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Incorporation of Chlorogenic Acids in Coffee Brew Melanoidins

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The incorporation of chlorogenic acids (CGAs) and their subunits quinic and caffeic acids (QA and CA) in coffee brew melanoidins was studied. Fractions with different molecular weights, ionic charges, and ethanol solubilities were isolated from coffee brew. Fractions were saponified, and the released QA and CA were quantified. For all melanoidin fractions, it was found that more QA than CA was released. QA levels correlated with melanoidin levels, indicating that QA is incorporated in melanoidins. The QA level was correlated with increasing ionic charge of the melanoidin populations, suggesting that QA may contribute to the negative charge and consequently is, most likely, not linked via its carboxyl group. The QA level correlated with the phenolic acid group level, as determined by Folin-Ciocalteu, indicating that QA was incorporated to a similar extent as the polyphenolic moiety from CGA. The QA and CA released from brew fractions by enzymes confirmed the incorporation of intact CGAs. Intact CGAs are proposed to be incorporated in melanoidins upon roasting via CA through mainly nonester linkages. This complex can be written as Mel═CA−QA, in which Mel represents the melanoidin backbone, ═CA represents CA nonester-linked to the melanoidin backbone, and −QA represents QA ester-linked to CA. Additionally, a total of 12% of QA was identified in coffee brew, whereas only 6% was reported in the literature so far. The relevance of the additional QA on coffee brew stability is discussed.

KEYWORDS: Coffee; melanoidins; phenolic; chlorogenic; quinic; caffeic acid; incorporation

INTRODUCTION

Coffee is one of the most important crops in the world; its worldwide production was almost seven million tons in 2005 (*1*). Green coffee beans are roasted; this process changes the chemical composition drastically. The Maillard reaction is one of the dominant reactions that occur during roasting. This reaction, in which reducing sugars react in a cascade of reaction steps with amino groups, leads to the formation of desired characteristic flavor and color compounds (*2, 3*). The brown-colored Maillard-reaction end-products are referred to as melanoidins, and they are often defined as brown-colored, high-molecular-weight (Mw), nitrogenous Maillard-reaction end-products (*4*). The melanoidin content in coffee brew is frequently determined by difference and may account for 25% of the dry matter (*5*). The chemical and functional properties of coffee melanoidins are not fully understood, which can be ascribed to the extremely complex chemical composition of these molecules. One of the important functional properties of melanoidins is its antioxidative activity (*6–9*), which is believed to be at least partly due to the incorporation of chlorogenic acids (CGAs) (*10–15*).

Many CGAs are present in coffee beans, and 5-caffeoylquinic acid is by far the most abundant one (*16*). Green Arabica coffee beans contain around 7% of CGA; this level decreases drastically upon roasting to levels as low as 0.2% for dark roasted beans (*17*). The fate of the disappearing CGAs upon roasting is not totally understood. It was suggested that part of the CGAs is converted into flavor compounds upon roasting (*10, 18*), whereas others reported that CGAs might be incorporated in coffee brew melanoidins. Already in 1971, Klöcking et al. identified the release of caffeic acid (CA) and ferulic acid (FA) from high-Mw (HMw) coffee material after alkaline pressure hydrolysis (*11*). More than a decade later, thermal degradation studies indicated the involvement of CGAs in melanoidin formation, because many phenolics were released from HMw coffee material (*12, 13*). Delgado-Andrade et al. (*14*) showed that part of the antioxidative compounds in coffee, which are supposedly phenolics, might be bound ionically to melanoidins.

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Leloup et al. (*10*) reported that roasted coffee beans contained ester-linked quinic acid (QA) as well as ester-linked CA and FA, which are not present within CGAs. Recently, Takenaka et al. (*19*) and Nunes and Coimbra (*15*) identified and quantified phenolic components that were released from HMw coffee brew fractions after alkaline fusion. As these fragments were indicative for CA and FA, they concluded that phenolic acids from CGAs were covalently linked to HMw coffee material (*15*). In previous research, we found that the melanoidin level correlated with the level of phenolic groups present in coffee fractions which were isolated by using different techniques (*4, 20*). Next to that, it was found that the negative charge on melanoidins could not only be caused by uronic acids in arabinogalactans but that there are most probably other negatively charged groups present as well (*20*). Incorporation of CGAs into melanoidins might explain these observations because the CA or FA moiety from CGAs might explain the observed correlation between melanoidins and phenolic groups level, whereas the QA moiety might provide the negative charge on melanoidins.

The aim of this research was to gain insight into the involvement of CGA in coffee melanoidin formation. To this end, series of coffee brew fractions differing in melanoidin content were investigated for their unbound and ester-bound CGA, QA, CA, and FA levels.

MATERIALS AND METHODS

Materials. Green and roasted Colombian coffee beans (*Coffea arabica*) were provided by a local factory. The degree of roast was 16.4% (w/w), which corresponds to 8.0% (w/w) on a dry matter basis.

Preparation of Coffee Brew. Roasted beans were ground, and a brew was prepared as described previously (*4*). The extraction yield was 20%. For characterization purposes, part of the brew was lyophilized, yielding Brew. The major part of the brew was used for further isolation.

Defatting of Coffee Samples. Lyophilized coffee brew samples were defatted by Soxhlet extraction by using a Soxtherm which was connected to a Multistat system (Gerhardt, Königswinter, Germany), as described previously (*20*). The solvent used for extraction was dichloromethane.

Extraction of 5-Caffeoylquinic Acid, QA, CA, and FA from Green and Roasted Beans. Green beans (frozen in liquid nitrogen) and roasted beans were ground by using a Retsch ZM200 rotor mill as described previously (*4*). Ground beans were lyophilized; this step was followed by a second milling step by using a Retsch MM2000 ball mill operating at maximum amplitude for 1 min, yielding a powder. A mixture of 6 g of water and 1 g of coffee powder was stirred for 30 min at room temperature. This step was followed by centrifugation for 5 min at 2500*g*. This procedure was repeated five times, and the supernatants were pooled, filtered over a Büchner funnel by using a S&S 595 filter (Schleicher and Schuell, Dassel, Germany), and subsequently lyophilized. Lyophilized green and roasted bean extracts were used for determination of unbound and total 5-caffeoylquinic acid (IUPAC), QA, CA, and FA levels.

Isolation of HMw Coffee Brew Material. HMw material was obtained from the brew by diafiltration as described previously (*4*). The retentate and dialysate were lyophilized, yielding a HMw fraction and a fraction with a lower Mw (DF-dialysate), respectively (**Figure 1**).

Isolation of Intermediate-Mw Coffee Brew Material. Intermediate-Mw (IMw) material was obtained from the defatted DF-dialysate sample by membrane dialysis. The DF-dialysate sample was dialyzed by using a Visking size 9 dialysis membrane with a cutoff of 12–14 kDa (Medicell International Ltd., London, U.K.) for 2 days against running tap water and 1 day against demineralized water with two water renewals. The retentate was lyophilized, yielding the IMw fraction (**Figure 1**).

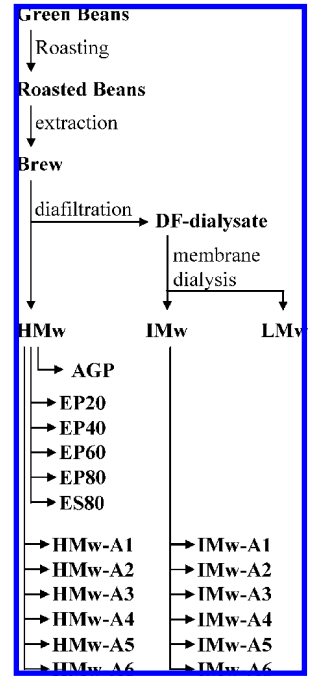

Figure 1. Scheme of the coffee brew isolation procedure (see text for abbreviations).

Isolation of Low-Mw Coffee Brew Material. The low-Mw (LMw) fraction was obtained from the defatted DF-dialysate sample by membrane dialysis. The DF-dialysate sample (500 mL, 100 mg/mL) was dialyzed by using a Visking size 9 dialysis membrane with a cutoff of 12–14 kDa (Medicell International Ltd.) for 3 days at 4 °C against 4.5 L of demineralized water with three water renewals. The dialysates were pooled and lyophilized, yielding the LMw fraction (**Figure 1**).

Arabinogalactan Protein Isolation. Arabinogalactan proteins (AGP) were isolated from the HMw fraction as described previously (*20*). The obtained material was lyophilized, yielding HMw AGP (**Figure 1**).

Ethanol Precipitation of the HMw Fraction. The HMw fraction obtained after diafiltration was subjected to ethanol precipitation, as described previously (*4*). Absolute ethanol was added to the HMw solution until the desired concentration was reached. The solution was left for precipitation and was subsequently centrifuged. The supernatant was subjected to further precipitation steps. Coffee fractions that precipitated at 20, 40, 60, and 80% ethanol were coded EP20, EP40, EP60, and EP80, respectively. The supernatant of 80% ethanol was coded ES80.

Preparative Anion-Exchange Chromatography of the HMw and IMw Fractions. Preparative anion-exchange chromatography was conducted as described previously (*20*). Briefly, HMw and IMw solutions were loaded on a strong anion exchanger. Coffee material was eluted in steps by elution with 5, 300, 600, and 2000 mM NaOAc, and subsequently with 1 and 2 M NaCl, yielding fractions A1, A2, A3, A4, A5, and A6, respectively. The obtained HMw and IMw fractions were coded HMw-A1−HMw-A6 and IMw-A1−IMw-A6, respectively (**Figure 1**).

Sample Preparation for Free and Total 5-Caffeoylquinic Acid, CA, and FA Determination. For determination of the free 5-caffeoylquinic acid, CA, and FA levels, a sample solution (5 mg/mL in water) was centrifuged and subsequently analyzed by reversed-phase high-performance liquid chromatography (HPLC). For determination of the total CA and FA levels, the sample was saponified by using a procedure that prevented oxidation of phenolics (*21*). To 750 μL of sample solution (12 mg/mL), 750 μL of 2 M NaOH solution containing 2% (w/w) ascorbic acid and 20 mM ethylenediaminetetraacetic acid was added. After incubation for 1 h at 30 °C, the mixture was quenched to pH $\approx$ 1 with 330 μL of 5 M HCl to precipitate most of the coffee material, preventing precipitation during further analysis. The mixture was stored for 2 h at 4 °C, the precipitate was removed by centrifugation, and the supernatant was analyzed by reversed-phase HPLC. Experiments were performed at least in duplicate.

Sample Preparation for Free and Total QA Determination. For free QA determination, a sample solution (5 mg/mL in water) was centrifuged and subsequently analyzed by ion-moderated partitioning HPLC or by gas chromatography (GC) after silylation, as described below. For total QA determination, the sample was saponified by using the same procedure as for the determination of the total CA and FA levels, although no ascorbic acid and ethylenediaminetetraacetic acid were added. The saponified solution was analyzed by ion-moderated partitioning HPLC or by GC after silylation. Experiments were performed at least in duplicate.

Enzyme Incubation of Coffee Material. Brew and HMw and IMw fractions were subjected to enzymatic degradation by using commercial chlorogenate esterase from *Aspergillus japonicus* (*22, 23*), kindly provided by Kikkoman Corp. (Tokyo, Japan). A sample of 10 mg was dissolved in 500 μL of 25 mM 4-morpholineethanesulfonic acid sodium salt buffer, pH 6. To this solution, 500 μL of 1 mg/mL chlorogenate esterase in 25 mM 4-morpholineethanesulfonic acid sodium salt buffer, pH 6, was added. After overnight incubation at 40 °C, enzymes were inactivated by heating for 10 min at 100 °C. Experiments were performed at least in duplicate. The quantities of released QA, CA, and FA were determined by ion-moderated partitioning and reversed-phase HPLC.

Determination of 5-Caffeoylquinic Acid, CA, and FA by Reversed-Phase HPLC. For determination of free and total 5-caffeoylquinic acid, CA, and FA levels, untreated and saponified samples (5 mg/mL) were analyzed by reversed-phase HPLC on a 150 × 4.6 mm i.d. XTerra MS C18 3.5 μm column (Waters, Milford, MA) in combination with a 20 × 3.9 mm i.d. XTerra MS C18 3.5 μm guard column (Waters). Elution took place at room temperature (0.5 mL/min) by using (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in methanol. The elution profile after injection (20 μL) was as follows: 1 min isocratic elution with 100% A, in 30 min to 63% B, in 10 min to 100% B, 1 min isocratic elution, in 1 min to 100% A, and 17 min re-equilibration. The absorbance of the eluate was measured at 325 nm by using a Spectra System UV3000 (Thermo Electron Company, Waltham, MA). Aqueous solutions of 5-caffeoylquinic acid, CA, and FA were used as reference compounds for determination of the free 5-caffeoylquinic acid, CA, and FA levels. Saponified CA and FA were used as reference compounds for determination of the total CA and FA levels. The recovery of CA and FA after saponification was 129 ± 2% and 114 ± 2% of the expected value, respectively. Saponification of 5-caffeoylquinic acid resulted in a recovery of 91 ± 2% of the CA. Enzymatic degradation of 5-caffeoylquinic acid resulted in complete degradation of 5-caffeoylquinic acid, and all CA was recovered.

Determination of QA by Ion-Moderated Partitioning HPLC. For determination of free and total QA levels, untreated and saponified coffee samples (~5 mg/mL) were analyzed for their QA levels based on the procedure described by Zeppa et al. (*24*). High-performance cation-exchange chromatography was performed by using a 300 × 7.8 mm i.d. Aminex HPX 87H column equipped with a cation H+ guard column filled with AG 50W-X4 (Bio-Rad, Hercules, CA). After injection (100 μL), isocratic elution took place with 5 mM sulfuric acid at 0.6 mL/min for 150 min at 40 °C. The eluate was monitored by a Spectra System RI-150 refractive index detector (Thermo Electron Company). The recovery of D(−)-QA (Fluka Chemie GmbH, Buchs, Switzerland) after saponification was 83 ± 5% of the expected value. Saponification of 5-caffeoylquinic acid resulted in a recovery of 75 ± 3% of the QA, which is similar to the recovery of saponified QA. Therefore, untreated QA and saponified QA were used as reference compounds for determination of the free and total QA levels, respectively. Enzymatic degradation of 5-caffeoylquinic acid resulted in a complete recovery of the QA.

Determination of QA by GC after Silylation. Untreated and saponified coffee samples were silylated on the basis of procedures described by Huang et al. (*25*) and Butts (*26*). A total of 200 μL of the untreated or saponified solution (~5 mg/mL) was transferred into a 1 mL reaction vessel with screw cap (Alltech, Deerfield, IL), subsequently frozen, and lyophilized. Pyridine (3 mL) (Aldrich, Steinheim, Germany) was added to 3 mL of trimethylsilyl 2,2,2-trifluoro-n-(trimethylsilyl)-acetamide containing chlorotrimethylsilane (99:1) (Supelco, Bellefonte, PA). A stirring bone and 600 μL of this solution were added to the lyophilized material. After incubation for 2 h at 125 °C under continuous stirring, the solution was cooled down, and 100 μL of 200 μg/mL dodecane (Merck, Darmstadt, Germany) in hexane was added as internal standard. The solution was mixed and centrifuged, and the supernatant was transferred into a GC vial. A GC Trace gas chromatograph (Thermo Finnigan, Waltham, MA) with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and a flame ionization detector was equipped with a 3000 × 0.25 mm i.d. DB-1 column, 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA). The sample (1.5 μL) was injected, and the oven temperature program was as follows: 3 min at 40 °C, in 4.5 min to 130 °C, 5 min at 130 °C, in 19.5 min to 325 °C, and 15 min at 325 °C. QA was used as reference compound.

Total Phenolic Groups Level. The total phenolic groups contents of the coffee samples were determined with the Folin-Ciocalteu assay as described previously (*4*). The reference compound was 5-caffeoylquinic acid.

Specific Extinction Coefficient of Coffee Material. The absorption of aqueous sample solutions (1 g/L) was determined at 405 nm by using a Hitachi U-3000 spectrophotometer (Hitachi, Tokyo, Japan). The specific extinction coefficient $K_{\mathrm{mix\ 405\ nm}}$ (L/g/cm) was calculated as described previously (*4*).

RESULTS AND DISCUSSION

5-Caffeoylquinic Acid, QA, CA, and FA in HMw and IMw Fractions. Brew fractions HMw and IMw were previously shown to be rich in melanoidins and free from LMw molecules (*20*). Therefore, HMw and IMw fractions were the first fractions that were screened for the presence of ester-bound QA, CA, and FA. The untreated HMw elution patterns (**Figure 2**) did not show unexpected peaks, from which it was concluded that this fraction did not indeed contain any free 5-caffeoylquinic acid, QA, CA, or FA (**Table 1**). The saponified HMw fraction elution patterns (**Figure 2**) showed peaks corresponding to 1.7% of QA and 0.5% of CA in the HMw fraction (**Tables 2** and **3**). It was found that a HMw fraction from green beans isolated by using the same conditions did not contain any free or ester-bound QA, CA, or FA. The IMw fraction elution patterns were similar to those of the HMw fraction. The trace amounts (<0.1%) of 5-caffeoylquinic acid in the IMw fraction (**Table 1**) might be explained by the fact that the isolation technique involved membrane dialysis, a passive and slow procedure, even though it was conducted for a prolonged time. The IMw fraction was saponified, and the levels of total QA, CA, and FA released are shown in **Table 2**. After correction for 5-caffeoylquinic acid, it was calculated that the IMw fraction contained 3.7% of QA, 0.9% of CA, and 0.1% of FA (**Table 3**). To the best of our knowledge, this is the first time that release of phenolic acids from HMw and IMw coffee materials by saponification is reported. Previously, Nunes and Coimbra did not detect any phenolic acids after saponification with sodium hydroxide (*15*). One possible explanation for our observations could be the high level of precautions taken in our experiments to prevent oxidation.

On the basis of these findings, it could be concluded that at least part of the CGAs present in green beans is incorporated into HMw and IMw coffee materials as a result of the roasting process. It was found that the ester-linked QA level was around 3−4 times higher than the ester-linked CA level, whereas QA and CA are present in a ratio of around 1:1 in CGAs. This observation might be ascribed to the fact that QA is less prone to oxidative changes during roasting (*27*).

QA, CA, and FA in the AGP−Melanoidin Complex. Previously, it was shown that AGPs isolated from roasted coffee brew were part of a melanoidin complex (*20*). Because of the specificity of the Yariv isolation procedure, it could be

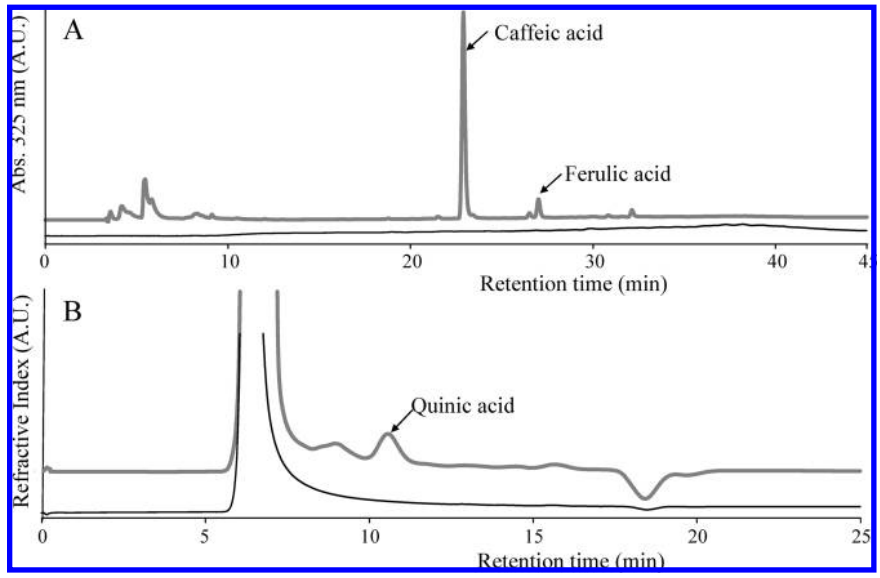

Figure 2. (**A**) Reversed-phase and (**B**) ion-moderated partitioning HPLC elution patterns of HMw (thin black line) and saponified HMw (thick gray line) fractions.

Table 1. Free 5-Caffeoylquinic Acid, QA, CA, and FA Levels in Various Coffee Fractions

	5-caffeoylquinic acid (%, w/w)[a]	QA (%, w/w)[b]	CA (%, w/w)	FA (%, w/w)
green beans	3.0	0.6	0.0	0.0
roasted beans	0.7	0.7	0.0	0.0
Brew	2.9	3.0	0.1	0.0
HMw fraction	0.0	0.0	0.0	0.0
IMw fraction	0.1	0.0	0.0	0.0
LMw fraction	4.3	4.6	0.1	0.1

[a] The coefficient of variation was 1% on average. [b] The coefficient of variation was 3% on average.

Table 2. QA, CA, and FA Levels after Saponification in Various Coffee Fractions

	QA (%, w/w)[a]	CA (%, w/w)[b]	FA (%, w/w)[b]
green beans	3.9	3.3	0.3
roasted beans	3.4	1.7	0.2
Brew	11.8	4.6	0.6
HMw fraction	1.7	0.5	0.0
IMw fraction	3.7	0.9	0.1
LMw fraction	15.1	6.4	0.8

[a] The coefficient of variation was 3% on average. [b] The coefficient of variation was 1% on average.

Table 3. Ester-Bound QA, CA, and FA Levels Not from CGAs in Various Coffee Fractions

	$K_{mix\ 405\ nm}$ (L/g/cm)	phenolic groups (%, w/w)[a]	QA (%, w/w)[b]	CA (%, w/w)[b]	FA (%, w/w)[b]
Brew	0.7	29			
HMw fraction	1.1	11	1.7	0.5	0.0
IMw fraction	1.5	18	3.6	0.8	0.1
LMw fraction	0.4	23			

[a] As 5-caffeoylquinic acid equivalents. [b] Calculated from **Tables 1** and **2**.

concluded that this fraction was a pure AGP−melanoidin complex. As expected, it was found that HMw AGPs did not contain any unbound 5-caffeoylquinic acid, QA, CA, or FA. After alkaline hydrolysis, the levels of ester-bound QA, CA, and FA (**Table 4**) were found to be 0.9, 0.2, and 0.02%, respectively. Because this fraction was a pure melanoidin population, and QA and CA were present in this fraction, the conclusion is that these ester-linked acids are incorporated in the melanoidin complex. It is not known how these CGA derivatives are bound within the AGP−melanoidin complex. Both covalent binding of phenolic compounds to sugar (*28*) and protein (*29*) were reported. Additionally, it is known that arabinose from arabinogalactan is quite susceptible to degradation upon roasting (*30, 31*). Therefore, arabinose might be a possible binding site for CGA derivatives. At this moment, it is unclear whether CGA is first split into QA and CA upon roasting and then incorporated in melanoidins or whether intact CGA is incorporated. In the case of intact CGA incorporation, it also remains to be determined whether the intact CGA molecule is connected through the QA or CA moiety to melanoidins.

To the best of our knowledge, this is the first time that it can be decisively concluded that CGAs are involved in melanoidin

Table 4. Ester-Bound QA, CA, and FA Levels Not from CGA in Various Coffee Fractions

	$K_{mix\ 405\ nm}$ (L/g/cm)	phenolic groups (%, w/w)[a]	QA (%, w/w)[b]	CA (%, w/w)[c]	FA (%, w/w)[b]
HMw AGP	1.1	10	0.92	0.19	0.02
HMw EP20	0.5	6	1.04	0.40	0.04
HMw EP40	0.7	8	0.97	0.36	0.03
HMw EP60	0.6	11	1.52	0.56	0.05
HMw EP80	1.2	17	2.12	0.63	0.06
HMw ES80	2.5	26	3.47	0.58	0.05
HMw A1	0.2	2	0.07	0.03	0.01
HMw A2	0.3	4	0.58	0.11	0.01
HMw A3	0.9	6	0.76	0.09	0.01
HMw A4	1.2	9	0.99	0.07	0.01
HMw A5	1.4	10	0.98	0.07	0.01
HMw A6	0.7	5	0.32	0.03	0.00
IMw A1	0.3	2	0.07	0.11	0.02
IMw A2	0.9	8	0.85	0.24	0.03
IMw A3	1.6	12	1.58	0.29	0.04
IMw A4	2.6	17	2.55	0.29	0.03
IMw A5	2.7	12	3.25	0.39	0.04
IMw A6	1.0	4	1.86	0.27	0.03

[a] As 5-caffeoylquinic acid equivalents. [b] The coefficient of variation was 3% on average. [c] The coefficient of variation was 1% on average.

formation upon roasting of coffee beans. Both building blocks of CGA, QA and CA, are incorporated in melanoidins although at different levels.

5-Caffeoylquinic Acid, QA, CA, and FA in Ethanol Precipitation Fractions. HMw Brew was previously fractionated by ethanol precipitation, and it was found that fractions which were soluble at high ethanol concentration had the highest melanoidin levels (*4*). Furthermore, a relation between the phenolic groups level and the melanoidin level was observed (*4*). The ethanol precipitation fractions did not contain any unbound 5-caffeoylquinic acid, QA, CA, or FA, which was expected. The ester-bound QA level varied significantly, ranging from 1% in HMw EP20 to 3.5% in HMw ES80 (**Table 4**). The ester-bound CA and FA levels were quite low and showed a less-pronounced differentiation over the ethanol precipitation fractions; the CA levels were between 0.36 and 0.63%, and the FA levels were between 0.03 and 0.06% (**Table 4**). Such low levels were not expected because these fractions contain many phenolic groups as measured by the Folin-Ciocalteu assay (**Table 4**). Because CGAs are the predominant source of phenolic acids in green beans, it is expected that the phenolic groups in HMw and IMw fractions are due to the incorporation of CA and FA. This CA and FA incorporation occurs in such a way that CA and FA are not ester-linked and might be due to oxidation of these polyphenolics upon roasting (*27*).

On the basis of the results found so far, several options for the CGA incorporation can be defined. First, CGA might be first hydrolyzed, QA might then be separately incorporated through ester linkages, and CA might be incorporated mainly through nonester linkages. Second, intact CQA might be incorporated via the QA moiety through an ester linkage, after which most of the CA is oxidized or degraded. Third, intact CQA might be incorporated via the CA moiety mainly through nonester linkages. The nonester linkage of CA to the melanoidin backbone might be the result of single or multiple condensation reactions. In the case where CA is condensated into the melanoidin complex, it might well be that the conjugated ring structure of CA contributes to the observed brown color of melanoidins. Furthermore, the pronounced distribution of QA suggests that the incorporation of QA from CGAs occurs at specific types of molecules.

5-Caffeoylquinic Acid, QA, CA, and FA in Anion-Exchange Chromatography Fractions. HMw and IMw brews were previously fractionated by anion-exchange chromatography, and it was found that brew melanoidins are negatively charged (*20*). The ester-linked 5-caffeoylquinic acid, QA, CA, and FA levels in these fractions were of special interest, because the incorporation of these acids might contribute to this negative charge. The levels of the ester-bound QA, CA, and FA, not present as free acids or in intact CGAs, are shown in **Table 4**. It is noticeable that the ester-linked acids levels in all anion-exchange fractions were lower than the levels in the parent fractions, indicating that part of the ester-bound acids was not recovered. Supposedly, this is due to the fact that these acids are linked to melanoidins that have such a strong negative charge that these complexes did not elute from the column. This is evident from the fact that the melanoidin recovery of this fractionation step was not optimal either, being 67% for HMw and 72% for IMw fractions (*20*). Nevertheless, the fractions differing in charge (A1−A6) represent the majority of the melanoidins and were still of special interest, because their ester-linked acid levels might provide information on the binding mechanism of the CGA incorporation. As can be seen in **Table 4**, similar results were obtained for both the HMw and IMw anion-exchange series. The ester-bound QA level initially

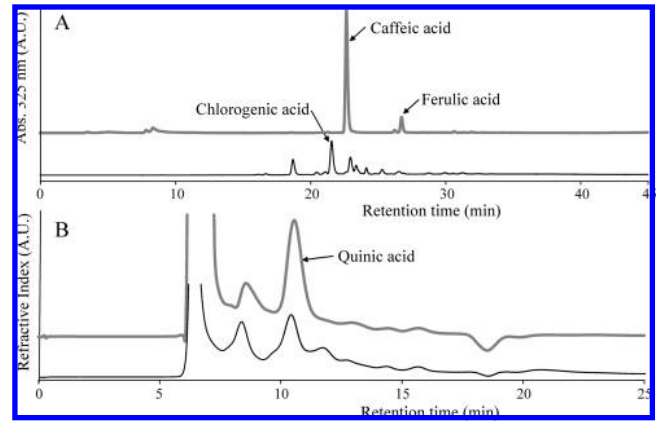

Figure 3. (**A**) Reversed-phase and (**B**) ion-moderated partitioning HPLC elution patterns of Brew (thin black line) and saponified Brew (thick gray line).

increased (A1→A4/A5) and subsequently decreased (A6). This increase confirmed that QA might indeed be, at least partly, responsible for the observed anionic properties of the melanoidins. This automatically implies that the carboxyl group of QA is not involved in CGA incorporation. The low level of ester-linked QA in fraction A6 indicated that a component other than QA is present in this fraction and provides the anionic character. As the melanoidin level in both A6 fractions was low too (*20*), it is expected that another negatively charged nonmelanoidin component is present in these fractions. With respect to the ester-linked CA and FA, observations similar to those for ethanol precipitation fractions from HMw fractions were made (**Table 4**): CA and FA levels were much lower than the QA levels and showed a less-pronounced differentiation over the fractions. Thus, CA and FA levels, again, did not match with QA levels.

These results indicate that the carboxyl group of QA is not primarily involved in CGA incorporation. On the basis of this finding, it can be stated that the second option given for CGA incorporation, that is, intact CGA incorporation via QA, is not the most prevalent reaction. Because CA levels were relatively low as well, it is most likely that intact CGA is incorporated into melanoidins via the CA moiety through nonester linkages, or that CGA is first split, and QA and CA are then separately incorporated.

Free 5-Caffeoylquinic Acid, QA, CA, and FA Levels in Green Beans, Roasted Beans, and Coffee Brew. The HMw and IMw fractions and subfractions were shown to contain ester-linked CGA derivatives in melanoidin structures. However, it should be realized that HMw and IMw fractions together represented 32% (w/w) of the Brew (*20*), leaving 68% (w/w) not studied so far. Furthermore, the LMw fraction still contained 40% of the melanoidins (*20*). To be able to place the values for HMw and IMw fractions in a broader perspective, the 5-caffeoylquinic acid, QA, CA, and FA levels in green beans, roasted beans, and Brew were determined as well. The reversed-phase HPLC elution pattern of the Brew (**Figure 3A**) showed several peaks, among which the largest peak was identified as 5-caffeoylquinic acid. The molecules causing the other peaks were not identified but were expected to be due to other CGAs (*16, 32*). The 5-caffeoylquinic acid levels was 3.0% in green beans, 0.7% in roasted beans, and 2.9% in the Brew (**Table 1**). These results were in agreement with the literature; Perrone et al. (*16*) found 3.5% 5-caffeoylquinic acid in green Arabica beans and 0.7% 5-caffeoylquinic acid in roasted Arabica beans (degree of roast, 16%). No or only trace amounts of free CA and FA were found in green beans, roasted beans, and the Brew, which was also in

Table 5. QA, CA, and FA Released after Enzymatic Degradation of Coffee Fractions with Chlorogenate Esterase[a]

	QA (%, w/w)[b]	CA (%, w/w)[c]	FA (%, w/w)[c]
Brew	2.3	3.8	0.3
HMw fraction	0.2	0.1	0.0
IMw fraction	0.1	0.4	0.0

[a] After correction for free acids level. [b] The coefficient of variation was 3% on average. [c] The coefficient of variation was 1% on average.

agreement with the literature (*33*). The free QA level was initially determined by ion-moderated partitioning HPLC, and the elution pattern of the Brew is shown in **Figure 3B**. The observed peak for QA corresponds to a level of 3.0% of QA in the Brew. The free QA level was also determined by GC after silylation because no baseline separation was obtained for QA by using ion-moderated partitioning HPLC. The free QA level in the Brew determined after silylation was calculated to be 3.3%. Thus, both techniques gave similar free QA levels, and the QA levels for the beans and the Brew were in agreement with the literature (*10, 34*).

Total QA, CA, and FA Levels in Green Beans, Roasted Beans, and Coffee Brew. The reversed-phase HPLC elution pattern of the saponified Brew (**Figure 3A**) showed two main peaks which were identified as CA and FA. The peaks observed in the elution pattern of the untreated Brew were not present in the elution pattern of the saponified Brew anymore, confirming that these peaks were indeed from other CGAs. The saponified green beans, roasted beans, and Brew contained 3.3, 1.7, and 4.6% CA and 0.3, 0.2, and 0.6% FA, respectively (**Table 2**). The total QA level was determined by ion-moderated partitioning HPLC, and the elution patterns of the saponified Brew is shown in **Figure 3B**. The total QA level was 3.9% in green beans, 3.4% in roasted beans, and 11.8% in Brew (**Table 2**). For confirmation, the total QA level in the Brew was determined by GC after silylation and was precisely 11.8% as well. For green beans, it stands out that the level of ester-linked QA (3.3%, total − free) was similar to the levels of ester-linked CA and FA (3.6%), indicating that these ester-linked acids were originating from CGAs. The 11.8% total QA was unexpectedly high; such levels have not been reported so far. The distribution of these acids over the various coffee components will be discussed after estimation of the CGAs level.

Enzymatic Degradation of Coffee Brew Material. The applied saponification procedure did not allow the detection of intact CGA in melanoidins because the internal ester bond in CGA is hydrolyzed as well. It was reasoned that, if intact CGA is incorporated in melanoidins, the internal ester linkage might still be intact. This binding might be selectively split by enzymes. As enzymes have high substrate specificity, a release of QA, CA, or FA from coffee melanoidin material would indicate that the binding between CGA's nonphenolic and phenolic moieties is still intact. This would then strongly indicate that intact CGA is incorporated in melanoidin structures. To this end, Brew and HMw and IMw fractions were incubated with chlorogenate esterase, and the quantities of QA, CA, and FA released were measured (**Table 5**). Even though the enzyme used accepts few other phenolic acid-based substrates as well, it is still quite specific for CGAs (*22, 23*). However, because CGAs are the only source of CA esters in coffee beans, a release of CA would strongly indicate that intact CGA is incorporated in melanoidins. It was found that the enzyme released 2.3% of QA, 3.8% of CA, and 0.3% of FA from the coffee Brew. The fact that no or negligible amounts of free CGAs were present after enzyme incubation of the Brew showed that CGAs were indeed a substrate that was effectively split. The enzyme incubation released 0.2% of QA, 0.1% of CA, and no FA from the HMw fraction. Thus, 12 and 20% of the ester-linked QA and CA were released, respectively (**Tables 3** and **5**). Enzymatic degradation of the IMw fraction yielded 0.1% of QA and 0.4% of CA, representing 3% of the QA and 44% of the CA that were present in the IMw fraction in ester linkages. The observed release of QA and especially CA from HMw and IMw fractions indicated that the ester linkage between the phenolic and nonphenolic moieties of CGA was still intact, and thereby, that intact CGA was incorporated. Relatively more CA than QA was released, whereas the QA level was larger than the CA level, indicating that the enzyme required ester-bound CA from CGA in its active cleft. This implies that a small part of the incorporated CGAs are linked via QA to the melanoidins.

It was shown by Delgado-Andrade et al. (*14*) that part of the antioxidant activity of melanoidins could be due to components that are ionically bound to melanoidins (*14*); these components might be CGAs. One could argue that the QA and CA released by the saponification procedure were not released because of the cleavage of ester bonds but were ionically bound and were released because of the high ionic strength of the 2 M NaOH solution. The fact that the enzyme treatment, conducted by using a low ionic strength, yielded both QA and CA shows that these molecules were really ester bound.

CGAs in Green Beans and Brew. To be able to discuss the fate of QA, CA, and FA present in coffee brew, it is necessary to estimate the extent in which these molecules are present in CGAs. Because the precise level of the different CGAs was unknown, estimations were made. The actual CGAs level in green beans was estimated by using the total and free QA, CA, and FA levels ($\sum$ total acids − $\sum$ free acids). This level for QA (3.3%) was similar to the combined CA and FA levels (3.6%), indicating that these ester-linked acids were solely from CGAs. On the basis of these values, the level of CGAs in green beans would be 6.9%, which is in agreement with values reported in the literature (*16*). It is impossible to calculate the CGAs level for the Brew in a similar manner, because a part of the QA, CA, and FA was present in CGAs, and a part was incorporated in melanoidins. Alternatively, as the enzyme treatment degraded almost all CGAs in the Brew, the actual level of CGAs could be estimated by using the level of QA released by the enzymes. The enzyme incubation released 2.3% of QA from the Brew, from which it was calculated that the the total CGAs level should be around 4.2% when all CGAs are monocaffeoylquinic acids.

Distribution of QA and CA in Coffee Brew over Various Components. It was never reported so far that the total QA level in coffee brew could be as high as 11.8%. The total QA in brew is present as free QA, QA in CGAs, QA incorporated in HMw and IMw materials, and quinides. Quinides are QA lactones that are formed during roasting and are present up to levels of 2.0% in roasted beans (*34*). However, the quinide level is rather low in coffee brew because of hydrolysis of the internal ester during the extraction process, leaving quinide levels of 0.2–0.4% in the coffee brew (*35*). HMw and IMw fractions each represented 16% (w/w) of the brew (*20*). By using these isolation yields as well as their ester-linked QA levels (**Table 3**), it was calculated that HMw and IMw fractions together account for 0.9% of QA in coffee brew (percent of brew solids). The 11.8% of total QA in brew is distributed as follows: (I) 3.0–3.3% of free QA, (II) 2.3% of QA from 4.2% CGAs, (III) 0.9% of QA ester-linked to HMw and IMw materials, and (IV) 0.2–0.4% of QA from quinides (*34, 35*). In total, 6.9% of QA in the Brew

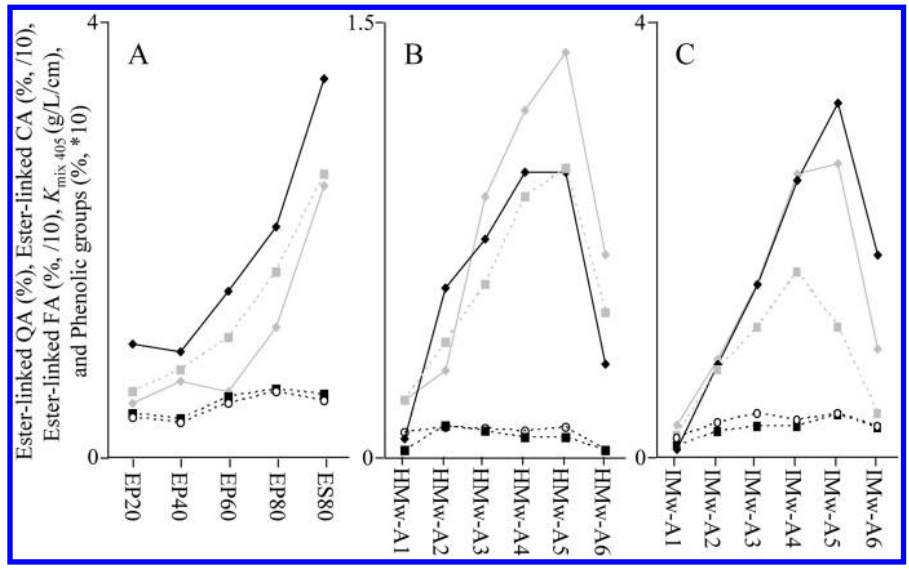

Figure 4. Ester-linked QA levels (black solid line, solid diamonds), ester-linked CA levels (black dashed line, solid squares), ester-linked FA levels (black dashed line, open circles), melanoidin levels (gray solid line, solid diamonds), and phenolic groups levels (gray dashed line, solid squares) in various Brew fractions plotted as a function of (**A**) ethanol solubility, (**B**) negative charge in HMw material, and (**C**) negative charge in IMw material.

was accounted for, and the remaining 4.9% should be ester linked to LMw coffee components such as melanoidins.

It was calculated that the ester-linked CA in HMw and IMw fractions together accounted for 0.2% of CA in coffee brew (percent of brew solids). The 4.6% of total CA in brew (**Table 2**) is distributed as follows: (I) 0.1% of free CA, (II) ~2.1% of CA from 4.2% CGAs, and (III) 0.2% of CA ester-linked to HMw and IMw materials (percent of brew solids). In total, 2.4% of CA was accounted for, and the remaining 2.2% should be ester-linked to LMw coffee components such as melanoidins.

Correlation of Melanoidin, Phenolic Groups, and CGA Derivatives Levels. It was shown that CA, FA, and especially QA were incorporated in HMw and IMw melanoidins through ester linkages. It was of importance to determine whether the level of these acids showed a correlation with the melanoidin level and/or the phenolic groups level. Therefore, the phenolic groups level, the melanoidin level ($K_{mix\ 405\ nm}$), and the ester-linked QA, CA, and FA levels were plotted as a function of the HMw ethanol precipitation fractions (**Figure 4A**) and HMw and IMw anion-exchange fractions (**Figure 4B**,**C**). These figures show that the ester-linked QA level correlates with the melanoidin level as well as with the phenolic groups level. Because CGAs are the predominant phenolics in coffee, it is believed that the presence of these phenolics in HMw and IMw fractions is the result of incorporation of CA and FA from CGAs. However, no correlation was observed between the ester-bound CA and FA levels and the phenolic groups level as determined by the Folin-Ciocalteu assay. Therefore, it is expected that CA and FA are responsible for the observed phenolic groups level, and also that these phenolic acids are mainly linked through nonester linkages. Because the ester-linked QA level correlated with the phenolic groups level, it is expected that the nonphenolic and phenolic moieties from CGAs are incorporated together. In other words, these findings indicate the incorporation of intact CGAs in coffee material. If CGAs were hydrolyzed first, the observed correlation between QA and the phenolic groups levels would indicate that both QA and CA or FA are incorporated in a similar extent in various series of fractions. Because QA and CA or FA have different chemical properties, it is rather unlikely that this occurred. Therefore, the proposed incorporation of intact CGAs seems more logical. The fact that the enzyme treatment with chlorogenate esterase released some QA and CA further strengthened the fact that the linkage between QA and CA in incorporated CGAs can survive roasting.

On the basis of these results, it can be proposed that the CGA incorporation mechanism comprises incorporation of intact CGA molecules into melanoidins via the CA moiety mainly through nonester linkages upon roasting of coffee beans. This complex can be written as Mel═CA−QA, in which Mel represents the melanoidin backbone, ═CA represents CA nonester linked to the melanoidin backbone, and −QA represents QA ester-linked to CA. Within these structures, the nonester-linked CA provides phenolic characteristics, whereas the free carboxyl group from QA contributes to the observed negative charge. However, it is unclear whether the part of QA that is ester-linked to the CA moiety is split off upon further roasting.

Comparison of the Proposed CGA Incorporation Mechanism with the Literature. Nunes and Coimbra (*15*) recently reported that alkaline fusion released ~3% of phenolics from a HMw coffee material. Their HMw fraction was comparable to the combined HMw and IMw fractions used in this study. The released phenolics by alkaline fusion were degradation products similar to those from CA, strongly indicating that CA was incorporated in HMw coffee material. The level of 3% of phenolics did not match with the relatively low level of CA released by saponification as reported herein. The difference between alkaline fusion and conventional saponification is that alkaline fusion hydrolyzes other linkages than ester linkages too, such as double bonds and ether bindings (*15, 19, 36*). On the basis of this difference, the results of Nunes and Coimbra actually complement the proposed hypothesis. The saponification step only releases ester-linked QA from Mel═CA−QA, whereas nonester-linked CA is hardly split off. The ester-linked QA level of the combined HMw and IMw fractions was 2.6%. Alkaline fusion is capable of releasing CA from Mel═CA because alkaline fusion degrades more types of bindings. If the combined HMw and IMw fractions were subjected to alkaline fusion, a CA level of 2.6% would be expected, which would be as large as the ester-linked QA level. This level is quite similar to the level of 2.7–3.2% that was reported by Nunes and Coimbra. Thus, their findings support the incorporation of CGA via CA through a nonester linkage.

In a mechanistic study on whole coffee beans, Leloup et al. (*10*) also proposed that intact CGA was incorporated in coffee bean material. However, Leloup et al. suggested that CGA was incorporated via the carboxyl group on QA. In their model, the low level of ester-linked CA, relative to the level of ester-linked QA, was ascribed to the fact that CGA incorporation was followed by hydrolysis of the internal CGA, yielding LMw phenols from CA. The results, on which their incorporation mechanism is based, are also in agreement with the incorporation mechanism proposed by us. However, in their study, no attention was paid to, for instance, the phenolic groups level and the nonester-linked CA and FA levels. Results presented in this study and results reported by Nunes and Coimbra point out that the phenolic moiety should be present in the melanoidin structure, and that it is therefore more likely that CGA is incorporated via CA. This is further strengthened by the fact that thermal degradation of HMw coffee material yielded a broad series of phenolic compounds as well (*12, 13*).

Influence of CQA Incorporation on Physico-chemical Properties of Coffee Brew. It was found that coffee brew contained 12% of QA, about half of which could be ascribed to free QA, quinides, and QA in CGAs. The additional 6% ester-linked QA was not reported so far and might have significant effects on the physico-chemical properties, such as coffee acidification. So far, acidification is ascribed mainly to a release of QA because of hydrolysis of CGAs and quinides upon storage. However, the increase in QA and other LMw acids can not fully account for the increase in acidity (*37, 38*). Now, with the additional 6% ester-linked QA in coffee brew, additional possibilities for coffee acidification are possible. For example, it might be that the ester-linked QA is actually a quinide, or that the ester-linked QA is covalently linked to CA as well as to another molecule via its carboxyl group. In both cases, hydrolysis that occurs upon storage leads to an increase of carboxyl groups and acidity, without affecting the free QA level. Therefore, it might well be that melanoidins are an important contributor to coffee acidification.

Further research, in which the releases of phenolics by saponification and alkaline fusion are compared, might lead to complete understanding of the CGA incorporation mechanism. Furthermore, investigation of the effect of the roasting degree on CGA incorporation in similar coffee brew fractions is expected to provide more insight in the kinetics of CGA incorporation in coffee brew melanoidins.

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