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Determination of Carotenoids, Total Phenolic Content, and Antioxidant Activity of Arazá (*Eugenia stipitata* McVaugh), an Amazonian Fruit

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ABSTRACT: The fruit of Arazá (*Eugenia stipitata* McVaugh) native to the Colombian Amazon is considered a potentially economically valuable fruit for the Andean economy due to its novel and unique taste. The fruit has an intense yellow color, but its chemical composition and properties have not been well studied. Here we report the identification and quantitation of carotenoids in the ripe fruit using high performance liquid chromatography (HPLC) with photodiode array detector (PDA) and atmospheric pressure chemical ionization (APcI) mass spectrometry (MS/MS). The qualitative carotenoid profile of the fruit according to maturity stage was also observed. Furthermore, antioxidant activity of the peel and pulp were assessed using the ferric reducing ability of plasma (FRAP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) methods, in addition to chemical indexes and total phenolic content. Multiple carotenoids were identified in the peel and pulp including four xanthophylls (free and esterified as their mono and diesters) and two carotenes. One of the xanthophylls was tentatively identified as zeinoxanthin, while the others were identified as lutein, zeaxanthin, and β-cryptoxanthin. Carotenes included α-carotene and β-carotene. The total carotenoid content was significantly higher in the peel (2484 ± 421 μg/100 g FW) than in the pulp (806 ± 348 μg/100 g FW) with lutein, β-cryptoxanthin, and zeinoxanthin as the major carotenoid components. The unique carotenoid composition of this fruit can differentiate it from other carotenoid-rich fruits and perhaps be useful in authentication procedures. Overall, results from this study suggest that Colombian Arazá may be a good edible source of carotenoids important in retinal health as well as carotenoids with provitamin A activity. Therefore, Arazá fruit can be used as a nutraceutical ingredient and in production of functional foods in the Colombian diet.

KEYWORDS: *Arazá, Eugenia stipitata McVaugh, carotenoids, lutein, antioxidant activity*

INTRODUCTION

Carotenoids are one of the most widespread groups of pigments in nature. Approximately 750 carotenoids have been identified to date.[1] However, only a fraction of these compounds are absorbed and utilized by humans, and only a small percentage serve as precursors of vitamin A. Carotenoids which contain an unsubstituted β-ionone ring, including β-carotene, α-carotene, β-cryptoxanthin, and α-cryptoxanthin, have the ability to be converted into vitamin A in vivo.[2] The biological effects of vitamin A include growth promotion, cellular differentiation, immune function, embryonic development, and gap junction communication.[3,4] Besides the well-recognized provitamin A activity, carotenoids have further potential health benefits such as prevention of chronic diseases, including certain types of cancer[5−8] and prevention of arterial plaque formation.[9] Human studies have also demonstrated that consumption of carotenoid rich fruits and vegetables increases low density lipoprotein oxidation resistance and decreases DNA damage.[10] In addition, studies of lutein and zeaxanthin suggest that the consumption of these compounds or food products that contain these xanthophylls in high concentrations, e.g., green leafy foods, some varieties of squash, broccoli, peas, and corn, may reduce the risk of macular degeneration[11−13] and cataract formation.[14−18] Some of these biological effects have been attributed to the antioxidant activity of carotenoids, through deactivation of free radicals and singlet oxygen quenching.[19−21] Additionally, recent research has linked increased brain levels of lutein with improved cognitive function in a geriatric population.[22]

Due to the scientific evidence supporting the health benefits of carotenoid consumption and the lack of fruit sources of lutein and zeaxanthin, new food sources of these pigments are being sought.

Arazá (*Eugenia stipitata* McVaugh) is a perennial tree from the Myrtaceae family and native to the Amazon rainforest. *Eugenia stipitata* McVaugh grows in the Colombian Amazon region. The tree (2−5 m tall) produces a spherical fruit (4−7 cm in diameter) with a delicate peel of 1 mm thickness, which accounts for 10% of the total fruit weight and 6 to 15 seeds per fruit. The fruit has an intense canary yellow color, and the juiciness, high acidity, unique sensory characteristics, and pectin content of the fruit make it suitable to produce juice, nectars, jams, and jellies. In Colombia, the interest in this fruit has

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increased in recent years because of the constant search for new products and exotic tastes, its traditional medicinal applications, and its potential for export.[23] Arazá is being introduced both fresh and processed in the local market of Colombia, and it is considered a potentially economically valuable fruit for the Andean economy.[24]

Besides brief reports in the literature about the carotene equivalence of Arazá,[25] the profile and level of individual carotenoids have not been reported. Considering the supporting information on the potential health benefits of carotenoids, it is important to characterize *Eugenia stipitata* McVaugh subspecies grown in the Colombian Amazon. Here we have characterized and reported levels of the carotenoids in the ripe fruit. Additionally, we have determined qualitative changes in the carotenoid profile of Arazá in the green and half-green maturity stages. Composition indices, total phenolic content (TPC), and antioxidant activity of the ripe fruit were also assessed.

MATERIALS AND METHODS

Plant Material. Arazá fruit originally from the Colombian Amazon was purchased in local markets in Bogotá (Colombia) at three stages of ripeness: green, half-green, and ripe, according to the description given by Hernández et al.[26] The fruit was separated into peel and pulp, and samples to be analyzed for carotenoid content, TPC, and antioxidant activity were lyophilized immediately. Fresh ripe samples were used to analyze moisture, titratable acidity (TA), pH, and total soluble solids (TSS).

Reagents. Gallic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), TPTZ (2,4,6-tripyridyl-*s*-triazine), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma Aldrich (St. Louis, MO). Sodium carbonate, potassium persulfate, and Folin−Ciocalteau reagent were from Merck (Darmstadt, Germany). Methanol, acetone, and liquids were from Fisher Scientific (Fair Lawn, NJ, USA).

Lutein, zeaxanthin, and α-carotene were purchased from Chromadex (Irvine, CA). β-Cryptoxanthin was purchased from Indofine Chemical Company (Hillsborough, NJ). β-Carotene was purchased from Sigma-Aldrich (St. Louis, MO). Methanol, methyl *tert*-butyl ether (MTBE), ammonium acetate, water, hexane, acetone, sodium sulfate, and potassium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ).

Determination of Compositional Indexes of the Fruit. Moisture content was determined using the official AOAC method 934.06.[27] TA was measured by titrating the sample (2 g of homogenate + 50 mL of CO_2-free distilled water) with standardized 0.1 N NaOH to pH 8.2 using a Schott Gerate pH meter, model CG820 (Mainz, Germany) and expressed as mg of malic acid/100 g of fruit.[26] TSS (°Brix) was assessed using a digital refractometer Abbe II (Reichert-Jung, Leica Inc., Buffalo, NY, USA). All determinations were done on six different lots.

Carotenoid Extraction. Extracts from freeze-dried samples of peel and pulp were obtained according to the method described by Ferruzzi et al.[28] with modifications. Approximately 0.25 g of powdered lyophilized sample was weighed into a centrifuge tube, to which methanol (5 mL) was added. The sample was homogenized using a Polytron homogenizer (Kinematica Polytron PT 3100, Bohemia, NY) at 5,000 rpm for 2 min, capped, and centrifuged at 300*g* for 10 min to pellet the solids. The methanol extract was then transferred to a separate vial, and 5 mL of hexane/acetone (1:1) was added to the pelleted material. The sample was homogenized and centrifuged again, and the hexane/acetone extract was removed and combined with the methanol extract. This hexane/acetone extraction was repeated twice more for pulp and three times more for peel.

The extracts were treated in two ways for carotenoid analysis. For qualitative analysis, 10 mL of 30% potassium hydroxide in methanol (w/v) was added to the pooled extracts, and the samples were saponified on a stir-plate at 60 °C for 1 h. Afterward, water was added to induce phase separation and the upper nonpolar layer was removed and dried under nitrogen gas and stored at −20 °C until analysis.

For quantitative analysis, 1 mL of an aqueous saturated sodium chloride solution and 5 mL of water were added to the pooled hexane/acetone/methanol extract to induce phase-separation. The centrifuge tube was gently inverted and vented multiple times, and then allowed to phase separate. The upper hexane layer was removed, dried over sodium sulfate, and brought up to 20 mL in a volumetric flask. A fraction of the extract was dried under nitrogen gas and stored at −20 °C until analysis. Determinations were done on three different lots.

Qualitative and Quantitative Analyses of Carotenoids. Samples were reconstituted in methanol/methyl *tert*-butyl ether (MTBE) (1:1, v/v) and filtered through a 0.4 μm nylon syringe filter. Separation and identification of carotenoids was done using high performance liquid chromatography−mass spectrometry (HPLC-PDA−MS/MS). The system was equipped with an Acquity HPLC interfaced with an Acquity eλ PDA detector (Waters Corp., Milford, MA). Separation was achieved using a YMC C-30 S-3 column (2.0 mm × 150 mm, 3 μm; Waters Corp, Mildford, MA). A 30 min gradient was applied with solvent A (80:18:2 MeOH/water/2% aqueous ammonium acetate) and solvent B (20:78:2 MeOH/MTBE/2% aqueous ammonium acetate) from 0 to 90% B over 28 min with a two minute re-equilibration. The flow rate was 0.4 mL/min, column temperature 40 °C, and injection volume 5 μL.

The HPLC eluate was interfaced with a triple quadrupole mass spectrometer (Quattro Ultima, Micromass UK Ltd., Manchester, U.K.) via an APcI source operated in negative ion mode. Multiple reaction monitoring (MRM) MS/MS was used to detect compounds according to their appropriate parent → daughter ion transitions. Instrument parameters included the following: corona current 30 μA, cone 35 V, desolvation 450 °C, desolvation gas 400 L/h with argon gas for collision induced dissociation.

Identity of α-carotene, β-carotene, nonesterified lutein, zeaxanthin, and β-cryptoxanthin was determined by retention time (RT), ultraviolet−visible (UV−vis) spectra, and parent ion m/z coincident with commercial standards. The ester forms of lutein and β-cryptoxanthin were identified based on PDA spectra, parent m/z, and daughter ions. The base carotenoid m/z for each ester was calculated from the difference between the parent m/z and the fatty acid masses minus the number of fatty acids multiplied by 18. Intermediate fragments such as listed in Table 2 for diesters (peaks 13−15) provide additional confirmation of fatty acid substitutions. Due to lack of commercial standards, tentative identification of anhydrolutein and anhydrozeaxanthin was determined by UV−vis spectra and MS/MS fragmentation patterns. Free zeinoxanthin was identified on the peel fraction using the methylation test on the monohydroxy fraction.[29] Approximately 10 g of the freeze-dried peel was extracted and saponified as described above. The extract was freeze-dried, subsequently reconstituted in 3 mL of MeOH/MTBE (1:1, v/v) and passed through a 0.2 μm nylon filter. The xanthophyll was then separated via preparatory HPLC using a Shimadzu ultrafast liquid chromatography (UFLC) system (Shimadzu Corp, Kyoto, Japan) equipped with a SIL-20A autosampler, and SPD-M20A PDA. Conditions for the mobile phase: (A) 88:5:5:2 MeOH/H_2O/MTBE/2% aqueous ammonium acetate and (B) 20:78:2 MeOH/MTBE/2% aqueous ammonium acetate. Gradient conditions were linear gradient from 0% to 73% B over 22.5 min, followed by an immediate increase to 100% B and holding for 2 min, and returning to 0% B. The column used for separation was a YMC C-30 (YMC America, Allentown, PA) (20 × 250 mm, 5 μm particle size). Flow rate was 15 mL/min, injection volume 1 mL. The fractions were combined, dried under nitrogen gas, and stored at −80 °C.

The methylation reaction was performed on lutein alone, on the monohydroxy fraction alone, and on the monohydroxy fraction spiked with lutein in a similar proportion to the putative α-cryptoxanthin/zeinoxanthin (i.e., as a positive control to monitor reaction efficacy). The monohydroxy fraction and the lutein standard were dissolved in 5 mL of methanol and placed in separate 11 mL vials. The vials were

flushed with nitrogen gas, and 150 μL of 0.2 M HCl in MeOH was added to each one. The methylation reaction proceeded for 6 h in the dark. Commercial orange juice was extracted using the method previously described and tested as a surrogate source of zeinoxanthin.[29]

The reaction was monitored using a model 2996 HPLC (Waters Corp., Milford, MA) connected to a model 2996 PDA (Waters Corp., Milford, MA) interfaced with a Q-Tof Premier quadrople time-of-flight hybrid mass spectrometer (Micromass UK Ltd., Manchester, U.K.) using an atmospheric pressure chemical ionization (APcI) source operated in negative ion mode. Additional settings were as follows: corona current, 30 μA; cone, 35 V; desolvation temp, 400 °C; desolvation gas flow, 300 L/h; and gas for collisionally induced dissociation (CID), argon at 4×10^{-3} mbar. The column used for separation was a C-30 (prepared by Lane Sander, National Institute of Standards and Technology, Gaithersburg, MD) (250 × 4.6 mm, 3 μm particle size). The composition of solvents was (A) 90:10 MeOH/0.7% aqueous ammonium formate solution at pH 3 and (B) 78:20:2 MTBE/MeOH/0.7% aqueous ammonium formate solution at pH 3. Gradient conditions were initially 0% B increased linearly to 50% B over 12 min, then linearly to 80% over 16 min, followed by a 3 min re-equilibration period. Injection volume was 20 μL, flow rate was 1.5 mL/min, and a column temperature of 30 °C was employed.

Quantification of free carotenoids was based on peak area at 450 nm by external calibration curves of lutein, zeaxanthin, α-carotene, β-carotene, and β-cryptoxanthin. Peak area from PDA was used to generate the calibration curves. Levels of all esterified xanthophylls were calculated using the calibration curve of the respective free xanthophylls, except for zeinoxanthin, whose esters were calculated as free lutein equivalents. Values are expressed as μg/100 g FW.

Comparison of carotenoid profile at the green, half-green, and ripe maturity stages was carried out by HPLC on a Waters 2695 gradient HPLC separation module (Waters Corp., Mildford, MA) equipped with a 996 PDA. The previously described HPLC parameters were used for this analysis.

Extraction of Compounds with Antioxidant Activity. Freeze-dried samples of peel or pulp (100 mg) were milled and extracted with 2 mL of 100% acetone for 1 h, followed by three re-extractions with 2 mL of the same solvent. Extracts were stored for two days at −20 °C until all the measurements for TPC and antioxidant activity were completed.

Determination of Total Phenolics Content. Total phenolics content was determined as gallic acid equivalents (GAE)/100 g FW using the method described by Waterhouse.[30] A 20 μL aliquot of extract or gallic acid standard (50 to 500 mg/L) was mixed with 1.58 mL of water followed by 100 μL of Folin−Ciocalteau's reagent. After vortexing and incubating at room temperature for 8 min, 300 μL of 20% aqueous sodium carbonate solution (w/v) was added. Samples were vortexed and held at room temperature for 2 h. Absorbance of the blue-colored solution was recorded at 765 nm on a Shimadzu UV visible spectrophotometer, model UV 160 U (Kyoto, Japan), using 1 cm disposable cells. Determinations were performed on six different lots of both ripe peel and ripe pulp.

Determination of Antioxidant Activity. Antioxidant activity was determined by the ABTS, FRAP, and DPPH assays. ABTS radical cation scavenging activity was assessed according to the method described by Re et al.[31] To obtain the $ABTS^{\bullet+}$ solution, a mixture of 7.0 mM ABTS and 2.45 mM potassium persulphate (final concentration) was prepared and stored in darkness for 16 h. The $ABTS^{\bullet+}$ solution was diluted with ethanol to reach an absorbance of 0.70 ± 0.01 at 734 nm of the working solution. Fresh $ABTS^{\bullet+}$ solution was prepared for each analysis. The reaction mixture consisted of 1 mL of the $ABTS^{\bullet+}$ working solution and 10 μL sample, placed in a cuvette incubated at 30 °C. Absorbance was measured every 30 s until it reached the plateau (60 min). The percentage $ABTS^{\bullet+}$ inhibition at 734 nm was calculated by the formula $I = [(AB - AA)/AA] \times 100$; where I = $ABTS^{\bullet+}$ inhibition %; AB = absorbance of a blank sample (t = 0 min); AA = absorbance of a tested extract solution at the end of the reaction. Standard curves using Trolox ranging in concentration from 250 μM to 1500 μM were run with each set of extracts. Samples were diluted to get $ABTS^{\bullet+}$ inhibition values within the calibration curve. ABTS values were expressed as μmol Trolox equivalents (TE)/g FW.

The FRAP assay was performed according to Benzie and Strain.[32] FRAP reagent was prepared by mixing 2.5 mL of TPTZ solution (10 mM in 40 mM HCl), 25 mL of acetate buffer (300 mM, pH 3.6), and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM). Reaction was started by mixing 900 μL of FRAP solution with 90 μL of distilled water and 30 μL of standards or extracts in a cuvette incubated at 37 °C. Standard curves using Trolox (250−1000 μM) were run with each set of extracts. The reaction of the samples was followed until it reached the plateau (45 min). Final results were expressed as μmol TE/g FW.

The total free radical scavenging capacity was determined and compared to that of Trolox according to the method described by Hsu, Coupar, and Ng.[33] The extract (100 μL) was mixed with 1.9 mL of 0.1 mM DPPH methanolic solution. The mixture was shaken vigorously and left to stand for 30 min at room temperature, and the absorbance was then measured at 517 nm against a blank until the reaction reached the plateau. The percentage scavenging effect was calculated as scavenging rate = $(A_1 - A_2/A_0)100$, where A_0 was the absorbance of the control (without extract), A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without DPPH. Results were expressed as μmol TE/g FW. All measurements were done on six different lots of both peel and pulp.

STATISTICAL ANALYSIS

Quantitative data are presented as mean values with the respective standard deviation. Significant ($P < 0.05$) differences between means (pulp and peel) were identified using the Student's two-sample t-test. All analyses were performed with Statgraphics plus, version 2.1.

RESULTS AND DISCUSSION

Compositional Indexes. As reported in Table 1, the Arazá pulp had low TSS (4.6 ± 1.1 °Brix). The TA was 2830 ± 500 mg malic acid/100 g FW, which is approximately 7-fold higher than the average content found in apples (394 mg malic acid/100 g FW).[34] The pH of the pulp was 2.6 ± 0.1, which is similar to the pH of lemon.[35] Values of pH and TSS are close to those reported for ripe Arazá fruits harvested on the Amazon Region in Brazil[36] and in Colombia.[26] The moisture value of the pulp was 93.6% ± 1.0, which is similar to the value (96%) found by Rogez et al.[36] for Brazilian Arazá. The high TA and low TSS values seem to explain the low sugar/acid ratio (1.6 ± 0.2) of this fruit.

Table 1. Compositional Indexes of Arazá Fruit (*Eugenia stipitata* McVaugh)[a]

compositional index	pulp	peel
TA[b]	2830 ± 500	ND
pH	2.6 ± 0.1	ND
TSS[c]	4.6 ± 1 0.1	ND
TSS/TA (sugar:acid ratio)	1.6 ± 0.2	ND
moisture content[d]	93.6 ± 1.0 a	89.0 ± 2.4 b

[a]Results are expressed as mean ± SD (n = 6). ND = not determined. Values within the same row with different letters show significant differences ($p < 0.05$). [b]Titratable acidity (TA) expressed in mg malic acid/100 g FW. [c]Total soluble solids (°Brix). [d]Expressed in g/100 g FW.

Carotenoid Analysis. A representative HPLC−MS chromatogram of carotenoids in ripe Arazá is shown in Figure 1 while mass spectrum data is shown in Table 2. After saponification, multiple parent carotenoids were identified

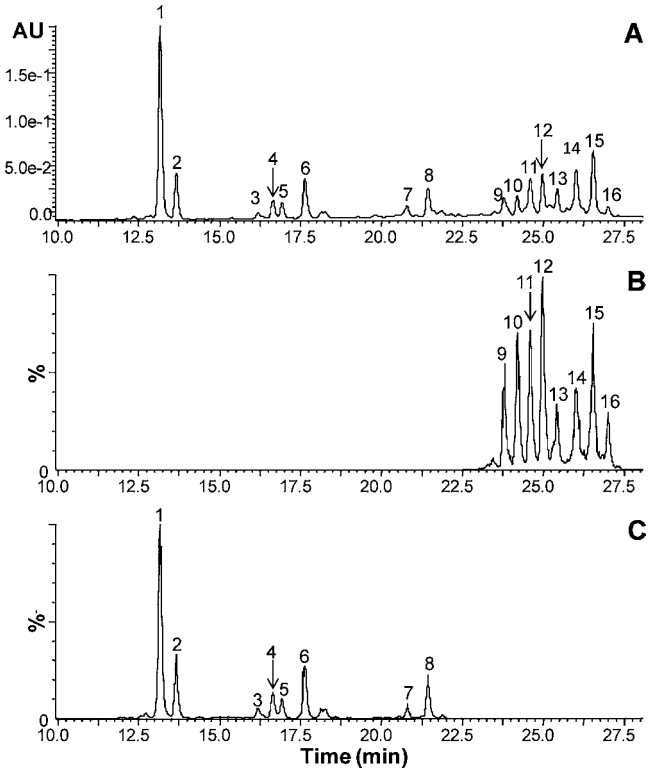

Figure 1. HPLC-PDA–MS chromatograms of carotenoids in Arazá fruit peel (*Eugenia stipitata* McVaugh). (A) PDA chromatogram at 445 nm. (B) Chromatogram of the sum of the parent molecular ions for the 5 carotenoid esters *m/z* 762.6, 790.6, 988.6, 1016.6, and 1044.6. (C) Chromatogram of the parent molecular ions for the free carotenoids *m/z* 568.4, 550.4, 552.4, and 536.4. Peaks are labeled as follows: 1 = lutein; 2 = zeaxanthin; 3 = anhydrolutein, 4 = anhydrozeaxanthin; 5 = zeinoxanthin; 6 = *β*-cryptoxanthin; 7 = *α*-carotene; 8 = *β*-carotene; 9 = zeinoxanthin myristate; 10 = *β*-cryptoxanthin myristate; 11 = zeinoxanthin palmitate; 12 = *β*-cryptoxanthin palmitate; 13 = lutein dimyristate; 14 = lutein myristoyl palmitate; 15 = lutein dipalmitate; 16 = unknown.

including lutein, zeaxanthin, zeinoxanthin, *α*-carotene, *β*-carotene, and *β*-cryptoxanthin. UV–vis spectra, RT, and parent *m/z* of lutein, zeaxanthin, *β*-cryptoxanthin, *α*-carotene, and *β*-carotene matched those of authentic standards. Saponification revealed the presence of these compounds as the primary carotenoids, in addition to putative anhydrolutein, anhydrozeaxanthin, and zeinoxanthin. The identification of anhydrolutein, anhydrozeaxanthin, and zeinoxanthin, in addition to the carotenoid esters, is detailed below.

Peaks 3 and 4 produced molecular ions at *m/z* 550.4 generated by the loss of water as result of in-source fragmentation (*m/z* 568 $[M - H_2O - H^+]^-$). However, the UV–vis spectrum of peak 3 matched that of lutein, while the UV–vis spectrum of peak 4 corresponded to that of zeaxanthin. As a result, peak 3 was tentatively identified as anhydrolutein, and peak 4 was tentatively identified as anhydrozeaxanthin. Previous publications report the presence of anhydrolutein while anhydrozeaxanthin has only been suggested as a carotenoid present in mussels.[38] Molnár et al.[39] detected anhydrolutein in sorrel (*Rumexrugosus*) after processing the vegetable with steam. Anhydrolutein has also been found in human plasma by Khachik et al.,[40] who postulated that its appearance is due to acid-catalyzed dehydration of the dietary lutein as it passes through the stomach. Based on this

Table 2. Main Characteristics Obtained by HPLC–MS/MS of Carotenoids from Arazá Fruit (*Eugenia stipitata* McVaugh)

peak	RT (min)	molecular ion M^- *m/z*	fragment ions in MS/MS *m/z*	UV–vis *λ* max (nm)	identity
1	13.16	568.4		445	lutein
2	13.65	568.4		452	zeaxanthin
3	16.19	550.4		445	anhydrolutein[a]
4	16.68	550.4		452	anhydrozeaxanthin[a]
5	16.95	552.4		445	zeinoxanthin[a]
6	17.67	552.4		452	*β*-cryptoxanthin
7	20.81	536.4		445	*α*-carotene
8	21.49	536.4		452	*β*-carotene
9	23.81	762.6	227	445	zeinoxanthin myristate[a]
10	24.19	762.6	227	449	*β*-cryptoxanthin myristate[a]
11	24.60	790.6	255	445	zeinoxanthin palmitate[a]
12	24.97	790.6	255	451	*β*-cryptoxanthin palmitate[a]
13	25.40	988.6	760, 227	445	lutein dimyristate[a]
14	26.02	1016.6	760, 788, 227, 255	445	lutein myristoylpalmitate[a]
15	26.54	1044.6	788, 255	445	lutein dipalmitate[a]
16	27.00	790.6	255	458	unknown

[a]Consistent with PDA and MS/MS data but tentative as authentic standards not available.

information, Molnár et al.[39] proposed that, in sorrel, the conversion of lutein to anhydrolutein might be catalyzed by the high concentration of oxalic acid in this product. Based on this affirmation, we hypothesize that the high acid content of Arazá, and the decompartmentalization of cells during the fruit processing step, may produce anhydrolutein.

Peak 5 produced a molecular ion at *m/z* 552.4, which matches the parent molecular ion of *β*-cryptoxanthin, but the UV–vis spectrum matched that of lutein. This information, in addition to the retention time, suggested that this compound was either *α*-cryptoxanthin or zeinoxanthin. Both of these carotenoids have identical spectra with a λ_{max} of 445 nm, and commercial standards are not available for either xanthophyll. Identification of zeinoxanthin versus *α*-cryptoxanthin is nutritionally important because *α*-cryptoxanthin possesses provitamin A activity and zeinoxanthin does not. The only point of differentiation between these two compounds is the ring on which the hydroxyl group is placed. Thus, these two xanthophylls have identical spectra, elute in the same region of the chromatogram, and have the same molecular weight.[2] A hydroxyl group on the *β* ring (zeinoxanthin) does not react to the methylation test, while the hydroxyl group on the *ε* ring (*α*-cryptoxanthin) reacts positively to the methylation test.[41] Lutein was used as a positive control as it contains a hydroxyl group on the *ε* ring.[29]

Figure 2A is the LC–MS profile of the isolated fraction obtained from Arazá peel via preparatory HPLC with carotenoid detected at *m/z* 552.4 and 452 nm absorbance. It was unclear whether this species was *α*-cryptoxanthin or possibly zeinoxanthin as reported in citrus.[29] As seen in Figure 2B, when this xanthophyll fraction (plus lutein as positive control internal standard) was subjected to a methylation reaction for one hour, the only change was appearance of two peaks with shorter RT and *m/z* 582.4. This *m/z* corresponds to

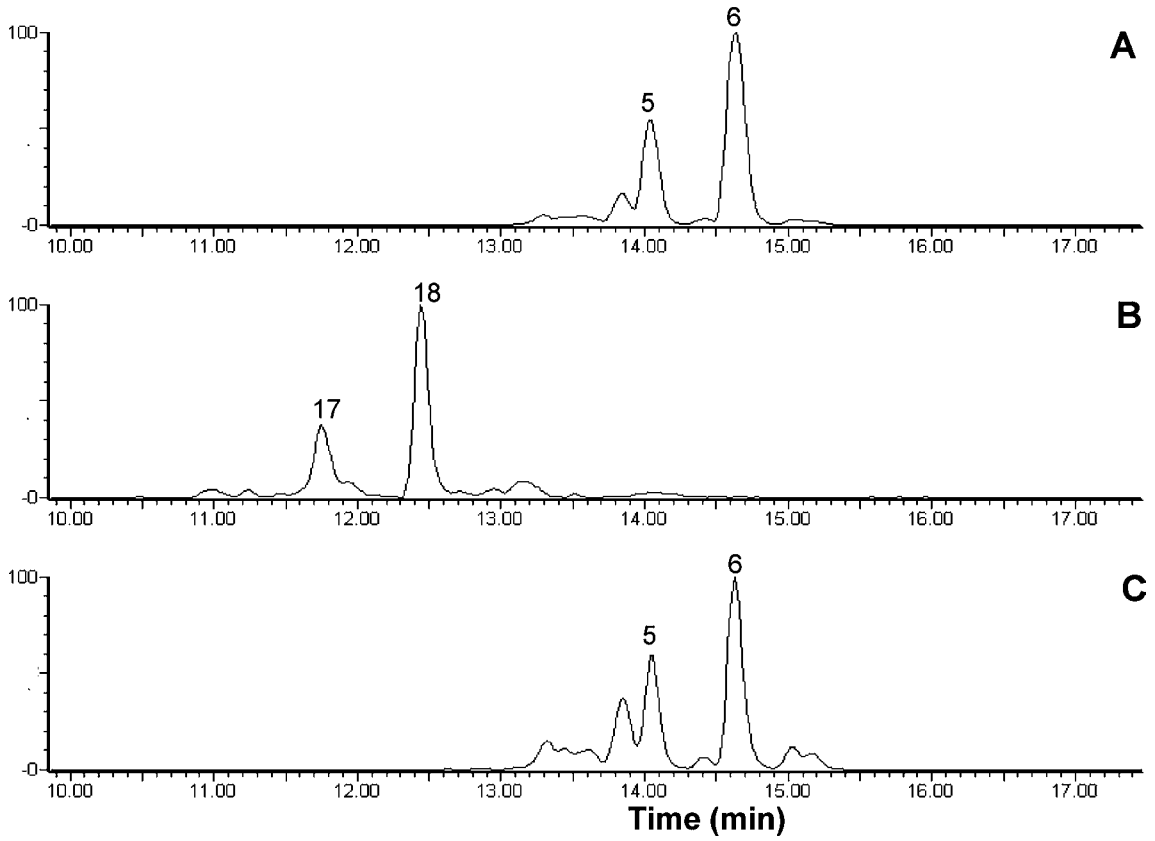

Figure 2. HPLC–MS chromatograms for methylation reaction of carotenoids in Arazá fruit (*Eugenia stipitata* McVaugh). (A) Monohydroxy carotenoid fraction before methylation reaction, m/z 552.4. (B) Monohydroxy fraction spiked with lutein, after methylation reaction, m/z 582.4. (C) Monohydroxy fraction spiked with lutein after methylation reaction monitored at m/z 552.4. Peaks are labeled as follows: 5 = zeinoxanthin; 6 = β-cryptoxanthin; 17, 18 = isomers of methylated lutein derivatives.

methylated lutein (internal standard) by both m/z and retention time of lutein reacted separately. The appearance of two peaks can be explained by the fact that the methylation reaction occurs through a carbocation transition state. This transition state can allow for the double bond in the ε ring to flip, which would give two methylated lutein derivatives with an identical mass but slightly different retention times.

Methylated monohydroxy carotenoid, expected m/z 566.4, was not observed. Furthermore, the unknown m/z 552.4 fraction exhibited the same original m/z 552.4 with no change after 6 h of methylating conditions (Figure 2C). Peak 5 was thus tentatively identified as zeinoxanthin since it was not methylated under these conditions. This result was further affirmed with an orange juice extract as the putative zeinoxanthin peaks in the orange juice extract had coincident retention times with the unknown m/z 552.4 peaks in Arazá.

Arazá also contained xanthophyll esters acylated exclusively with saturated fatty acids. The mass spectrum revealed that the relative molecular weight of carotenoids 9 and 10 was 781 according to parent ion with m/z 762.6 $[M - H_2O - H^+]^-$. The fragment with m/z 227 represented the elimination of myristic acid. The UV–vis spectra of both carotenoids matched those of peaks 5 and 6, and thus, they were labeled as zeinoxanthin myristate and β-cryptoxanthin myristate, respectively. Similarly, fragments with m/z 790.6 for peaks 11 and 12 represented the elimination of one H_2O molecule from cryptoxanthin esters. Since the fragments at m/z 255 correspond to palmitic acid $[M - H^+]^-$ and based on comparison with standards, compound 11 was tentatively identified as zeinoxanthin palmitate and compound 12 was labeled as β-cryptoxanthin palmitate.

Peak 13, with parent ion 988.6, corresponds to the [lutein dimyristoyl $- H_2O - H^+]^-$, further confirmed by the daughter m/z 760, which corresponds to the loss of one myristic acid molecule, and the 227 m/z fragment of $[\text{myristoyl} - H^+]^-$. Accordingly, peak 13 was identified as lutein dimyristate.

The molecular ion (m/z 1016.6) corresponding to peak 14 was fragmented into four major fragments at m/z 760, 788, 227, and 255. Previous publications[42] report that the fragment at m/z 760 corresponds to the loss of one molecule of palmitic acid from the protonated molecular lutein ion. The fragment ions at m/z 227 and 255 represent $[\text{myristoyl} - H^+]^-$ and $[\text{palmitoyl} - H^+]^-$ ions. Then, peak 14 was identified as lutein myristoyl palmitate.

Peak 15 was labeled as lutein dipalmitate. It yielded a parent ion at m/z 1044.6 and two daughter ions at m/z 788 and 255 formed by the loss of palmitic acid (256 Da). The UV–visible spectra characteristics indicated it was a lutein derivative.

The UV–vis spectra and MS/MS data of the unknown peak (16) suggest that this compound may be rubixanthin-palmitate. However, this was not confirmed.

The concentration of carotenoids in the peel and pulp of Arazá fruit is summarized in Table 3. The total carotenoid content of the peel (2484 ± 421 μg/100 g FW) was approximately 3-fold higher than the carotenoid content of the pulp (806 ± 348 μg/100 g), and the concentration of all

Table 3. Concentration of Carotenoids in the Peel and Pulp of Ripe Arazá Fruit (*Eugenia stipitata* McVaugh)[a]

carotenoid	carotenoid content (μg/100 g FW)	
	peel	pulp
α-carotene	96 ± 20 a	31 ± 14 b
zeinoxanthin[b]	81 ± 23 a	25 ± 20 b
zeinoxanthin myristate[b]	103 ± 6 a	38 ± 16 b
zeinoxanthin palmitate[b]	187 ± 24 a	54 ± 22 b
β-carotene	143 ± 25 a	44 ± 16 b
β-cryptoxanthin	142 ± 45 a	47 ± 27 b
β-cryptoxanthin myristate[c]	77 ± 10 a	43 ± 22 b
β-cryptoxanthin palmitate[c]	153 ± 32 a	92 ± 38 b
lutein	756 ± 116 a	154 ± 107 b
anhydrolutein[b]	136 ± 25 a	63 ± 24 b
lutein dimyristate[b]	101 ± 14 a	47 ± 9 b
lutein myristate palmitate[b]	235 ± 32 a	99 ± 17 b
lutein dipalmitate[b]	256 ± 46 a	91 ± 18 b
zeaxanthin	114 ± 49 a	17 ± 9 b
total	2484 ± 421	806 ± 348

[a]Calculated as μg/100 g FW. Results are expressed as mean ± SD (n = 3). Values within the same row with different letters show significant differences ($p < 0.05$). [b]Calculated as free lutein. [c]Calculated as free β-cryptoxanthin.

carotenoids was significantly higher in the peel of the fruit ($p < 0.05$). Our results agree with those of other groups who observed higher carotenoid concentrations in fruit peel as compared to pulp in several fruits. Kreck et al.[43] found higher content of carotenoids in pumpkin peel as compared to pulp. Similarly, Gross et al.[44] have reported higher concentration of β-carotene (10.8%) and lutein (55.8%) in avocado peel as compared to pulp (4% and 25%, respectively). Rodriguez-Amaya et al.[45] reported a similar observation in *Cyphomandra betacea* fruits where the peel contained higher concentrations of β-carotene and zeaxanthin than the pulp. The total carotenoid content of the Arazá pulp is comparable to that of Andean naranjilla fruit pulp (*Solanum quitoense* Lam) (794 μg/100 g),[46] and is within the range reported for several cultivars of papaya (*Carica papaya*) ranging from 793 to 5134 μg/100 g.[47]

Lutein in free and esterified forms was the most abundant carotenoid in both peel (1349 ± 156 μg/100 g FW) and pulp (392 ± 136 μg/100 g FW) accounting for a total of 489 ± 115 μg/100 g FW in the edible part of the fruit. The lutein content in the peel is within the range reported for vegetables such as broccoli (707−3300 μg/100 g FW) and European lettuce (100−4780 μg/100 g FW) and higher than the content of good sources of lutein such as egg yolk (384−1320 μg/100 g FW) as reported by Maiani et al.[48] The total level of lutein in the fruit as well as in the pulp is comparable to the level observed in squash (*Curcubita pepo*) (460 μg/100 g FW) from Brazil and within the range reported for a total of 141 Brazilian fresh and processed fruits (20 and 620 μg/100 g FW).[49] The total lutein content in the edible part of Arazá is higher than the content in cooked corn (239 μg/100 g FW),[50] which is considered a significant source of this xanthophyll in the American diet.[51−53]

Total β-cryptoxanthin levels were close to the zeinoxanthin levels. The β-cryptoxanthin content was 199 ± 91 μg/100 g FW in the pulp and 421 ± 93 μg/100 g FW in the peel for a total of 221 ± 72 μg/100 g FW in the edible part. The content of this carotenoid in the Arazá fruit is comparable to that obtained by Rodriguez-Amaya et al.[49] for Brazilian canned peaches, which have 250 μg/100 g FW, and for chili (*Capsicum frutescents* L),[54] whose content is 260 μg/100 g FW. When quantifying carotenoids in food products, special attention is dedicated to β-cryptoxanthin as it has provitamin A activity.

The fourth most abundant carotenoid found in Arazá was β-carotene, accounting for 143 ± 25 μg/100 g FW in the peel, 44 ± 16 μg/100 g FW in the pulp, and 53 ± 14 μg/100 g FW in the edible part, which is comparable to the content in cabbage (*Brassica oleracea*) (42 μg/100 g FW)[37] and higher than the content in yellow pepper (38 μg/100 g FW) as reported by Perry et al.[50] Zeaxanthin accounted for 114 ± 49 μg/100 g FW in the peel, 17 ± 9 μg/100 g FW in pulp, and 28 ± 5 μg/100 g FW in the whole fruit. This value is within the range for fresh White Shoepeg and Golden Whole Kernel corn (28.5 ± 5.2 to 209 ± 12 μg/100 g FW) grown in Minnesota.[55] Corn is considered to contain significant amounts of lutein and zeaxanthin, and to be a good source of these xanthophylls in the American diet. In general, zeaxanthin is considered a minor food carotenoid as compared to its parent β-carotene. We observed this pattern in the pulp, but not in the peel, where the content of both carotenoids was comparable. Zeaxanthin and lutein are two of the most studied carotenoids in terms of health promoting effects because together they comprise the yellow pigments in the macula of the human retina.[56] Dietary intake and plasma levels of these carotenoids have been found to have statistically significant inverse relation with the risk of macular degeneration,[13] the principal cause of irreversible blindness in the elderly. Zeaxanthin has also been consistently associated with a reduction in the risk for cataracts[57] and recently with improved cognitive function in a geriatric population.[22]

The α-carotene content (96 ± 20 μg/100 g FW in the peel and 31 ± 14 μg/100 g FW in the pulp) was comparable to the β-carotene content in the peel and in the pulp.

All in all, the carotenoid profile of Arazá mimics the constant carotenoid profile of green leafy vegetables, often referred to as the chloroplast carotenoid pattern, whose main characteristic is the high lutein concentration (about 45%).[2] However, the xanthophylls in leafy vegetables are not esterified, as they are in Arazá fruit.[2]

The general pattern of variation in carotenoid profiles at the green and half-green maturity stages of Arazá as determined by HPLC analysis is shown in Figure 3. The predominant carotenoid in the green pulp (Figure 3A) was lutein. The chromatogram representing the carotenoid profile for the half-green pulp (Figure 3B) showed a decrease in the peak areas corresponding to free lutein along with the appearance of carotenoid esters. In contrast, the evolution of carotenoids in pulp from half-green to ripe Arazá (Figure 3C) showed an increase in both free and esterified lutein.

Total Phenolics Content and Antioxidant Activity. Results on TPC and antioxidant activity in both peel and pulp of Arazá fruit are shown in Table 4. The TPC was approximately 5 times higher in peel than in pulp, and the peel exhibited significantly higher ($p < 0.05$) DPPH radical-scavenging activity, ABTS radical cation reducing activity, and ferric ion reducing ability than the pulp. These results agree with previous studies on TPC and antioxidant activity of other fruits. Jiménez-Escrig et al.[58] found that the TPC and antioxidant activity of guava fruit as measured by the FRAP and DPPH assays were 2-fold higher in peel than in pulp. Similarly, Guo et al.[59] showed that the FRAP value in 28 different fruits was between 2- and 27-fold higher in the peel than in the pulp. Peel of apple, citrus, and mango also

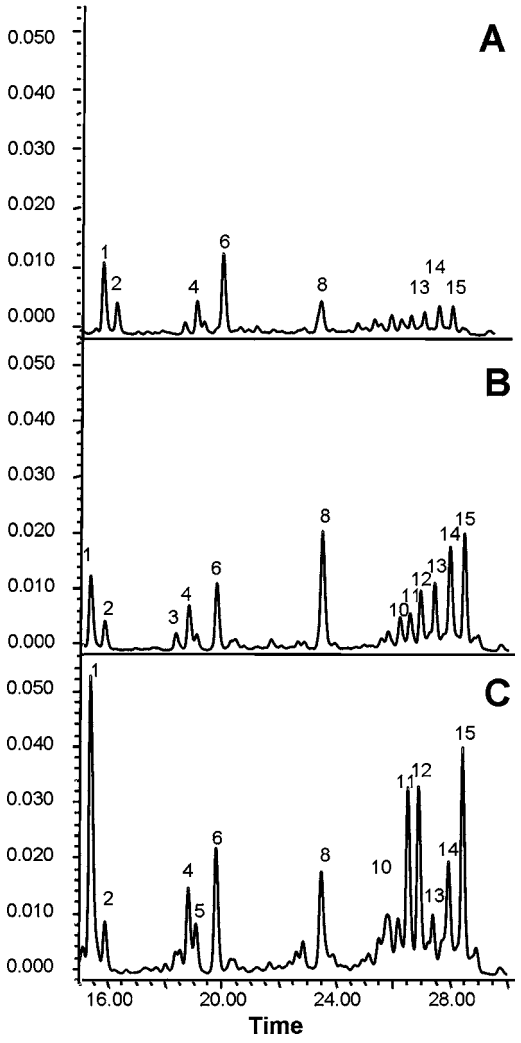

Figure 3. HPLC chromatograms of carotenoids in pulp of Arazá fruit (*Eugenia stipitata* McVaugh) detected at 450 nm: (A) green, (B) half-green, (C) ripe. Peaks are labeled as follows: 1 = lutein; 2 = zeaxanthin; 3 = anhydrolutein; 4 = anhydrozeinoxanthin; 5 = zeinoxanthin; 6 = β-cryptoxanthin; 8 = β-carotene; 10 = β-cryptoxanthin myristate; 11 = zeinoxanthin palmitate; 12 = β-cryptoxanthin palmitate; 13 = lutein dimyristate; 14 = lutein myristoyl palmitate; 15 = lutein dimyristate. Common *Y*-axis scales are used to convey typical changes in species levels with ripening.

contained higher levels of phenolic compounds and exhibited stronger antioxidant properties as reported by Lakshminarayana et al.[60] and by Lata.[61] In a recent study, Huang et al.[62] observed that the peel samples from the edible and medicinal fruit plants presented higher TPC and antioxidant activity than whole fruits and other parts of the same species. The higher TPC and antioxidant activity of fruit peel has been attributed to its high concentration of carotenoids, polyphenols, and vitamin C, which play an important role as defense against the outside world to fulfill evolutionary and biological demands of the plant.

Table 4. Total Phenolics and Antioxidant Activity in Peel and Pulp of Arazá Fruit (*Eugenia stipitata* McVaugh)[a]

	peel	pulp	edible part[b]
total phenolics[c]	124.3 ± 87.3 a	19.3 ± 5.1 b	34.1 ± 9.8
ABTS[d]	11.0 ± 5.3 a	1.2 ± 0.3 b	2.6 ± 0.7
FRAP[d]	12.4 ± 7.7 a	3.5 ± 0.9 b	4.7 ± 1.4
DPPH[d]	9.0 ± 8.6 a	0.8 ± 0.3 b	2.0 ± 0.8

[a]Results are expressed as mean ± SD (n = 6). Values within the same row with different letters show significant differences ($p < 0.05$). [b]Peel and pulp. [c]Expressed as mg GAE/100 g FW. [d]Expressed in μmol Trolox equivalents/g FW.

Regarding the TPC in the edible part of Arazá, which includes fruit and pulp (34.1 ± 9.8 mg GAE/100 g FW), we found a significantly lower value than the one reported when phenolic compounds were extracted with other solvents. Lizcano et al.[23] detected 157 mg GAE/100 g FW when extracting with boiling water while Genovese et al.[63] reported 87 mg GAE/100 g FW when extracting with methanol/water/acetic acid (70:30:5). Likewise, the TPC we found in the pulp (19.3 ± 5.1 mg GAE/100 g FW) was lower than the value reported in pulp extracted with 80% methanol (35.0 mg GAE/100 g).[64] The TPC value for the Arazá pulp is comparable to that for strawberries (*Fragaria* × *ananassa* Duchvar Pink Camino Real) extracted with acetone (18.5 ± 1.2 mg GAE/100 g FW) as reported by Pineli et al.[65]

The higher FRAP antioxidant activity of the Arazá extracts as compared to the ABTS activity may also be related to their high concentration of xanthophylls. When comparing antioxidant activities of carotenoids, Müller et al.[66] observed that xanthophylls were more effective than carotenes in reducing ferric ions, which was hypothesized to be due to steric hindrance and the low chemical reactivity of cyclic carotenes and their carbonyl substituted derivatives. Regarding the ABTS assay, these authors demonstrated that α-carotene and β-carotene were more efficient quenchers of $ABTS^{\bullet+}$ than most of the xanthophylls. On the other hand, neither xanthophylls nor carotenes showed any activity to scavenge DPPH radicals, which supports the low DPPH values found in our study. Wang et al.[67] have explained the very low scavenging activity against DPPH radicals from carotenoids like zeaxanthin due to the presence of a β-ionone ring, which might decrease the resonance effect of pi electrons due to steric hindrance and hence lower free radical-scavenging activity.

As a conclusion, this study shows for the first time that the fruit of *Eugenia stipitata* McVaugh can be differentiated from other fruits by its unique carotenoid pattern, e.g., by the high proportions of lutein. This information may be useful in the proper identification and authentication of products derived from this fruit. In addition, the fact that many of the carotenoids found in Arazá, such as lutein, zeaxanthin, α-carotene, and β-carotene, have been shown to have beneficial health effects suggests that Arazá fruit can be used as a nutraceutical ingredient in the production of functional foods in the Colombian diet.

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Notes

The authors declare no competing financial interest.

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