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Flavonoids, Proanthocyanidins, Vitamin C, and Antioxidant Activity of *Theobroma grandiflorum* (Cupuassu) Pulp and Seeds

Alexandre G. Pugliese,[†] Francisco A. Tomas-Barberan,[‡] Pilar Truchado,[‡] and Maria I. Genovese*[,†]

[†]Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes 580, Bloco 14, 05508-900 São Paulo, SP, Brazil

[‡]Research Group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, P.O. Box 164, Espinardo, Murcia, 30100, Spain

ABSTRACT: The ascorbic acid, flavonoids, and proanthocyanidins content and in vitro antioxidant activity of fresh pulps and seeds of cupuassu, harvested at three different times of the year, and in commercial frozen pulps were evaluated. Lipids, total phenolics contents, and antioxidant activities were the highest in the seeds, followed by fresh and commercial frozen pulps, respectively. The latter also showed a lower content of ascorbic acid (9−13 mg/100 g DW) when compared to fresh pulps (96−111 mg/100 g DW). The 8-*O*-*β*-D-glucuronides and the corresponding 3″-sulfates of isoscutellarein (5,7,8,4′-tetrahydroxyflavone), hypolaetin (5,7,8,3′,4′-pentahydroxyflavone), and 8-hydroxychrysoeriol (5,7,8,4′-tetrahydroxy-3′-methoxyflavone), also known as hypolaetin 3′-methyl ether, were identified and quantified (31 mg/g DW) in cupuassu seeds. The same flavonoid profile was present in pulps although in much lower concentrations (0.5 to 2 mg/g DW). The two 8-hydroxychrysoeriol glycosides had not been previously reported in cupuasssu. The content of proanthocyanidin oligomers in seeds (23 mg/g DW), mainly of the epicatechin type, and the mean degree of polymerization (5.5) were calculated. No discernible effect of the harvesting period on the evaluated chemical aspects could be identified. Commercial frozen pulps contained a smaller amount of all these compounds than the fresh pulp, suggesting that these compounds were potentially degraded during processing/storage.

KEYWORDS: *cupuassu, Theobroma grandiflorum, vitamin C, flavonoids, antioxidant capacity*

INTRODUCTION

The cupuassu tree, *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum., is naturally distributed in Brazilian rainforests, especially in the states of Amazonas, Pará, Maranhão, Rondônia, and Acre, while commercial crops can be found in other states, e.g., Bahia. Other tropical countries such as Costa Rica, Colombia, Ecuador, French Guyana, Guyana, and Surinam also cultivate this tree. This plant belongs to *Theobroma* genus, composed of 22 species. One of those is *Theobroma cacao*, cocoa tree, with great economical importance and largely studied for the flavor of the processed seeds.[1]

Cupuassu is a large tree whose fruits have a yellowish-white pulp. This pulp has a strong flavor and is very much appreciated by local communities, and also international markets, as an ingredient in fruit juice drinks. The rising commercial interest of this fruit has led to the development of new industries and has encouraged research on this food product. Noteworthy, this tree grows in synergy with other native rainforest species, offering an ecological approach for sustainable agroforestal management and preservation.[2]

Cupuassu pulp can be consumed as juices, drinks, ice-creams, jellies, and candies. It is not usually consumed directly due to its strong acidity. The seeds contain high amounts of fat and may be used in food products and in a variety of cosmetics. The seeds can also be processed to yield a chocolate-like product called "cupulate", first described and patented by the Brazilian Research Institute Embrapa (Empresa Brasileira de Pesquisa Agropecuária).[3]

It is well-known that cocoa seeds, cocoa powder, and liquor contain a wide range of monomeric flavan-3-ols and their oligomeric derivatives, called proanthocyanidins. Those flavonoids are associated with potential health benefits like antioxidant, anticancer, and anti-inflammatory activities, cardiovascular protective properties, modulation of immune response, endothelial function, and antimicrobial and enzyme inhibitory activities.[4−12] One activity reported is related to lipoxygenase inhibition by flavan-3-ols, which prevents oxidative cell damage by diminishing lipid peroxidation catalysis.[4]

Kuskoski et al.[13] previously reported the presence of phenolic compounds in cupuassu pulp in similar amounts as those found in other tropical fruits, like passion fruit. Nine known flavonoids were found in cupuassu seeds: (+)-catechin, (−)-epicatechin, isoscutellarein 8-*O*-*β*-D-glucuronide, hypolaetin 8-*O*-*β*-glucuronide, quercetin 3-*O*-*β*-D-glucuronide, quercetin 3-*O*-*β*-D-glucuronide 6″-methyl ester, quercetin, kaempferol, and isoscutellarein 8-*O*-*β*-D-glucuronide 6″-methyl ester. Two new sulfated flavonoid glycosides, theograndins I and II, were also described.[1]

In general, information about cupuassu polyphenolic composition, distribution of these compounds in the fruit and over different crops, and ascorbic acid content and antioxidant activities is scarce or lacking. Therefore, the present work aimed to study fresh pulps and seeds, harvested at three different

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times of year, and three commercial frozen pulps, in order to compare to the fresh pulp and evaluate the effect of processing.

MATERIALS AND METHODS

Materials. Samples (total of ca. 5 kg, corresponding to three different fruits at the mature stage having average weight 1.6−2.0 kg) of cupuassu fruit (variety Mamorana) were collected in a commercial plantation in Bahia (Brazil) at three different harvesting periods: (A) Autumn, May 2008; (B) Winter, August 2008; and (C) Summer, February 2009. Fully mature fruits were collected randomly after falling to the ground (usual way of harvesting cupuassu). There is no crop of cupuassu during spring. Pulp and seeds were manually separated after liquid nitrogen freezing and lyophilization. Liquid nitrogen frozen parts were ground in a mortar with a pestle and stored. In addition, three commercial frozen pulps, namely, Brasfrut Frutos do Brasil Ltd.a. (Feira de Santana, Bahia) (X), Icefruit Comércio de Alimentos Ltd.a. (Tatuí, São Paulo) (Y), and Ricaeli (Cabreúva, São Paulo) (Z), were bought at the Central Market in São Paulo (CEAGESP) and immediately freeze-dried. All samples were stored at −80 °C until the time of analysis. All chemicals and solvents were reagent or HPLC grade.

Chemical Composition. Moisture, ashes, proteins and lipids were evaluated according to AOAC[14] methods. Protein content was calculated using the factor 6.25 (cocoa products). Carbohydrates content was measured through Dubois et al.[15] methodology. The absorbance was measured at 490 nm using a model Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech). The results were calculated based on a glucose standard curve. All the results were given in percentage.

Total Ascorbic Acid. The determination was performed according to Pasternak et al.,[16] with some modifications. Ascorbic acid was extracted with metaphosphoric acid (6% w/v) and analyzed by reversed-phase HPLC in a Hewlett-Packard 1100 system with autosampler and quaternary pump coupled to a diode array detector. Separation was achieved on a NucleoSil 100 C18 column (150 × 3.6 mm, 5 μm; Macherey-Nagel, Germany) under isocratic elution with 2 mM potassium chloride buffer (pH 2.5) at 0.8 mL/min, monitoring at 245 nm. Total AA was estimated after reduction of dehydroascorbic acid (DHA) with 10 mM dithiothreitol. The results were expressed as mg/100 g sample dry weight (DW).

Total Phenolics. The determination was performed by means of Folin−Ciocalteu reagent according to Singleton et al.,[17] after extraction in a solvent mixture comprising methanol/water (70:30, v/v). Briefly, 0.5 g of sample was weighed, added to 20 mL of this mixture, and extracted at speed 4 for 1 min (Brinkmann homogenizer, Polytron; Kinematica GmbH), while cooled in ice, and then filtered through filter paper (Whatman No. 1). The absorbance was measured at 750 nm using a model Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech). The results were expressed as mg of catechin/100 g sample (DW).

Total Proanthocyanidins Content. It was determined according to Porter et al.,[18] after extraction of 0.5 g of sample in 20 mL of methanol:acetic acid (99:1). A reagent comprising 154 mg of $FeSO_4{\cdot}7H_2O$ per liter of *n*-butanol:hydrochloric acid (3:2) was prepared. A total of 250 μL of each sample extract and 2.5 mL of the described reagent were incubated at 90 °C for 15 min. The blank consisted of 2.5 mL of the reagent and 250 μL of methanol:acetic acid (99:1). The absorbance was measured at 540 nm using a model Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech). Capped bottles were used in this assay in order to avoid evaporation of the solvents. The results were expressed as mg of quebracho tannin/100 g sample (DW).

DPPH Radical Scavenging Activity. The extracts obtained above for quantification of total phenolics were used to assess the antioxidant capacity by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging method according to Brand-Williams et al.[19] A 50 μL aliquot of the extract previously diluted with methanol and 250 μL of DPPH (0.5 mM) were shaken and after 25 min the absorbance was measured at 517 nm using a microplate spectrophotometer (Benchmark Plus, Biorad, Hercules, CA). The antioxidant capacity was calculated based on a standard curve obtained with a methanolic solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at different concentrations. Results were expressed as μmol Trolox equivalents/g sample (DW).

Oxygen Radical Absorbance Capacity (ORAC). The antioxidant activity was determined by an assay from Dávalos et al.[20] A total of 200 μL of each sample extract, obtained as described above for total phenolics determination, and 1200 μL of 70 nM fluorescein solution in 75 mM sodium phosphate buffer (pH 7.4) were incubated at 37 °C for 15 min. After preincubation, 600 μL of a 24 mM 2,2′-azobis(2-amidinopropane) dihydrochloride solution in 75 mM sodium phosphate buffer (pH 7.4) was added. Fluorescence was measured at 485 nm_{ex}/525 nm_{em} using a spectrophotometer (F-3010, Hitachi, Japan) in a quartz cuvette each 10 min, up to 80 min. The same phosphate buffer was used as blank. The control consisted of 200 μL of methanol added to fluorescein solution. The results were expressed as μmol of Trolox equivalents/g sample (DW).

Flavonoids Extraction. One gram of powdered sample was homogenized in Ultraturrax T-25 equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24000 rpm for 1 min after the addition of 25 mL of extraction solution (methanol/water/acetic acid, 70/29.5/0.5, v/v/v). The extracts were centrifuged at 5000*g* for 5 min in an Eppendorf centrifuge. The supernatant was evaporated under reduced pressure at 40 °C to remove the methanol phase. The aqueous phase was extracted with hexane (1:1) and further filtered through a Sep-Pak cartridge (reverse phase C-18 cartridge; Waters Millipore, United States), which retained phenolic compounds and removed other highly hydrophilic compounds. The cartridges were previously activated with 10 mL of methanol and 10 mL of water. The remaining volume in each cartridge was eluted with 2 mL of methanol. The methanolic fractions of each cartridge were collected, filtered through a 0.45 μm PVDF filter, and injected in the HPLC−DAD.

Flavonoid identification by HPLC−DAD−Tandem Mass Spectrometry (MS−MS). The samples were analyzed using an Agilent HPLC 1100 series instrument equipped with a diode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (G1312 A), an auto sampler (G1313 A), a degasser (G1322 A), and photodiode-array detector (G1315 B) controlled by software (v. A08.03). Separations of phenolic compounds were achieved on a C18 Mediterranea Sea column (Teknokroma, Barcelona, Spain) (250 × 4 mm; 5 μm particle size). The mobile phase was water/formic acid (99:1, v/v) (solvent A) and HPLC grade acetonitrile (solvent B) at flow rate of 1 mL/min. Elution was performed with a gradient starting with 5% B in A, to reach 12% B in A at 10 min, 25% B in A at 20 min, 35% B in A at 35 min, 70% B in A at 35 min, 90% B in A at 36 min and then became isocratic for 5 min. UV chromatograms were recorded at 280 and 360 nm. The mass detector was an ion trap spectrometer (G2445A) equipped with an electrospray ionization (ESI) system and controlled by software (v. 4.1). The nebulizer gas was nitrogen. The pressure and the flow rate of the dryer gas were set at 65 psi and 11 L/min, respectively. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS−MS) spectra were measured from m/z 100 up to m/z 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative mode. The phenolic compounds were identified according to their UV spectra, molecular weights, retention time, and their MS−MS fragments; when possible, the compounds were compared with authentic standards previously isolated and identified in our group from *Gratiola officinalis*.[21]

UPLC-QTOF Analysis of the Flavonoid Extracts. The same samples as analyzed above were also analyzed by UPLC-Q-TOF (Agilent) in order to further confirm the phenolic compounds identified by MS Trap. The Q-TOF equipment had the following conditions: ESI gas temperature 280 °C, drying gas 9 L/min, nebulizer 35 psig, sheath gas temp 400 °C, sheath gas flow 12 L/min. MS TOF fragmentor 100 V, mass range 100−1500, negative mode. The column

Table 1. Chemical Composition (%) and Total Ascorbic Acid (mg/100 g DW Sample) of Cupuassu Fresh Pulps and Seeds and Commercial Frozen Pulps[a]

sample	moisture	ashes	lipids	proteins	carbohydrates	total ascorbic acid
fresh seed A	53 ± 0 b	1.47 ± 0.02 d	22 ± 1 c	4.2 ± 0.2 c	13.6 ± 0.9 b	ne[b]
fresh seed B	52 ± 1 b	1.50 ± 0.01 d	21 ± 1 c	3.8 ± 0.1 d	15.9 ± 1.0 c	ne
fresh seed C	53 ± 0 b	1.40 ± 0.10 e	22 ± 1 c	4.9 ± 0.1 e	17.9 ± 0.6 d	ne
fresh pulp A	83 ± 1 a	0.94 ± 0.02 a	2.2 ± 0.3 a	1.1 ± 0.1 a	10.6 ± 0.7 a	98 ± 5 a
fresh pulp B	82 ± 3 a	0.81 ± 0.01 b	2.3 ± 0.3 a	0.8 ± 0.0 b	10.2 ± 0.6 a	111 ± 7 a
fresh pulp C	84 ± 1 a	1.03 ± 0.03 c	1.7 ± 0.1 ab	1.2 ± 0.0 a	11.0 ± 0.9 a	96 ± 8 a
commercial pulp X	90 ± 1 c	0.46 ± 0.01 f	1.0 ± 0.1 b	0.7 ± 0.0 b	5.4 ± 0.1 e	9 ± 1 c
commercial pulp Y	89 ± 1 c	0.46 ± 0.01 f	1.0 ± 0.1 b	0.7 ± 0.0 b	6.3 ± 0.6 e	12 ± 1 c
commercial pulp Z	90 ± 1 c	0.47 ± 0.02 f	1.0 ± 0.1 b	0.7 ± 0.0 b	5.2 ± 0.3 e	13 ± 1 c

[a]Means in the same column with equal letters are not significantly different ($p < 0.05$). [b]Not evaluated.

was Poroshell 120, EC-C18, 2.7 μm, 30 × 100 mm (Agilent); the eluents were 0.1% formic acid in water (A) and acetonitrile acidified with 0.1% formic acid (B). The separation gradient started with 1% B in A at 0 min, 9% at 3 min, 48% at 20 min, and 95% at 23 min, followed by washing and conditioning steps. The volume injected was 2 μL, and the flow rate was 0.4 mL/min. The determinations were carried out in triplicate.

Quantification of Phenolic Compounds by HPLC–DAD. The HPLC analyses were performed on an L-6200 liquid chromatograph (Merck-Hitachi, Darmstadt, Germany) equipped with a Shimadzu SPD-M6A UV diode array detector. The mobile phase was water with 5% formic acid (solvent A) and HPLC grade acetonitrile (solvent B) at a flow rate of 1 mL/min. The column and gradient were the same as indicated above. Chromatograms were recorded at 280 and 360 nm. Flavonoids were quantified in the chromatograms recorded at 360 nm, using the external standard hypoaletin-8-*O*-glucuronide previously isolated from *Gratiola officinalis*.[21] The results were expressed in mg of each compound per g of sample.

Phloroglucinolysis. Cupuassu flavan 3-ols were determined according to the method described by Kennedy and Jones[22] with modifications.[23] A solution of 0.1 N HCl in methanol, containing 50 g/L phloroglucinol and 10 g/L ascorbic acid, was prepared (phloroglucinolysis reagent). 50 mg of freeze-dried extract was reacted with the phloroglucinolysis reagent (800 μL) in a water bath for 20 min at 50 °C. The reaction was stopped by placing the vials in an ice bath and by diluting the reaction medium with 1 mL of a 40 mM sodium acetate solution. The HPLC–DAD–MS–MS used to analyze the phloroglucinol adducts was indicated above, and chromatographic separations were carried out in a C18 Atlantis column (250 × 4.6 mm, 5 μm particle size) protected by a 4 × 4 mm C18 Atlantis guard column composed of the same material, all purchased from Waters (Barcelona, Spain). The method utilized a binary gradient with water containing 2.5% v/v aqueous acetic acid (mobile phase A) and acetonitrile (mobile phase B) at flow rate of 1 mL/min. The linear gradient started with 3% B, at 5 min 5% B, at 15 min 16% B, at 45 min 50% B, at 52 min 3% B, and then it was maintained isocratic up to 57 min. Flavan-3-ol cleavage products were estimated using their response factors relative to catechin (Sigma St. Louis, USA), which was used as the quantitative standard. To calculate the apparent mean degree of polymerization (mDP), the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, mg) was divided by the sum of all flavan-3-ol monomers (mg).[22]

Hydrolysis with Glucuronidase and Sulfatase. The flavonoid extracts were hydrolyzed with *Helix pomatia* enzymes to release the aglycons. Glucuronide and sulfate metabolites were hydrolyzed by incubating 500 μL of a solution containing 1 mg of extract/mL of methanol–water (1:1, v:v) in 250 μL of 0.1 M sodium acetate buffer (pH 5.2) with β-glucuronidase (88,500 U/mL) and sulfatase (404 U/mL) (aqueous solution from *Helix pomatia*, type H-2, G-0876, EC 3.2.1.31, Sigma) at 37 °C for 8 h. The reaction mixture was extracted with 600 μL of ethyl acetate, vortexed, and centrifuged at 1000*g* for 1 min. The supernatant fraction was dried under N_2 and the residue dissolved in 100 μL of methanol.

Isolation of Flavones. Flavones were extracted with methanol–water (1:1; v:v) and isolated by a combination of Sephadex LH-20 chromatography with methanol, and semipreparative HPLC (same conditions as reported above for analytical purposes).

NMR Analyses. ^{1}H NMR spectra were recorded using a Brüker 400 MHz in d_6-DMSO. Analytical conditions were similar to those reported previously.[1]

Statistical Analysis. All analyses were run in triplicate and were expressed as mean ± standard deviation. Statistical analysis was done by using the Statistic software package version 5.0 (StatSoft, Inc., Tulsa, OK). Differences between means were first analyzed by ANOVA test and then followed by LSD, least significant difference ($p < 0.05$).

RESULTS AND DISCUSSION

Chemical Composition. The pulp and the seed samples from cupuassu showed some similarity in their chemical compositions, over the different harvest seasons (Table 1). There were statistical differences ($p < 0.05$) between the three different pulps comparing the quantity of ashes. The seeds, on the other hand, showed statistical differences in the carbohydrate and protein amount. In spite of those differences, cupuassu had a constant composition over the year. This regularity may be due to the fact that the harvested fruits were those naturally fallen on the ground and, therefore, at the same stage of maturation. In addition the uniform climatic conditions at tropical level may also have an influence, as temperatures and light incidence are rather uniform in transequatorial zones. Average moisture content (ca. 83%) of fresh pulps was higher than for seeds (ca. 52%), while protein content was lower (ca. 1% compared to 4%). Seeds also concentrated lipids (ca. 22%). Chemical composition of commercial frozen pulps was significantly different, but similar between the three brands. The chemical composition found for the cupuassu pulps is similar to that found in TACO (*Tabela brasileira de Composição dos Alimentos, or Brazilian Food Composition Tables*),[24] and the composition of the seeds is similar to that reported by Aragão et al.[25] Minor differences might be due to variation of the soil, type of cupuassu (mamorana and roundish), and genetic variations under a specific type. The higher moisture content of the frozen pulps may be related to the type of cupuassu used in their production and to the depulping process itself, which results in the elimination of the fibrous residue.

Ascorbic Acid. A high content of ascorbic acid was found in fresh pulps of cupuassu (ca. 102 mg/100 g DW sample, or 17 mg/100 g FW), but a drastic loss seems to occur during processing, as the content of commercial frozen pulps was of only ca. 11 mg/100 g (Table 1). Oxidative reactions during pulp separation and thermal processing such as pasteurization

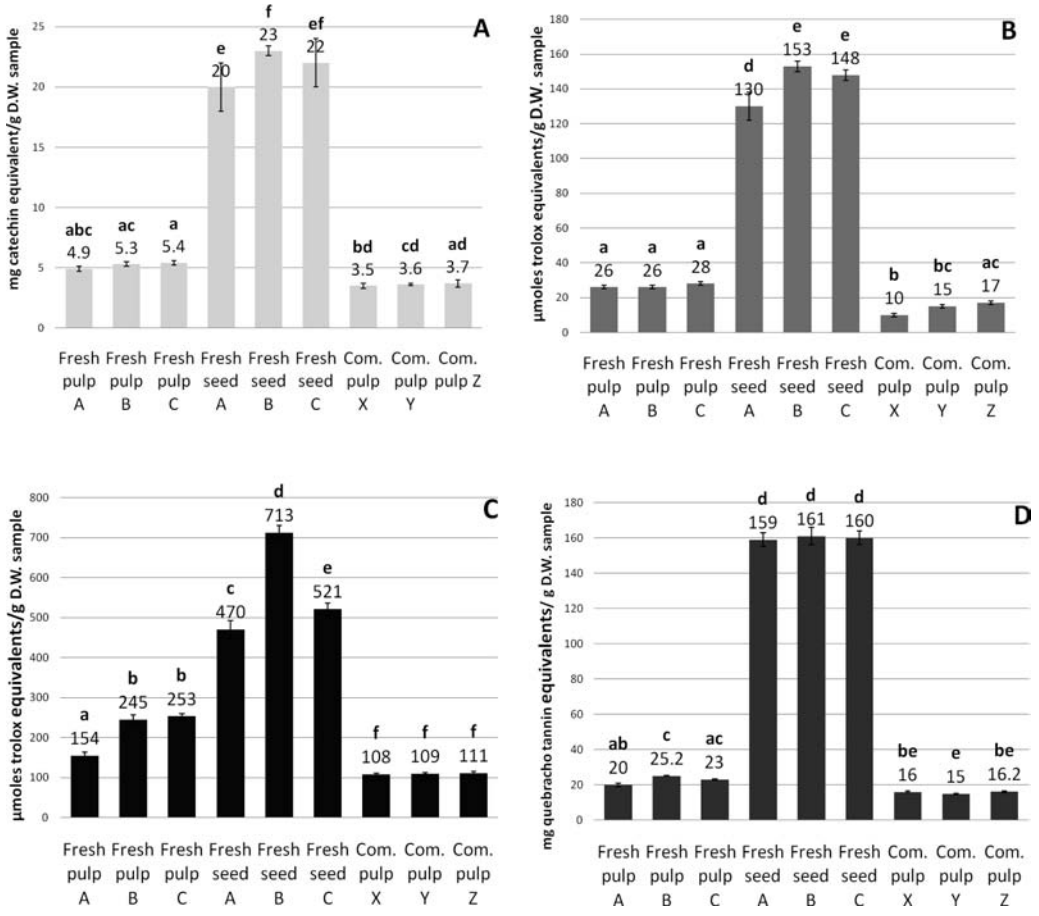

Figure 1. Total phenolics (mg catechin equivalent/g DW sample) (A), antioxidant capacity determined by DPPH scavenging activity (μmol Trolox equivalents/g DW sample) (B) and ORAC method (μmol Trolox equivalents/g DW sample) (C), and proanthocyanidins (mg quebracho tannin equivalents/g DW sample) of cupuassu fresh pulps and seeds and commercial frozen pulps. Means with different letters are significantly different ($p < 0.05$).

are known to diminish fresh pulp vitamin C.[26] Ascorbic acid content of commercial frozen pulps had statistical similarity among the three different brands. Fresh pulps also presented similar contents through the different seasons (Table 1). The amount of ascorbic acid in frozen pulps was similar to that reported by Freire et al.[26] According to TACO,[24] cocoa pulp (13.6 mg/100 g FW) has less ascorbic acid than cupuassu (24.5 mg/100 g FW); acerola, on the contrary, has almost 40-fold the quantity (941 mg/100 g FW) found in cupuassu.

Total Phenolics, Proanthocyanidins, and Antioxidant Activity (DPPH• and ORAC). Commercial frozen pulps presented, in general, lower total phenolics, total proanthocyanidins, and antioxidant activity than fresh pulps (Figure 1). Those differences might be a consequence of the oxygen exposure during the fruit opening and manipulation of the pulp, freezing, and storage. Those factors could lead to degradation of polyphenolics and ascorbic acid, diminishing the antioxidant capacity. Cupuassu fresh seeds, however, had 4- to 5-fold the amount of phenolics and 6- to 8-fold the amount of proanthocyanidins found in fresh pulps. Harvesting time had no influence on fresh pulps; commercial frozen pulps, equally, had no statistical difference among the evaluated brands (Figure 1). Fresh seeds, on the contrary, presented statistically different antioxidant activities according to the season: fresh seed A presented the lower values of ORAC and DPPH scavenging activity although presenting same amounts of proanthocyanidins. This result indicated the possibility of differences in flavonoid contents, which was next confirmed (see below). Fresh pulp presented a considerable amount of total phenolics and antioxidant activity superior to, for example, strawberry (*Fragaria ananassa* X Dutch)[27] and similar to other Brazilian native fruits like "araçá-boi" (*Eugenia stipitata* Mc. Vaugh) and jaracatiá (*Jaracatia spinosa* (Aubli) A. DC.).[28] Those values were also higher than those reported by Spada et al.[29] for the same fruit pulp.

Flavonoid Identification. The HPLC−DAD−MS−MS chromatogram of the extract allowed the detection of nine flavone derivatives (Figure 2). The different compounds were characterized by their UV spectra, and their MS spectra recorded with an ion-trap MS−MS detector (Table 2), and the exact mass analysis (UPLC-Q-TOF) allowed the determination of their elemental formulas (Table 3). Compound **1** was only present as trace amounts and had a UV spectrum characteristic of an apigenin (5,7,4‴-trihydroxyflavone) derivative, in which a sugar residue was C−C linked to that carbon 8 of the flavonoid nucleus. The MS analysis (Table 2) showed a pseudomolecular ion at m/z 431 consistent with an apigenin mono-*C*-hexoside. The MS−MS fragmentation yielded the M − 90 [11] and the M − 120 [100] fragments, which are characteristic of an 8-*C*-hexosylflavone.[30] This suggested that compound **1** was apigenin-8-*C*-hexoside and most probably apigenin-8-*C*-glucoside also known as vitexin, and this was confirmed with an authentic standard. The HR MS−MS analysis confirmed the elemental formula and the structure of this flavonoid.

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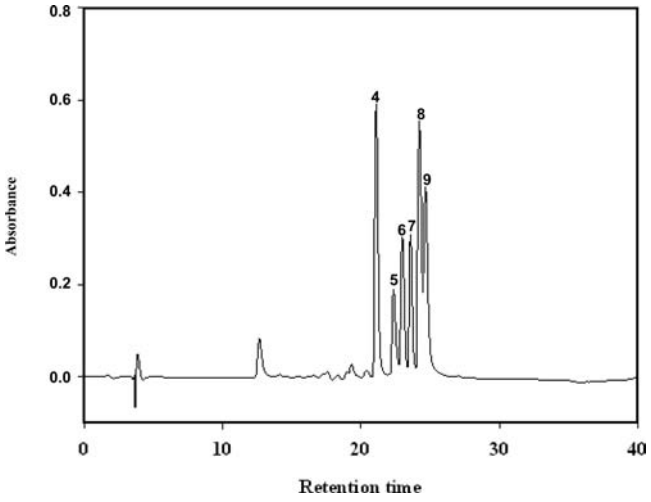


Figure 2. HPLC chromatogram (360 nm) of cupuassu seeds from Brazil: (**4**) hypolaetin 8-*O*-*β*-D-glucuronide; (**5**) hypolaetin 8-*O*-*β*-D-glucuronide 3″-*O*-sulfate (theograndin II); (**6**) isoscutellarein 8-*O*-*β*-D-glucuronide; (**7**) hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide; (**8**) isoscutellarein 8-*O*-*β*-D-glucuronide 3″-*O*-sulfate (theograndin I); (**9**) hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide 3″-*O*-sulfate. Compounds **1**, **2**, and **3** were not detected in HPLC chromatogram.

Compound **2** has a UV spectrum similar to that of hypolaetin-8-glucoside,[31] and a pseudomolecular ion at *m*/*z* 639 consistent with a hypolaetin-glucuronide-hexoside. The MS2 showed fragment ions at *m*/*z* 477 [34] corresponding with hypolaetin glucuronide and *m*/*z* 463 [71] consistent with hypolaetin-glucoside, suggesting that the glucoside and the glucuronide moieties were linked to different phenolic hydroxyls. The HR MS−MS analysis confirmed the elemental formula and the structure of this flavonoid diglycoside. The small amount of compound present prevented the complete identification of this metabolite that was characterized as hypolaetin-*O*-glucuronide-*O*-glucoside (**2**). Compound **3** was also a minor metabolite with the UV spectrum of a quercetin 3-*O*-glycoside.[32] Its MS spectrum showed a pseudomolecular ion at *m*/*z* 433 and a fragment at *m*/*z* 301 for the aglycon consistent with a quercetin *O*-pentoside that was confirmed by HR-MS−MS. Compound **4** showed a UV spectrum like that of hypolaetin 8-glucoside[33] and a pseudomolecular ion at *m*/*z* 477 that coincided with the previously published hypolaetin 8-glucuronide. After enzymatic hydrolysis with a *β*-D-glucuronidase/sulfatase preparation, the aglycon hypolaetin was identified by its mass spectrum and UV spectrum[34] (Figures 3 and 4). This coincided with an authentic sample isolated and identified previously from *Gratiola officinalis*,[21] and the structure was confirmed by HR-MS−MS. Compound **5** showed a UV spectrum identical to that of compound **4**, and its MS analysis showed a pseudomolecular ion at *m*/*z* 557, and fragments at M − 80 (M − sulfate) and M − 80 − 176 (M-sulfate-glucuronide) showing the sequential losses of the sulfate and glucuronide residues to lead to the aglycon hypolaetin (*m*/*z* 301). A fragment at *m*/*z* 254 corresponding to the sulfoglucuronide fragment was also detected (Table 2). This compound coincided with theograndin II previously reported from the same source.[1] An intermediate coincident with compound **4** was detected confirming its structure, and this was also confirmed by HR-MS−MS analysis. Compound **6** showed a UV spectrum similar to that of apigenin but with a BII at 272 nm that indicated an additional substitution on the A-ring of the flavone nucleus. The UV spectrum coincided with that of isoscutellarein 8-*O*-glucuronide previously reported.[1] Its MS spectrum showed a pseudomolecular ion at *m*/*z* 461 and an aglycon fragment at *m*/*z* 285 that coincided with that expected for isoscutellarein 8-*O*-*β*-D-glucuronide. The glucuronidase/sulfatase hydrolysis yielded isoscutellarein (Figure 4), and the HR-MS−MS analyses confirmed the structure (Table 3). Compound **7** showed a UV spectrum similar to that of hypolaetin 8-glucuronide (**4**). Its MS analysis showed that the molecular weight of compound **7** was 15 mass units higher than that of compound **4**, suggesting a methyl ether derivative of hypolaetin 8-glucuronide. The MS2 analysis showed that the aglycon had *m*/*z* 315, which indicated that the methyl ether was located on the aglycon and not on the glucuronide residue. The presence of an ion at *m*/*z* 301 (9%) confirmed that the aglycon was a hypolaetin methyl ether, and that the methyl was most probably located on the hydroxyl at the 3′-position. Enzymatic

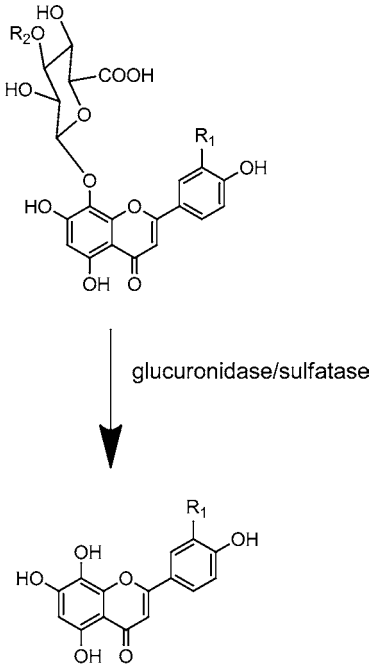
Table 2. t_R and MS $[M - H]^-$ and −MS2 $[M - H]^-$ Data of Glycosyl Flavones of Cupuassu Seeds[a]

				−MS2 $[M - H]^-$ losses *m*/*z* (%)							
peak	compound	t_R (min)	$[M - H]^-$ *m*/*z*	15	80	90	120	162	176	302	[aglycon − H]$^-$
1	apigenin 8-*C*-hexoside	13.3	431			341 (11)	311 (100)				
2	hypolaetin 8-*O*-*β*-D-glucuronide glucoside	15.6	639					477 (34)	463 (71)		301 (100)
3	quercetin 3-*O*-pentoside	17.1	433								301 (100)
4	hypolaetin 8-*O*-*β*-D-glucuronide	18.0	477								301 (100)
5	hypolaetin 8-*O*-*β*-D-glucuronide 3″-*O*-sulfate	19.9	557		477 (100)					254 (48)	301 (41)
6	isoscutellarein 8-*O*-*β*-D-glucuronide	20.7	461								285 (100)
7	hypolaetin 3″-methyl ether 8-*O*-*β*-D-glucuronide	21.2	491	301 (9)							315 (100)
8	isoscutellarein 8-*O*-*β*-D-glucuronide 3″-*O*-sulfate	22.0	541		461 (35)					254 (100)	285 (5)
9	hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide 3″-*O*-sulfate	22.8	571		490 (27)					254 (100)	315 (12)

[a]Main observed fragments. Other ions were found, but they have not been included.

Table 3. HR-MS−MS UPLC QTOF Analysis and Content (mg/g DW) of Flavone Glycosides in Cupuassu Seeds

peak	compound	mass found	mass theor	formulas	score	content mg/g
1	apigenin 8-*C*-hexoside	431.0988	431.0984	$C_{21}H_{20}O_{10}$	97.85	traces
2	hypolaeytin glucuronide glucoside	639.1204	639.1203	$C_{27}H_{28}O_{18}$	98.32	traces
3	quercetin 3-*O*-pentoside	433.1354	433.1351	$C_{18}H_{26}O_{12}$	98.88	traces
4	hypolaetin 8-*O*-*β*-D-glucuronide	477.0684	477.0675	$C_{21}H_{18}O_{13}$	97.96	7.49 ± 0.39
5	hypolaetin 8-*O*-*β*-D-glucuronide 3″-sulfate	557.0254	557.0243	$C_{21}H_{18}O_{16}S$	95.94	2.61 ± 0.02
6	isoscutellarein 8-*O*-*β*-D-glucuronide	461.0734	461.0725	$C_{21}H_{18}O_{12}$	98.23	3.92 ± 0.09
7	hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide sulfate	491.0834	491.0831	$C_{22}H_{20}O_{13}$	99.35	3.19 ± 0.40
8	isoscutellarein 8-*O*-*β*-D-glucuronide sulfate	541.0298	541.0294	$C_{21}H_{18}O_{15}S$	97.59	7.20 ± 0.35
9	hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide sulfate	571.0407	571.0399	$C_{22}H_{20}O_{16}S$	97.61	6.69 ± 0.17
					total	31.08



Compound	R_1	R_2
4	OH	H
5	OH	SO_3H
6	H	H
7	OCH_3	H
8	H	SO_3H
9	OCH_3	SO_3H

Figure 3. Aglycons produced after enzymatic hydrolysis of cupuassu flavonoids with glucuronidase-sulfatase.

hydrolysis produced an aglycon with the characteristic UV spectrum of hypolaetin 3′-methyl ether (8-hydroxychrysoeriol),[34] in which the hydroxyl at the 8-position was now free (Figure 4). This compound was also detected when the samples were extracted with water, and therefore could not be produced as an artifact when extracting with methanol. Therefore, **7** was identified as hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide (8-hydroxychrysoeriol 8-glucuronide). This compound had not been previously reported in cupuassu but had been identified by our group in *Gratiola officinalis*.[35] Compound **7** coincided chromatographically with the authentic standard isolated from *Gratiola officinalis*,[35] and the structure was confirmed by HR-MS−MS (Table 3). Compound **8** showed a UV spectrum similar to that of isoscutellarein 8-glucuronide (**6**), and its MS analysis showed a pseudomolecular ion at *m*/*z* 541, and fragments at M − 80 (M − sulfate) and M − 80 − 176 (M − sulfate − glucuronide) showing the sequential losses of the sulfate and glucuronide residues to lead to the aglycon hypolaetin (*m*/*z* 285). A fragment at *m*/*z* 254 corresponding to the sulfoglucuronide fragment was also detected (Table 2). The hydrolysis with glucuronidase/sulfatase yielded isoscutellarein, and the structure was confirmed by HR-MS−MS analyses (Table 3). This compound coincided with theograndin I previously reported from the same source.[1] Compound **9** showed a UV spectrum very similar to that of compound **4**, and its MS analysis showed a pseudomolecular ion at *m*/*z* 571, and fragments at M − 80 (M − sulfate) and M − 80 − 176 (M − sulfate − glucuronide) showing the sequential losses of the sulfate and glucuronide residues to lead to the aglycon hypolaetin methyl ether (*m*/*z* 315). A fragment at *m*/*z* 254 corresponding to the sulfoglucuronide fragment was also detected (Table 2). After enzymatic hydrolysis with glucuronidase/sulfatase (Figure 3), the aglycon produced had the characteristic UV spectrum of hypoaletin 3′-methyl ether,[34] in which the hydroxyl at the 8-position was now free (compared with the original 8-*O*-glycoside) (Figure 4). Compound **7** was detected as an intermediate when treated with mild acidic conditions, confirming the presence of a sulfate, and the HR-MS−MS analysis confirmed the structure. Therefore, **9** was characterized as hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide, 3″-sulfate (8-hydroxychrysoeriol 8-*O*-*β*-D-glucuronide, 3″-sulfate). This compound had not been previously reported in cupuassu. The structure was confirmed by ^{1}H NMR, showing a spectrum similar to that reported for theograndin II[1] but with an additional singlet at 3.791 ppm (3H) corresponding to the methyl ether on the hydroxyl 3′-position. This compound was also detected when the samples were extracted with water, and therefore could not be produced as an artifact when extracting with methanol.

The flavone glycosides present in cupuassu were quantified as 8-hydroxychrysoeriol 8-*O*-glucuronide (same as compound **7**) previously isolated from *Gratiola officinalis*[21] in the HPLC−UV analyses. The quantification showed that the main flavonoids were the glucuronide sulfates, with a total flavone

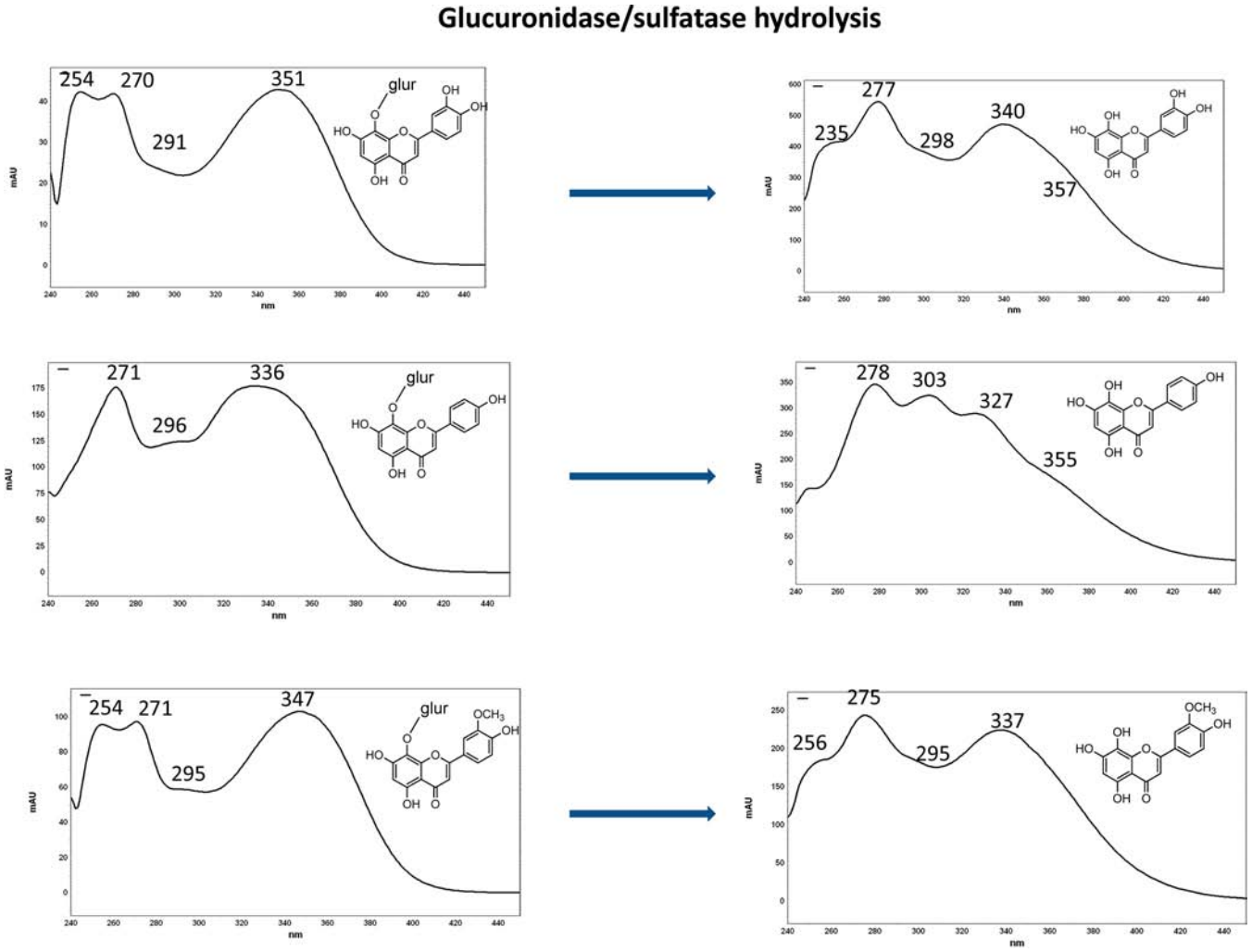


Figure 4. UV spectra of the flavone glycosides hypolaetin 8-*O*-*β*-D-glucuronide 3″ sulfate (**5**), isoscutellarein 8-*O*-*β*-D-glucuronide sulfate (**8**), and hypolaetin 3′-methyl ether 8-*O*-*β*-D-glucuronide sulfate (**9**), and the aglycons obtained after glucuronidase/sulfatase hydrolysis. The spectra of the aglycons denote that the glycosidic moiety was linked to the hydroxyls at the 8-position in the original glycosides.[34].

concentration above 31 mg/g of unfermented cupuassu seeds (Table 3). Similar flavonoid profiles were observed for fresh and commercial frozen pulps, but varying in flavonoid quantities. Commercial frozen pulps presented lower contents than did fresh pulps, on average 0.5 and 2.2 mg/g, respectively. The seeds showed the highest contents of flavonoids, almost 15 times those of fresh seeds (results not shown), corroborating results from total phenolics analyses.

Quantification of Proanthocyanidins. Cupuassu also contained proanthocyanidins as other *Theobroma* species, in particular *Theobroma cacao*. The proanthocyanidin content was evaluated using an acid-catalyzed degradation reaction previously reported.[22] The analysis showed that both epicatechin and catechin were present as terminal units in the proanthocyanidin oligomers, although epicatechin was 6 times more abundant than catechin (Table 4). The extension units were only epicatechin, in a similar way to what was reported in cocoa.[35] The total content was close to 23 mg/g of cupuassu seeds. The mean degree of polymerization was 5.5 calculated as reported previously.[22]

This study showed that the chemical composition, the ascorbic acid, and the total phenolic contents of cupuassu pulps had few variations according to the harvesting time, and this

Table 4. Proanthocyanidin Characterization and Content (mg/g DW) of Cupuassu Seeds

compound	mg/g	%[a]
extension units		
epicatechin	18.33 ± 1.17	100
terminal units		
catechin	0.64 ± 0.03	14
epicatechin	4.09 ± 0.20	86
total	23.06	
DPn[a]	5.5	

[a]DPn, mean degree of polymerization.

was also the case for the cupuassu commercial frozen pulps from different brands. This regularity may be due to the fact that the harvested fruits were those naturally fallen on the ground and, therefore, at the same stage of maturation. Cupuassu showed a high amount of phenolics, especially on seeds, and of ascorbic acid on pulps. Those compounds resulted in a high in vitro antioxidant activity, being higher on cupuassu seeds. The analysis of the flavone glycosides has revealed that vitexin (**1**), hypolaetin 8-glucuronide-glucoside (**2**), hypolaetin 3′-methyl ether 8-*β*-D-glucuronide (**7**), and the corresponding 3″-sulfate (**9**) had not been previously reported

in cupuassu and the last one (**9**) is a novel conjugate in nature to the best of our knowledge. In this study we did not detect the presence of isoscutellarein 8-*O*-*β*-D-glucuronide 6″-methyl ester and quercetin 3-*O*-*β*-D-glucuronide 6″-methyl ester, which were previously reported from cupuassu.[1] This could be explained by different chemotypes that are feasible in nature, or to the production of the glucuronide methyl esters during the extraction method used, as these esters are easily produced when warming in methanol medium. Cupuassu did not show any anthocyanidins, which are common in cocoa seeds. The total proanthocyanidin content of unfermented cupuassu seeds (23 mg/g) is a bit lower in comparison with those reported for cocoa powder (32−48 mg/g) but was higher compared to those of milk chocolate (2−3 mg/g).[36] The content of flavones, however, is much higher in unfermented cupuassu, with contents above 31 mg/g, when these compounds (mainly quercetin glycosides) are only present in very low concentration in cocoa powder.[35] These 8-hydroxyflavone glycosides are unusual plant metabolites, that are present in some medicinal plants and for which anti-inflammatory activity has been previously reported.[37,38] The lower contents of ascorbic acid, phenolics, and antioxidants in general of commercial frozen pulps indicate that the effect of depulping and storage should be studied further.

AUTHOR INFORMATION

Corresponding Author

*Tel: +55 11 3091 1525. Fax: +55 11 3815 4410. E-mail: genovese@usp.br.

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