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Evolution of Green Coffee Protein Profiles with Maturation and Relationship to Coffee Cup Quality

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Coffee flavor is the product of a complex chain of chemical transformations. The green bean has only a faint odor that is not at all reminiscent of coffee aroma. It contains, however, all of the necessary precursors to generate the unmistakable coffee flavor during roasting. The levels and biochemical status of these precursors may vary in relation to genetic traits, environmental factors, maturation level, postharvest treatment, and storage. To improve our understanding of coffee flavor generation, the sensory and biochemical impact of maturation was assessed. Maturation clearly favored the development of high-quality flavor in the coffee brew. A specific subclass of green coffee beans, however, generated high-quality coffee flavor irrespective of maturation. Biochemical aspects were examined using a dynamic system: immature and mature green coffee suspensions were incubated under air or argon. On the analytical side, a specific pool of flavor precursors was monitored: chlorogenic acids, green coffee proteins, and free amino acids. A link between maturation, the redox behavior of green coffee suspensions, and their sensory scores was identified. Compared to ripe beans, unripe beans were found to be more sensitive to oxidation of chlorogenic acids. Aerobic incubation also triggered the fragmentation or digestion of the 11S seed storage protein and the release of free amino acids.

KEYWORDS: Green coffee; maturation; sensory evaluation; redox status; two-dimensional polyacrylamide gel electrophoresis

INTRODUCTION

Typical sensory attributes have been ascribed to Arabicas (*Coffea arabica*) and Robustas (*Coffea canephora*, genetic aspects) or to immature and mature cherries (metabolic aspects), for a long time. Even species-specific (Arabica versus Robusta) concentrations of impact odorants of the brew were identified (*1*). In fact, coffee aroma may be composed by >800 volatile compounds (*2*−*4*). Precursors responsible for the generation of these many volatile compounds have been identified: sugars, amino acids, peptides, trigonelline, chlorogenic acids, organic acids, lipids, and carotenoids (reviewed in refs 5−8). Interactions between reducing sugars and amino groups of amino acids, peptides, and even proteins (known as the Maillard reaction) have been extensively studied in model systems and also in complex beverages such as coffee (*7*−*11*). This type of reaction is recognized as essential for coffee aroma development. Along this line, free amino acid and peptide profiles were investigated in green coffee beans (*12*, *13*). The contribution of mixtures of these nitrogenous compounds to the development of coffee flavor was suggested by model roasting trials.

On the other hand, the redox status of the green coffee appears to control the final quality of the beverage (*14*). Polyphenols are well-known redox-active compounds that are found in the coffee bean. In fact polyphenols can be oxidized enzymatically by the phenolase complex (*15*, *16*). Another pathway toward oxidation should draw our attention: autoxidation (*17*, *18*). Autoxidation, as suggested by its designation, does not require the intervention of redox enzymes such as polyphenol oxidases or peroxidases. Autoxidative processes spontaneously occur in the presence of oxygen, if the redox compounds have the adequate reducing potential to reduce oxygen. Once generated, initial oxidation products (semiquinones and quinones) proceed with new reactions, which can be of enzymatic or nonenzymatic origin. For instance, quinones may react further with amino acids and proteins or polymerize (*19*−*22*).

One of the richest sources of a specific type of phenolic compound, the chlorogenic acids (CGAs), is actually the coffee bean. CGAs constitute a family of esters formed between certain transcinnamic acids (caffeic and ferulic) and quinic acid. Considering the content of chlorogenic acids in coffee [7−10 and 5−7.5% on a dry matter basis for Robustas and Arabicas, respectively (*23*)], autoxidation of CGAs might be expected during coffee processing. In fact, the importance of such reactions has long been recognized in wine aging (*24*) or staling and haze formation in beer (*25*, *26*).

The biochemical and chemical mechanisms responsible for the specificity and uniqueness of coffee flavor are still not

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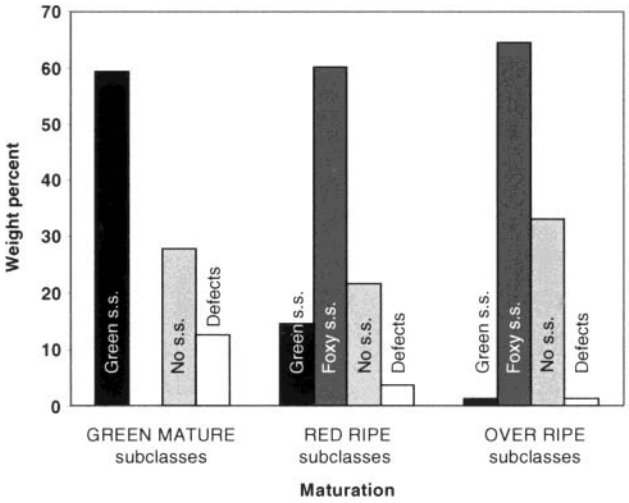

Figure 1. Quantitative distribution of subclasses within maturation classes (green mature, red ripe, and over-ripe, 100 g/class). Segregation criteria for subclasses were absence of silver skin (no s.s.) or presence of silver skin [either green (green s.s.) or reddish-brown (foxy s.s.)]. Broken and malformed beans (e.g., shell beans and black beans) were considered as defects.

understood. From the literature mentioned here, it appears that nitrogenous compounds (free amino acids, peptides) and the redox status of chlorogenic acids contribute to the development of coffee flavor and quality during roasting. During maturation, the redox status is essentially determined by metabolic aspects and the expression and activation of redox enzymes. By incubating green coffee aqueous suspensions under anaerobic and aerobic conditions, we hoped to challenge the metabolic machinery corresponding to specific maturation stages. We followed the losses of free CGAs, the evolution of protein profiles, and the release of free amino acids during anaerobic and aerobic incubation. Sensory profiles of the maturation stages were also investigated.

MATERIALS AND METHODS

Origin, Harvesting, and Preparation of Samples. *Harvesting in Relation to Maturation (Maturation Classes).* Robusta green coffee samples (*C. canephora* Pierre ex A. Froehner, Rubiaceae) were obtained from Cagayan, The Philippines. Cherries were harvested according to color from open-pollinated Robusta clones. Harvesting was completed on three consecutive days by manual picking of the cherries. Dark to red brown (over-ripe), yellow orange to red (red ripe), and light green (green mature) cherries were selectively harvested on the first, second, and third day of harvesting, respectively. Cherries with characteristic color were harvested regardless of size and shape.

Postharvest Treatment. Cherries were then processed according to the dry method. The average duration of drying was 15 days. The exact drying period, however, varied according to maturity stages. Green cherries were dried longer than over-ripe ones. No sorting or preclassification based on size and shape was made.

In the absence of a huller for such small quantities, the beans were hulled manually, using a pestle and mortar.

Extra Sorting of Original Samples (Subclasses). A high degree of heterogeneity was observed in all three green coffee samples (maturation classes) received from The Philippines. Hence, we decided to create two subclasses according to the color (green and red brown) of the integument, usually called silver skin (**Figure 1**). Another subclass was created for the beans that did not have a silver skin. Broken, malformed beans (e.g., shell beans) and black beans were classified under "defects" (**Figure 1**). Quantitative values were determined on 100 g (~1200 beans) of green coffee of each maturation stage.

Aerobic and Anoxic Incubations. Two ripe samples (red ripe foxy silver skin and red ripe no silver skin) and two unripe samples (green mature green silver skin and red ripe green silver skin) were milled under liquid nitrogen. One gram of each powder was incubated for 48 h at 37 °C in 10 mL of 0.05 M sodium borate, pH 8.5 (degassed and flushed with argon for anaerobic incubations), containing trypsin (1 mg/mL). Incubation proceeded with agitation under air or argon. After incubation, samples were freeze-dried and stored at −20 °C.

Roasting and Sensory Evaluation. *Roasting.* Green coffee beans were roasted at 180−200 °C for 20 min in a laboratory-scale commercial roaster (*Probat*, BRZ 2) and ground (Retsch type ZM1 mill). The temperature was adjusted by regulating the heating current and the flow of air through the roasting drum.

To estimate the roasting level, the Neuhaus Colortest was applied by using a Neuhaus electronic reflectometer. A sample of roasted and ground coffee (ground on an Olympia mocca express mill set at 5.5), evenly distributed on a circular surface (diameter = 7 cm), was illuminated by a monochromatic infrared source. The reflected light was expressed in Colortest numbers (CTn), based on a linear scale ranging from 0 (black) to 200 (white). For our tasting sessions, a CTn of ~100 was adopted.

Tasting. After milling, 6 g of roasted and ground coffee was poured into a cup, and 150 mL of water (Vittel Bonne Source from Perrier Vittel, France) at ~90 °C was added. After 2 min of extraction, 12 trained tasters rated the brews according to 11 descriptors (coffee aroma, Robusta flavor, body, bitter, rubbery, fruity, green, chemical/phenolic, earthy, fermented, and moldy/musty). Each descriptor was scored on an intensity scale from 0 (none) to 5 (high). Final ranking of each sample into Robusta quality classes was performed by summing the sensory scores of each descriptor and taking into account relative indices set for each descriptor. All of the samples were evaluated by comparison with a high-quality Robusta used as reference.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE). *Apparatus.* Isoelectric focusing (IEF) separation was performed with an ISOBOX (Hoefer Scientific Instruments, San Francisco, CA) connected to a MultiDrive XL power supply from Amersham Pharmacia Biotech (Europe GmbH). For the high voltage run, a Consort power supply E 752 was used. The ISOBOX was modified so that the tray could accommodate Immobiline DryStrips (Amersham Pharmacia Biotech, Europe GmbH). SDS-PAGE was performed on vertical slab gels (160 × 200 × 1.5 mm) using a Protean II xi 2-D multicell (Bio-Rad). The gels were cast in a Protean II multigel casting chamber (Bio-Rad) using a model 395 gradient former (Bio-Rad). The gels were run using Bio-Rad's 3000xi power supply. Gels were scanned with the Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA) and analyzed on a SUN Sparc Station 10 and Melanie II software from Bio-Rad.

Reagents. Reagents were from the following sources: Resolyte 3.5−10 from BDH (Pool, U.K.); acrylamide 2X from Serva (Heidelberg, Germany); piperazine diacrylamide (PDA), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and ammonium persulfate from Bio-Rad (Glattbrugg, Switzerland); glycine and sodium dodecyl sulfate (SDS) from Fluka; tris(hydroxymethyl)aminomethane, 1,4-dithioerythritol (DTE), and urea from Merck; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) from Sigma; and naphthalenedisulfonic acid disodium salt (NDS) from Kodak. All other chemicals were of analytical grade.

Protein Extraction. Green beans (n = 30/subclass) were milled under liquid nitrogen, and 200 mg samples were mixed with 3 mL of solubilization/lysis buffer (64 mM DTE, 65 mM CHAPS, 9 M urea, 35 mM Tris) and extracted for 2−3 h at room temperature with occasional vortexing. Freeze-dried coffee suspensions (200 mg) were solubilized and extracted in a similar way. After extraction, the samples were centrifuged (20000*g*, 30 min, 15 °C), and the supernatants were used immediately for electrophoresis or stored at −20 °C until use.

Electrophoresis. (a) Standard Gels. The 2D-PAGE was performed with an immobilized pH gradient (IPG) strips (Immobiline DryStrip gels, Amersham Pharmacia Biotech; see ref 27) as described by Hochstrasser et al. (*28*).

Fifty microliters of protein extracts was loaded onto rehydrated IPG strips with a nonlinear pH gradient (Immobiline DryStrip, pH 3−10NL, 18 cm).

A 300 V potential was applied and increased linearly to 3500 V within 3 h and then overnight at 5000 V (total of ~100 kVh). The temperature was maintained at 10 °C. The IPG strips were then equilibrated in 100 mL of 0.05 M Tris-HCl buffer, pH 6.8, containing 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol, and 129 mM DTE. After 12 min, the procedure was repeated with another 100 mL of equilibration buffer containing 135 mM iodoacetamide and 250 μL of bromophenol blue solution (0.5% w/v) instead of DTE (*29*). After equilibration, the IPG strip gels were transferred to 9−16% gradient polyacrylamide gels (*29*, *30*). SDS-PAGE was performed at 40 mA per gel at a constant temperature of 8 °C. Then the gels were washed, fixed, and silver-stained as described elsewhere (*30*).

(b) Gelatin Gels. To identify proteins that could not be silver-stained (negative staining), a modified 2D-PAGE procedure was applied. Gelatin was added to the polyacrylamide matrix to generate a dark background against which negatively staining proteins appeared as white, unstained, spots. Gelatin A (15 mg %) was allowed to copolymerize with the PDA−polyacrylamide gel. After the second dimensional run and prior to staining, the gels were washed in 2.5% Triton X-100 (30 min at room temperature) and then in Tris-HCl buffer (50 mM, pH 7.4; 5 mM $CaCl_2$) at 40 °C during 120 min. The gels were then silver-stained as usual.

Phenol Staining. To reveal polyphenols on two-dimensional gels, Folin−Ciocalteu's reagent (Sigma) was used. After electrophoresis, gels were fixed in 12% trichloroacetic acid, washed in H_2O, and incubated with Folin−Ciocalteu's solution according to Jennings method (*31*) until a blue coloration appeared.

Free Chlorogenic Acids. *Sample Preparation.* Green coffee powders (500 mg) were extracted with 70% methanol, using the Dionex ASE-200 system (ASE = accelerated solvent extractor). This fully automated extraction system was validated prior to routine use. Each sample was mixed with enough sand (extra pure sea sand from Merck) to fill the stainless steel extraction cell. Two successive extractions were performed (10 min at 40 °C). Each extraction cycle included a 5 min heating period to reach thermal equilibrium and a 5 min static period. Applied pressure was 1500 psi. The extraction cell was then flushed with a 100% volume of fresh solvent and purged for 2 min with nitrogen. Both extracts were pooled, and the total volume was adjusted to 100 mL.

Determination of Caffeoylquinic Acids by Reversed-Phase HPLC. Ten microliters of sample extract was injected onto a Nucleosil-100, C-18 column, 250 × 4 mm id., 5 μm (Macherey-Nagel). The column was eluted (1.0 mL/min) using an aqueous trifluoroacetic acid (TFA, 0.1%)/0−51% acetonitrile gradient (detection at 325 nm). 5-Caffeoylquinic acid (Fluka) was used as an external standard. A correction factor of 1.36 was applied to quantify dicaffeoylquinic acids (no commercial standard was available).

Free Amino Acids. Samples were finely ground and extracted with *n*-hexane, precolumn derivatized with *o*-phthalaldehyde (OPA) and 9-fluorenylmethoxycarbonyl (FMOC), and analyzed by reversed-phase HPLC.

Apparatus. A Hewlett-Packard AminoQuant HP 1090M liquid chromatograph equipped with a binary solvent delivery system was used. This system was combined with an HP 1046A fluorescence detector.

A Hypersil ODS separation column (5 μm, 250 × 4.0 mm, HP catalog no. 799260D-584) and a LiChrospher 100 RP-18 precolumn (5 μm, 4 × 4 mm, HP catalog no. 799250D-504) were adapted to the system.

Reagents. Reagents were from the following sources: *o*-phthalaldehyde from Serva; potassium borate and tetrahydrofuran (THF) from Pierce; mercaptopropionic acid from Fluka; ethylenediaminetetraacetic acid (EDTA) and *n*-hexane from Merck; and CH_3CN from Baker.

The standards used were HP amino acid standards of 10, 25, and 100 pmol/μL in 0.1 N HCl and a calibration reference standard no 2.

Preparation of the Coffee Samples and Derivatization. Ground green coffee was defatted with *n*-hexane during 2 h at 70 °C. Freeze-dried green coffee aqueous suspensions were not defatted. Five hundred milligrams of either defatted green coffee or freeze-dried suspensions were mixed with 9 mL of a MeOH/H_2O (4:6) mixture and ultrasonicated for 30 min in an ice-cold water bath. An adequate amount of norvaline (internal standard, e.g., 200 μL of a 2.5 μg/mL norvaline solution) was added, and the volume was adjusted to 10 mL with H_2O. This mixture was finally centrifuged at 10000 rpm for 10 min.

Derivatization was performed on-line using OPA for primary amino acids and FMOC for secondary amino acids.

HPLC Separation and Detection. Separation by HPLC was performed using the following mobile phases.

Mobile Phase A. One liter of sodium acetate (25 mM) was mixed with 40 mg of EDTA and 90 μL of triethylamine (TEA). The pH was adjusted to 7.20 ± 0.05 by adding a few drops of 1−2% acetic acid. This solution was filtered over a Waters HA filter, and 6 mL of THF was added.

Mobile Phase B. Two hundred milliliters of sodium acetate (100 mM) was mixed with 40 mg of EDTA. The pH was adjusted to 7.20 ± 0.05 by adding a few drops of 1−2% acetic acid. This solution was then filtered over a Waters HA filter, and 800 mL of acetonitrile was added.

Fluorescence detection (230/450 and 266/305) was used.

RESULTS AND DISCUSSION

Postharvest Processing and Maturation. Beans alone are required for the preparation of coffee. Skin, pulp, mucilage, parchment, silver skin (to some extent), and water must be removed before storage and roasting. Basically, that is what postharvest processing is all about. There are three main processes commonly used in the coffee world: dry, semidry, and wet. The dry process is the oldest and simplest; it essentially consists of drying the cherries, most usually in the sun, before hulling. Beans treated according to this method are slightly lighter in color because of the silver skin that has not been removed. The uneven and slow maturation of coffee cherries (6−8 and 9−11 months for the species of economic value, *C. arabica* and *C. canephora*, respectively) results in the coexistence of green (unripe), red (ripe), and dark red (over-ripe) cherries on the same tree. Strip harvesting, a method of harvesting that is typical of the dry process, does not include any form of classification or selection. Dry-processed coffee is consequently composed of beans representing various maturation stages unless, as required for this study, a preselection according to the color of the cherry was performed.

During the wet and semidry processes, pulping is the essential operation by which the soft pulpy part of the cherry together with the skin is removed. To be effective, pulping machines have to be fed with cherries of the right texture corresponding to ripe cherries. Hence, to evaluate the sensory contribution of unripe and over-ripe cherries altogether with ripe cherries, we had to adopt the dry process.

Maturation Classes and Subclasses. The replacement of chlorophyll in the pericarp by red flavonoid pigments indicates maturity. Hence, the color of the cherry is a good marker of maturation. We used this marker to segregate harvested cherries into three maturation classes, which were called green mature, red ripe, and over-ripe (corresponding to a light green, yellow orange to red, and dark red to brown pericarp, respectively).

After postharvest treatment, we observed heterogeneity among the green beans corresponding to each maturation class. Some beans failed to display any integument, commonly called silver skin. They belong to the "no silver skin" subclass. Among the beans harboring a silver skin, green and red-brown silver skins were observed. They belong to the "green silver skin" and "foxy silver skin" subclasses, respectively. The quantitative distribution of subclasses within maturation classes suggests that the green silver skin turns into foxy silver skin upon contact with the red flavonoid pigments that appear with maturity (**Figure 1**). The green silver skin subclass was best represented among green mature beans (59.5%), whereas the foxy silver skin subclass

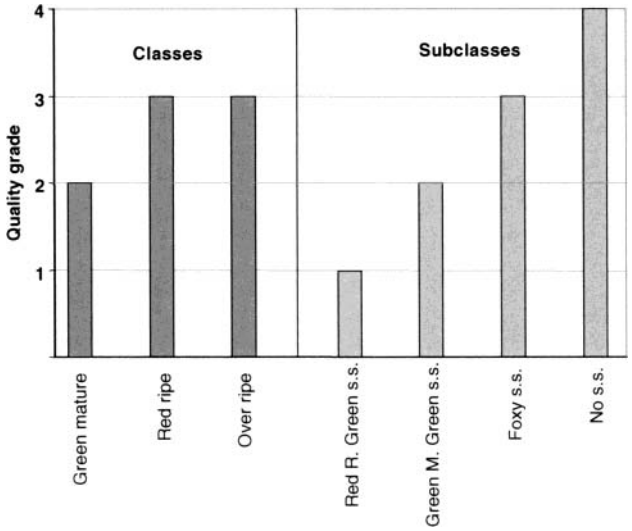

Figure 2. Quality grading of classes and subclasses. Samples were ranked among four quality grades (1 = very low, 2 = low, 3 = good, 4 = very good). Descriptors were coffee aroma, Robusta flavor, body, bitter, rubbery, fruity, green, chemical/phenolic, earthy, fermented, and moldy/musty.

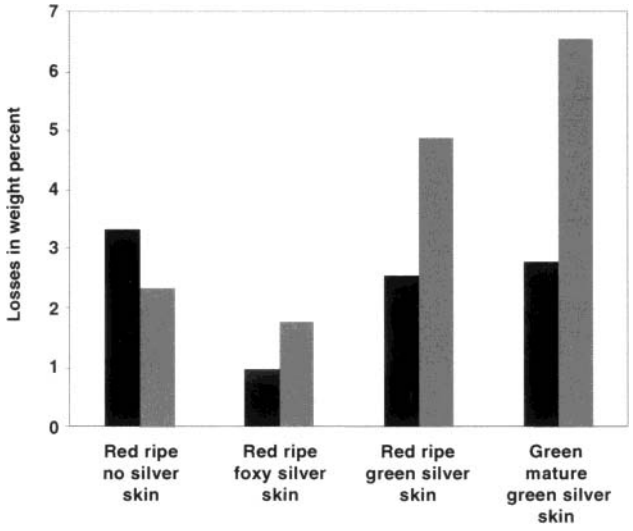

Figure 3. Absolute quantitative losses of CGAs during incubation of milled green bean suspensions in borate buffer as described under Materials and Methods: (black bars) incubation under argon; (gray bars) incubation under air. Two ripe (red ripe no silver skin and red ripe foxy silver skin) and two unripe (red ripe green silver skin and green mature green silver skin) samples were selected for investigation.

was found exclusively among red ripe and over-ripe beans (60 and 64%, respectively). The no silver skin subclass was, however, present in significant amounts in all three maturation classes (21−33%), suggesting that this trait resulted from genetic differentiation rather than maturation. In fact, Robustas are strictly allogamous with a gametophytic system of self-incompatibility that favors genetic variability (*32*).

Quality Grades of Classes and Subclasses. Four quality grades were defined according to sensory scores (grade 1 = very low, grade 2 = low, grade 3 = good, and grade 4 = very good) (**Figure 2**). As expected, quality improved with maturation. Quality grading of each type of subclass was remarkably consistent across maturation classes (**Figure 2**). Surprisingly, among all subclasses, the no silver skin subclass achieved highest quality regardless of maturation (**Figure 2**). This trait is of great interest because it confers the potential to produce quality coffee on immature beans. Due to geographical and cost considerations, Robustas are usually dry-processed. Immature beans are not removed during this process, leveling the final quality of the cup.

CGAs and Maturation. We selected a borate buffer to perform our incubation experiments. A basic pH was chosen to favor autoxidation reactions and boric acid−diol interactions. This type of interaction is favored by the 1,2-*cis*-diol configuration found in catechols and leads to the formation of borate−polyol esters (*33*). Such complexes should prevent undesirable oxidation reactions with any residual oxygen that might still be present during anoxic incubations. Under aerobic conditions, there is enough oxygen to compete with boric acid and oxidation of CGAs can proceed.

Upon incubation, losses of CGAs were observed (**Figure 3**). As expected, losses were triggered by aerobic incubation, hence, by oxidation of these phenolic compounds. Oxidation of CGAs generates reactive semiquinones and quinones. Absolute (**Figure 3**), as well as relative, losses of CGAs were more prominent in unripe samples than in ripe samples. This observation suggests that protective mechanisms against oxidation are more effective in mature beans. Alternatively, mature beans may contain less oxidase (polyphenol oxidase and peroxidase, for instance).

Under anaerobic conditions, losses may be best explained by the reaction of existing quinones with CGAs (**Figure 3**). Once formed, quinones do not require oxygen to react with nucleophiles. A quinone (for instance, an oxidized CGA) can react as an electrophile with the nucleophilic center of a CGA and generate a dimer. Consistent with our previous observation, mature beans seem to contain lower amounts of oxidized phenols. This indicates that the redox status of the green bean varies with maturation and that variations may be ascribed to the efficiency of the bean's protective system to escape from oxidative injury.

The sample without silver skin displayed an atypical behavior as far as losses of CGAs were more important under anaerobic conditions than under aerobic conditions. One may speculate that a subclass-specific set of enzymes or an atypical expression pattern of enzymatic activities might cause this behavior. For the time being, we cannot provide an explanation for this observation.

Coffee Storage Protein. The coffee storage protein (11S) was characterized by Rogers et al. (*34*). Its physiological role is to provide a source of amino acids and nitrogen for seed germination. Storage protein accounts for ∼45% of total proteins in the coffee bean (*34*). Under reducing conditions, the mature precursor form generates one high molecular weight subunit (α-component, ∼32 kDa) and one low molecular weight subunit (*β*-component, ∼22 kDa). Isoforms of each subunit constitute the α- and *β*-arms of each subunit. On the basis of the publication of Rogers et al. (*34*), subunits of coffee storage protein can easily be recognized on two-dimensional profiles of green coffee proteins.

Unripe green coffee samples displayed smeared profiles and fuzzy spots when compared to the ripe samples (**Figure 4**). Isoforms of the α-arm (rectangles in **Figure 4**) appeared to be especially prone to this phenomenon. In our experiments, polyphenols were not removed prior to electrophoresis. Reactive quinones in coffee samples are likely to generate smearing by interacting with migrating proteins and with the polyacrylamide matrix. Hence, samples with a higher quinone content, as might be expected for unripe beans, should lead to stronger smearing.

During incubation under argon, fuzzy spots of the α-arm tended to disappear first (**Figure 4A** versus **Figure 4B**). The

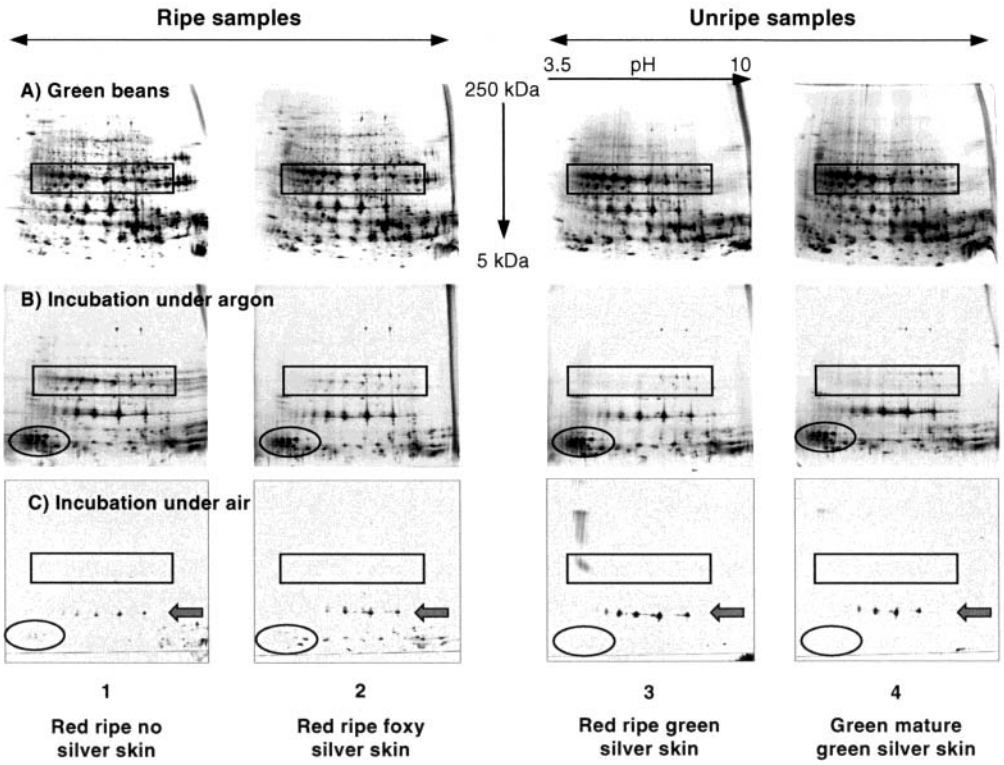

Figure 4. Green coffee protein profiles (obtained by 2D-PAGE) before (A) and after incubation under either anaerobic (B) or aerobic conditions (C) (as described under Materials and Methods). Two ripe and two unripe samples were selected for investigation. The α- and β-arms of the 11S coffee storage protein are highlighted by a rectangle and an arrow, respectively. After incubation, new acidic low molecular weight proteins appeared on the gels (ovals).

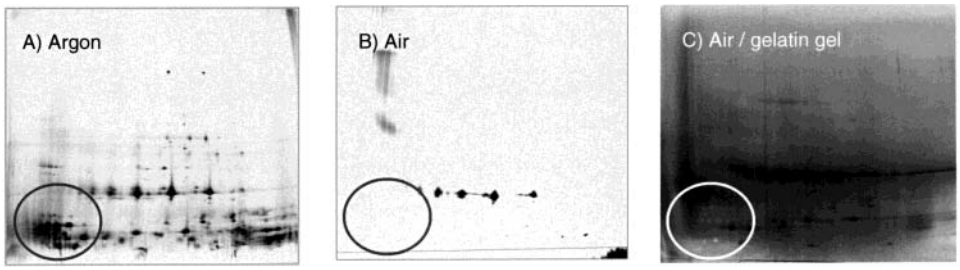

Figure 5. Identification of negatively staining proteins in gelatin gels (2D-PAGE procedure and silver staining as described under Material and Methods). Gelatin A (15 mg %) was included in the polyacrylamide matrix to generate a dark background upon silver staining (C). Aerobic (B, C) incubation promoted negative staining of acidic protein fragments (circles) that became visible in a gelatin gel (C versus B). Incubation under argon restored the usual staining behavior of proteins (A).

β-arm, which is known to be less hydrophilic than the α-arm (*34*), was much less responsive to this process. Trails of acidic low molecular weight proteins also appeared during the anaerobic incubation (ovals in **Figure 4B**), most probably as a consequence of protein fragmentation or digestion.

When incubation was performed under aerobic conditions, the acidic low molecular weight proteins and the α-arm totally disappeared, whereas the β-arm was partially preserved (**Figure 4C**). When gelatin was included in the gels (**Figure 5C** versus **Figure 5B**), a dark background was generated. The acidic low molecular weight fragments that produced a black stain after anaerobic incubation (**Figure 5A**) reappeared here as white spots. The α-arm, however, was still missing, pointing to fragmentation or digestion of these proteins during aerobic incubation. At this point, no correlation between fragmentation or digestion kinetics and maturation could be identified, because the α-arm had already disappeared in all samples.

Like degradation of storage protein isoforms, release of free amino acids during incubation relied on the presence of oxygen (**Figure 6**). Unripe samples, however, displayed lower levels of free amino acids after aerobic incubation. Accordingly, release of free amino acids might be less efficient in unripe beans. Differences in free amino acid concentrations may also simply reflect the levels of reactive quinones in the samples. In unripe samples, which appeared to be more prone to oxidation, the formation of free amino acid−polyphenol adducts should be favored, reducing the apparent levels of released free amino acids.

If we consider the acidic low molecular weight fragments, it appears that redox conversion controlled the observed dual staining behavior (**Figure 5**). Redox-sensitive protein−polyphenol adducts are strong candidates to explain such atypical staining behavior. In fact, negatively staining proteins and storage proteins were the only proteins that responded to Folin−Ciocalteu's phenol reagent (data not shown). This observation should be confirmed by a more precise technique such as mass spectrometry. Mass spectrometry might also provide interesting structural data about protein−polyphenol adducts.

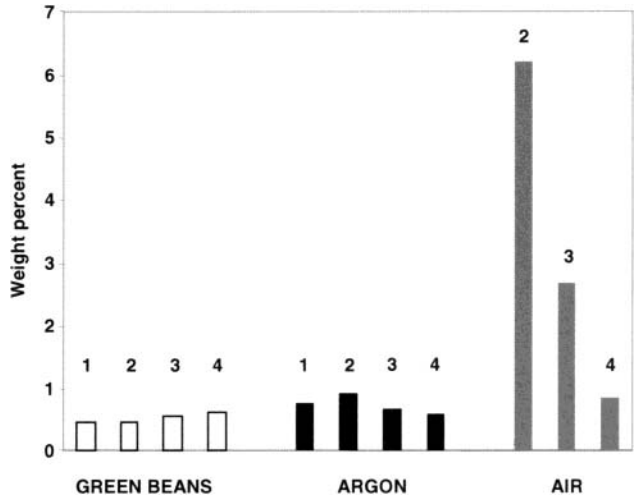

Figure 6. Release of free amino acids during incubation of green bean suspensions in borate buffer (as described under Materials and Methods): (1) red ripe no silver skin; (2) red ripe foxy silver skin; (3) red ripe green silver skin; (4) green mature green silver skin; (white bars) free amino acids in green coffee samples; (black and gray bars) free amino acids after incubation under argon and air, respectively. Red ripe no silver skin sample (1, air) was not investigated due to lack of material.

At the molecular level, this staining behavior can be explained as follows. The silver stain is based on the redox chemistry of silver (*35*). The dark color of the silver stain is due to molecular silver that forms upon reduction of protein-bound silver ions. The conversion of putative phenol-proteins into quinone-proteins by O_2 conveys oxidative strength to the adducts that potentially outweighs the oxidative strength of protein-bound silver ions. When exposed to the developing solution (citric acid and formaldehyde), putative quinone-proteins may sequester the electrons supposed to reduce protein-bound silver ions to dark molecular silver, causing negative staining.

Oxidative or Enzymatic Fragmentation of Coffee Storage Protein. Ludwig and colleagues (*13*) detected endogenous protease activities in green coffee beans. The use of antioxidants to block the reaction of plant phenols with coffee proteins and polyvinylpolypyrrolidone to remove polyphenols appeared to be a prerequisite to observe proteolytic activity in green coffee extracts. Identified proteolytic activities could not be assigned to known families of proteases, precluding the efficient use of protease inhibitors, and did not release single amino acids. To clarify the situation as to the mechanism of protein degradation, we decided to add trypsin (1 mg/mL) to the incubation buffer, guessing that it would be inactivated. Actually, oxidative conditions should trigger the inhibition of trypsin and other proteases. However, in reality, we observed that aerobic incubation favored storage protein degradation, suggesting that oxidative fragmentation rather than proteolytic digestion was occurring. If we assume that a redox-driven fragmentation process is generating the observed negatively staining fragments, protein−polyphenol adducts might play an important role in this process.

One should, however, bear in mind that oxidation of proteins may alter their conformation and increase their sensitivity toward active proteases. In the case of CGA−albumin adducts, for instance, covalent attachment of CGAs to albumin hampers tryptic digestion of that protein, then it is promoted upon attachment of additional CGAs (*36*). Hence, a sequential synergy between oxidation and proteolytic activity cannot be totally excluded.

This study defined the sensory contribution to the coffee brew of specific Robusta green coffee maturation classes and subclasses. Ripe beans provided a better quality brew. A specific subclass, the no silver skin subclass, provided optimal quality irrespective of maturation. This subclass is of great interest for further investigations aiming at the identification of quality makers in green coffee. The sensitivity of ripe and unripe samples toward oxidation differed, suggesting that defense mechanisms against oxidative stress become more efficient during maturation and remain effective in green coffee suspensions. Alternatively, oxidases may be less efficient in ripe samples. This also suggests that the redox status of the green coffee bean may influence the development of quality in the brew. The oxidative degradation process of coffee storage protein subunits may contribute to coffee flavor generation by providing nitrogenous flavor precursors. Its relevance, in particular during roasting, has to be further examined.

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