Comparison of Different Extraction Techniques and Conditions for Optimizing an HPLC-DAD Method for the Routine Determination of the Content of Chlorogenic Acids in Green Coffee Beans

Mohammed D. Y. Oteef

Department of Chemistry, College of Science, Jazan University, Jazan 82817-2820, Saudi Arabia; moteef@jazanu.edu.sa

Abstract: Chlorogenic acids (CGAs) are the main phenolic compounds found in green coffee beans. They are receiving more attention recently due to the proven health and nutrition benefits they offer, in addition to their role as markers for coffee quality. A relatively large number of studies are reported in the literature that are based on the analysis of these compounds. However, very limited research is dedicated to the evaluation of the performance of the analytical methods used, particularly the extraction procedures. Therefore, this work was dedicated to the comparison of different extraction techniques and conditions in order to evaluate their influence on the measured content of the three main CGAs in green coffee beans, namely, chlorogenic acid (5-CQA), neochlorogenic acid (3-CQA) and cryptochlorogenic acid (4-CQA). Five simple extraction techniques with affordable equipment were compared in order to develop a routine method suitable for most analytical and food analysis laboratories. The compared extraction techniques provided relatively similar extraction efficiency for the three compounds. However, due to the merits of ultrasonic-assisted extraction as a fast, effective, green, and economical technique, this was selected for comparing the extraction variables and developing an optimized routine method. The extraction solvent, temperature, time, solid-to-solvent ratio, and grinding treatments were the variables that were investigated. The extraction solvent and the solid-to-solvent ratio were found to be the most influencing variables that may improve the extraction efficiency to up to 50%. Based on this thorough investigation, an optimized method for the routine determination of the content of chlorogenic acids in green coffee beans was developed. The developed method is simple, fast, and efficient in the extraction of the three main CGAs.

Keywords: green coffee beans; chlorogenic acids; extraction techniques; ultrasonic-assisted extraction; HPLC-DAD

1. Introduction

Coffee is the second largest traded commodity, and one of the most widely consumed beverages globally due to its aroma, taste, stimulating effects and health benefits [1,2]. The chemical composition of green coffee beans is an important factor in characterizing the quality of the coffee [3]. Chlorogenic acids (CGAs) are common chemical constituents associated with coffee quality, taste and flavor [4,5]. CGAs are the main group of phenolic compounds found in coffee beans. Chemically, they are the esters of quinic acid with either caffeic, ferulic, or p-coumaric acids, and are found in plants as a mixture of the various esters. The main subgroups include caffeoylquinic acids (CQAs), dicaffeoylquinic acids (diCQAs), feruloylquinic acids (FQAs), and p-coumaroylquinic acids (pCoQAs) [6-7].

Research has concluded the importance of CGAs as markers to relate coffee varieties to their genetic and geographical origins [4,5,8]. In addition, CGAs play a vital role in the composition of coffee due to their beneficial health effects as antioxidants, antimicrobial, antiviral, antispasmodics, hypoglycemic, hepatoprotectants, and neuroprotectants.
top of that, they are also inhibitors of the HIV-1 integrase and of the mutagenicity of carcinogenic compounds [7,9,10]. Recent studies also revealed the benefit of CGAs in fighting obesity and reducing body weight and fat deposition [9,11]. Due to these health benefits, more people become attracted to the consumption of coffee as the main natural source of CGAs. Furthermore, new products and applications of CGAs are introduced in pharmaceuticals, foodstuffs, food additives, and cosmetics [9].

The content of CGAs in green coffee beans is relatively high. The levels are in the range of 2.1 to 7.8 g per 100 g (dry weight basis; d.w) for the Coffea arabica species with levels of 4 to 6 g/100 g (d.w.) being more common, and 2.3 to 14.4 g/100 g (d.w.) in the Coffea canephora species, with 7 to 8 g/100 g (d.w.) more commonly reported [3]. Some extreme ranges were also reported in the literature from as low as 0.6% to up to 26.4% [12]. Similar to the other bioactive compounds in coffee, the content of CGAs is affected by many factors including genetics, degree of maturation, agricultural practices, climate, and soil properties [3,7,13].

Despite more than 300 major and minor CGAs and related compounds being described in coffee beans and other plants, three isomers of caffeoylquinic acids (Figure 1) are the major contributors to the content of CGAs in green coffee beans. These isomers are chlorogenic acid (5-O-caffeoylquinic acid, 5-CQA; CAS: 327-97-9); neochlorogenic acid (3-O-caffeoylquinic acid, 3-CQA; CAS: 906-33-2) and cryptochlorogenic acid (4-O-caffeoylquinic acid, 4-CQA; CAS: 905-99-7) [3,13]. CQA compounds represent approximately 70–85% of total CGAs contained in green coffee beans, with 5-CQA alone accounting for about 60%, and 3-CQA and 4-CQA each comprising approximately 10% [3,14,15].

![Chemical structures of the three main chlorogenic acids in green coffee beans.](image)

Figure 1. Chemical structures of the three main chlorogenic acids in green coffee beans.

In the roasting process, CGAs play a role in the color, flavor, and aroma of coffee [3,16]. Due to the thermal instability of CGAs, the intense heat conditions used during roasting reduce the level of the total content of CGAs in the coffee beans compared to their original levels in green coffee. A drop in the total content of CGAs—from 30% to up to 90% of the original levels—was observed during the roasting process, depending on the roasting conditions and coffee variety [16,17]. In roasted coffees, CQA compounds represent around 78% of the total content of CGAs in seeds, with 5-CQA alone accounting for about 40% and 3-CQA and 4-CQA contributing approximately 16% and 20%, respectively [3]. Despite
the large drop in the content of CGAs in roasted coffee, substantial amounts survive to be extracted into coffee brews [18].

In brewing coffee, a 200 mL cup was estimated to provide from 20 mg to up to 675 mg of CGAs, depending on the variety of coffee, roasting level, and the brewing method used [18–20]. CGAs and derivatives contribute to the final acidity of the coffee brews and to the coffee bitterness characteristic [16,19]. Higher levels of the content of CGAs were reported to be associated with lower scores in coffee cup quality, which may explain the superiority of *Coffea arabica* [3,21,22]. However, some reports concluded that there seems to be an acceptable level of CGAs (particularly 5-CQA); above which, cup quality decreases [3].

Due to the role of CGAs in coffee quality and human health and nutrition, a relatively large number of research studying these compounds are found in the literature. A major proportion of these reports is dependent on the qualitative and quantitative data produced by the analytical methods applied in the determination of CGAs. The quality of data produced by these methods clearly affected the conclusions of these studies. Typically, analytical methods used in determining the content of CGAs in green coffee beans include a sample preparation step, where CGAs are extracted into a solution followed by a cleanup procedure prior to the measurement by instrumental techniques.

Various extraction techniques were employed to extract CGAs from green coffee beans including soaking (or maceration) [23–25]; Soxhlet extraction (or automated Soxhlet extraction) [26,27]; shaking at room temperature [6,28–32], in a heated water bath [15,33–35] or with cooling [36]; ultrasonic-assisted extraction [4,37–42]; microwave-assisted extraction [43,44]; pressurized solvent extraction [45] and supercritical-fluid extraction [46].

The extracts produced by the above techniques will include, in addition to the targeted CGAs, different amounts of carbohydrates, proteins, lipids and other untargeted constituents that may interfere with the measurement procedure used. Although a number of published CGA determination methods did not report any cleanup method other than filtration or centrifugation [4,13,23,25,28,32–34,37,39–41,44], it is always recommended to add a cleanup step after the extraction and before the measurement steps to reduce the possibilities of interference and protect the instrumental techniques used in the measurements.

A simple, effective and very frequently used method for cleaning up extracts of green coffee beans is by adding Carrez solutions [6,15,24,27,29–31,36,47]. Other methods were also reported in the literature such as adding lead acetate solution [35,43,45] or QuEChERS extraction [38].

Quantitative measurement of CGAs in the extracts of green coffee beans is mainly performed by chromatographic techniques. The various chromatographic techniques employed for this purpose include HPLC-UV [4,6,15,23,24,26,32,39,40,46,47]; HPLC-DAD [30,31,36,37,41,44]; HPLC-MS [25,28,33,48]; UPLC-UV [13]; UPLC-DAD [29]; TLC [23] and high-performance gel filtration chromatography [34]. In addition, some spectroscopic techniques have also been used such as UV-Vis spectrophotometry [35,38,43,45,49] and NIR [13,21].

There is a wide range of levels of the content of CGAs reported in the literature, from as low as 0.6% to up to 26.4% [12]. This wide range is, in part, due to the natural variations caused by environmental and post-harvest processes. However, the variations in the efficiency and performance of the analytical methods used are also thought to contribute to the discrepancies in the levels reported for these compounds [3,18,50]. The extraction step is usually a major contributor to this inconsistency [51].

Therefore, the aim of this work was to compare different extraction techniques and conditions and investigate their effects on the measured content of the three main CGAs in green coffee beans. At the end of this thorough investigation, an optimized method for the routine determination of the content of chlorogenic acids in green coffee beans was developed. The extraction procedure was designed to use simple laboratory techniques and reagents that are commonly available in analytical and food analysis laboratories.
2. Experimental

2.1. Collection and Processing of Coffee Sample

The *Coffea arabica* sample used in this study was obtained from three trees belonging to the same genotype, planted in Jazan University Coffee Germplasm Repository (Fayfa, Saudi Arabia) at an altitude of 850 m above sea level. A sample of ripe cherries were picked and sun-dried for three weeks in accordance with the traditional method of drying coffee by local growers. Dried cherries were hulled with an electric bench-top cherry huller (673 S200 model, Coffee Laboratory, White Stone, VA, USA). To minimize the effect of the moisture content of coffee samples on the measured levels of CGAs, coffee beans were kept in an oven at 40 °C for 24 h before and after grinding. The ground samples were then stored in sealed plastic containers in a desiccator. The moisture content of ground green coffee beans was determined using the method described in the ISO 6673 standard [52]. However, it was not used to correct the results since all samples showed similar levels of moisture content (5.6 ± 0.26%).

2.2. Materials and Reagents

All organic solvents used in this study were HPLC-grade (Sigma–Aldrich, St. Louis, MO, USA). The distilled water used for extraction was obtained from a GFL Water Still (2004 model, Burgwedel, Germany). The ultrapure (type 1) water used for the HPLC mobile phase and preparation of standard solutions was obtained through a Direct-3 water purification system (Millipore, Darmstadt, Germany).

Chlorogenic acid (Certified Reference Material, 5-O-caffeoylquinic acid; 5-CQA; CAS No.: 327-97-9), neochlorogenic acid (≥98.0%; 3-O-caffeoylquinic acid; 3-CQA; CAS No.: 906-33-2), and cryptochlorogenic acid (≥98.0%; 4-O-caffeoylquinic acid; 4-CQA; CAS No.: 905-99-7) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The recommended IUPAC numbering system for chlorogenic acids was used throughout this manuscript.

Carrez clarification solutions I and II were obtained as a ready-to-use kit (Sigma-Aldrich, St. Louis, MO, USA). Magnesium oxide, MgO (heavy) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Instruments

Different techniques were applied to compare the extraction of chlorogenic acids from green coffee beans. The ultrasonic bath used (Powersonic 405, Hwashin Instrument Co., Ltd., Seoul, Korea) has a frequency of 40 KHz and a digital control for time (1 to 99 min), temperature (ambient to 70 °C) and strength of ultrasonic wave (low, medium and high). The highest strength of ultrasonic waves was used throughout this study. The orbital shaker was the SSL1 model from Stuart, UK, with a speed range from 30 to 300 rpm (digital). The linear shaking water bath (India) was controlled by analog controls for temperature and shaking speed. Throughout this study, it was run at the maximum shaking speed. The four-hole water bath (HWS-24, China) used has digital controls for both temperature and time but without a shaking option. Samples were swirled manually every 5 min. Green coffee beans were ground in a blade coffee grinder (Homeco, China).

The HPLC analyses were carried out on a Shimadzu LC-20A HPLC (Kyoto, Japan) equipped with an LC-20AD pump, DGU-20A5R degassing unit, SIL-20A auto-sampler, CTO-20A column oven and an SPD-M20A photodiode array detector (DAD). The LC-Solution software was used in controlling the system and in processing the chromatographic data.

2.4. HPLC Separation Method

The HPLC separation of natural CGA isomers found in coffee requires the use of high-efficiency HPLC columns due to a large number of these isomers in addition to the other components present in the extracts. Therefore, several HPLC columns and conditions were compared in this work to obtain the optimum separation of the three major CGAs. The columns evaluated were a C18 column (Thermo Scientific Hypersil GOLD™, 150 × 4.6 mm,
5 µm); a C8 column (Thermo Scientific Acclaim™ 120, 150 × 2.1 mm, 3 µm); a phenyl-hexyl column with core-shell silica particles (Phenomenex Kinetex®, 50 × 4.6 mm, 2.6 µm) and a biphenyl column with core-shell silica particles (Restek Raptor™, 50 × 3.0 mm, 2.7 µm). The optimization of HPLC separation included mobile phase composition, flow rate, and column temperature as the main factors.

The optimum separation of the three CGAs (3-CQA, 4-CQA, and 5-CQA) targeted in this study was achieved on the biphenyl column. For this separation, the mobile phase consisted of a 1.0% formic acid solution in water (component A) and methanol (component B). A gradient program was used in the separation process with a total flow rate of 0.5 mL/min. The program started at 10% methanol; this was maintained for 10 min to separate the three chlorogenic acids within this time frame. By the end of 10 min, the methanol content was increased to 100% for 10 min to allow the elution of caffeine and any strongly retained compounds from the coffee extract. After that, a re-equilibration step of 15 min with 10% methanol was programmed to get the column ready for the next injection. Five microliters of sample were injected into the HPLC and the total runtime for each injection was 35 min. The column oven temperature was maintained at 30 °C. The DAD detector collected data in the range from 190 to 400 nm, and chromatograms were extracted at 324 nm for quantitation. UV spectra were used to help in the confirmation of the peaks’ identity and purity.

At these conditions, 3-CQA, 4-CQA, and 5-CQA retention times were 2.5 min, 5.9 min, and 6.9 min, respectively. The concentrations of chlorogenic acids in the sample were calculated using five-point linear calibration graphs constructed by authentic standards with concentrations ranging from 6 to 50 mg/L for 3-CQA and 4-CQA and from 40 to 300 mg/L for 5-CQA. The limit of detection (LOD) and the limit of quantification (LOQ) of the method were calculated based on the standard deviation of the linear response and the slope, following the ICH guidelines [53] as follows:

$$\text{LOD} = 3.3\sigma / S$$

$$\text{LOQ} = 10\sigma / S$$

where $\sigma$ = the standard deviation of y-intercepts of the calibration curve (n = 5).

$S$ = the slope of the calibration curve.

The LOD were found to be 0.09, 0.14 and 0.79 µg/mL, and the LOQ were 0.27, 0.43 and 2.39 µg/mL for 3-CQA, 4-CQA and 5-CQA, respectively. The content of chlorogenic acids in green coffee beans was expressed as a percentage by mass (g/100g). This method was used for all HPLC analyses of green coffee bean extracts in this study.

### 2.5. Optimization of Extraction Procedure

#### 2.5.1. Preliminary Extraction Experiments

The starting method was a modification of previously published methods [6,42,50]. Briefly, 0.125 g of coffee powder was transferred into a 50 mL conical centrifuge tube, and about 20 mL of 40% (v/v) methanol/water mixture was added. The tube was placed in an ultrasonic bath maintained at a temperature of 4 °C and extracted for 30 min. The extract was then clarified by adding 0.25 mL each of Carrez solutions I and II and kept standing for about 10 min before centrifuging at 10,000 rpm for 10 min. The supernatant was transferred into a 25 mL volumetric flask along with several washings of distilled water and made up to volume. A portion was filtered through a 0.45 µm syringe filter into a HPLC vial and used for HPLC analysis.

To simplify this method, the various steps used in the above extraction procedure were evaluated. The extraction was tested without centrifugation by weighing the coffee powder directly into a volumetric flask and conducting the extraction and Carrez clarification in the same flask.

To check the possibility of using the caffeine extraction procedure described in the ISO 20481:2008 standard [54] for the simultaneous extraction of CGAs and caffeine, MgO was
used to compare to the Carrez solutions as the clarification method. The extraction was conducted in a water bath set at the boiling temperature (97 ± 2 °C) for 30 min using water as the extractant.

In addition to these preliminary experiments, several extraction conditions were compared in order to plan for the rest of the optimization experiments. Combinations of the main variables (extraction solvents, temperature and time) were compared at this stage.

As a result of this preliminary work, all the subsequent extraction experiments were carried out in 50 mL volumetric flasks with Carrez solutions as the clarification method and without centrifugation.

2.5.2. Effect of the Extraction Technique

There were five extraction techniques that were compared in this study. The simplest technique was soaking (or macerating) the coffee grounds in the extractant at ambient room temperature without mixing. The other techniques were heating (50 ± 2 °C) in a four-hole water bath with occasional hand mixing, heating (50 ± 2 °C) in a shaking water bath with continuous linear shaking, orbital shaking at room temperature, and ultrasonic-assisted extraction in an ultrasonic bath.

In this comparison, the extraction was carried out in 50 mL volumetric flasks using the same extractant (40 mL of 40% [v/v] methanol/water mixture) and 0.250 g of coffee powder for a duration of 30 min (except for soaking for 14 h).

2.5.3. Effect of the Extraction Solvent

Six extraction solvents were compared after fixing all the other conditions. The extractants compared here were water; 40% (v/v) methanol/water; 60% (v/v) methanol/water; methanol; 40% (v/v) ethanol/water and 60% (v/v) ethanol/water.

The extraction was carried out in an ultrasonic bath at 50 ± 2 °C for 10 min using 0.250 g of coffee powder and 40 mL of the specified extractant.

2.5.4. Effect of the Extraction Temperature

To evaluate the effect of the extraction temperature, 0.250 g of coffee powder was extracted with 40 mL of 60% (v/v) methanol/water in the ultrasonic bath for 10 min. The temperature of the ultrasonic bath was controlled using the digital thermostat in the instrument to increase the temperature or by adding iced water to the bath to decrease it. It was maintained at the specified temperature ±2 °C and monitored by a digital thermometer. The compared temperatures were 4 °C, 20 °C, 50 °C, and 70 °C.

2.5.5. Effect of the Extraction Time

The effect of the extraction time was examined by extracting 0.250 g of coffee powder with 40 mL of 60% (v/v) methanol/water in the ultrasonic bath at 50 ± 2 °C for various durations. Due to the high extracting efficiency of ultrasonic waves, short extraction times were included. The extraction times tested here were 5, 10, 20, 30, 40, and 60 min.

2.5.6. Effect of the Solid-to-Solvent Ratio

For the purpose of optimizing the solid-to-solvent ratio, various coffee powder amounts were extracted by 40 mL of 60% (v/v) methanol/water in the ultrasonic bath at 50 ± 2 °C for 10 min. The solid-to-solvent ratios examined were 1:40 (1.000 g of coffee powder in 40 mL of extractant), 1:80 (0.500 g of coffee powder in 40 mL of extractant), 1:160 (0.250 g of coffee powder in 40 mL of extractant), and 1:320 (0.125 g of coffee powder in 40 mL of extractant).

2.5.7. Effect of the Grinding Treatments

There were various grinding treatments were applied to prepare green coffee beans powder for the analysis of CGAs, as reported in the literature. One of the main ones is freezing green coffee beans in liquid nitrogen or in freezers at −80 °C before grinding, and
the second is sieving with different mesh sizes. To evaluate the effect of these two steps in the procedure, the sample of green coffee beans was split into two parts for grinding. A portion was ground without adding liquid nitrogen, and the rest were ground with it in order to evaluate the effect of cooling with liquid nitrogen on the content of CGAs. The first portion was passed through a 600 micron sieve (VWR, West Chester, PA, USA). To study the influence of particle size on the content of CGAs, the second portion that was ground with liquid nitrogen was split into three parts to be sieved with different sizes. Three sieve sizes were used here: 1 mm (Gilson Co., Lewis Center, OH, USA); 600 µm and 425 µm (VWR, Radnor, PA, USA). The 425 µm and the 1-mm powders were only used to study the influence of particle size on the measured content of CGAs while the 600-µm powder was used for all the rest of the optimization experiments in this study. The extraction to evaluate the effect of grinding treatments was carried out in the ultrasonic bath at 50 ± 2 °C for 10 min using 0.250 g of coffee powder and 40 mL of 60% (v/v) methanol/water.

2.6. Statistical Analysis

The contents of CGAs for each treatment were presented as the mean ± standard deviation of five replicate extractions. The OriginPro 9.9 software (OriginLab Corporation, Northampton, MA, USA) was used to carry out the statistical analysis for all experimental data. One-way analysis of variance (ANOVA) and Tukey’s test was used to determine the differences among the means. p-values < 0.05 were considered to be significantly different.

3. Results

Natural sources that are rich in chlorogenic acids are recently receiving increased interest due to their health and nutrition benefits. As a result of this interest, more research activities are dedicated to the extraction, purification, and determination of these compounds in plants and other natural products [55]. However, due to the large number of naturally occurring CGAs with very similar properties and their instability during extraction, their analysis is usually challenging and requires careful optimization of the analytical procedure [9]. Therefore, this work aimed to investigate the effect of various extraction conditions and techniques on the measured levels of the three main naturally occurring CGAs in green coffee beans to develop an optimized method for the routine analysis of these compounds. The analysis was based on HPLC-DAD as the final separation and measurement technique. Hence, this work started with the evaluation of the HPLC separation of the three CGAs on various columns and with the use of different separation conditions.

3.1. Optimization of HPLC Separation Method

A number of HPLC separation methods for the analysis of CGAs in green coffee beans are reported in the literature. HPLC columns with different stationary phases and separation mechanisms were employed in the separation of CGAs. Most separation methods use low-pH mobile phases to increase the retention of CGAs, improve peak shape, and reduce tailing. HPLC columns with C18 stationary phase are more frequently used [6,13,15,17,30,40,56]. Therefore, the initial separation conditions in this work used a C18 column (Thermo Scientific Hypersil GOLD™, 150 × 4.6 mm, 5 µm) and a mobile phase acidified with formic acid. Different compositions of mobile phase, flow rates, and column temperatures were evaluated to optimize the separation of the three CGAs. However, despite the various separation conditions examined, 4-CQA did not have enough separation from the major compound, 5-CQA, at the optimum conditions for this column as is shown in the chromatogram in Figure 2.
Figure 2. HPLC chromatograms for the separation of the three CGAs on various columns. The elution order of peaks was 3-CQA, 5-CQA then 4-CQA in all columns except the biphenyl column where the order was 3-CQA, 4-CQA then 5-CQA. The upper chromatogram is for a green coffee bean extract separated on the biphenyl column.

A better separation was achieved on the C8 column (Thermo Scientific Acclaim™ 120, 150 × 2.1 mm, 3 µm) at the optimum separation conditions as shown in Figure 2. The compounds 4-CQA and 5-CQA were separated just to the baseline. However, this separation required about 20 min for the elution of the three CGAs only. The other strongly retained compounds found in the green coffee extracts will require more time in order to be eluted out of this column. The column will also require a re-equilibration step before the next injection. Therefore, it was decided to find a column with better efficiency that will provide the optimum resolution of the three CGAs in a shorter time.

HPLC columns with core-shell particles (also called superficially porous particles) can provide exceptional performance in terms of separation efficiency and speed compared to conventional fully-porous particles [57]. These columns are usually short (5 cm long) in order to provide fast separations and packed with sub-3 µm efficient core-shell particles [57,58].

Two columns with core-shell particles were examined here for the separation of the three main CGAs in green coffee beans extracts, a phenyl-hexyl column (Phenomenex Kinetex®, 50 × 4.6 mm, 2.6 µm) and a biphenyl column (Restek Raptor™, 50 × 3.0 mm, 2.7 µm). The phenyl-hexyl column provided enough separation for all the three CGAs but with a very low content of methanol in the mobile phase (5% v/v), which resulted in high retention times, above 20 min, for 4-CQA and 5-CQA as can be seen in Figure 2. This of course eliminated the main advantages of using core-shell columns. In contrast, the biphenyl column provided an exceptional performance for the separation of the three CGAs...
in terms of separation efficiency and speed. With 2.7 µm core-shell particles, the Raptor biphenyl column provided high resolution with very narrow peaks for all three CGAs in less than 8 min as shown in Figure 2. In addition, as can be seen in the chromatogram for the coffee extract in Figure 2, this column also provided a good separation of the three targeted CGAs from the other CGAs that are present at minor levels in the extracts of green coffee beans. The biphenyl stationary phase is known to provide greater aromatic selectivity compared to the phenyl-hexyl stationary phase [59]. The superior performance of this column has been reported previously for different separations [60,61]. Therefore, this column was selected in this study for all the subsequent HPLC analyses and for the final optimized method.

3.2. Optimization of the Extraction Procedure

The extraction procedure is a very crucial part of any analytical method used for the determination of CGAs. This step has a great effect on the levels of CGAs that are measured at the end of the analytical method. The levels of CGAs in green coffee beans reported in the literature have some wide discrepancies ranging from as low as 0.6% to up to 26.4% [12]. The extraction techniques and conditions can greatly affect the reported levels of the analytes and contribute to this high variation [3,18,50,51]. Therefore, it was vital to study the effect of the various steps in the extraction procedure in order to select optimum conditions.

3.2.1. Preliminary Extraction Experiments

Various extraction techniques and conditions were previously employed to analyze the CGAs in green coffee beans. However, most of the published literature do not include analytical comparisons or performance evaluations of the extraction procedures used. The majority are either based on previously published methods or using new extraction procedures without enough analytical evaluation of these methods in terms of extraction efficiency and analytical validity.

The optimization of the extraction procedure in this work started with a modification of previously published methods [6,42,50] using an ultrasonic bath as the extraction technique and the set of conditions detailed in Section 2.5.1. A simplification of the analytical methods can usually lead to an improvement in results [50]. Therefore, the various steps in the starting method were evaluated in order to simplify the method. The extraction was tested without centrifugation by weighing the coffee powder directly into a volumetric flask and conducting the extraction and Carrez clarification in the same flask. By doing this, the requirement to transfer the extract quantitatively from the centrifuge tube to the volumetric flask was eliminated. This led to an improvement in the measured amounts of the three CGAs in addition to achieving better precision between replicates. Therefore, all subsequent extractions were conducted directly in the volumetric flasks and without centrifugation.

After extraction, it is common to add a cleanup step, particularly for complex matrices such as plant and food extracts. However, because of the relatively high concentrations of the three CGAs in the green coffee bean extracts, in addition to the use of chromatographic techniques, simple cleanup procedures were adequate. Clarification using Carrez solutions [6,15,24,27,29–31,36,47] or lead acetate solution [35,43,45] are common practices. However, MgO is the clarification material used in the ISO 20481:2008 standard [54] for the determination of caffeine content in coffee. Therefore, it was decided to evaluate the possibility of using the caffeine extraction procedure as described in the ISO 20481:2008 standard for the simultaneous extraction of CGAs and caffeine. For this purpose, the extraction was conducted in a water bath set at a boiling temperature of 97 ± 2 °C for 30 min using water as the extractant, and MgO was compared to Carrez solutions as the clarification method. The result of this compassion is shown in Table 1.
Table 1. Effect of the clarification methods on the extracted amount of 3-CQA, 4-CQA, and 5-CQA.

| Treatment        | Extracted Amount (g/100g); Mean ± SD (n = 5) |
|------------------|-----------------------------------------------|
|                  |                  3-CQA          4-CQA          5-CQA          |
| MgO              | 0.018 ± 0.005 b 1 | 0.011 ± 0.003 b | 0.011 ± 0.001 b |
| Carrez Solutions | 0.807 ± 0.070 a  | 0.678 ± 0.086 a | 2.147 ± 0.207 a |

1 Means that do not share a letter are significantly different (p < 0.05).

The results shown in Table 1 clearly indicate that the MgO clarification method is not suitable for extracts intended for the determination of CGAs. The use of MgO removed almost all the extracted CGAs (97.8% of 3-CQA, 98.4% of 4-CQA and 99.5% of 5-CQA). This effect is in agreement with a previous report [62].

At this stage of the work, several scouting experiments were conducted to evaluate some extraction techniques and conditions in order to draw a comprehensive plan for the optimization of the extraction procedure as discussed below.

3.2.2. Effect of the Extraction Technique

Various classical or sophisticated instrumental extraction techniques are available for the solvent extraction of bioactive compounds from green coffee beans. However, when developing a routine analytical method, it is advantageous to use simple techniques that are available in most analytical laboratories. Therefore, five simple solvent extraction techniques were compared in this study. The simplest was by soaking coffee powder in the extractant at ambient room temperature without any mixing for 14 h. The other techniques were heating in a water bath at 50 ± 2 °C with occasional hand mixing, heating in a shaking water bath at 50 ± 2 °C with continuous mechanical linear shaking, orbital shaking at room temperature and, the last one was using ultrasonic-assisted extraction in an ultrasonic bath. All extractions were carried out in 50 mL volumetric flasks using the same extractant (40 mL of 40% [v/v] methanol/water mixture), 0.250 g of coffee powder, and for a duration of 30 min (except for soaking, 14 h). The results of this comparison are shown in Figure 3a–c for the three CGA compounds.

The five extraction techniques showed relatively comparable extraction efficiency for the three compounds at the used extraction conditions. However, the one-way ANOVA test showed that the soaking technique extracted significantly lower amounts of both 3-CQA and 5-CQA than the other four techniques. For 4-CQA, the lowest extraction efficiency was obtained by the shaking water bath. However, despite the statistically significant difference, all five extraction techniques provided enough extraction efficiency for the three CGAs to be used in developing a routine quantitative analytical method, yet each technique has its own advantages and limitations. Soaking is the simplest technique and does not require the analyst’s attendance. However, it requires the longest extraction time (14 h here). The other four techniques require the availability of extraction equipment. Nevertheless, the equipment required for the four extraction techniques (water bath, shaking water bath, shaker, and ultrasonic bath) is usually available in most analytical laboratories or can be obtained at reasonable costs.

Advanced extraction techniques that require the use of more sophisticated instruments such as microwave-assisted extraction [43,44], pressurized solvent extraction [45], and supercritical-fluid extraction [46] have also been used for the extraction of CGAs from coffee beans. However, in addition to the high cost of such instruments, they require careful optimization of more parameters to avoid the degradation and transformation of CGAs [63].

Ultrasonic-assisted extraction is getting more attention recently as a fast, effective, green, and economical extraction technique for routine methods. Conventional ultrasonic baths are cheap and readily available in most analytical laboratories, and they allow treating large numbers of samples simultaneously [64]. Due to the high extraction efficiency of this technique, extraction times and temperature, in addition to solvent consumption, can be substantially reduced [65,66]. Ultrasound waves help to improve the extraction rate by
increasing the mass transfer rates and possible rupture of the cell wall due to the formation of microcavities [66]. Several reports used analytical methods based on ultrasonic-assisted extraction for the determination of CGAs [4,37–42] but with limited details on the extraction parameters and their optimization. Therefore, this technique was selected for the rest of the detailed optimization experiments in this work.

Figure 3. Effect of the extraction techniques on the extracted amount of 3-CQA (a), 4-CQA (b), and 5-CQA (c). Each data point represents the mean of five measurements. Error bars represent the standard deviation of the measurements. Means that do not share a letter are significantly different ($p < 0.05$). Abbreviations: SONIC = ultrasonic-assisted extraction; WTR BTH = heating in a water bath at 50 ± 2 °C; ORP SHAKE = orbital shaking at room temperature; SHAKE WTR BTH = heating in a shaking water bath at 50 ± 2 °C; SOAKING = soaking coffee powder in the extractant at room temperature.

3.2.3. Effect of the Extraction Solvent

The extraction solvent (extractant) showed the greatest effect on the extraction efficiency for the three CGA compounds. An increase of up to 50% in the extracted amount of CGAs was obtained by just selecting the optimum extraction solvent. Six extraction
solvents were compared here after fixing all the other conditions. The extractants compared were water, 40% (v/v) methanol/water, 60% (v/v) methanol/water, methanol, 40% (v/v) ethanol/water, and 60% (v/v) ethanol/water. Water and alcoholic solvents were selected because CGA compounds contain several hydroxyl groups, and therefore, have high solubility in water and alcohols [67]. Figure 4a–c illustrate the extraction efficiency of the six extractants for the three CGA compounds.

Pure water and pure methanol extracted significantly lower amounts of the three CGA compounds while water mixtures with either methanol or ethanol provided better extraction efficiencies. The mixtures of 60% (v/v) methanol/water and 60% (v/v) ethanol/water extracted the highest amounts of the three acids with no significant difference between the two extractants. However, the HPLC chromatograms for the aqueous ethanolic extracts
showed broader and distorted peaks that may affect the reproducibility of the quantitative HPLC determinations. Figure 5 illustrates this effect for the 60% (v/v) ethanol/water extract compared to the aqueous methanolic extract. Aqueous methanolic extracts are more compatible with the mobile phases that are commonly used in HPLC. Therefore, a 60% (v/v) methanol/water mixture was selected as the optimum extractant for the rest of the optimization experiments. This extraction mixture was also found to be the most effective extractant for phenolic compounds (including chlorogenic acid) from apple peel and pulp [9,68]. Aqueous methanolic mixtures are the frequently reported extractants for chlorogenic acids in green coffee beans [4,6,47,48,50,69,70] probably due to their availability, good extraction efficiency, and compatibility with chromatographic methods used for the measurements.

![HPLC chromatograms of green coffee beans extracts in 60% (v/v) methanol/water and 60% (v/v) ethanol/water using the same separation method.](image)

**Figure 5.** HPLC chromatograms of green coffee beans extracts in 60% (v/v) methanol/water and 60% (v/v) ethanol/water using the same separation method.

3.2.4. Effect of the Extraction Temperature

Temperature is an important variable that governs the extraction efficiency of natural compounds from plants [71,72]. An optimized extraction temperature allows for better extraction efficiency and shorter extraction time [64]. Usually, higher temperatures help in increasing extraction efficiency, but it is important to choose an extraction temperature based on the target compounds to avoid their degradation [64]. Chlorogenic acids are known to easily transform/degrade, particularly in the presence of water. Heating of 5-CQA in the presence of water was reported to cause its isomerization to 3-CQA and 4-CQA, in addition to other transformations such as esterification and reaction with water [9,55,73,74]. These processes can occur during the extraction of CGAs from plant materials at elevated temperatures [55], and therefore, it is vital to optimize the extraction temperature to avoid such effects.

To evaluate the effect of the extraction temperature, the temperature of the ultrasonic bath was controlled at the specified temperature ±2 °C and monitored by a digital thermometer. Temperatures of 4 °C, 20 °C, 50 °C and 70 °C were compared in this study. This comparison is demonstrated in Figure 6a–c.

Figure 6 shows that the optimum temperature that extracted the highest amounts of CGAs was 50 °C. At this temperature, significantly higher amounts of 5-CQA were extracted compared to all the other examined temperatures. For 3-CQA and 4-CQA, extraction at 70 °C shows no significant difference from 50 °C. However, due to the possible risk of 5-CQA degradation/transformation at higher temperatures, in addition to the ease of maintaining the ultrasonic bath at 50 °C, this temperature was selected as the optimum temperature in the extraction of the three CGAs. It should be noted that most commercial ultrasonic baths with temperature controls have temperature ranges from ambient to 80 °C, but during continuous running, the water temperature in the ultrasonic bath increases...
since the ultrasonic irradiation of a liquid produces heat [75]. This effect is greater at lower temperatures compared to higher ones, such as 50 °C, and increases with longer running times. An increase of 10 to 15 °C above the set temperature over a one-hour run is common [72]. This was noticed in one of the preliminary experiments conducted in this study where the temperature of the ultrasonic bath was not controlled. In this experiment, the temperature was found to rise from 18 °C to as high as 31 °C over a period of 30 min. In contrast, when the ultrasonic bath temperature was set at 50 °C, the maximum temperature rise was only 5 °C over the 30 minute runtime. Therefore, the extraction temperature of 50 °C was selected for the subsequent optimizations, and extraction time was optimized to be as short as possible in order to minimize the effect of the temperature variations.

Figure 6. Effect of the extraction temperature on the extracted amount of 3-CQA (a), 4-CQA (b), and 5-CQA (c). Each data point represents the mean of five measurements. Error bars represent the standard deviation of the measurements. Means that do not share a letter are significantly different (p < 0.05).
3.2.5. Effect of the Extraction Time

One of the main advantages of ultrasonic-assisted extraction that attracted researchers and analysts is the reduction in extraction time and the higher processing throughput compared to conventional extraction methods [64,76]. The typical extraction time for this technique is 10 to 60 min instead of the several hours required by conventional techniques such as Soxhlet extraction or mechanical agitation [77]. Therefore, due to the high extracting efficiency of ultrasonic waves, short extraction times were compared in this study. The extraction times examined here were 5, 10, 20, 30, 40, and 60 min, and their influence is demonstrated in Figure 7a–c.

Figure 7. Effect of the extraction time on the extracted amount of 3-CQA (a), 4-CQA (b), and 5-CQA (c). Each data point represents the mean of five measurements. Error bars represent the standard deviation of the measurements. Means that do not share a letter are significantly different ($p < 0.05$).
The results in Figure 7 show that 10 min duration was enough to provide efficient extraction of the three CGA compounds. Longer times (20, 30, 40, and 60 min) showed no significant increase in the extracted amounts of the three CGAs. Therefore, this amount of time was selected as the optimum extraction time. A number of previous reports used extraction times close to this optimum time [4,37,41]; though, some methods reported much longer extraction times despite using ultrasonic-assisted extraction [38,39,42,78].

3.2.6. Effect of the Solid-to-Solvent Ratio

The solid-to-solvent ratio was the second most effective variable on the extraction efficiency of the three CGAs. By optimizing this variable, an increase of up to 34% in the extracted amount of CGAs was achieved. Solid-to-solvent ratios of 1:40 (1.000 g of coffee powder in 40 mL extractant), 1:80 (0.500 g of coffee powder in 40 mL extractant), 1:160 (0.250 g of coffee powder in 40 mL extractant) and 1:320 (0.125 g of coffee powder in 40 mL extractant) were compared here. Figure 8a–c demonstrates the effect of these different ratios on the amount of CGAs extracted.

Figure 8 shows that the decrease in the solid-to-solvent ratio provided significantly higher extraction efficiency up to the ratio of 1:160 (0.250 g of coffee powder in 40 mL extractant); that was the optimum ratio. The ratio of 1:320 extracted significantly lower amounts of the three CGA compounds, which indicates that the optimum solid-to-solvent ratio is 1:160. This effect can be explained by the higher concentration gradient of the 1:160 solid-to-solvent ratio compared to the other ratios of 1:40 and 1:80. When increasing the quantity of solvent used to extract a fixed amount of the solid, the concentration gradient between the interior of the plant cells and the external solvent will increase, and hence, a faster extraction rate can be expected. However, adding more solvent will make the solution very diluted. Since an increased quantity of solvent would not lead to a sufficient increase in the concentration gradient, the increase in extraction yield would be limited [79,80].

A very wide range of the solid-to-solvent ratios was reported in the literature for methods using ultrasonic-assisted extraction for the extraction of CGAs from green coffee beans, starting from 1:10 (g/mL) to up to 1:714 (g/mL) [4,37–42,78]. This shows the importance of optimizing the solid-to-solvent ratio during the development of routine methods. In addition to achieving the highest extraction efficiency, the optimization of the solid-to-solvent ratio will also help in the efficient use of solvents for a greener method, also reducing the cost of the disposal of solvents.

3.2.7. Effect of the Grinding Treatments

Size reduction and homogenization are common practices in the sample preparation procedures for food analysis. Analytical methods for the analysis of bioactive compounds (including CGAs) in green coffee beans usually include grinding and sieving of the ground coffee beans to obtain a homogenous coffee powder with standardized particle size. Various grinding techniques and practices were used in the preparation of green coffee powder. Freezing with liquid nitrogen [4,13,21,48–50,70,81–83] or in freezers at $−80{^\circ}C$ [42,84,85] before grinding were reported to facilitate the grinding of green coffee beans [42] and also to minimize the degradation of CGAs due to the heat generated during this process [36,50,86]. After grinding, sieves of different mesh sizes ranging from 0.046 mm to up to 2 mm were used to standardize the ground green coffee powder used for the extraction of CGAs [21,25,48,84].

To evaluate the effect of these practices on the content of extracted CGAs, the sample of green coffee beans was split into two portions; one was ground without adding liquid nitrogen, and the other was ground with liquid nitrogen. To study the effect of particle size on the content of CGAs, the second portion that was ground with liquid nitrogen was split into three parts for sieving with different mesh sizes, namely, 425 µm, 600 µm, and 1 mm. The effect of these grinding practices is compared in Figure 9a–c.
Figure 8. Effect of the solid-to-solvent ratio on the extracted amount of 3-CQA (a), 4-CQA (b), and 5-CQA (c). Each data point represents the mean of five measurements. Error bars represent the standard deviation of the measurements. Means that do not share a letter are significantly different (p < 0.05).
Figure 9. Effect of the grinding treatments on the extracted amount of 3-CQA (a), 4-CQA (b), and 5-CQA (c). Each data point represents the mean of five measurements. Error bars represent the standard deviation of the measurements. Means that do not share a letter are significantly different ($p < 0.05$). The abbreviation “No N2” = coffee beans ground without adding liquid nitrogen.

The results in Figure 9 show that the compared grinding treatments extracted similar amounts of the three CGAs with no statistically significant difference except for the 450 µm
sieved coffee powder that showed the significantly lowest content of the CGAs. This result was surprising as size reduction is usually used in sample preparation to increase the total surface area of the particles, and, hence, enhance the extraction efficiency by increasing surface contact between the solid and the extractant. The lower content of CGAs extracted from the 450 µm sieved coffee powder is probably because the distribution of CGAs inside the coffee bean is not homogenous. Therefore, the contribution of certain parts of the beans that may contain less CGAs (e.g., the silverskin) in the 450 µm sieved fraction is more. Some authors related such an effect to the possibility of the agglomeration and compactness of finely ground solids during the extraction process and, therefore, reducing the contact between the solid and the extractant, which results in lower extraction efficiency [87–89]. However, more work is required to explain this in more detail.

It was interesting to find that freezing green coffee beans with liquid nitrogen has no significant effect on the extracted amounts of CGAs, contrary to what was previously reported in the literature [36,50,86]. The use of liquid nitrogen or the freezing of green coffee beans before grinding was suggested in methods developed about two decades ago. It is possible that the grinding machines used at that time required more time to produce finely ground coffee powder and, therefore, generate more heat compared to modern high-speed grinders. Omitting this sample preparation treatment will help in simplifying the analytical method. Nevertheless, freezing green coffee beans does help in facilitating the grinding process, particularly with certain grinding techniques.

3.3. Optimized Method for the Quantitative Determination of CGAs

At the end of the thorough investigation of the effect of the main factors on the extraction of the three dominant CGAs in green coffee beans—namely 3-CQA, 4-CQA and 5-CQA—the following method can be suggested for the routine quantitative determination of CGAs in green coffee beans.

Green coffee beans are ground in a suitable grinder (with liquid nitrogen if needed to facilitate the grinding process). The ground coffee is passed through a 600 µm sieve, then a test sample of 0.250 g is taken for extraction. Three replicates should be prepared for each coffee sample. Each test sample is transferred into a 50 mL volumetric flask, and about 40 mL of 60% (v/v) methanol/water mixture is added. The flask is placed in an ultrasonic bath maintained at a temperature of 50 °C and extracted for 10 min. The extract is then clarified by adding 0.5 mL of each of Carrez solutions I and II. The flask is then made up to volume with distilled water and kept standing for about 10 min to obtain the clear extract. Finally, a portion is filtered through a 0.45 µm membrane syringe filter into an HPLC vial and used for HPLC analysis. The sample is analyzed using the HPLC separation method detailed in Section 2.4.

4. Conclusions

This work reveals the importance of reviewing old analytical methods and updating them based on the recent advances in analytical technologies. Simplifying analytical methods is helpful in achieving better performance and higher throughput. Though there was a large number of published analytical methods that were employed for the determination of CGAs in green coffee beans, the discrepancy in the reported results and the wide range of the content of CGAs are indications of the necessity for a standardized analytical method. Therefore, this study was dedicated to investigating the effect of the main variables influencing the extraction of three CGAs (3-CQA, 4-CQA and 5-CQA) from green coffee beans in order to optimize them and develop a simple method that is suitable for routine analysis. Five simple extraction techniques with affordable equipment were compared and found to provide relatively similar extraction efficiencies for the three compounds. Ultrasonic-assisted extraction was selected for the optimization of the rest of the variables due to its merits for routine analysis. The extraction solvent and the solid-to-solvent ratio were found to have the greatest influence on the extraction efficiency compared to the other variables such as the extraction temperature, time, and grinding treatments.
By optimizing these variables, an increase of up to 50% in the extracted amounts of CGAs can be achieved, which proves the importance of optimizing the extraction procedure for accurate and consistent results. Optimized methods will also help to reduce the analysis time, effort, and cost, which are important factors in a routine analytical method.

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