

Characterization and Estimation of Proanthocyanidins and Other Phenolics in Coffee Pulp (*Coffea arabica*) by Thiolysis–High-Performance Liquid Chromatography

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Fresh and 3-day-old coffee pulp of the Arabica variety were analyzed for polyphenol composition followed by characterization by two different methods. The first method consisted in subjecting coffee pulp powder to direct thiolysis. For the second method, coffee pulp was subjected to successive solvent extractions, followed by thiolysis. Quantification of phenolic compounds was then achieved by high-performance liquid chromatography (HPLC) analysis of thiolysis products. Four major classes of polyphenols were identified: flavan-3-ols (monomers and procyanidins), hydroxycinnamic acids, flavonols, and anthocyanidins. Differences in concentration of procyanidins were observed between fresh and 3-day-old coffee pulp. Constitutive units were mainly epicatechin, representing more than 90% of the proanthocyanidin units, with average degrees of polymerization in the range of 3.8–9.1. Monomer to hexamer units of flavan-3-ols from fresh coffee pulp were separated by normal-phase HPLC. Molecular size of oligomeric proanthocyanidins was obtained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Results obtained confirm the presence of oligomers of the flavan-3-ol (–)epicatechin.

KEYWORDS: Coffee pulp; Arabica; polyphenol; proanthocyanidin; thiolysis; MALDI-TOF MS

INTRODUCTION

Coffee is the major cash crop for many developing countries such as Brazil, Venezuela, and Mexico. Coffee pulp is the major byproduct of the coffee industry. It represents 40% of the fresh weight of coffee cherries (1). In recent years, there has been an increasing trend toward efficient utilization and value addition of agroindustrial residues such as coffee pulp. These include its use as animal feed or as a substrate for enzyme production by solid-state fermentation (2). Polyphenol composition in coffee pulp has been studied because various components, including caffeine, low molecular mass phenols, and tannins have been blamed for antinutritive or toxic effects when the pulp was tested as animal feed (3–5). Information about phenolic compounds in coffee pulp is scarce and often contradictory. Zuluaga-Vasco and Tabacchi (6) found that fresh coffee pulp contained no condensed tannins. In 1991, however, Clifford and Ramirez-Martinez (7) estimated that Arabica coffee pulp contained 1%

condensed tannins. More recently, Gonzalez de Colmenares et al. (8) isolated dimer-rich and oligomer-rich fractions of specific condensed tannins, also known as proanthocyanidins, establishing their presence in coffee pulp.

Proanthocyanidins (condensed tannins) are polymers of flavan-3-ol units linked by carbon–carbon bonds; they are naturally occurring polyphenolic compounds that are present in fruit and leaves of many higher plants (9). Flavan-3-ols are present either as individual monomers or as oligomeric units. Many plant species contain relatively high concentrations of tannins, which are thought to reduce dietary quality by reducing protein and carbohydrate digestibility, with direct physiological effects on herbivores. When absorbed, condensed tannins can be toxic to some species of mammals (10, 11). In fruit-derived products (wine, ciders) and more generally in many foodstuffs, proanthocyanidins largely contribute to organoleptic qualities such as bitterness and astringency (12, 13). More recently, proanthocyanidins have attracted interest because of their antioxidant properties (14, 15).

Thiolysis, alone or coupled with high-performance liquid chromatography (HPLC) analysis of the reaction mixture, has been used to characterize and quantify proanthocyanidins in

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plants and fruits (16–19). The method consists of heating proanthocyanidins in the presence of HCl and a nucleophile such as toluene- α -thiol. Lower units (terminal units) are released by hydrolysis, whereas the upper units (extension units) yield benzyl thioether derivatives (20, 21). Properties of proanthocyanidins vary according to the nature and number of their constitutive units. Thiolysis can also be used to characterize the mean degree of polymerization (DP_n), giving valuable information regarding the oligomeric structure of proanthocyanidins. The DP_n is calculated by HPLC analysis after thiolysis. Normal-phase HPLC is used to separate proanthocyanidin oligomers according to their molecular weight (22–24). Mass spectrometry (MS), particularly when coupled with electrospray or matrix-assisted laser desorption ionization (MALDI) sources, is a powerful technique for oligomeric proanthocyanidins analysis and identification (25–30).

This study investigated the quantification and characterization of native phenolic compounds in fresh coffee pulp as compared to those of 3-day-old pulp. The aim of the present work was to evaluate changes in polyphenolic compounds that may occur between harvest and potential consumption of coffee pulp.

MATERIALS AND METHODS

Plant Material. Red coffee cherries (*Coffea arabica*) of the Arabica variety were collected from different trees and divided into two lots. The seeds of the first one were removed and the pulp was immediately frozen in liquid nitrogen. After removal of the seeds, the pulp of the second lot was sun-dried for 72 h and frozen in liquid nitrogen. Frozen pulp from each lot was lyophilized and ground into fine powders and kept dry and in the dark until use.

Solvents and Phenolic Standards. Methanol, acetone, and acetonitrile were HPLC-grade (Darmstadt, Germany). Hexane and acetone were analytical-grade (Merck). Deionized water was obtained with a Milli-Q Waters system (Millipore, Bedford, MA). (+)-Catechin, (–)-epicatechin, caffeoylquinic acid, and *p*-coumaric acid were purchased from Sigma (St. Louis, MO). Hyperoside (quercetin-3-galactoside) and ideain were purchased from Extrasynthese S.A. (Lyon, France). Caffeine was obtained from Merck (Darmstadt, Germany). *p*-Coumaroylquinic acid and (–)-epicatechin benzyl thioether were kindly provided by the Laboratoire de Recherches Cidricoles, INRA, Rennes, France.

Successive Solvent Extraction Procedure. Five grams of freeze-dried material from each lot was first extracted with 3×50 mL of hexane to remove carotenoids and other lipophilic compounds. The remaining solids were treated with methanol (3×50 mL, containing 2.5% acetic acid) to further extract sugars and phenolic compounds. The insoluble material was then subjected to three successive extractions with 50 mL of aqueous acetone (2:3) containing 2.5% acetic acid. The resulting suspension from each step was filtered through a G3 sintered glass filter. The hexane filtrate was not analyzed. The methanol filtrates were combined and concentrated to 20 mL by evaporation under vacuum at 35 °C. Deionized water (60 mL) was added and evaporation of methanol was completed. The aqueous acetone filtrates were combined, evaporated, and freeze-dried, as were the methanol extracts, and stored prior to analysis.

Thiolytic Reaction. Coffee pulp dry powders were directly analyzed by thiolysis (31), as were the dry extracts after successive solvent extractions (18). For thiolysis reactions, 30 mg of each freeze-dried coffee pulp powder, or extract, or extraction residue, was used as starting material. Toluene- α -thiol (5% in methanol, 800 μ L) was added to each sample. This was followed by the addition of 400 μ L of 0.4 N HCl in methanol. Each reaction tube was closed and incubated at 40 °C for 60 min with mixing on a vortex mixer every 10 min. The tubes were put on ice to stop the reaction. The mixture, 200 μ L, was immediately filtered through a polytetrafluoroethylene (PTFE) membrane (0.45 μ m) into a vial closed with a Teflon cap and stored at 4 °C for reverse-phase HPLC analysis (18).

Native Monomers. Native (–)-epicatechin and (+)-catechin already present in fresh and 3-day-old coffee pulp were analyzed by reversed-phase HPLC without thiolysis as described by Guyot et al. (31).

Reverse-Phase HPLC Analysis. Thiolysis media (10 μ L) from fresh or 3-day-old pulp samples were injected into a Waters analytical HPLC equipped with an autosampler, a cooling system set to 4 °C, and a model 996 photodiode array detector. The column was a 250 \times 4 mm i.d., 60 Å, 5 μ m Purospher RP18 (Merck, Germany). The temperature of the column was maintained at 30 °C. Solvents for elution were acetonitrile (solvent A) and aqueous acetic acid (2.5% v/v; solvent B). The solvents were filtered through 0.45 μ m PTFE membranes. The flow rate was maintained at 1 mL/min, and elution of phenolic compounds was monitored at 280, 320, 350, and 515 nm. Data processing was done by Millennium software. The elution conditions were as follows: initial condition 3% A; 0–5 min, 9% A linear; 5–15 min, 16% A linear; 15–45 min, 50% A linear; 45–48 min, 90% A linear; and 48–51 min, 90% A. The column was then washed and reconditioned (18, 31).

Polyphenol Characterization. Flavanols [(+)-catechin, (–)-epicatechin, and (–)-epicatechin benzyl thioether] were monitored at 280 nm in reference to the corresponding standards and their retention times as well as by their respective UV–visible spectra. Caffeoylquinic acid, *p*-coumaroylquinic acid, and other hydroxycinnamic acids were quantified at 320 nm, flavonols at 350 nm, and anthocyanidins at 515 nm (18, 36). Flavan-3-ols, caffeoylquinic acid, and *p*-coumaroylquinic acid were compared to available standards. Concentrations are expressed in grams per kilogram of dry weight, and all experiments were conducted in triplicate. The total concentration of proanthocyanidins was obtained by summing all catechins present on the HPLC chromatogram and by subtracting the amount of native catechins assayed without thiolysis (31).

Polyphenol Purification. Elimination of sugars from the methanol and aqueous acetone extracts of the fresh and 3-day-old coffee pulps was carried out as follows: 150 mg of dry extract was suspended in 40 mL of dilute acetic acid (2.5% v/v) and purified on 5-g C18 Sep-Pak cartridges (Waters, Milford, MA). The cartridges were conditioned with 20 mL of methanol and then equilibrated with 40 mL of 2.5% acetic acid (v/v). The sample was eluted, and the cartridge was rinsed with 80 mL of acetic acid (2.5% v/v) to remove sugars. Polyphenols were eluted with 20 mL of acetone–water (6:4) containing 2.5% acetic acid (v/v). The eluate was collected, evaporated, and lyophilized in preparation for normal-phase HPLC and MALDI time-of-flight (TOF) MS analyses.

Normal-Phase HPLC Fractionation of Procyanidin Oligomers of Coffee Pulp Extracts. Coffee pulp polyphenols from the methanol and aqueous acetone extracts (fresh or 3-day-old coffee pulps) were analyzed as follows. Five milligrams of Sep-Pak purified extract was dissolved into 200 μ L of solution containing MeOH/CH₂Cl₂/H₂O/HCOOH (7:41:1:1). The mixture was sonicated and filtered through a PTFE membrane (0.45 μ m), and 10 μ L was injected into the normal-phase HPLC system. Chromatographic analyses were performed on an Agilent 1100 HPLC system with a binary HPLC pump, a 10 μ L injection loop, and a detector set at 280 nm. Compounds were separated by a gradient elution system on a 150 \times 3.9 mm i.d., 4 μ m, Nova-Pak silica column (Waters, Milford, MA), at room temperature, with a flow rate of 1 mL/min.

The eluents were MeOH/CH₂Cl₂/H₂O/HCOOH (43:5:1:1) for solvent A and MeOH/CH₂Cl₂/H₂O/HCOOH (7:41:1:1) for solvent B. Compounds were separated by a linear gradient: initial, 100% B; 0–30 min, 20% A; 30–35 min, 25% A; 35–40 min, 100% A; and 40–42 min, 100% B isocratic.

MALDI-TOF Mass Spectrometry of Procyanidin Oligomers. The MALDI-TOF mass spectra were collected on a Hewlett-Packard LDI 1700XP time-of-flight mass spectrometer, equipped with delayed extraction and N₂ laser, set at 337 nm. For positive-ion linear mode spectra, an accelerating voltage of 30 kV, an extractor voltage of 9 kV, and a pressure of 8×10^{-7} Torr were used. The lyophilized extracts of coffee pulp were reconstituted in methanol to give a final concentration of 500 mg/L and mixed with a matrix solution of 10 g/L 2,5-dihydroxybenzoic acid in methanol. Sample and matrix were mixed in

Table 1. Quantification of Phenolic Compounds Present in Fresh and 3-Day-Old Pulps, Analyzed by HPLC^a

compounds	fresh	3-day-old
flavan-3-ols		
monomers	2.0 ± 0.6	0.6 ± 0.07
proanthocyanidins	20.1 ± 0.7	15.0 ± 0.4
hydroxycinnamic acids		
caffeoylquinic acid	11.8 ± 0.01	12.8 ± 0.3
caffeoylquinic acid derivatives	2.4 ± 0.8	3.1 ± 0.1
<i>p</i> -coumaroylquinic acid	0.6 ± 0	0.08 ± 0
flavonols	0.6 ± 0.02	0.5 ± 0.05
anthocyanidins	0.4 ± 0.22	0.5 ± 0.2
total	37.9 ± 1.0	32.6 ± 1.2

^a Quantities are given as grams per kilogram of dried material, with standard deviation ($n = 3$).

a relation of 1:1 (v/v). The mix (1 μ L) was put on a stainless steel target and crystallized at room temperature before analysis.

RESULTS AND DISCUSSION

Considerable progress has been made in recent years in the field of polyphenol assay and analysis. The most widely used assays are colorimetric (32). However, depolymerization reactions in the presence of a nucleophile (thiolysis reactions) have become powerful tools, mainly due to their sensitivity and specificity. Thiolysis, when associated with HPLC, has proven to be a useful method, not only to characterize and quantify polyphenols in general but also to allow the precise characterization and quantification of specific groups of polyphenols, namely, proanthocyanidins. HPLC before and after thiolytic treatment of coffee pulp samples was used successfully to separate and analyze polyphenols present in coffee pulp.

Composition of Phenolic Compounds in Fresh and 3-Day-Old Coffee Pulp. The polyphenols of fresh and 3-day-old coffee pulp were characterized by thiolysis coupled with reverse-phase HPLC and also by direct HPLC analysis without thiolysis. Phenolic compounds were identified according to their retention time and their UV-visible spectra. Quantification was performed by use of the response factors of the corresponding standard compounds. The total concentration of phenolic compounds coming from fresh coffee pulp, as determined by thiolysis, was 37.9 g/kg (Table 1). The result is higher than previously reported (3, 6–8). However, it is important to note that all previous data of polyphenols present within coffee pulp resulted from either methanol extracts (3), or acetone extracts (7, 8), giving partial estimates. Phenolic compounds identified in fresh coffee pulp were grouped into four classes: flavan-3-ols including monomeric catechins and proanthocyanidins, hydroxycinnamic acids (caffeoylquinic acid, caffeoylquinic acid derivatives, and *p*-coumaroylquinic acid), flavonols, and anthocyanidins. Flavan-3-ols were the predominant group, representing over 58.3% of total polyphenols identified. Monomeric flavan-3-ols account for 5.3% of total phenolic compounds present in fresh coffee pulp. All classes of polyphenols were present both in fresh pulp and in 3-day-old pulp. Quantitative data are presented in Table 1. Results show an overall decrease by 14% of total polyphenolics (from 37.9 to 32.6 g/kg), on a dry weight basis, when coffee pulp was left to dry for 3 days. Flavan-3-ols accounted for most of the observed decrease.

The total concentrations of hydroxycinnamic acids were 14.8 and 16.0 g/kg, respectively, for fresh and 3-day-old coffee pulp. After proanthocyanidins, hydroxycinnamic acids correspond to the second most abundant polyphenol class in coffee pulp, as also reported in apples (36). They account for 39% of polyphenols

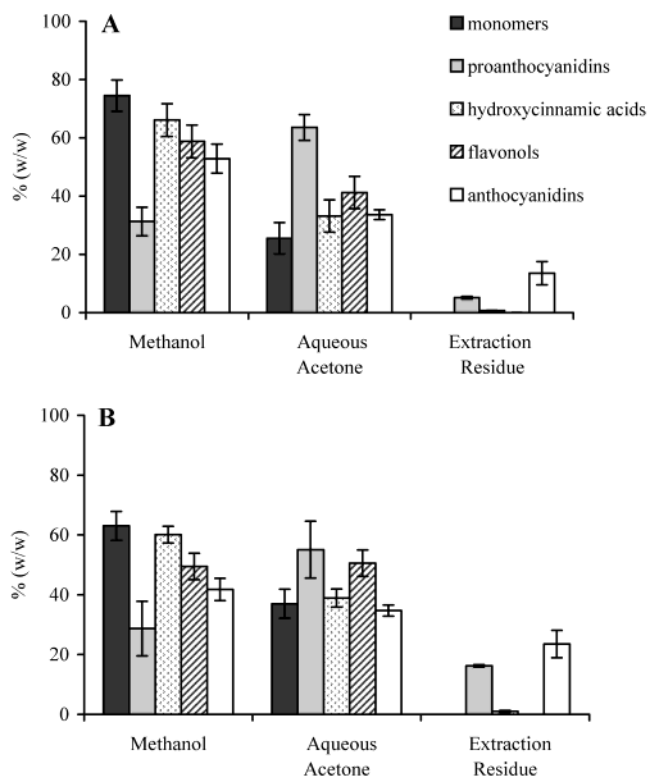


Figure 1. Percentage distribution of phenolic compounds of (A) fresh and (B) 3-day-old coffee pulp present in the methanol extract, aqueous acetone extract, and extraction residue.

nols in fresh coffee pulp and 49% of polyphenols in 3-day-old coffee pulp. Caffeoylquinic acid did not decrease in 3-day-old coffee pulp. It is known to have a greater redox potential than catechins and may play an important role in proanthocyanidin oxidation. Moreover, caffeoylquinic acid is known to enhance the degradation rates of epicatechin in apples (34) and pears (35), thereby explaining the observed decrease in coffee pulp (Table 1). In addition, higher amounts of proanthocyanidins were present in the non-organic-extractable residue of 3-day-old coffee pulp, as compared to fresh coffee pulp (Figure 1). As pointed out previously by Ramirez-Martinez (3), flavan-3-ols and hydroxycinnamic acids together account for over 97.3% of total identified polyphenolic compounds. Flavonols and anthocyanidins can be considered as minor classes in coffee pulp, representing 1.6% and 1.1%, respectively, of total polyphenol concentration. However, the identity of the compounds within these classes remains unknown.

Fractionation of Coffee Phenolic Compounds. To further characterize polyphenolic constituents present within coffee pulp, freeze-dried pulp (fresh and 3-day-old) was successively fractionated into hexane, methanol, and aqueous acetone extracts, leaving a final insoluble residue. Methanol extracted more material from fresh coffee pulp as compared to 3-day-old pulp (46% and 26% respectively, of the 5 g of lyophilized material subjected to the various extraction steps). On the contrary, acetone extracted more material from 3-day-old coffee pulp (23% and 13%, respectively). Finally, the residue from 3-day-old pulp was more important (46% in 3-day-old pulp versus 36% in fresh pulp).

For fresh coffee pulp, most of the tissue constituents were extracted by methanol. Three-day-old coffee pulp yielded larger quantities of insoluble residual matter, accounting for over 40% of total freeze-dried material. Only methanol and aqueous

Table 2. Distribution of the Constitutive Units and Mean Degree of Polymerization of Proanthocyanidins, Present in Coffee Pulp and Coffee Organic Solvent Extracts^a

sample	CTt (%)	ECt (%)	ECe (%)	\overline{DPn}
Fresh Coffee Pulp				
pulp	2.9 ± 0.1	14.7 ± 0.07	82.2 ± 2.4	5.7 ± 0.8
methanol	9.5 ± 0.4	8.5 ± 4.9	81.8 ± 4.4	5.6 ± 1.2
acetone	1.8 ± 0.3	9.2 ± 1.4	88.9 ± 1.6	9.1 ± 1.4
residue	0.8 ± 0.07	14.1 ± 1.3	85.2 ± 0.8	6.7 ± 0.4
3-Day-Old Coffee Pulp				
pulp	4.3 ± 0.4	22.0 ± 2.1	73.6 ± 1.7	3.8 ± 0.2
methanol	7.9 ± 0.6	6.2 ± 2.2	85.7 ± 4.2	7.3 ± 2.1
acetone	2.4 ± 1.6	10.9 ± 0.4	86.6 ± 1.9	7.6 ± 1.2
residue	1.2 ± 0.6	16.3 ± 2.1	82.3 ± 2.8	5.7 ± 0.8

^a CT_t, (+)-catechin as terminal unit; EC_t, (-)-epicatechin as terminal unit; EC_e, (-)-epicatechin as extension unit; \overline{DPn} , mean degree of polymerization. Standard deviations ($n = 3$) are shown.

acetone extracts were further characterized, as the hexane extract was known not to contain polyphenolic compounds (18).

The distribution of proanthocyanidins and simple phenolic compounds in the extract was studied. The distribution within the methanol extract, aqueous acetone extract, and extraction residue is presented in **Figure 1**. The methanol extracts from both fresh and 3-day-old pulp solubilized a majority of monomeric flavan-3-ol (75% and 63%, respectively) as well as hydroxycinnamic acids (66% and 60%, respectively). The majority of proanthocyanidins were solubilized in the acetone extract from fresh (63.5%) and 3-day-old (55%) coffee pulp. The average degree of polymerization of proanthocyanidins in the different extracts from fresh coffee pulp indicated that, with methanol, there was solubilization mainly of lower \overline{DPn} (5.6) as compared to acetone extracts (9.1), as shown in **Table 2**. These observations are in agreement with similar results reported for apple (18) and pear (36). Additionally, for fresh coffee pulp, the DP values are intermediate between methanol extracts and acetone extracts. However, for 3-day-old pulp, the DP is much lower than that of both methanol and acetone extracts. This could be due to transformations in pulp constituents upon sun drying as suggested by Clifford et al. (33), which could result in an altered reactivity toward thiolysis.

(-)-Epicatechin was the major structure found in both coffee pulp and solvent extracts. Catechin was present exclusively as a terminal unit. The same proanthocyanidin structure was reported in apple (18, 36) and pear (37), in which (-)-epicatechin represented more than 90% of total units with a small proportion of (+)-catechin as terminal units.

HPLC Separation of Coffee Pulp Proanthocyanidins.

Separation of proanthocyanidins of fresh coffee pulp, present within the methanol extract, was achieved by normal-phase HPLC (**Figure 2A**). Hydroxycinnamic acids eluted first, followed by caffeine, which coeluted with catechin monomers. Oligomeric proanthocyanidins eluted with increasing \overline{DPn} from dimers to hexamers. This chromatogram demonstrates the presence of a series of proanthocyanidins in coffee pulp. Likewise, separation of proanthocyanidins from the methanol extract of 3-day-old coffee pulp showed a similar elution profile from monomers to pentamers (**Figure 2B**).

To further confirm the observed oligomeric structures in fresh coffee pulp separated by normal-phase HPLC, the same sample was subjected to MALDI-TOF mass spectrometry.

Mass Spectrometry of Proanthocyanidin Oligomers. Structural information regarding the molecular mass of coffee pulp proanthocyanidins was obtained by MALDI-TOF MS. Spectra

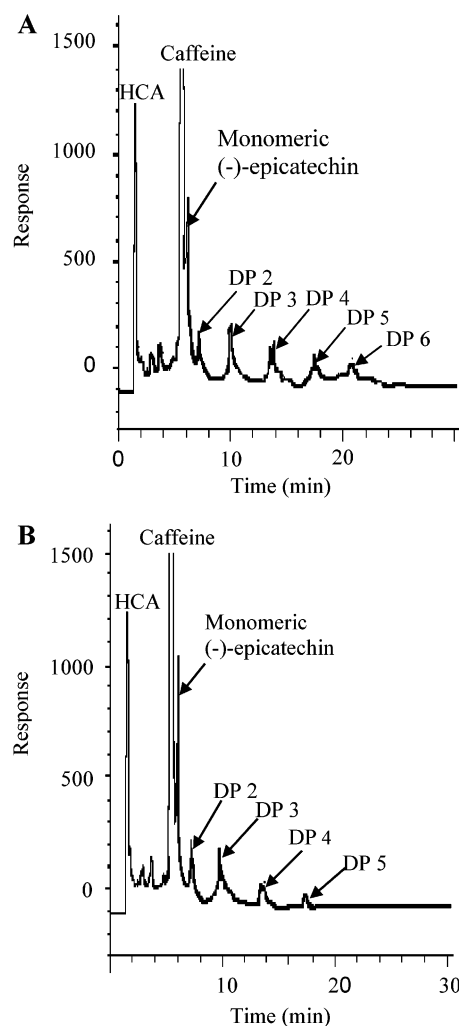


Figure 2. Normal-phase HPLC chromatogram of methanol extracts from (A) fresh and (B) 3-day-old coffee pulp. Detection was at 280 nm. DP, degree of polymerization; HCA, hydroxycinnamic acids.

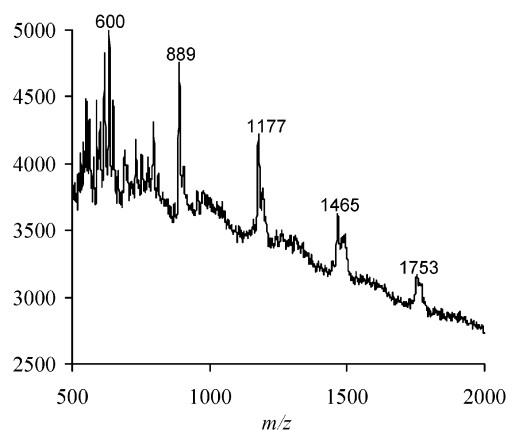


Figure 3. MALDI-TOF mass spectrum in positive linear mode, showing molecular ions of polymerized coffee pulp proanthocyanidins in the presence of dihydroxybenzoic acid as matrix.

of oligomeric proanthocyanidins show a series of peaks separated by 288 or 289 Da, corresponding to the incremental mass for epicatechin and extending up to hexamers. Molecular ions (m/z) obtained in the positive mode were as follows: 600 for the dimeric unit, 889 for the trimer, 1177 for the tetramer, 1465 for pentamer, and 1753 for hexameric units (**Figure 3**). Results show ionization of the dimeric form with sodium [M

+ Na⁺]. Such ionization patterns have been reported previously by Ricardo da Sylva et al. (38), characterized by the formula $290 + 288(c - 1) + 23$,

where 290 is the molecular weight of the catechin/epicatechin unit, c is the degree of polymerization of catechin/epicatechin units, and 23 is the molecular weight of sodium. Ricardo da Sylva et al. (38) had observed the same ionization pattern for catechin dimers isolated from grape. Similar molecular forms and ionization patterns for grape proanthocyanidins were also reported by Krueger et al. (29).

In conclusion, thiolysis coupled to HPLC was shown to be a powerful tool to characterize coffee polyphenols. The two major classes accounting for over 97% of total polyphenols were flavan-3-ols and hydroxycinnamic acids. Flavan-3-ols were further characterized by normal-phase HPLC and MALDI-TOF mass spectrometry, identifying proanthocyanidin monomers through hexamers present in fresh coffee pulp. Polyphenols present in fresh and 3-day-old coffee pulp were compared and the composition was found to be similar. However, proanthocyanidins were probably oxidized over time, rendering them partially unextractable.

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LITERATURE CITED

- (1) Ramirez-Martinez, J. R.; Pernía, R. D.; Bautista E. O.; Clifford, M. N.; Adams M. R. Producción y caracterización de la pulpa de café Ensilada. In *Pulpa de café ensilada, producción caracterización y utilización en alimentación animal*; Ramirez-Martinez, J. R., Ed.; Ediciones Universidad de Tachira: San Cristobal, Venezuela, 1999; pp 11–29.
- (2) Perraud-Gaime, I.; Saucedo-Castañeda, G.; Augur, C.; Roussos, S. Adding value to coffee solid byproducts through biotechnology. In *Coffee Biotechnology and Quality*; Sera, T., Soccol, C. R., Pandey, A., Roussos, S., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2000; pp 437–446.
- (3) Ramirez-Martinez, J. R. Phenolic compounds in coffee pulp: Quantitative determination by HPLC. *J. Sci. Food Agric.* **1988**, *43*, 135–144.
- (4) Clifford, M. N.; Ramirez-Martinez, J. R. Phenols and caffeine in wet-processed coffee beans and coffee pulp. *Food Chem.* **1991**, *40*, 35–42.
- (5) González de Colmenares N.; Ramírez-Martínez, J. R.; Aldana, J.-O.; Ramos-Niño, M. E.; Clifford, M. N.; Pékerar, S.; Méndez, B. Isolation, characterisation and determination of biological activity of coffee proanthocyanidins. *J. Sci. Food Agric.* **1998**, *77*, 368–372.
- (6) Zuluaga-Vasco, J.; Tabacchi, R. Contribution á l'étude de la composition chimique de la pulpe de café. In *Neuvième Colloque Scientifique International sur le café*; ASIC: Paris, 1979; pp 335–344.
- (7) Clifford, M. N.; Ramirez-Martinez, J. R. Tannins in wet-processed coffee beans and coffee pulp. *Food Chem.* **1991**, *40*, 191–200.
- (8) González de Colmenares, N.; Ramírez-Martínez, J. R.; Aldana, J.-O.; Clifford, M. N. Analysis of proanthocyanidins in coffee pulp. *J. Sci. Food Agric.* **1994**, *65*, 157–162.
- (9) Hemingway, R. Reactions at the interflavanoid bond of proanthocyanidins. In *Chemistry and significance of condensed tannins*; Hemingway, R. W., Karchesy, J. J., Eds.; Plenum Press: New York and London, 1988; pp 265–283.

- (10) Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 875–883.
- (11) Wollgast, J.; Anklam, E. Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Res. Int.* **2000**, *33*, 423–447.
- (12) Lea A. G.; Arnold, G. M. The phenolics of ciders: Bitterness and astringency. *J. Sci. Food Agric.* **1978**, *29*, 478–483.
- (13) Haslam, E.; Lilley, T. H. Natural astringency in foodstuffs — a molecular interpretation. *Crit. Rev. Food Sci. Nutr.* **1988**, *27*, 1–11.
- (14) Ricardo da Sylva, J. M.; Darmon, N.; Fernandez, Y.; Mitjavila, S. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. *J. Agric. Food Chem.* **1991**, *39*, 1549–1552.
- (15) Ursini, F.; Rapuzzi, I.; Toniolo, R.; Tubaro, F.; Bontempelli, J. Characterization of antioxidant effect of procyanidins. Flavonoids and other polyphenols. *Methods Enzymol.* **2001**, *335*, 338–350.
- (16) Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509–512.
- (17) Matthews, S.; Mila, I.; Scalbert, A.; Pollet, B.; Lapiere, C.; Catherine, L. M.; Du Penhoat, H.; Rolando, C.; Donnelly, D. M. X. Method for estimation of proanthocyanidins based in their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem.* **1997**, *45*, 1195–1201.
- (18) Guyot, S.; Marnet, N.; Laraba, D.; Sanoner, P.; Drilleau, J.-F. Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (*Malus domestica* var. Kermerrien). *J. Agric. Food Chem.* **1998**, *46*, 1698–1705.
- (19) Guyot, S.; Marnet, N.; Drilleau, J.-F. Thiolysis–HPLC Characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *49*, 14–20.
- (20) Thompson, R. S.; Jaques, D.; Haslam, E.; Tanner, R. J. N. Plant procyanidins Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc. Perkins., Perkin Trans. 1* **1972**, 1387–1399.
- (21) Rigaud, J.; Perez-Illzarre, J.; Ricardo da Sylva, J. M.; Cheynier, V. Micro method for the identification of proanthocyanidins using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr.* **1991**, *540*, 401–405.
- (22) Rigaud, J.; Escribano-Bailon, M. T.; Prieur, C.; Souquet, J. M.; Cheynier, V. Normal-phase high-performance liquid chromatographic separation of procyanidins from cacao beans and grape seeds. *J. Chromatogr. A* **1993**, *654*, 255–260.
- (23) Cheynier, V.; Souquet, J. M.; Le Roux, E.; Guyot, S.; Rigaud, J. Size separation of condensed tannins by normal-phase high-performance liquid chromatography. *Methods Enzymol.* **1999**, *299*, 178–184.
- (24) Hammerstone, J. F.; Lazarus, S. A.; Mitchell, A. E.; Rucker, R.; Schmitz, H. H. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem.* **1999**, *47*, 490–496.
- (25) Lazarus, S. A.; Hammerstone, J. F.; Adamson, G. E.; Shmitz, H. High-performance liquid chromatography/mass spectrometry, Analysis of proanthocyanidins in food and beverages. *Methods Enzymol.* **2001**, *335*, 46–57.
- (26) Guyot, S.; Doco, T.; Souquet, J.-M.; Moutounet, M.; Drilleau, J.-F. Characterization of highly polymerized procyanidins in cider apple (*Malus sylvestris* var. Kermerrien) skin and pulp. *Phytochemistry* **1997**, *44*, 351–357.
- (27) Ohnishi-Kameyama, M.; Yanagida, A.; Kanda, T.; Nagata, T. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 31–36.

- (28) Fulcrand, H.; Guyot, S.; Le Roux, E.; Remy, S.; Souquet, J.-M.; Doco, T.; Cheynier, V. Electrospray contribution to structural analysis of condensed tannin oligomers and polymers. In: *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Gross, G. G., Hemingway, R. W., Yoshida, T., Branham, S. J., Eds.; Basic Life Sciences series; Kluwer Academic/Plenum Publishers: New York, 1988; pp 223–244.
- (29) Krueger, C. G.; Dopke, N. C.; Treichel, P. M.; Folts, J.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ol in grape seed extract. *J. Agric. Food Chem.* **2000**, *48*, 1663–1667.
- (30) Wollgast, J.; Pallaroni, L.; Agazzi, M. E.; Anklam, E. Analysis of procyanidin in chocolate by reversed-phase high-performance liquid chromatography with electrospray. Ionization mass spectrometric and tandem mass spectrometric detection. *J. Chromatogr. A* **2001**, *926*, 211–220.
- (31) Guyot, S.; Marnet, N.; Sanoner, P.; Drilleau, J.-F. Direct thiolysis on crude apple materials for high-performance liquid chromatography. Characterization and quantification of polyphenols in cider apple tissues and juices. *Methods Enzymol.* **2001**, *335*, 57–70.
- (32) Scalbert, A. Quantitative methods for the estimation of tannins in plant tissues. In *Plant Polyphenols, Synthesis, Properties, Significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; pp 259–280.
- (33) Clifford, M. N., De Colmenares, N. G., Ramirez-Martinez, J. R., Adams, M. R., and De Menzes, H. C. Tannins in the sun-dried pulp from wet-processing of arabica coffee beans. In *14th Colloque Scientifique International sur le Café*; ASIC: San Francisco, CA, 1991; pp 230–236.
- (34) Rouet-Mayer, M. A.; Philippon, J.; Ralambosoa, J. Roles of o-quinones and their polymers in the enzymatic browning of apples. *Phytochemistry* **1990**, *29*, 435–440.
- (35) Amiot, M. J.; Aubert, S.; Nicolas, J.; Goupy, P.; Aparicio, P. Phenolic composition and browning susceptibility of various apple and pear cultivars at maturity. *Bull. Liaison-Gruppe Polyphenols* **1992**, *16*, 48–51.
- (36) Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J. F. Polyphenols profiles of French cider apple varieties. *J. Agric. Food Chem.* **1999**, *47*, 4887–4853.
- (37) Ferreira, D.; Guyot, S.; Marnet, N.; Delgadillo, I.; Renard, C. M. G. C.; Coimbra, M. A. Composition of phenolic compounds in Portuguese pear (*Pirus communis* L. var. S. Bartolomeu) and changes after sun-drying. *J. Agric. Food Chem.* **2002**, *50*, 4537–4544.
- (38) Ricardo da Sylva, J. M.; Rigaud, J.; Cheynier, V.; Cheminat, A.; Moutounet, M. Procyanidin dimers and trimers from grape seeds. *Phytochemistry* **1991**, *30*, 1269–1264.

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