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Performance review of a fast HPLC-UV method for the quantification of chlorogenic acids in green coffee bean extracts

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ABSTRACT

The aim of this study was to test the performance of a HPLC method, designated for rapid quantification of chlorogenic acids (CGA) in green coffee extract (GCE). The precision statistics associated with the method were assessed using three independent laboratories with five samples analyzed in triplicate. Seven main CGA isomers (3-CQA, 5-CQA, 4-CQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA) were quantified. The concentration of total CGA in the samples varied from 32.24% to 52.65% w/w. The repeatability and reproducibility standard deviations for the determination of individual isomers varied, respectively, from 0.01 to 0.28 and 0.05–1.59. The repeatability and reproducibility standard deviations of the calculated total CGA, corresponding to the sum of the seven main CGA isomers, varied respectively, from 0.17 to 0.58 and 0.55–2.01. The fast HPLC method evaluated in this study was considered precise and appropriate for the determination of CGA in GCE.

1. Introduction

Chlorogenic acids (CGA) are a family of esters formed between certain phenolic acids (*trans*-cinnamic acids) and quinic acid. The main subgroups of CGA found in green coffee beans are caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloylquinic acids (FQA), each group having at least three isomers [1]. Similar to other phenolic compounds, CGA are generally thought of as secondary plant products present to protect against environmental stress [2]. There is growing scientific evidence to support the health benefits of CGA to humans. Particularly, studies indicate that CGA act as protective agents, inhibiting or reducing the oxidative stress of cell structures and functions, and promoting health [3,4]. Furthermore, CGA have also been investigated for their positive effect on blood pressure [5] and glucose regulation [6].

Although CGA are naturally occurring in a variety of plants, the green coffee bean is somewhat unusual since it contains a much higher CGA content than most other plants. This high content, which can vary from 5.5% to 10% [2], makes roasted brewed coffee one of the main sources of polyphenols consumed in the human diet [7], even though up to 60% of total CGA in the green coffee beans can be lost with conventional roasting conditions [8]. The potential health benefits of coffee motivated the nutrition industry to develop and market a variety of "green coffee extracts" (GCE's), which are the product obtained from the water, solvent or CO_2 extraction of green coffee beans, thus yielding a process that retains high levels of CGA.

Given the increase in consumer interest towards GCE rich food and nutraceutical products, quality challenges that include developing a standardized method for quantification of CGA for GCE use by suppliers, industry, and regulatory bodies have emerged. For quantitative analysis of bioactive compounds in foods and beverages, several techniques have been employed; however high-performance liquid chromatography (HPLC) remains one of the most common for accurate results [9–12]. Conventional HPLC methods for the determination of CGA and its isomers in coffee have reported separation times that vary from 35 min to 95 min [13–15]; a situation which limits their applicability for use in the industry.

A recent advancement in HPLC analysis of food materials has been the development of fused-core particles within the column, which has enabled analysts to achieve faster and more sensitive

Abbreviations: 3-CQA, 3-Caffeoyquinic Acid; 3,4-diCQA, 3,4-Dicaffeoylquinic Acid; 3,5-diCQA, 3,5-Dicaffeoylquinic Acid; 4-CQA, 4-Caffeoyquinic Acid; 4,5-diCQA, 4,5-Dicaffeoylquinic Acid; 5-CQA, 5-Caffeoyquinic Acid; 5-FQA, 5-Feruloylquinic Acid; CGA, chlorogenic acids; CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; FQA, feruloyilquinic acid; GCE, green coffee extract; HPLC, high-performance liquid chromatography

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and accurate separations. Fused-core particles are composed of porous shells that are fused to solid core particles. This technology addresses the slow mass transfer of solute molecules inside conventional particles by providing a small diffusion path into and out of the stationary phase; thereby reducing the time molecules spend inside the particles. Physical characteristics of the molecules also allow an increase in flow rate of the mobile phase to achieve faster separation without generating high column backpressure [9,16]. In particular, recent studies report the use of fused-core particles to the determination of CGA in instant coffee, green coffee beans and spent coffee grounds, respectively [9,17,18].

The objective of this study was to evaluate the performance of a fast HPLC method with UV detection for the quantification of CGA in GCE. The precision parameters were obtained from three participating laboratories, each generating test results on five identical GCE samples.

2. Material and methods

2.1. Chemical and reagents

Acetonitrile (ACN), methanol (MeOH), trifluoroacetic acid (TFA) and formic acid were HPLC grade. The 5-caffeoyquinic acid (5-CQA) standard was purchased from Chromadex (Irvine, United States of America). The 3-caffeoyquinic acid (3-CQA), 4-caffeoyquinic acid (4-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA) and 4,5-dicaffeoylquinic acid (4,5-diCQA) standards were purchased from Cerilliant Corporation (Round Rock, Texas, USA) and Chengdu Must Bio-Technology Co., Ltd (Chengdu, Sichuan, China).

2.2. Samples

Five green coffee extracts provided by Applied Food Sciences, Inc. were used in this study. All extracts were produced with Arabica green coffee beans from different farms in India. Samples 1 and 4 were produced by supercritical CO_2 extraction, and samples 2, 3 and 5 by ethanol extraction.

2.3. Diluent and mobile phase preparation

The diluent used was 2.0% formic acid in 10% ACN (aq) prepared by thorough mixing 50 mL of ACN, 10 mL of formic acid and 440 mL of Milli-Q water. The mobile phase A, 0.1% TFA in water, was prepared by adding 1 mL of TFA to ~900 mL of Milli-Q water in a 1000 mL volumetric flask and mixing well. The flask was brought to volume with Milli-Q water and transferred to a mobile phase container.

2.4. Standard and sample preparation

The standard solution was prepared by weighing approximately 5 mg of the standard into a 10 mL volumetric flask, adding 10 mL of diluent, mixing the solution and vialing for analysis. The green coffee extract samples were prepared by weighing approximately 250 mg of sample into a 50 mL volumetric flask and adding 40 mL of diluent. The solutions were sonicated for 10 min, cooled to room temperature, brought to volume with Milli-Q water and mixed well. Aliquots of the solutions were filtered using 0.45 μm PTFE syringe filters and the filtrates were collected in HPLC vials for analysis.

2.5. HPLC analyses

Laboratories A and C used Agilent 1100 Series HPLC system, and Laboratory B used a Shimadzu LC2010CHT. All HPLC systems were equipped with Phenomenex Kinetex C18 (100 × 4.8 mm, 5 μm) column and UV–vis detector. The temperature was maintained at 25 °C. Mobile phase A was 0.1% TFA in water and mobile phase B was ACN. The injection volume was 3 μL. The flow rate was set as 1.5 mL/min. The gradient mode was set as indicated in Table 1. The detector was set at 330 nm.

Table 1
HPLC gradient program.

General gradient		
Time (Min)	A (%)	B (%)
0	95	5
10	80	20
12	80	20
15	95	5
15.1	5	95
16.5	5	95
16.6	95	5
19	95	5

The identification of the CGA isomers was confirmed by the analysis of the 3-CQA, 5-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA standard solutions. The concentration of each CGA isomer in the GCE samples was calculated as 5-CQA equivalent using an external standard method through calibration curves. The total CGA was calculated as the sum of the main CGA isomers: 3-CQA, 5-CQA, 4-CQA, 5-feruloylquinic acid (5-FQA), 3,4-diCQA, 3,5-diCQA and 4,5-diCQA.

2.6. Interlaboratory study and statistical analyses

Three laboratories located in the United States, Canada and India participated in the study. The interlaboratory organizer was not one of the participating laboratories. Laboratories were asked to analyze each of the five green coffee extract samples in triplicate following the methodology described in Sections 2.3–2.6. Results were analyzed according to the standard practice for conducting interlaboratory study by ASTM E691–14 [19]. The data consistency statistics, *h* and *k*, and precision under repeatability and reproducibility conditions were explored in this study.

3. Results and discussion

3.1. Chromatogram analysis and pilot run

Fig. 1 shows the chromatograms of the 5-CQA standard and one GCE sample. The peak profiles were consistent between the three participant laboratories and followed the same pattern reported by Aqeel et al. [20] in GCE and Rostagno et al. [9] in instant coffee.

Seven main CGA isomers were clearly identified, including 3-CQA, 5-CQA, 4-CQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA. Other minor related compounds were also identified (e.g. 3-FQA and 4-FQA). The elution times were consistent between the laboratories (Fig. 2).

The linearity was evaluated by building an external calibration curve for 5-CQA at a concentration range of 0.00969–1.299 mg/mL. The analyte peak area response was plotted versus its concentration at eight different levels. From this, a coefficient of determination (R^2) of 0.99989 was achieved, thus demonstrating that the peak area response was linear and related to the 5-CQA concentration; enabling this approach to be used to quantitate CGA. The method used in this study has been validated for the quantification of 5-CQA in GCE by Phenomenex [20]. The LOQ and R^2

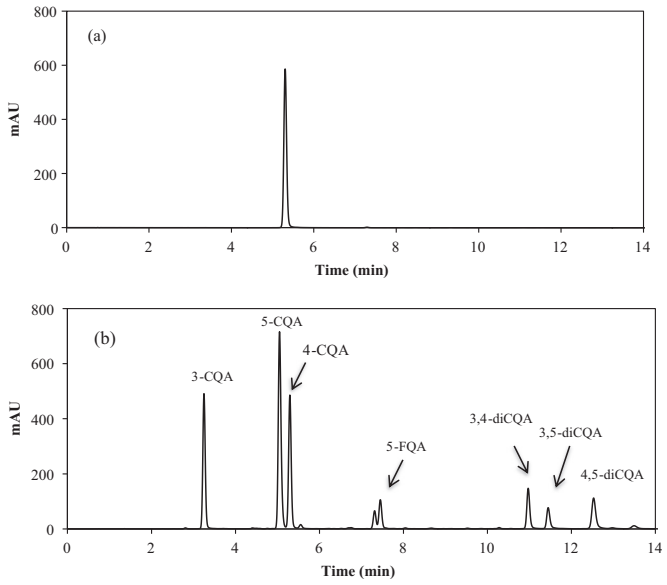

Fig. 1. Typical chromatogram of (a) 5-CQA standard and (b) a green coffee extract sample.

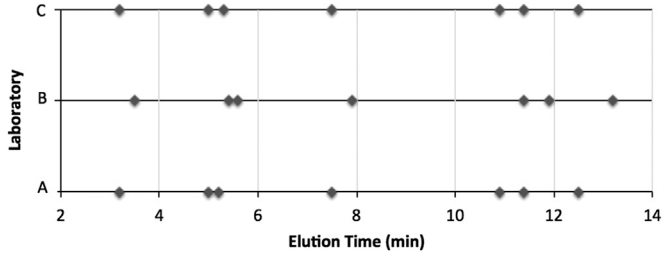

Fig. 2. Elution time of CGA reported by three independent laboratories. The elution order is: 3-CQA, 5-CQA, 4-CQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA.

were found to be, respectively, 1.25 μg/mL and 0.9997. Rostagno et al. [9] also validated this method, with minor modifications, for the purpose of quantitating 5-CQA in instant coffee, teas and mate. Resolution, selectivity, peak symmetry and limits of detection were reported by the authors.

3.2. Data consistency

Before initiating the statistical analysis, a careful examination of individual laboratory reports was made to identify possible errors associated with the sample identification and abnormal results reported for sample replicates.

Data consistency was evaluated based on the *h* and *k* statistics [19], which explains the overall character of the variability of the HPLC method as well as singling out particular laboratories or outlier cells that should be investigated further. The statistic *h* is algebraically identical to one of Grubbs' outlier statistics, and *k* is algebraically related to the Cochran's C. However, Grubbs and Cochran's statistics apply only to an extreme value or observation, while the *h* and *k* statistics apply to each cell-average and cell-standard deviation [21]. The data consistency was calculated with the total CGA values for each sample and laboratory.

3.2.1. Between-laboratory consistency

The between-laboratory consistency statistic *h* was used as an indicator of how one laboratory's cell average, for a particular sample, compares with the average of the other laboratories. Small *h* values indicate proximity to the average over all laboratories, while high *h* values indicate the opposite [21]. The results are

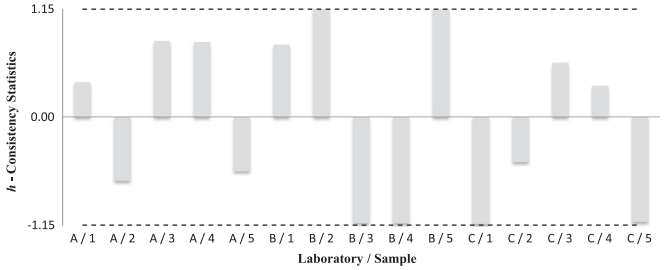

Fig. 3. Between-laboratory consistency statistics, *h*, for the determination of chlorogenic acids in green coffee extract. A, B and C correspond to the laboratory; 1, 2, 3, 4 and 5 correspond to the sample number.

shown in Fig. 3. The critical values of the *h* consistency statistics were calculated at a $P < 0.05$ significance level. For a collaborative study with three laboratories, the *h* critical value was determined to be 1.15. All laboratories were within the limits for each of the five samples analyzed. Fig. 3 shows that all laboratories had both positive and negative *h* values among the materials. This pattern indicates that the nature of the test method variability is normal and does not require investigation of any particular laboratory.

3.2.2. Within-laboratory consistency

The consistency statistic *k* is an indicator of how one laboratory's within-laboratory variability, under repeatability conditions, on a particular material, compares with all of the laboratories combined. Values of *k* larger than 1 indicate greater within-laboratory variability than the average for all laboratories. Since such variation among laboratories is expected, critical values of *k* have been calculated at a P-value of 0.05 significance level, to assist in deciding whether the cell standard deviation of one laboratory is sufficiently different from the rest of the laboratories as to require investigation. In this inter-laboratory study with three laboratories analyzing samples in triplicate, a *k* critical value was determined to be 1.67. The results shown in Fig. 4 indicate that Laboratories B and C exhibited at least one *k* value larger than one. Laboratory C had three *k* values over the recommended *k* critical value, which were, 1.71, 1.72 and 1.70, associated with the determination of total CGA in samples 2, 3 and 5. Due to the closeness of these values to the *k* critical value, the results were judged acceptable.

3.3. Intra- and inter-laboratory precision

Table 2 shows the precision statistics associated with the quantification of each CGA isomer and total CGA. The total CGA levels were calculated as the sum of the levels of the seven main CQA isomers (3-CQA, 5-CQA, 4-CQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA). Samples 1 and 4, which correspond to supercritical CO_2 extracts, exhibited respectively 32.24% and 34.21% w/w

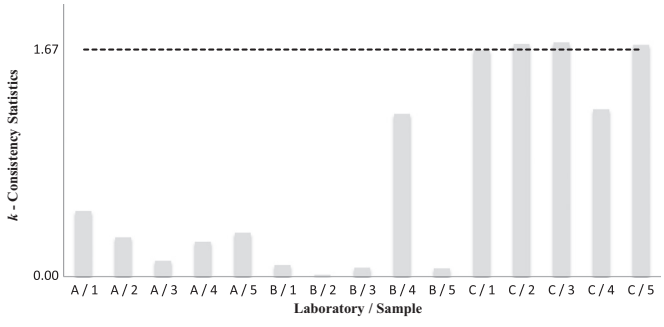

Fig. 4. Within-laboratory consistency statistics, *k*, for the determination of chlorogenic acids in green coffee extract. A, B and C correspond to the laboratory; 1, 2, 3, 4 and 5 correspond to the sample number.

Table 2
Precision statistics for the quantification of CGA in GCE expressed as % w/w.

Sample	Isomer	Average	sx	sr	sR
1	3-CQA	6.57	0.17	0.03	0.17
	5-CQA	12.57	1.58	0.11	1.59
	4-CQA	7.05	0.31	0.08	0.32
	5-FQA	1.25	0.31	0.01	0.31
	3,4-diCQA	1.77	0.84	0.04	0.84
	3,5-diCQA	1.12	0.52	0.04	0.52
	4,5-diCQA	1.90	0.97	0.05	0.97
	Total	**32.24**	**0.74**	**0.17**	**0.75**
2	3-CQA	8.73	0.38	0.07	0.38
	5-CQA	22.88	0.62	0.28	0.66
	4-CQA	9.85	0.64	0.23	0.67
	5-FQA	1.72	0.06	0.01	0.18
	3,4-diCQA	2.32	0.40	0.10	0.41
	3,5-diCQA	1.76	0.11	0.13	0.15
	4,5-diCQA	2.86	0.40	0.18	0.43
	Total	**50.12**	**1.48**	**0.51**	**1.54**
3	3-CQA	7.66	0.11	0.08	0.13
	5-CQA	26.36	0.31	0.30	0.40
	4-CQA	9.62	0.06	0.29	0.25
	5-FQA	1.95	0.06	0.03	0.06
	3,4-diCQA	2.26	0.02	0.09	0.17
	3,5-diCQA	1.91	0.02	0.12	0.16
	4,5-diCQA	2.88	0.07	0.17	0.17
	Total	**52.65**	**0.41**	**0.52**	**0.59**
4	3-CQA	6.72	0.08	0.07	0.10
	5-CQA	11.15	0.92	0.15	0.24
	4-CQA	7.16	0.05	0.05	0.06
	5-FQA	1.72	0.07	0.02	0.07
	3,4-diCQA	2.71	0.10	0.13	0.15
	3,5-diCQA	1.79	0.13	0.12	0.16
	4,5-diCQA	2.96	0.22	0.18	0.27
	Total	**34.21**	**0.60**	**0.58**	**0.77**
5	3-CQA	8.53	0.39	0.04	0.39
	5-CQA	20.08	0.92	0.09	0.92
	4-CQA	10.19	0.38	0.06	0.38
	5-FQA	1.87	0.05	0.02	0.05
	3,4-diCQA	2.62	0.13	0.09	0.15
	3,5-diCQA	1.84	0.03	0.09	0.09
	4,5-diCQA	3.14	0.19	0.14	0.22
	Total	**48.27**	**1.98**	**0.41**	**2.01**

s_x, standard deviation of cell averages; s_r, repeatability standard deviation; s_R, reproducibility standard deviation.

of total CGA, respectively, while the levels in the ethanolic extract samples 2, 3 and 5 ranged from 48.27% to 52.65% w/w. The lower level of total CGA measured in those samples extracted by supercritical CO_2 can be explained by the fact that polyphenols are moderately polar compounds, while CO_2 is relatively non-polar, thus being a preferred solvent for lipid, fat and non-polar substances [22,23].

Besides the seven main CQA isomers, other isomers such as 3-FQA and 4-FQA were identified in the samples. These isomers were not quantified due to their lower resolution in the chromatograms, which would lead to ambiguity in their determination between the laboratories. The total CGA levels reported in this study are interpreted as the minimum levels of CGA in the samples. As a comparison, Phenomenex [20] reported concentrations of total CGA and 5-CQA in GCE were found to be 45% w/w and 18.8% w/w, respectively, which is similar to the concentrations found in this study. In the study by Henry-Vitrac et al. [24] the concentration of CGA in a commercial decaffeinated GCE was evaluated. The sum of the seven main isomers (3-CQA, 5-CQA, 4-CQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA) was reported to be 41.36% w/w, and the total CGA, including minor compounds, was 47.66% w/w. The 5-CQA was found to represent 30% w/w of total CGA.

Plots of the standard deviation versus the CGA concentration levels were generated to evaluate if the precision statistics were linear and dependent on the concentration of the analyte. A trend could be observed, but the coefficients of determination in both repeatability ($R^2=0.7102$) and reproducibility ($R^2=0.4477$) standard deviation versus concentration plots indicated that this relationship was not significant.

The seven CGA isomers determined were precisely quantified. The repeatability and reproducibility standard deviations for the determination of individual isomers varied, from 0.01 to 0.30 and 0.06–1.59, respectively. The repeatability and reproducibility standard deviations of the calculated total CGA varied, respectively, from 0.17 to 0.58 and 0.59–2.01. From this information we conclude that the fast HPLC method evaluated in this study was precise and appropriate for the determination of CGA in GCE.

This study was aimed at determining the precision statistics of a fast HPLC method that quantifies CGA in GCE. While this method has been previously validated and used in other studies with a few modifications [9,20], its interlaboratory precision estimation has not been reported in the literature. There are examples where a quantification method of food components is not fully accepted until a valid interlaboratory performance study has been conducted [25]. Our results therefore have relevance to suppliers, industry, and regulatory bodies that are involved in the fast growing GCE market.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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