On the other hand, reproducibility was determined by subtracting at 100%, the average of %CV of all concentration levels of the calibration curve for the 5CQA. According to the results obtained (Table 1), HPLC offers excellent repeatability in the range of concentrations presented, since %CV ranged between 0.18% and 3.07%, where the percentage of variation is less than 5% and the reproducibility of the equipment for 5CQA was found at 98.98%.Therefore, the international acceptance criterion for precision was met and, since recoveries found take in account matrix effect, the identification and quantification is more reliable.

Table 1. Quantitative parameters of analytical data

Compound	Precision		LOD[2], µg/mL	LOQ[3], µg/mL
	CV_o[1], %	Reproducibility, %		
5CQA	1.02	98.98	3.311	11.037

[1] CV_o-overall coefficient of variation, [2] LOD-limit of detection, [3] LOQ-limit of quantification

3.2.3. Detection and Quantification Limits

The homoscedasticity of the variances was evaluated by applying the statistical test 'Fisher's F'. The analysis showed that at least one variance, of the experimental points of the calibration curve, was significantly higher at higher concentrations since the calculated Fisher F values exceeded the value of $F_{tables\ 2,2}$ = 19.0, at a confidence level of 95% (p = 0.05) (data not shown). This suggests a heteroscedastic behavior of the experimental data and, therefore, the use of a weighted linear regression method. The parameters of the instrumental calibration curve of the 5CQA were estimated, obtaining a weighted slope, b_w = 69,422.37, the weighted intercept, a_w = -105,263.92 and, the weighted standard deviation, $S_{(y/x)w}$ = 78,744.71.

The instrumental detection (LOD) and quantification (LOQ) limits for 5CQA (Table 1) were determined based on the signal-to-noise ratio of 3 and 10, respectively, using the weighted parameters [15], thus obtaining an LOD of 3.311 µg/mL and LOQ of 11.037 µg/mL.

3.3. 5CQA content in CS

The concentration of 5CQA was determined following the three methodologies, by determining the responses by means of peak area and correcting the concentration obtained with the calculated recovery.

In the chromatograms obtained for the M1 method, a shoulder can be seen at the chlorogenic acid (Figure 1a), which could mean that there is another compound that overlaps with the signal of the 5CQA. For the other methods, the peak is resolved, as can be seen in Figure 1b. The M2 method proved to be a simple and low-cost extraction process that can be easily used for the determination of 5CQA. However, many compounds present in food have been efficiently extracted by ultrasound, since it reduces the use of solvents, facilitates the release of extractable compounds and improves mass transfer [18], as could be corroborated by the M3 procedure.

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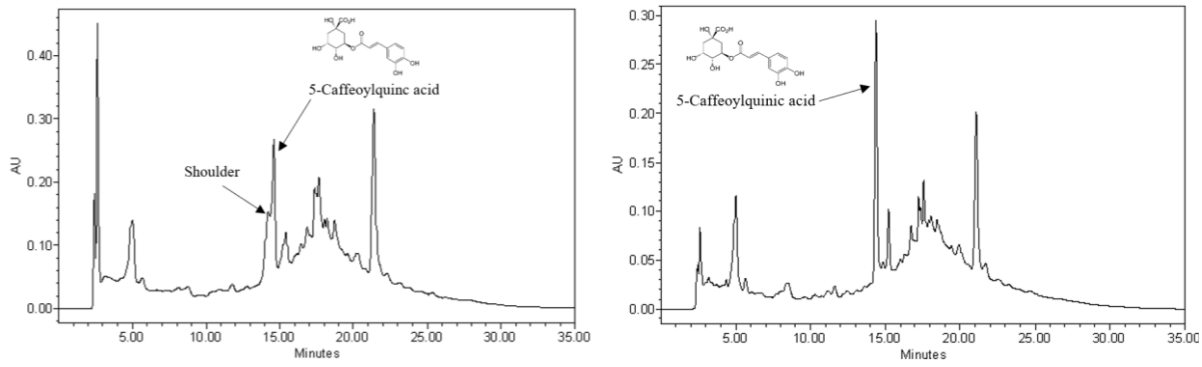

(**a**) (**b**)

Figure 1. Chromatogram of the CS extract obtained by method (**a**) M1 and (**b**) M3.

An ANOVA was performed for the different extraction methods, showing significant difference between the extraction methods, with a 95.0% of confidence level. In the Multiple Range test, three homogeneous groups were obtained, so each method belongs to a different group. As it is desired to maximize the recovery, the use of the M3 procedure is recommended. Table 2 shows the concentrations of 5CQA extracted from CS, which are in the range of 57.46 (M2) to 224.09 (M3) mg of 5CQA / kg of CS (db). Bresciani et al. (2014) [4] obtained 198.9 ± 6.6 mg of chlorogenic acid / 100 g of CS, which is 8 times higher than the concentration obtained in this work. While Narita & Inouye (2012) [10] quantified a content of 1.0 ± 0.0 to 1.7 ± 0.1 mg of chlorogenic acid / g of CS; Iriondo et al. (2019) [11] obtained 9.4 ± 2.6 mg of 5CQA / g extract of CS; and, Regazzoni et al. (2016) [12] determined a concentration of 89.83 ± 0.64 mg of 5CQA / g of dry extract of CS. The difference in the content of 5CQA in the CS could be due to the nature of the coffee bean, its origin. As well as the process of coffee roasting. During this process, when the temperature is higher than 160°C, a series of exothermic and endothermic reactions take place; the bean become light brown, their volume increases considerably, and the detachment of CS occurs. The chemical reactions responsible for aroma and flavor of roasted coffee are triggered at approximately 190°C. These reactions are interrupted at the desired point based on bean color or programmed time [19-20]. At temperatures between 150 and 170°C the decrease in 5CQA content starts to speed up [21]. Therefore, as the beans (and the CS) stay more time in the roaster, where high temperatures are present, the content of 5CQA considerably diminishes. This could explain the concentration of 5CQA obtained in the CS.

Table 2. Determination of the average concentration of 5CQA in CS, with the different methods proposed

Method	Concentration (mg of 5CQA[1] / kg CS[2], db[3])
M1	196.12 ± 8.03
M2	57.46 ± 3.6
M3	224.09 ± 7.72

[1] 5CQA-5-Cafeoylquinic acid, [2] CS-Coffee Silverskin, [3] db-dry basis

4. Conclusions

Due to the little evidence that exists about the determination of chlorogenic acid in coffee silverskin, this work constitutes a guide for its determination in a reliable way, in this by-product of the coffee industry. Thus, it can be used when designing functional foods where coffee Silverskin is incorporated.

The analytical conditions of the method to identify and quantify 5CQA have been satisfactorily evaluated. The method by ultrasonic bath extraction is proposed for quantification by HPLC of chlorogenic acid from the coffee silverskin, because its recovery is acceptable, the resolution of the peak is adequate and is an easy extract to obtain and handle. However, it will be necessary to optimize the extraction parameters.

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