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The Antioxidant and Chlorogenic Acid Profiles of Whole Coffee Fruits Are Influenced by the Extraction Procedures

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ABSTRACT: Commercial whole coffee fruit extracts and powder samples were analyzed for chlorogenic acids (CGA), caffeine and antioxidant activities. CGA and caffeine were characterized by LC−MS^n and HPLC accordingly, and quantified by UV absorbance. ORAC, HORAC, NORAC, SORAC and SOAC (antioxidant capacities) were assessed. Three caffeoylquinic acids, three feruloylquinic acids, three dicaffeoylquinic acids, one *p*-coumaroylquinic acid, two caffeoylferuloylquinic acids and three putative chlorogenic lactones were quantified, along with a methyl ester of 5-caffeoylquinic acid (detected in one sample, the first such report in any coffee material). Multistep whole coffee fruit extracts displayed higher CGA content than single-step extracts, freeze-dried, or air-dried whole raw fruits. Caffeine in multistep extracts was lower than in the single-step extracts and powders. Antioxidant activity in whole coffee fruit extracts was up to 25-fold higher than in powders dependent upon the radical. Total antioxidant activity of samples displayed strong correlation to CGA content.

KEYWORDS: chlorogenic acid, antioxidant activity, coffee fruits, green coffee, extract, caffeine

INTRODUCTION

Chlorogenic acids are a family of esters formed between quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, ferulic and *p*-coumaric.[1] Structures of some of the major components are shown in Figure 1. It is now known that chlorogenic acids are widely distributed in plants[1] but the coffee bean is remarkably rich (up to *ca.* 10% dry basis) including at least 45 chlorogenic acids that are not acylated at C1 of the quinic acid moiety.[2] Coffee beans also contain up to seven cinnamoyl−amino acid conjugates.[3]

There have been extensive studies of chlorogenic acid transformation during coffee roasting,[1] and it is well-established that they are destroyed progressively with increasing roast severity, but even the darkest roasts retain a significant amount. For people who consume coffee beverages regularly, whether prepared from soluble ("instant") powders or freshly brewed, coffee will almost certainly be their major dietary source of chlorogenic acids, with daily CGA intakes of 500 mg or even more being easily achieved.[1]

Due to growing evidence that diets rich in phenols and polyphenols may have potential health benefits for consumers, the nutritional supplement and food industries have been developing numerous products that feature enhanced phenolic content. In some cases, these higher phenolic sources have been represented as being "natural additives" when in fact they have been recovered from food-processing wastes or byproduct. Generic soluble green coffee extracts are readily available to use in encapsulated supplements, in ready-to-drink beverages, or in conventional soluble coffee products in order to enhance CGA content. However, in contrast to green and roasted coffee beans and instant coffees, there are scant data for the chlorogenic acid contents of whole coffee fruit ("whole fruit" meaning the husk, the pulp, the mucilage and the seed or "bean" inclusive) and of soluble whole coffee fruit extracts.[4]

To date, we are unaware of any studies comparing effects of the manufacturing process and the nature of the raw materials on the compositions of the resultant extracts and coffee fruit powders. Accordingly, we report here the analysis of the CGA contents of these coffee fruit extracts and powders, along with their various respective antioxidant scavenging properties. The antioxidant activity of any foodstuff or beverage is a good indicator of its phenolic content. The use of any single method for measurement of antioxidant activity can yield rather misleading results[5] (for example, carotenoids appear to be poor at scavenging peroxyl radicals, but score highly for quenching of singlet oxygen species). Here, we used five different assays to demonstrate the ability of the extracts to scavenge a range of free radical species commonly found *in vivo*. Industry-standard methods were used for measurement of radical scavenging for peroxyl radicals (ORAC), hydroxyl radicals (HORAC), peroxynitrite radicals (NORAC), super oxide anions (SORAC) and singlet oxygen (SOAC). Since the whole coffee fruit extracts and powders are intended for use as nutritional supplements or as food additives, the caffeine levels were determined, keeping in mind the known guidelines for recommended daily intake, especially in pregnant women. The influence of different extraction methods on the CGA and the caffeine content of the extracts, as well

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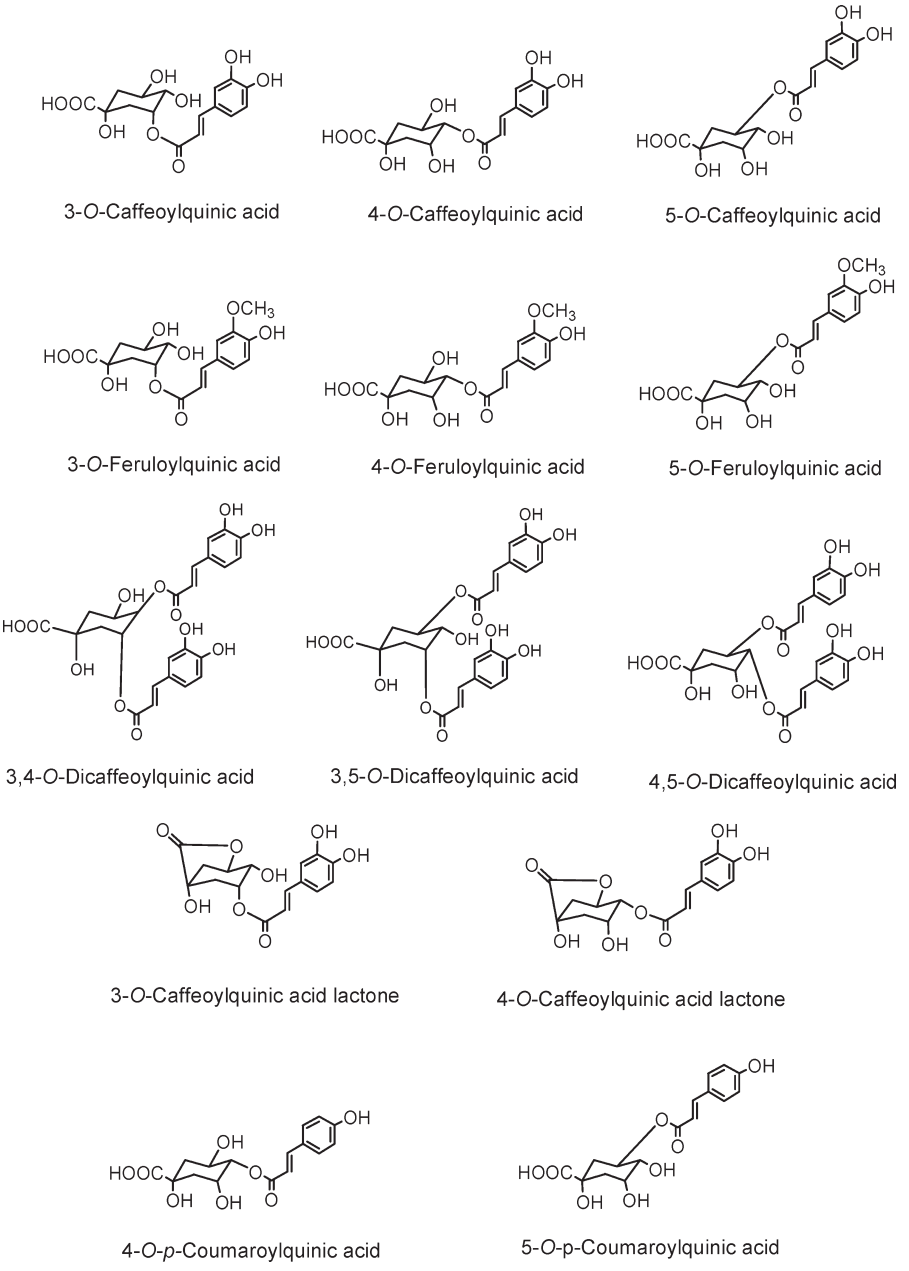

Figure 1. Structure of major hydroxycinnamates detected in coffee fruit samples.

as their antioxidant activities, was further documented in this study.

MATERIALS AND METHODS

Chemicals. 5-*O*-Caffeoylquinic acid was obtained from AASC Ltd. (Southampton, U.K.). Methanol and acetonitrile were obtained from Rathburn Chemicals (Walkburn, Scotland). Formic acid was obtained from Fisher Scientific (Loughborough, U.K.). All coffee samples, commercially marketed as "CoffeeBerry" whole coffee fruit products, were supplied by FutureCeuticals, Inc. (Momence, IL, USA). Caffeine primary standard was obtained from USP (Rockville, MD, USA). Perchloric acid (HPLC grade), acetonitrile (HPLC grade), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and dihydrorhodamine 123 (DHR-123) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). 3-Morpholinosydnonimine, hydrochloride (SIN-1) was purchased from Toronto Research Chemicals (North York, ON, Canada). Primary Sorbent Amine (PSA) was obtained from Supelco Inc. (Bellefonte, PA, USA). Hydroethidine fluorescent stain (5-ethyl-5,6-dihydro-6-phenyl-3,8-phenanthridinediamine) was purchased from Polysciences, Inc. (Warrington, PA, USA).

Whole Coffee Fruit Samples. The whole coffee fruit based materials (*Coffea arabica*) were supplied as (a) samples extracted using a proprietary multistep ethanol extraction and purification method (coffee fruit extracts 1, or CFE-1); (b) samples extracted with a proprietary single-step ethanol extraction and purification method and supplied as powders (coffee fruit extracts 2, or CFE-2); (c) commercially available air-dried intact whole coffee fruits that were subsequently crushed and sieved to produce a fine powder and prepared by dissolving in 50% ethanol/water (coffee fruit powder 1, or CFP-1); (d) freeze-dried whole coffee fruits that were ground in the laboratory and prepared by extraction with 50% ethanol/water (coffee fruit powder 2, or CFP-2).

All coffee samples were supplied by FutureCeuticals, Inc. (Momence, IL, USA); samples CFE-1, CFE-2 and CFP-1 are commercially available and marketed as "CoffeeBerry" whole coffee fruit products.

Sample Preparation for Chlorogenic Acid Analysis. Three ca. 10 mg aliquots of each sample CFE-1, CFE-2 and CFP-1 were accurately weighed into a 10 mL volumetric flask. The volume was made up to the mark with 50% aqueous ethanol. All samples were thoroughly mixed and then five 1 mL aliquots removed into Eppendorf vials and centrifuged at 13500*g* for 10 min. Aliquots of the supernatant were taken and diluted 10-fold with 50% aqueous methanol prior to analysis.

Approximately 5 g of freeze-dried whole coffee fruits was ground to a fine powder in a coffee grinder (CFP-2). Three aliquots (between 10 and 20 mg) of CFP-2 were accurately weighed into 15 mL centrifuge tubes. The powders were then repeatedly extracted with 50% ethanol/water, until no CGAs could be detected in extraction solvent (seven extraction replicates). The final volume of the extracts was made up to 50 mL and then analyzed directly.

Chlorogenic Acid Measurement by HPLC-PDA−MS^2. Analysis was carried out on a Thermo Surveyor HPLC system (Thermo Fisher Scientific, Hemel Heampstead, U.K.) comprising an autosampler with sampler cooler maintained at 6 °C and a photodiode array detector scanning from 200 to 600 nm. Samples (5 or 10 μL) were injected onto a 250 × 2.0 mm C_{18} RP polar column (Phenomenex, Macclesfield, U.K.) maintained at 40 °C and eluted with a 5−10−35% gradient of 1.0% formic acid and acetonitrile at 300 μL/min over 0−20−60 min. After passing through the absorbance detector, the eluant was directed to the electrospray interface of the Advantage ion trap mass spectrometer (Thermo Fisher Scientific, Hemel Hampstead, U.K.). Samples were run in negative ionization mode. The scan range was from 150 to 750 amu. Identifications were based on cochromatography using authentic standards, where available, absorbance spectra, and published MS^2 mass spectral data.[2,6] Quantification was by comparison to an authentic standard of 5-*O*-caffeoylquinic acid, range 5 to 750 ng at 325 nm.

Standard and Sample Preparation for Caffeine Analysis. An aliquot of about 10 mg of caffeine primary standard was accurately weighed into a 50 mL volumetric flask. The volume was made up to 25 mL with mobile phase (90% of 0.1% perchloric acid and 10% acetonitrile) and sonicated for 5 min. The final volume was made to the mark with mobile phase. About 500 mg of each sample was weighed into a 100 mL volumetric flask. The mobile phase was added up to 50 mL and sonicated for 5 min. The supernatant was then diluted to the mark with mobile phase. About 1 g of PSA was weighed into a centrifuge tube. The prepared sample (5 mL) was dispensed into a tube containing PSA, vortexed for 3 min and filtered through a 0.45 μm PTFE syringe filter for analysis.

Caffeine Analysis by HPLC-DAD. Analysis was carried out on a HPLC Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector at 275 nm. Samples (5 or 10 μL) were injected onto a 150 × 3.0 mm, 2.7 μm Supelco Ascentis Express Phenyl-Hexyl Column (Supelco Inc., Bellefonte, PA) maintained at 25 °C and eluted with a 90−10% isocratic of 0.1% perchloric acid and acetonitrile at 800 μL/min over 15 min. Quantification was by comparison to an authentic primary standard of caffeine.

Sample Preparation for Antioxidant Measurements. Approximately 20 mg of each sample was weighed and extracted with 20 mL of ethanol/water (70:30 v/v) for 1 h at room temperature on an orbital shaker. The extracts were centrifuged at 5900 rpm, and the supernatant was used for the total antioxidant capacity assay. The total antioxidant capacity was determined by calculating the sum of the individual results against five free radicals, namely, peroxyl radicals, hydroxyl radicals, peroxynitrite, superoxide anions, and singlet oxygen. All results were expressed as μM Trolox equivalent per gram (μM TE/g). Ethanol (HPLC grade) and 6-hydroxy-2,5,7,8 tetramethyl-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Peroxyl Radical Scavenging Capacity (ORAC assay). The ORAC assay was conducted on the basis of a report by Ou and co-workers,[7] modified for the FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The FL600 microplate fluorescence reader was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The plate reader was controlled by software KC4 3.0. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) is used as the source for the peroxyl radical, which is generated as a result of the spontaneous decomposition of AAPH at 37 °C. AAPH was obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA). Fluorescein is the chosen fluorescent marker attached to a protein, with loss of fluorescence an indicator of the extent of damage from its reaction with the peroxyl radical. The protective effect of the antioxidants is measured by comparing the fluorescence time/intensity area under the curve of the sample compared with a control assay with no antioxidant compounds present. Trolox, a water-soluble analogue of vitamin E, was used as the calibration standard. Fluorescence readings are taken every min for up to 35 min after the addition of AAPH.

Hydroxyl Radical Scavenging Capacity (HORAC Assay). The assay is based on a report by Ou and co-workers and modified for the FL600.[8] Fluorescein (FL) was used as the probe. The fluorescence decay curve of FL is monitored in the absence or presence of antioxidants, the area under the fluorescence decay curve (AUC) is then integrated, and the net AUC is calculated by subtracting the AUC of the blank from that of the sample antioxidant.

Peroxynitrite Scavenging Capacity (NORAC Assay). Peroxynitrite ($ONOO^-$) scavenging was measured by monitoring the oxidation of DHR-123 according to a modification of the method of Chung et al.[9] Briefly, a stock solution of DHR-123 (5 mM) in dimethylformamide was purged with nitrogen and stored at −80 °C. A working solution with DHR-123 (final concentration, fc, 5 μM) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 μM (fc) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. $ONOO^-$ scavenging by the oxidation of DHR-123 was measured with a microplate fluorescence reader FL600 with excitation and emission wavelengths of 485 and 530 nm, respectively, at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with or without SIN-1 (fc 10 μM) or authentic $ONOO^-$ (fc 10 μM) in 0.3 N sodium hydroxide. Oxidation of DHR-123 by decomposition of SIN-1 gradually increased, whereas authentic $ONOO^-$ rapidly oxidized DHR-123 with its final fluorescent intensity being stable over time.

Superoxide Anion Scavenging Assay (SORAC Assay). The SORAC assay was based on the previously described method by Zhang and co-workers.[10] Simply, hydroethidine was used as a probe in measuring $O_2^{-\bullet}$ scavenging capacity. Nonfluorescent hydroethidine was oxidized by $O_2^{-\bullet}$ generated by the mixture of xanthine and xanthine oxidase to form a species of unknown structure that exhibit strong fluorescence signal at 586 nm. Addition of SOD inhibits the hydroethidine oxidation.

Singlet Oxygen Scavenging Assay (SOAC Assay). The SOAC assay was based on the previously described method by Zhang and co-workers.[10] Singlet oxygen was generated from the mixture of H_2O_2 and MoO_4^{2-}. Hydroethidine was used as a probe to singlet oxygen. Hydroethidine was prepared in *N*,*N*-dimethylacetamide (DMA) to make 40 μM solution, 2.635 mM Na_2MoO_4 and 13.125 mM H_2O_2 working solutions were also prepared in DMA. HE solution (125 μL) was added to a well followed by addition of 25 μL of 2.635 mM $Na_2MoO_4^{2-}$ and 25 μL of 13.125 mM H_2O_2, respectively. The plate was then transferred to a microplate fluorescence reader FL600 with excitation and emission wavelengths of 530 and 620 nm to record the change of fluorescence

Table 1. Peak Numbers, Spectral Properties and Identities of Compounds in Coffee Fruit Samples

peak no.	t_R	$[M - H]^-$ (m/z)	MS^2 (m/z)	compd
1	8.5	353	191, 179	3-*O*-caffeoylquinic acid
2	15.0	353	173, 179	4-*O*-caffeoylquinic acid
3	15.8	353	191	5-*O*-caffeoylquinic acid
4	17.5	367	193 (191 trace)	3-*O*-feruloylquinic acid
5	24.6	337	173, 193	5-*O*-*p*-coumaroylquinic acid
6	27.9	367	191, 173	4-*O*-feruloylquinic acid
7	28.4	335	161, 135, 179	putative 3-*O*-caffeoylquinic lactone
8	29.7	335	161, 135, 179	putative 4-*O*-caffeoylquinic lactone
9	30.6	367	191	5-*O*-feruloylquinic acid
10	32.9	367	179	unknown: putative methyl-5- caffeoylquinic acid
11	42.1	515	353	3,4-*O*-dicaffeoylquinic acid
12	43.7	515	353	3.5-*O*-dicaffeoylquinic acid
13	45.9	515	353	4,5-*O*-dicaffeoylquinic acid
14	48.5	529	367, 335, 193, 179	3-*O*-feruloyl-4-*O*-caffeoylquinic acid
15	52.2	529	353, 367, 335, 173	4-*O*-caffeoyl-5-*O*-feruloylquinic acid
16	54.4	497	335, 161	putative dicaffeoylquinic lactone

intensity at 37 °C for 35 min. Addition of the samples inhibits the oxidation of hydroethidine induced by singlet oxygen.

Data Analysis. All compounds analyzed are presented as mean ± SD. Data normality was tested using the Shapiro−Wilk test in SPSS (PASW Statistics 18, SPSS Inc.). All data but NORAC, ORAC and total ORAC were normally distributed. Relationships between chlorogenic content, caffeine content and antioxidant capacities were tested using Spearman's correlation. Differences between extraction methods were tested using Kruskall−Wallis for nonparametric variables, followed by the Mann−Whitney *U*-test for pairwise comparisons (adjusted for Bonferroni's correction), and with ANOVA for parametric variable (with Tamhane post hoc test).

RESULTS

Identification of Chlorogenic Acids and Related Compounds. The compounds described here are listed in Table 1, the chromatographic separation is shown in Figure 2 and the mass spectral data used to identify these compounds are shown in Figure 3.

Peaks 1, 2 and 3. All had similar absorbance spectra with λ_{max} at 325 nm. Mass spectral analysis revealed they also had the same negatively charged molecular ion ($[M - H]^-$) at m/z 353. MS^2 fragmentation produced 3 different mass spectra (Figure 4) which allowed identification of these compounds as the 3-, 4- and 5-*O*-caffeoylquinic acid respectively.[6] In addition, peak 3 cochromatographed with the authentic standard.

Peaks 4, 6, 9 and 10. All had a $[M - H]^-$ at m/z 367 indicating the presence of feruloylquinic acids. In MS^2 analysis peak 4 fragmented to produce a base ion at m/z 193 with no other major ions present (Figure 5). This pattern is in keeping with a 3-*O*-feruloylquinic acid conjugate. Peak 6 fragmented to produce a base ion at m/z 173 with another major ion at m/z 193. This pattern is in keeping with a 4-*O*-feruloylquinic acid. In MS^2 the parent ion in peak 9 produced a single fragment at m/z 191 which is in keeping with that previously described for 5-*O*-feruloylquinic acid.[6] Peak 10 was only found in coffee fruit extract CFE-1. MS^2 fragmentation of the m/z 367 parent ion produced a daughter ion at m/z 179, which is indicative of the presence of a caffeoyl-containing compound, rather than a feruloylquinic acid conjugate. The identity of this compound is unknown, but its chromatographic and mass spectral behavior corresponds to the methyl ester of 5-caffeoylquinic acid, and is quite different from that observed for the methyl esters of 3-caffeoylquinic acid and 4-caffeoylquinic acid (N. Kuhnert, personal communication and ref 11). Such methyl esters have been reported in various plants and are of some pharmacological interest.[12,13]

Peak 5. This peak had a $[M - H]^-$ parent ion at m/z 337 and was a minor component in coffee fruit samples CFE-1 and CFP-1. An additional analysis of a more concentrated sample revealed the MS^2 fragmentation only produced an ion of m/z 191, typical of 5-*O*-*p*-coumaroylquinic acid. This compound has previously been reported in coffee bean extracts.[6]

Peaks 7 and 8. Both produced a $[M - H]^-$ at m/z 335. Both also had base daughter ions in MS^2 at m/z 161 and minor ions at m/z 135 and 179. These data would indicate that these compounds are putative caffeoylquinic lactones. The height of the daughter ions relative to the base ion would suggest that peak 7 is the 3-caffeoyl-γ-quinide and peak 8 the 4-caffeoyl-γ-quinide, as previously reported.[14]

Alternatively, there is a chance that these compounds are caffeoyl shikimates since both classes of compounds show similar retention times and MS spectra.[15]

Peaks 11, 12 and 13. All had a $[M - H]^-$ at m/z 515 indicating the presence of dicaffeoylquinic acids. Fragmentation spectra in MS^2 analysis and elution profile matched that previously seen in coffee fruit extracts.[6] The identity of peak 11 was 3,4-*O*-dicaffeoylquinic acid, peak 12 was the 3,5-*O*-dicaffeoylquinic acid and peak 13 the 4,5-*O*-dicaffeoylquinic acid

Peaks 14 and 15. Both had the same $[M - H]^-$ at m/z 529. This parent ion is indicative of a caffeoyl-feruloyl quinic acid, of which six have been previously reported. Fragmentation spectra in MS^2 analysis revealed the base daughter ion in peak 14 to be m/z 367 with additional minor fragment ions at m/z 335, 193, and 173. This would suggest that this is the 3-*O*-feruloyl-4-*O*-caffeoylquinic acid. Peak 15 differed in its MS^2 spectra in that the base daughter ion was m/z 353 with minor ions at m/z 367, 335, and 173 indicating that this peak may be 4-*O*-caffeoyl-5-*O*-feruloylquinic acid. Like peaks 7 and 8 without authentic standards this identification must be seen as tentative.

Peak 16. This peak had a $[M - H]^-$ at m/z 497. Like peaks 7 and 8 this parent ion is typical of that seen in a dicaffeoylquinic

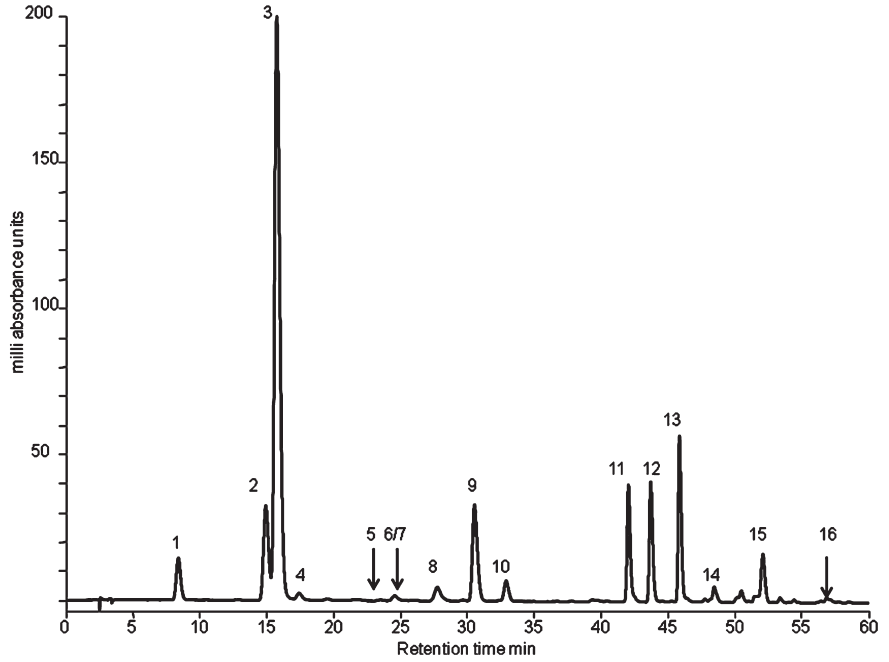

Figure 2. Gradient reverse phase HPLC absorbance analysis of coffee fruit samples, containing peaks 1 to16 listed in Table 1.

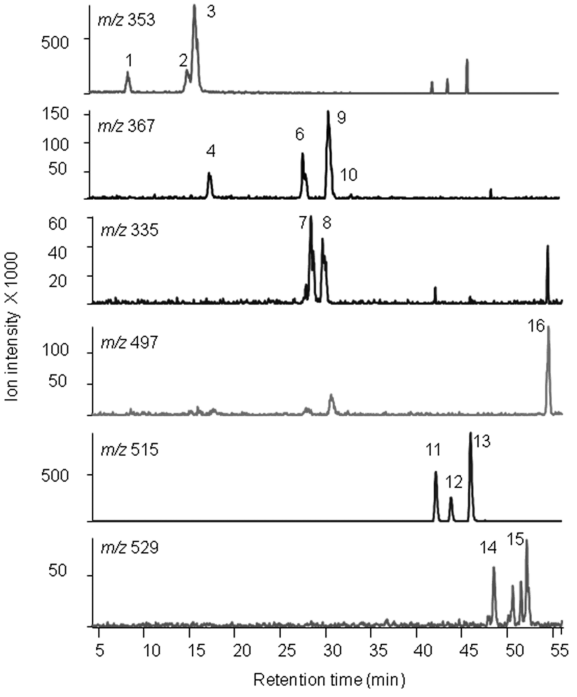

Figure 3. Gradient reverse phase HPLC mass spectrometric analysis of coffee fruit samples.

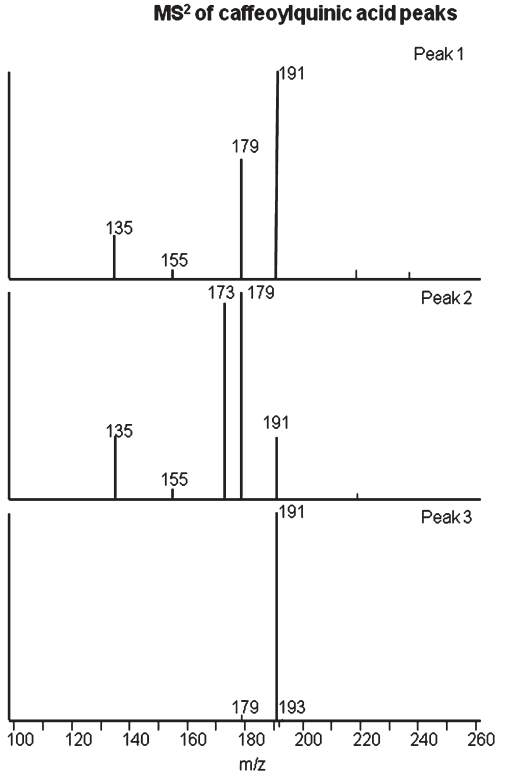

Figure 4. MS^2 analysis of caffeoylquinic acid in peaks 1−3.

lactone. Fragmentation in MS^2 analysis revealed daughter ions at m/z 335 and 161 which are in keeping with the ions seen in the monocaffeoylquinic lactones in peaks 7 and 8. Peak 16 was therefore identified as a dicaffeoylquinic lactone. Alternatively, there is a possibility that this compound is a dicaffeoyl shikimate since both classes of compounds show similar retention times and MS spectra.[15]

Quantification of Chlorogenic Acids and Caffeine in Coffee Fruit Extracts. Quantitative analysis of the chlorogenic acid compounds corresponding to peaks 1 to 16 is summarized in Table 2 (expressed in mg/g 5-CQA equivalent ± SD). Samples extracted using the multistep purification (CFE-1) had the highest CGA content (765 ± 99 mg/g), followed by the single-step samples (CFE-2, 450 ± 66 mg/g), air-dried whole coffee fruit powders (CFP-1, 88 ± 14 mg/g, and freeze-dried coffee fruits, CFP-2, 44 ± 5 mg/g) (Figure 6). 5-*O*-Caffeoylquinic acid

was the main chlorogenic acid present in all extracts, representing between 37 and 69% of the total chlorogenic acid content. Specific chlorogenic acids, including 3-*O*-caffeoylquinic lactone, 4-*O*-caffeoylquinic lactone, the putative methyl-5-caffeoylquinic acid and dicaffeoylquinic lactone, were detected only in the multistep proprietary coffee fruit extracts (CFE-1). 3-*O*-Feruloylquinic acid was only detected in proprietary CFE-1 and CFE-2. 4,5-*O*-Dicaffeoylquinic acid was present in higher proportions in proprietary extracts (8.4 ± 0.4% and 7.5 ± 0.1% of total chlorogenic acid content for CFE-1 and CFE-2, respectively) compared to air-dried coffee powders and coffee fruits (5.4 ± 0.2% and 1.6 ± 0.2% of total chlorogenic acid content for CFP-1 and CFP-2, respectively) ($p < 0.05$).

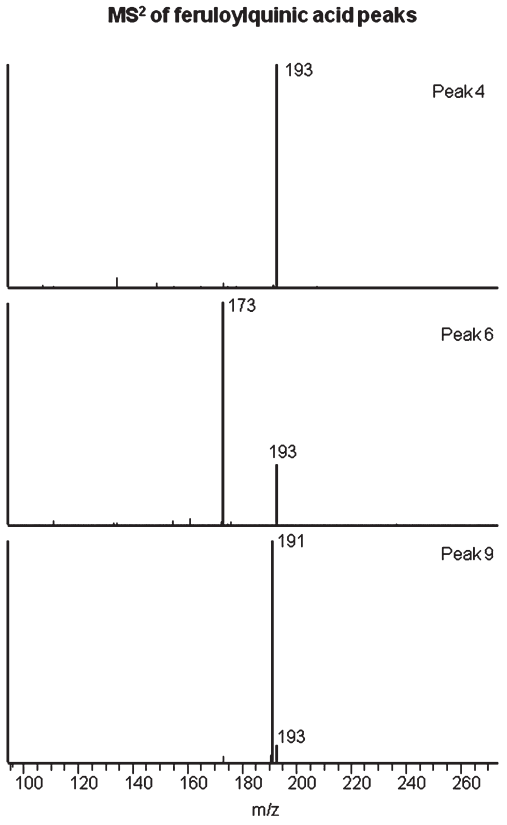

Figure 5. MS^2 analysis of feruloylquinic acids in peaks 4, 6 and 9.

Caffeine content of the extract ranged from 0.44 ± 0.14% for CFE-1, to 1.03 ± 0.12% in CFP-1. Caffeine was lowest ($p < 0.001$) in samples extracted with the multistep method (CFE-1, 0.44 ± 0.12%), followed by samples extracted with the single-step extraction method (CFE-2, 0.69 ± 0.03%), air-dried whole coffee fruit powder (CFP-1, 1.03 ± 0.15%) and freeze-dried coffee fruits (CFP-2, 0.67 ± 0.02) (Figure 7). Caffeine content was inversely correlated to the total chlorogenic acid content of the coffee fruit samples ($p < 0.01$).

Antioxidant Capacity of Coffee Fruit Extracts. The antioxidant capacity of the coffee fruit extracts is summarized in Table 3. Antioxidant capacities for HORAC, NORAC, SOAC, ORAC and total ORAC were between 7- and 25-fold higher in proprietary CFE-1 and CFE-2 compared to CFP-1 and CFP-2. The total

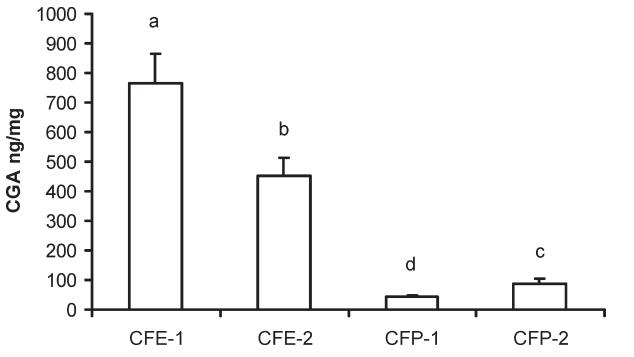

Figure 6. Comparison of total CGA (ng/mg) levels in coffee fruit samples (CFE-1, multistep proprietary extraction; CFE-2, single-step proprietary extraction; CFP-1, air-dried whole coffee fruit powder; CFP-2, freeze-dried, powdered raw coffee fruits).

Table 2. Peak Numbers and Concentration of Compounds in Coffee Fruit Samples[a]

no.	compd	CFE-1	CFE-2	CFP-1	CFP-2
1	3-*O*-caffeoylquinic acid	37.1 ± 8.9	60.5 ± 8.5	2.9 ± 0.9	2.6 ± 0.5
2	4-*O*-caffeoylquinic acid	84.1 ± 13.1	63.4 ± 8.7	3.7 ± 0.9	5.1 ± 1.1
3	5-*O*-caffeoylquinic acid	376.7 ± 67.1	167 ± 22.7	28.9 ± 3.4	60.8 ± 12.5
4	3-*O*-feruloylquinic acid	7.1 ± 2.5	10.7 ± 2.1	nd[b]	nd
5	5-*O*-*p*-coumaroylquinic acid	3.2 ± 2.8	nd	0.3 ± 0	0 ± 0
6	4-*O*-feruloylquinic acid	13 ± 0.4	12.9 ± 2	0.3 ± 0	0.4 ± 0.1
7	putative 3-*O*-caffeoylquinic lactone	7.7 ± 6.7	nd	nd	nd
8	putative 4-*O*-caffeoylquinic lactone	7 ± 6.1	nd	nd	nd
9	5-*O*-feruloylquinic acid	49.9 ± 16.6	28.6 ± 4.1	2.2 ± 0.1	4 ± 0.8
10	unknown: putative methyl-5-caffeoylquinic acid	4.3 ± 7.4	nd	nd	nd
11	3,4-*O*-dicaffeoylquinic acid	44 ± 10.5	29.8 ± 4.2	1.4 ± 0.3	1.5 ± 0.3
12	3.5-*O*-dicaffeoylquinic acid	43.7 ± 11.3	19.9 ± 2.7	2 ± 0.6	12.1 ± 2.5
13	4,5-*O*-dicaffeoylquinic acid	64.1 ± 9.8	33.6 ± 4.7	2.3 ± 0.3	1.4 ± 0.3
14	3-*O*-feruloyl-4-*O*-caffeoylquinic acid	6 ± 2.9	5 ± 0.7	1.4 ± 1.9	nd
15	4-*O*-caffeoyl-5-*O*-feruloylquinic acid	12.8 ± 11.5	18.9 ± 2.6	nd	nd
16	putative dicaffeoylquinic lactone	4.1 ± 3.6	nd	nd	nd
	total CGA content	765 ± 93.6	450.4 ± 62.2	45.3 ± 7.4	87.9 ± 18

[a] Data expressed in μg/g 5-CQA equivalents ± SD ($n = 3$). [b] Not detected.

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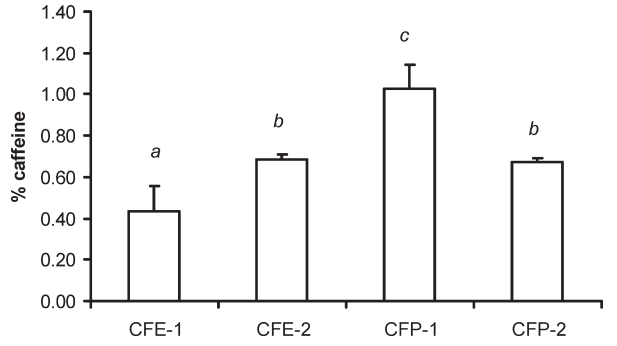

Figure 7. Comparison of caffeine levels in coffee fruit samples (CFE-1, multistep proprietary extraction; CFE-2, single-step proprietary extraction; CFP-1, air-dried raw coffee fruit powder; CFP-2, freeze-dried, powdered raw coffee fruits).

Table 3. Antioxidant Analysis of Coffee Fruit Samples[a]

antioxidant test	CFE-1	CFE-2	CFP-1	CFP-2
ORAC	15,264 ± 453	6,097 ± 225	823 ± 89	734 ± 48
HORAC	41,389 ± 3447	18,709 ± 426	2,140 ± 125	3,520 ± 287
NORAC	1,317 ± 104	527 ± 52	75 ± 11	57 ± 7
SORAC	2,193 ± 1591	860 ± 24	123 ± 62	271 ± 14
SOAC	3422 ± 355	2,042 ± 185	239 ± 12	311 ± 13
total ORAC	64,354 ± 2584	28,237 ± 782	3,439 ± 134	4,768 ± 285

[a] Results expressed as μmol Trolox equivalent/g ± SD.

Table 4. Correlation (Spearman's Rho) between Chlorogenic Acid Content, Antioxidant/Scavenging Capacities of the Raw Coffee Fruits

	total CGA content of samples	
	correlation coefficient[a]	*p* value
ORAC	0.893*	0.007
HORAC	0.929*	0.003
NORAC	0.893*	0.007
SORAC	0.607	0.148
SOAC	0.893*	0.007
total ORAC	0.964*	<0.001

[a] (*) Significant at $p < 0.01$.

antioxidant activity of the four samples was mainly attributable (>60%) to the hydroxyl scavenging capacity (HORAC assay). The total CGA content of the extracts was strongly correlated ($p < 0.005$) with all of the antioxidant capacity measures but SORAC (Table 4).

DISCUSSION

For many people, brewed coffee is the major dietary source of chlorogenic acids. Due to the large amount of coffee being consumed, and taking into account its high chlorogenic acid content, coffee is possibly the major dietary source of plant polyphenols.[1] Recently, extracts of green coffee beans (the nonviable seed) and, even more recently, extracts of whole coffee fruit (the whole fruit or cherry) have become available and are marketed as functional ingredients or health-supporting dietary supplements.[16,17]

The chlorogenic acid content of green and roasted coffee beans and also of instant coffee powders has been extensively studied and the data have been reviewed.[18] Data for proprietary green whole coffee fruit are scarce by comparison,[4] and to the best of our knowledge there are no such data for proprietary coffee fruit extracts. Accordingly, we present here data for the 16 most abundant chlorogenic acids in two proprietary whole coffee fruit extracts and in a proprietary whole coffee fruit powder as compared to a coffee fruit laboratory extract. The results obtained are consistent with previous reports for the composition of green coffee beans, with approximately 50% of the total CGA derived from 5-CQA ranging from 69% of the total for the freeze-dried berry extracts (CFP-2) to 37% for the single-step proprietary extracts (CFE-2). Total caffeoylquinic acids (i.e., the sum of the three monocaffeoylquinic acids and the three dicaffeoylquinic acids) accounted for 83% to 95% of the total CGA and was highest in freeze-dried coffee fruits (CFP-2), followed by air-dried coffee fruit powders (CFP-1), multistep coffee extract CFE-1, with single-step coffee fruit extract CFE-2 having the lowest CQA content ($p < 0.05$). The CGA contents, by weight, represent 80% for CFE-1, 42% for CFE-2, 4.5% for CFP-1, and 8.8% for the freeze-dried CFP-2.

Higher long-term coffee consumption has consistently been associated epidemiologically with a reduced incidence of type 2 diabetes, but a causative relationship has not been proven.[19,20] Various mechanisms have been hypothesized, mostly invoking a slowing of glucose uptake in the duodenum and/or effects on carbohydrate metabolism.[17,19] Epidemiology also suggests that coffee consumption might inhibit inflammation and thereby reduce the risk of cardiovascular and other inflammatory diseases in postmenopausal women.[21]

Similarly, higher consumption of other classes of polyphenols have been associated with better long-term health, such as reduced incidence of vascular disease and some cancers, although the mechanism(s) remain obscure, and the epidemiology is less consistent than observed for diabetes risk.[1,18] The health promoting potential of chlorogenic acid-rich extracts has been illustrated in some volunteer studies. In a placebo-controlled randomized trial in humans exhibiting with mild hypertension, one group of patients was treated with 140 mg/day of CGA for 12 weeks. During the treatment period, significant reductions in both systolic and diastolic blood pressure were observed in the group fed CGA, but not in the placebo group, with no other measured differences between the two groups.[16] Similar results, obtained with the same dose level, were reported by Ochiai et al.,[22] who speculated that green coffee bean extract improved vasoreactivity.

The *in vitro* antioxidant scavenging potential of polyphenols has been proposed as one of several potential *in vivo* mechanisms for disease prevention, despite limited evidence in human studies.[18] Nevertheless, studying the scavenging potential of specific polyphenols and foods has the potential to stimulate better research and product formulation. Our results showed a statistically significant correlation between the total CGA content of the coffee fruit samples (extracts and powders) and their ability to scavenge peroxyl radical, hydroxyl radical, peroxynitrite and singlet oxygen, in keeping with previous reports.[23] This correlation was however not true for scavenging of the superoxide anion (SORAC assay). These various free radicals, produced by phagocytes during the oxidative burst, are important for innate immunity but also are very toxic to tissues. The hydroxyl radical in particular is highly reactive and toxic, and our data

confirm an inverse correlation between total CGA content and its scavenging. These results also suggest that coffee fruit extracts and powders could protect lipid-rich formulations from oxidative rancidity during storage.

Meanwhile, the lack of significant correlation between scavenging of superoxide anion (SORAC assay) and increased chlorogenic acid content could be explained by the small sample size tested, or possibly by the caffeic acid content of the different extracts, which may also contribute to superoxide anion scavenging. Superoxide becomes highly toxic when it combines with nitric oxide to form peroxynitrite, for which scavenging (NORAC assay) was significantly higher in extracts richest in total CGA.

When volunteers consumed coffee, very little of the dominant chlorogenic acid, 5-caffeoylquinic acid, was absorbed intact.[24] Plasma concentrations did not exceed a transient ~2 nM maximum. Three feruloylquinic acids, caffeic acid and ferulic acid, and two chlorogenic acid lactones were also found in plasma and urine, the cinnamic acids and lactones as sulfate conjugates,[24] but the other monocaffeoylquinic acids and the dicaffeoylquinic acids were not detected in any form.

The major metabolites found in volunteer plasma and urine have been those produced by the gut microflora, especially dihydroferulic acid (DHFA) and dihydrocaffeic acid (DHCA), both of which occur in the free form and as sulfate conjugates. Volunteer studies have established that these two phenolic acids account for 33 and 37% of the chlorogenic acid dose with plasma maxima at least 2 orders of magnitude higher than that seen for 5-CQA.[24] The relevant mean (n = 11) AUC values are 142 and 2648 nmol/h/L for DHCA and its sulfate and 2333 and 1193 nmol/h/L for DHFA and its sulfate, respectively.

The absorption and metabolism of DHCA are well-defined,[25] and, increasingly, it is these metabolites of dietary polyphenols produced by the gut microflora upon which attention is being focused in order to identify possible mechanisms that may explain the benefits suggested by epidemiological studies.[26] There is, for example, some evidence that these phenolic acids can downregulate cholesterol biosynthesis[27] and factors associated with cellular inflammation,[28] and exhibit antithrombotic properties.[29] Concentrations of such phenolic acids in fecal water can be even higher than in plasma, totaling perhaps 2 mM,[30] and there is some potential for a prebiotic effect. For example, the levels achieved have in some cases exceeded the IC_{50} for the inhibition *in vitro* of the opportunistic pathogen, *Listeria monocytogenes*.[31]

In this study we observed, in CFE-1 prepared from arabica coffee berries, three minor components that were tentatively identified on the basis of their retention time and fragmentation as two caffeoylquinic lactones and one dicaffeoylquinic lactone. Our results establish that these putative lactones are present in the multistep coffee fruit extract CFE-1, at a level of approximately 14 mg/g, approaching the levels reported by Chu et al. in regular roasted coffees (23.27 g/kg) and surpassing those in roasted decaffeinated coffees (6.86 g/kg).[32] Farah et al. while investigating the formation of various chlorogenic lactones during the roasting of green coffee beans unexpectedly found traces of feruloylquinic and dicaffeoylquinic lactones, but not caffeoylquinic lactones, in green robusta coffee beans and suggested that the formation was associated with heating during the processing of the green coffee beans. These lactones were not detected in green arabica coffee beans.[33] It should be noted that caffeoylshikimic acids and caffeoylquinic lactones have very similar chromatographic and mass spectrometric behavior,[15] and further investigation is required for the unequivocal characterization of these minor components, as well as other minor chlorogenic acids known to occur in green coffee beans.[34,35] It has been suggested that the low polarity of chlorogenic acid lactones relative to the acids themselves has positioned said lactones as compounds that might permeate the blood−brain barrier, potentially enhancing their bioavailability and site-specific action as potential neuroprotective agents, potentially via modulation of the oxidative stress to neural cells as demonstrated *in vitro*.[32] It is also interesting to note that the coffee fruit samples analyzed in this study are richer in feruloylquinic acids than other commonly consumed coffee beverages.

The chlorogenic acid-rich coffee fruit extracts could be superior to conventional coffee beverages in situations where limited caffeine intake is required. For example, recent U.K. government guidelines advise pregnant women to limit their intake of caffeine to less than 300 mg per day, equivalent to approximately 2 or 3 cups of coffee, when instant coffee delivers from 21 to 120 mg per cup, and ground coffee from 15 to 254 mg per cup (Food Standard Agency, 2004).

In contrast, the low caffeine content of the multistep proprietary whole coffee fruit extract (CFE-1) is therefore attractive, since a 1 g extract would provide more than 10-fold the total CGA content of a regular 200 mL cup of brewed roasted coffee (approximately 70 mg), and only 1.5% of the recommended maximum daily caffeine dose.

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